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Editors

The Prokaryotes

Gammaproteobacteria

Fourth Edition

Springer Reference

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With 108 Figures and 143 Tables

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Foreword

The purpose of this brief foreword is unchanged from the first edition; it is simply to make you, the reader, hungry for the scientific feast that follows. These 11 volumes on the prokaryotes offer an expanded scientific menu that displays the biochemical depth and remarkable physiological and morphological diversity of prokaryote life. The size of the volumes might initially discourage the unprepared mind from being attracted to the study of prokaryote life, for this landmark assemblage thoroughly documents the wealth of present knowledge. But in confronting the reader with the state of the art, the Handbook also defines where more work needs to be done on well-studied bacteria as well as on unusual or poorly studied organisms.

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

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

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

To some readers, this edition of *The Prokaryotes* may indicate that the field is now mature, that from here on it is a matter of filling in details. I suspect that this is not the case. Perhaps we have assumed prematurely that we fully understand microbial life. Van Niel pointed out to his students that—after a lifetime of study—it was a very humbling experience to view in the microscope a sample of microbes from nature and recognize only a few. Recent evidence suggests that microbes have been evolving for nearly 4 billion years. Most certainly, those microbes now domesticated and kept in captivity in culture collections represent only a minor portion of the species that have evolved in this time span. Sometimes we must remind ourselves that evolution is actively taking place at the present moment. That the eukaryote cell evolved as a chimera of certain prokaryote parts is a generally accepted concept today. Higher as well as lower eukaryotes evolved in contact with prokaryotes, and evidence surrounds us of the complex interactions between eukaryotes and prokaryotes as well as among prokaryotes. We have so far only scratched the surface of these biochemical interrelationships. Perhaps the legume nodule is a pertinent example of nature caught in the act of evolving the "nitrosome," a unique nitrogen-fixing organelle. The study of prokaryotes is proceeding at such a fast pace that major advances are occurring yearly. The increase of this edition to four volumes documents the exciting pace of discoveries.

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

Happy hunting!

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Preface

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

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

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

Volumes 6–11, organized by Erko Stackebrandt and Fabiano Thompson, contain 265 chapters in total on each of the ca. 300 known prokaryotic families, in some cases even higher taxa. Each chapter presents both the historical and current taxonomy of these taxa, mostly above the genus level; molecular analyses (e.g., DDH, MLSA, riboprinting, and MALDI-TOF); genomic and phenetic properties of the taxa covered; genome analyses including nonchromosomal genetic elements; phenotypic analyses; methods for the enrichment, isolation, and maintenance of members of the family; ecological studies; clinical relevance; and applications.

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

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

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

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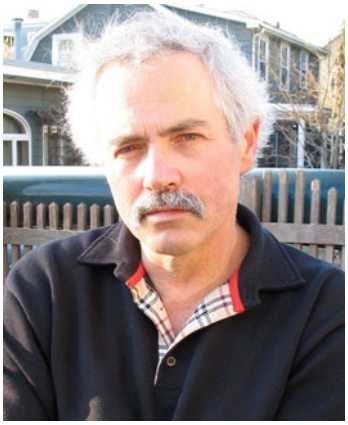
Edward DeLong received his bachelor of science in bacteriology at the University of California, Davis, and his Ph.D. in marine biology at Scripps Institute of Oceanography at the University of California, San Diego. He was a professor at the University of California, Santa Barbara, in the Department of Ecology for 7 years, before moving to the Monterey Bay Aquarium Research Institute where he was a senior scientist and chair of the science department, also for 7 years. He has worked for the past 10 years as a professor at the Massachusetts Institute of Technology in the Department of Biological Engineering, and the Department of Civil and Environmental Engineering, and in August 2014 joined the University of Hawaii as a professor of oceanography. DeLong's scientific interests focus primarily on central questions in marine microbial genomics, biogeochemistry, ecology, and evolution. A large part of DeLong's efforts have been devoted to the study of microbes and microbial processes in the ocean, combining laboratory and field-based approaches. Development and application of genomic, biochemical, and metabolic approaches to study and exploit microbial communities and processes is his other area of interest. DeLong is a fellow in the American Academy of Arts and Science, the U.S. National Academy of Science, and the American Association for the Advancement of Science.



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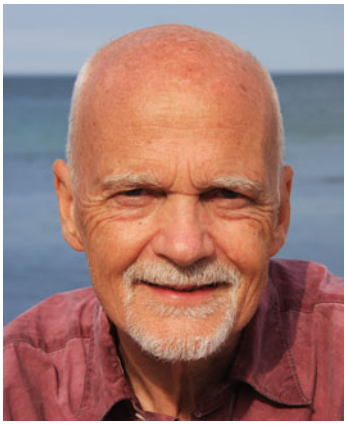
Stephen Lory received his Ph.D. degree in microbiology from the University of California in Los Angeles in 1980. The topic of his doctoral thesis was the structure-activity relationships of bacterial exotoxins. He carried out his postdoctoral research on the basic mechanism of protein secretion by Gram-negative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



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

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Fabiano Thompson became a professor of the Production Engineer Program (COPPE-UFRJ) in 2014 and the director of research at the Institute of Biology, Federal University of Rio de Janeiro (UFRJ), in 2012. He was an oceanographer at the Federal University of Rio Grande (Brazil) in 1997. He received his Ph.D. in biochemistry from Ghent University (Belgium) in 2003, with emphasis on marine microbial taxonomy and biodiversity. Thompson was an associate researcher in the BCCM/LMG Bacteria Collection (Ghent University) in 2004; professor of genetics in 2006 at the Institute of Biology, UFRJ; and professor of marine biology in 2011 at the same university. He has been a representative of UFRJ in the National Institute of Metrology (INMETRO) since 2009. Thompson is the president of the subcommittee on the Systematics of Vibrionaceae–IUMS and an associate editor of *BMC Genomics* and *Microbial Ecology*. The Thompson Lab in Rio currently performs research on marine microbiology in the Blue Amazon, the realm in the southwestern Atlantic that encompasses a variety of systems, including deep sea, Cabo Frio upwelling area, Amazonia river-plume continuum, mesophotic reefs, Abrolhos coral reef bank, and Oceanic Islands (Fernando de Noronha, Saint Peter and Saint Paul, and Trindade).

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1 The Family *Achromatiaceae*

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Abstract
The family *Achromatiaceae* (comprising the single genus *Achromatium*) is a group of morphologically conspicuous, single-celled, free-living, colorless sulfur-oxidizing bacteria which forms a coherent phylogenetic group within the *Gammaproteobacteria*. They have yet to be grown in laboratory culture, but ecophysiological measurements suggest they gain energy from sulfide oxidation and may use oxygen and/or nitrate as a terminal electron acceptor. Some *Achromatium* can fix inorganic carbon, but it is not clear if these are solely autotrophs as they have been shown to also incorporate carbon from acetate. In common with other large sulfur bacteria, they deposit elemental sulfur globules, but *Achromatium* spp. are unique among bacteria in that they precipitate intracellular calcium carbonate as intracellular inclusions. Recent taxonomic revisions indicate that this trait is ubiquitous to all *Achromatium* spp. The role of calcium carbonate precipitation and indeed many other aspects of the physiology of *Achromatium* remains, however, largely a matter of speculation without laboratory cultures.

First described by Schewiakoff (1893, Über einen neuen bakteriennähnlichen Organismus des Süßwassers. Habilitationsschrift, Heidelberg) as *Achromatium oxaliferum*, these organisms have been consistently observed in diverse freshwater and to a lesser extent brackish and marine sediments. In such environments, and in contrast to mat and veil forming sulfur bacteria, *Achromatium* communities are distributed throughout the zone of sulfate reduction. Here "free sulfide" may be present only in sub-micromolar concentrations. The organisms are responsive to redox changes and are able to migrate within the sediment column by a characteristic rolling jerky motion. Despite the absence of a cultured representative, the physical characteristics of *Achromatiaceae* (size and calcite inclusions) have facilitated culture-independent studies of communities using molecular biological methods. These studies have provided useful insights into the phylogeny, diversity, and phenotype of these organisms and ultimately the ecological mechanisms (redox zonation, environmental selection) and physical variation which may facilitate the coexistence and distribution of different species.

Taxonomy, Historical and Current

Short Description of the Family

A.chrom.a.ti.a'ce.ae. N.L. fem. n *Achromatium* type genus of the family; *-aceae* ending to denote a family; N.L. fem. Pl. n. *Achromatiaceae* the *Achromatium* family (Van Niel 1948).

Type genus: *Achromatium* (Schewiakoff 1893).

A.chro.ma'ti.um. Gr. Pref. a not; Gr. N. *chromatium* color, paint; M.L. net. N. *Achromatium* that which is not colored (Babenzien et al. 2005).

The inability to cultivate a representative of the *Achromatiaceae* has been a handicap to past taxonomic classification. Nevertheless, based on distinctive morphological and behavioral characteristics (size, sulfur inclusions, presence of calcium carbonate inclusions, and observed rolling motility), members of the family have been variously named *Achromatium oxaliferum* (Schewiakoff 1893), *Modderula hartwigi* (Frenzel 1897), *Achromatium gigas* (Nadson 1913), *Hillhousia mirabilis* (West and Griffiths 1909), and *Hillhousia palustris* (West and Griffiths 1913). From the middle of the twentieth century onward, the *Achromatium*-like organisms listed above have been united in the family *Achromatiaceae* containing a single genus *Achromatium* comprising two species (Van Niel 1948). At this time the genus *Achromatium* included the calcium carbonate containing *Achromatium oxaliferum* (formerly named *Achromatium Schewiakoff*) and a marine species *Achromatium volutans* (formerly named *Thiophysa volutans*) which does not contain calcium carbonate inclusions (Hinze 1903). In the first edition of *Bergey's Manual of Systematic Bacteriology*, the genus was placed with the non-fruiting gliding bacteria as a genus incertae sedis by virtue of its observed means of locomotion (La Rivière and Schmidt 1989). In the second edition of

E. Rosenberg et al. (eds.), *The Prokaryotes – Gammaproteobacteria*, DOI 10.1007/978-3-642-38922-1_406,

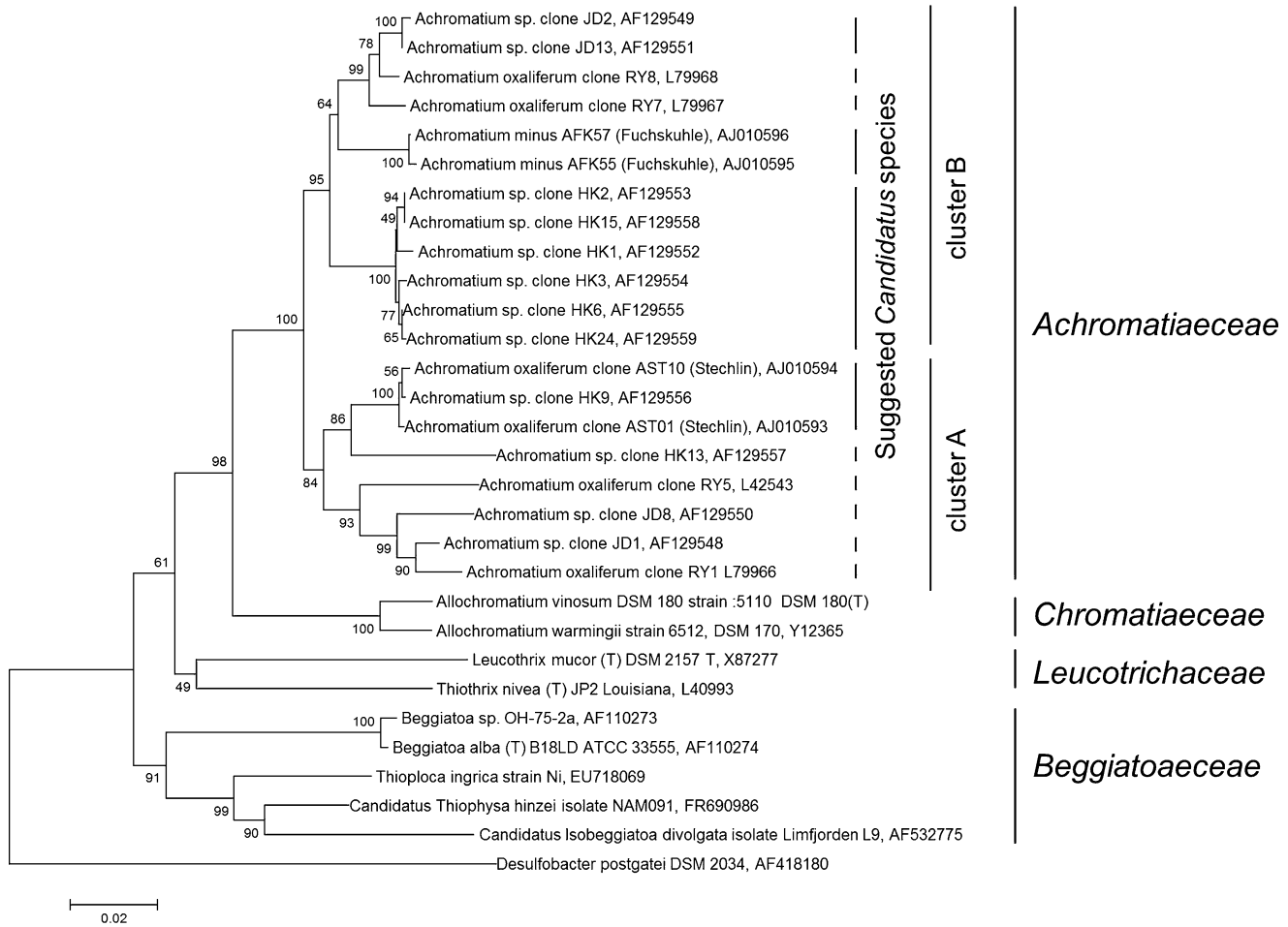

Fig. 1.1

Phylogenetic tree of *Achromatiaceae* 16S rRNA gene sequences. The tree was inferred using the neighbor-joining method. The evolutionary distances were computed using the Jukes-Cantor method based on a total of 1,152 positions using the MEGA 5 software package. The tree was rooted with respect to the *Desulfobacter postgatei* DSM 2034 and the scale bar denotes 2 % sequence divergence. Values at the nodes indicate the percentage of bootstrap trees (1,000 replicates) that contained the cluster to the right of the node. Bracketed sequences discriminate lineages which share less than 97.5 % sequence identity (genospecies)

Bergey's manual, *Achromatium* was incorporated into the family *Thiotrichaceae* on the basis of 16S rRNA sequence data (Garrity et al. 2005). In this most recent classification, the combined family name *Thiotrichaceae* (meaning sulfur hair family) was introduced to include the genera *Beggiatoa*, *Thioploca*, *Thiomargarita*, *Thiothrix*, *Leucothrix*, *Achromatium*, *Thiobacterium*, and *Thiospira*. However, this polyphyletic assemblage comprises physiologically and phylogenetically divergent bacteria of the previously validly named families *Beggiatoaceae*, *Leucotrichaceae* and *Achromatiaceae*. With respect to *Achromatium* spp., none are known to produce multicellular trichomes, and on the basis of 16S rRNA analysis of purified cell preparations, it is clear that the family *Achromatiaceae* as originally proposed by van Niel (1948) actually represents a distinct monophyletic lineage of morphologically conspicuous sulfur bacteria within the *Gammaproteobacteria* (Head et al. 1996; *Fig. 1.1*). A recent update on the phylogenetic position of *Achromatium volutans* revived the genus name *Thiophysa* and placed *A. volutans* in the candidate genus Candidatus "*Thiophysa*" within the family *Beggiatoaceae* (Salman et al. 2011). Accordingly, the family *Achromatiaceae* now provides a coherent, phylogenetically and morphologically robust home for the single genus *Achromatium* restricted to organisms that deposit intracellular calcium carbonate.

This chapter provides an overview of the family *Achromatiaceae* using morphological information, data from field observations, and ecophysiological and molecular biological analyses. Such approaches have been used to discriminate in situ activities, broad- and fine-scale spatial distribution, and different size classes of *Achromatium* species.

Molecular Analyses

Given the lack of cultured representatives of *Achromatium*, culture-independent molecular biological analyses have been

conducted on purified preparations of cells obtained from surface mud and sediment samples. To date *Achromatium* spp. from three locations in the UK and two in Germany have been studied using such a targeted molecular approach (e.g., Head et al. 1996; Glöckner et al. 1999; Gray et al. 1999a); nevertheless, it is clear even from this limited sampling of environments that *Achromatium* species form a coherent phylogenetic group within the *Gammaproteobacteria*. Critically, this deep branching lineage (*Fig. 1.1*) is delineated from the other families which comprise morphologically conspicuous chemoautotrophic sulfur-oxidizing bacteria (e.g., *Beggiatoaceae* and *Thiothricaceae*) and is most closely related to the *Chromatiaceae* which comprise anoxygenic photosynthetic sulfur bacteria, e.g., *Allochromatium vinosum*. Since uncultured *Achromatium* spp. are incompletely described, species are described here based only on 16S rRNA gene sequences. Molecular studies of natural populations have shown that individual sediments contain multiple coexisting *Achromatium* spp. which have complicated the description of individual taxa (Gray et al. 1999a). However, since the advent of whole cell-based phylogenetic techniques, links between genotypes, morphotypes, and phenotypes have been possible to some extent. Further characterization of natural communities of *Achromatium* will solidify the evolving taxonomy of the *Achromatiaceae*.

Genus *Achromatium* (Schewiakoff 1893). The family *Achromatiaceae* contains a single genus (*Achromatium*), which includes bacteria that produce large, nonfilamentous, motile, spherical, or cylindrical cells with round ends. Cells contain large calcium carbonate inclusions (typically 5–6 μm) and smaller sulfur inclusions 0.5–2.0 μm (*Fig. 1.2*). Cells range in width from 5 to 40 μm and from 15 to more than 100 μm in length (Head et al. 1996). Based on 16S rRNA gene sequence analysis, *Achromatium*-like cells form a distinct monophyletic group (Head et al. 1996) covering a sequence divergence of 10 %. Within this clade, two well-supported clusters are evident, and these exhibit sequence divergence between 7 % and 10 % (*Fig. 1.1*). The clusters are also distinguished by differences in a secondary-structure element in the V6 region of their 16S rRNA. Cluster A contains a characteristic deletion (Head et al. 1996) in helix 38 of the 16S rRNA secondary structure (positions 1024–1036, *E. coli* numbering), while cluster B has no such secondary-structure deletion (Glöckner et al. 1999; Gray et al. 1999a). 16S rRNA sequences from *Achromatium* spp. do not include self-splicing introns, a feature reported for other giant sulfide-oxidizing bacteria including *Thiophysa volutans* formerly accommodated in the genus *Achromatium* (Salman et al. 2012). Within-genus phylogenetic clusters are apparent in many families of morphologically conspicuous sulfur-oxidizing bacteria represented by a large proportion of uncultured organism, and some of these have been used to define new candidate genera within the *Beggiatoaceae* (Salman et al. 2011). The level of sequence divergence observed for the two *Achromatium* clusters would be consistent with the occurrence of two distinct genera within the *Achromatiaceae*. For instance, *Candidatus* "*Thiophysa*" and *Candidatus* "*Thiopilula*" 16S rRNA sequences share ≤95.9 % and ≤94.9 % identity with the 16S rRNA sequence from the most closely related type species, *Thiomargarita namibiensis*. Further, 16S rRNA sequences from members of *Candidatus* "*Marithioploca*," previously assigned to the genus *Thioploca*, exhibit ≤92.8 % divergence from the 16S rRNA of *Thioploca schmidlei*, the most closely related *Thioploca* type sequence. Definition of *Candidatus* taxa is not simply based on phylogenetic analysis but additional distinctive characteristics, e.g., cell shape, size, environmental origin, or behavior. At present no such obvious characteristics can be considered to delineate the family *Achromatiaceae* into more than one genus.

Each *Achromatium* 16S rRNA cluster contains sequences from several distinct species based on16S rRNA sequence identity of less than 97.5 % (Stackebrandt and Goebel 1994). For instance, *Achromatium* cluster A contains the original published nearly full-length sequence assigned to *Achromatium oxaliferum* from Rydal Water, Cumbria, UK (L42543). This was discriminated in a community of *Achromatium* species by whole cell fluorescence in situ hybridization (FISH; Head et al. 1996). This cluster contains several other near full-length 16S rRNA sequences which collectively share 90–100 % sequence identity with an average distance of 94 ±0.5 %. The organisms from which these additional sequences have been derived have also been discriminated by FISH (Gray et al. 1999a) and are distributed across four locations: Rydal Water (L79966); Jenny Dam pond, Cumbria, UK (AF129548 and AF129550); Hell Kettles pond, County Durham, UK (AF129556 and AF129557); and Lake Stechlin, Brandenburg-Mecklenburg lake district, Germany (AJ010593 and AJ010594). Cluster B sequences collectively share 94–100 % sequence identity with an average distance of 96 ± 0.2 % and have been discriminated by FISH in sediments from Rydal Water (L79967 and L79968), Jenny Dam (AF129548 and AF129550), Hell Kettles (AF129556 and AF129557), and Lake Fuchskuhle, Brandenburg-Mecklenburg lake district, Germany (AJ010595 and AJ010596). It seems, however, based on certain inconsistencies with FISH data (cells which were either un-hybridized or hybridized to probes not matched to recovered sequences), that the current inventory of *Achromatium* species in individual locations is incomplete (Glöckner et al. 1999; Gray et al. 2004). Indeed, additional *Achromatium* phylotypes from these sites have been inferred by sequencing of DNA fragments excised from denaturing gel electrophoresis (DGGE) gels or clones obtained by targeted PCR amplification with primers selective for sequences from the genus *Achromatium* (Gray et al. 2004, 2007). Surprisingly, a recent survey (January 2014) of the GenBank database using both keyword and BLAST searches identified only two additional partial 16S rRNA gene sequences recovered from DNA extracted from other geographical locations (clone pLW-25 (DQ066949), Lake Washington, USA (Nercessian et al. 2005), and clone Td4-9 (GU20836), Lake Dong ping, China (Song et al. 2012)), which cluster within the *Achromatium* lineage represented (*Fig. 1.1*). Despite the widely reported occurrence of *Achromatium* spp. in sediments from across the globe, this outcome of GenBank searches suggests that *Achromatium*-like sequences will be identified rarely in non-targeted DNA-based

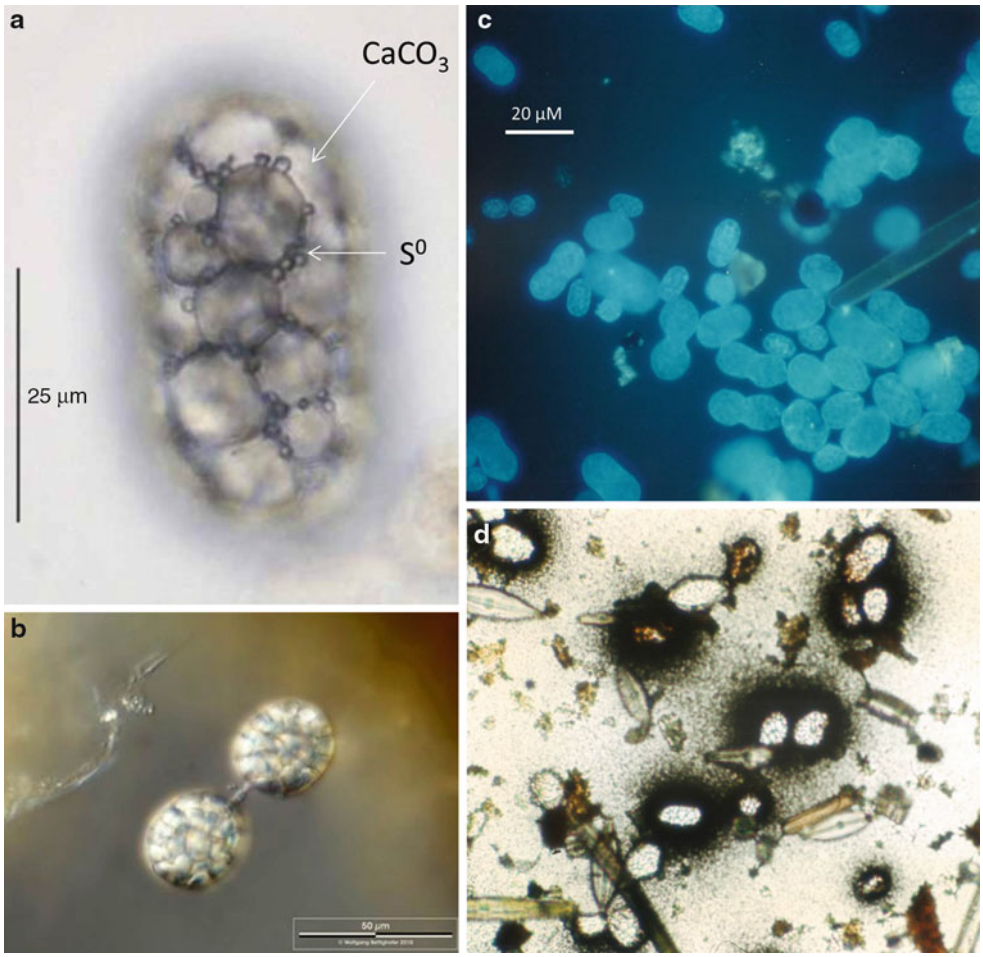

Fig. 1.2

Images of *Achromatium* cells. (**a**) *Achromatium* cell collected from the sediment of a stagnant freshwater pond near Boise, Idaho, USA, showing a characteristic rounded cylindrical cell and large inclusions of calcite and smaller inclusions of sulfur (Reproduced with the kind permission of William Bourland). (**b**) *Achromatium* cell in the final stage of division with two spherical daughter cells connected only by a thin tube of cell envelope (Collected from a pond situated in the vicinity of Lake Constance (Bodensee, Southern Germany). The image is reproduced with the kind permission of Wolfgang Bettighofer and was built up using several photomicrographic frames with manual stacking technique). (**c**) A DAPI (4′,6-diamidino-2-phenylindole)-stained preparation of a crudely purified suspension of *Achromatium* cells collected from the English Lake district. A heterogeneous distribution of the DAPI stain can be seen in some cells, which has been interpreted as the presence of discrete accumulations of DNA within the cells (Head et al. 2000a). This observation is consistent with the original description of *Achromatium* (Schewiakoff 1893) where cells were stained blue or violet with hematoxylin. Note for comparison the presence of a diatom in the field of view. (**d**) Microautoradiographic analysis of *Achromatium* cells incubated in sediments amended with ^{13}C-labeled acetate. Most cells show the characteristic halo of silver grains indicating uptake of radiolabel. Cells were exposed to the photographic emulsion only after acid dissolution of calcite inclusions

surveys of bacterial diversity. Presumably this is because *Achromatium* cell numbers rarely exceed 10^5 cells cm^{-3} and thus only ever represent a small proportion of total bacterial cell abundances even when the population accounts for a high proportion of the biomass. Nevertheless high-throughput sequencing surveys may yet reveal that *Achromatium* spp. are more widely distributed than current data suggest.

The use of FISH has shown that *Achromatium* phylotypes fall into distinct size classes (Glöckner et al. 1999; Gray et al. 1999a, 2004). On this basis Glöckner et al. proposed the apparently homogenous population with a uniformly small size (average 10 µm cell diameter) present in the acidic Lake Fuchskuhle as *Candidatus* "*Achromatium minus*." Morphological differentiation of putative *Achromatium* species has since been extended on the basis of statistically significant differences in mean cell diameter in genetically distinct *Achromatium* cells that coexist in single sediments and in populations from different geographical locations (*Figs. 1.3* and *1.4*, Gray et al. 1999a). The measurement of cell diameters of genetically distinct *Achromatium* spp. identified using FISH partially resolves an apparent contradiction in early descriptions of these organisms. For instance, West and Griffiths (1913) differentiated two separate species

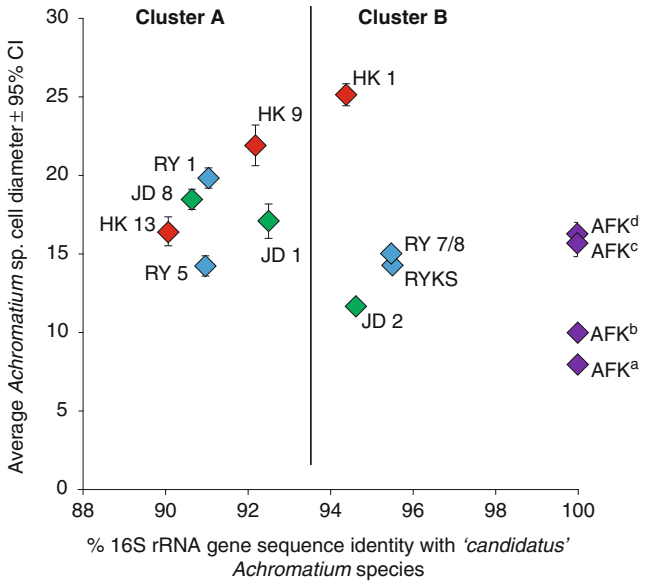

Fig. 1.3

16S rRNA sequence identities of individual *Achromatium* spp. relative to the *Candidatus* species *Achromatium minus* identified in Lake Fuchskuhle (*AFK*) plotted against average cell diameters. Error bars represent ± 95 % CI. The *vertical black line* indicates the division of *Achromatium* spp. into Clusters A and B. Rydal Water (RY, *light blue diamonds*), Jenny Dam (JD, *green diamonds*), Hell Kettles (HK, *red diamonds*), *Candidatus Achromatium minus* (AFK, *purple diamonds*). There is some discrepancy in reports of the diameter of *Achromatium minus* cells so multiple values have been presented, i.e., AFK^{a} cell diameter values reported in Babenzien 1991, i.e., 8 × 15 µm, and AFK^{b} cell diameter value reported in Glöckner et al. 1999, i.e., 10 × 15 µm; AFK^{c} cell diameter values were also measured from a micrograph with inset scale bar Glöckner et al. 1999, and AFK^{d} Cell diameter values were measured from micrograph with inset scale bar Babenzien et al. 2005

(*Hillhousia mirabilis* and *Hillhousia palustris*) principally based on cell size and unspecified habitat differences. Nadson and Visloukh (1923), however, described several different size classes of *Achromatium oxaliferum* cells (clearly similar organisms to *Hillhousia*) to discriminate what they took to be variations within a single species. To this end they employed the epithets *-minus*, *-medium*, *-majus*, *-elongatum*, and *-gigas*. Subsequently, Ellis (1932) concluded that size variation had no "genetic significance" and "a new specific name is called for on the score of differences in size only when a distinct gap appears in the grading." A wide but continuous size range (*Fig. 1.4*) is exactly what has been recorded for a mixed *Achromatium* population in a single pond (Gray et al. 1999a); however, based on 16S rRNA sequence identity between *Achromatium* spp. identified with FISH, it appears that both West and Griffiths and Nadson and Visloukh were each partly correct. For example, in sediments from Hell Kettles, a pond in northern England, FISH analysis and cell size measurements for cells that were the source of sequences HK1, HK 9, and HK13 showed that each fell into distinct size classes, albeit that these size classes overlapped. Intriguingly, however, there is no correlation at all between 16S rRNA sequence identity and cell size and/or geographical origin even in comparisons spanning the different 16S rRNA-defined clusters (*Fig. 1.3*). For instance, *Achromatium* sp. RYKS (14.3 ± 0.26 µm, mean diameter ± 2SE) which is most closely related to *Achromatium minus* (both in Cluster B) was not significantly different in mean cell diameter to that of the distantly related cluster A *Achromatium* sp. RY5 (14.28 ± 0.58 µm), both of which are present in Rydal Water sediments.

List of Species of the Genus *Achromatium*

Achromatium oxaliferum (Schewiakoff 1893). Originally identified in mud from Neuhofener Altrhein, Germany. Occurs as single spherical to cylindrical cells with an average size of 15 × 29 µM. Cells have a rolling motility.

Type strain: none isolated.

"Candidatus Achromatium minus" (Glöckner et al. 1999). Identified in the acidic Lake Fuchskuhle, Germany. Described as single coccoid cells with an average size of 10 × 15 µM.

Type strain: non-isolated.

GenBank accession number of 16S rRNA gene sequences: AJ010595, AJ010596.

Taxonomic Note: The genus *Achromatium* is not represented by pure cultures; its type specie *A. oxaliferum* remains uncultured and incompletely characterized. Such a combination is usually characteristic of a *Candidatus* group; however, the genus *Achromatium* retains its valid status due to historical precedent. *Achromatium* have been identified in various freshwater and brackish sediments in Europe, Asia, Africa, and North America and identified by a range of synonyms, i.e., *Hillhousia mirabilis* (West and Griffiths 1909) and *Hillhousia palustris* (West and Griffiths 1913). Based on 16S rRNA phylogeny, each Achromatium subcluster contains 11 branches which share sequence homologies of less than 97.5 % suggesting that each of these lineages represent *Candidatus* species (*Fig. 1.1*) with differing cell diameters (*Fig. 1.4*).

Phenotypic Analyses

Phenotypic Characteristics of the *Achromatiaceae* (Genus *Achromatium*)

Sulfur Oxidation

As for all morphologically conspicuous sulfur bacteria, a ubiquitous feature of the *Achromatiaceae* is the presence of intracellular sulfur grains which become more visible on treatment of cells with dilute acid to remove calcium carbonate inclusions (de Boer et al. 1971). Sulfur grains are themselves diagnostically removable by treatment with methanol and have been confirmed in cells by energy-dispersive

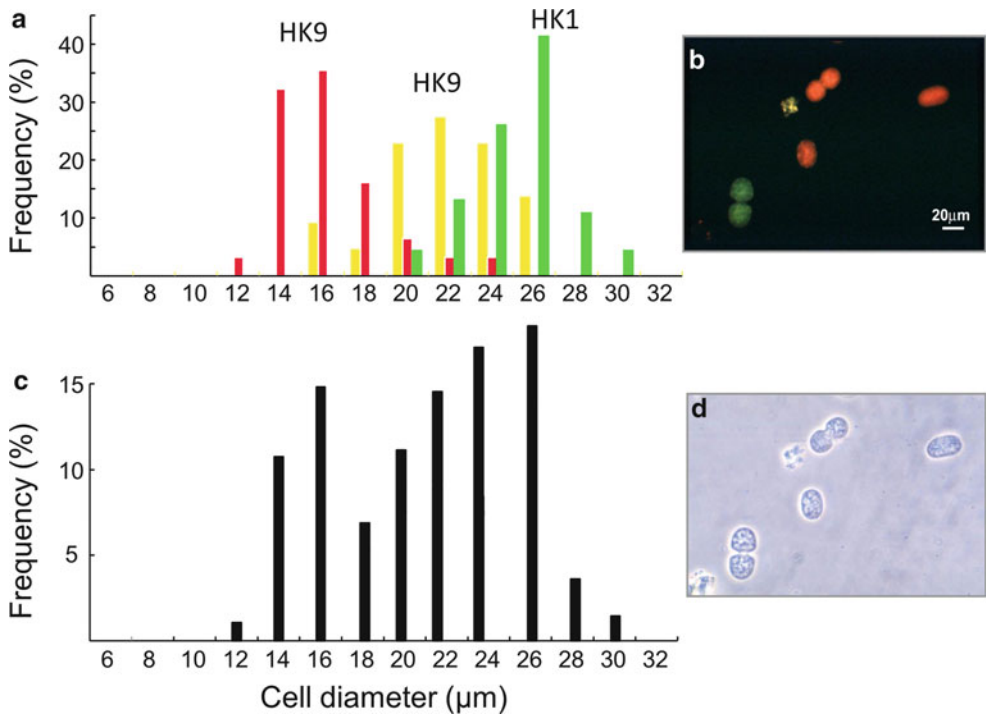

Fig. 1.4

Frequency distributions for *Achromatium* cell diameters from Hell Kettles pond. (a) Frequency distributions of different genotypes discriminated with fluorescence-labeled oligonucleotide probes specific for the different *Achromatium* "species," i.e., HK1, 9, and 13. (b) Epifluorescence micrograph of FISH discriminated cells. (c) Frequency distribution calculated from the same data without species discrimination. (d) Phase contrast micrograph (Gray et al. 1999a)

X-ray microanalysis (EDX) linked to scanning electron microscopy (SEM) (Head et al. 1996). The presence of intracellular sulfur and the distribution of *Achromatium* throughout the zone of sulfate reduction in sediments (see below) strongly suggest *Achromatium* spp. have a role in the oxidative component of a tightly coupled sulfur cycle. Evidence that phylogenetically mixed populations of *Achromatium* species are capable of oxidizing reduced sulfur species completely to sulfate has been obtained through correlation of rates of sulfate production in sediment microcosms augmented with physically purified preparations of cells (Gray et al. 1997). Further, incubation of *Achromatium*-bearing sediment cores where active sulfate reduction was occurring, with ^{35}S-labeled sulfate, demonstrated that *Achromatium* cells were capable of rapidly incorporating sulfide produced by sulfate reduction into intracellular elemental sulfur (Gray et al. 1999b). It has also been shown that the same *Achromatium* population may catalyze the AMP-dependent oxidation of sulfite to APS (adenosine-5′-phosphosulfate reductase) and ultimately sulfate using APS reductase since APS-reductase genes could be amplified from DNA extracts of purified *Achromatium* cells (Head et al. 2000b). The presence of high-affinity sulfide uptake systems in *Achromatium* has been inferred from the often observed high abundances of *Achromatium* cells in sediments in which free sulfide levels were below detection limits (Gray and Head 2005; Gray 2006). Such systems likely render *Achromatium* capable of effectively competing with reactive metal sinks for dissolved sulfide common in sediments.

Carbon Metabolism

Collectively, *Achromatium* spp. exhibit a range of trophic activities, i.e., facultative chemolithoautotrophy, mixotrophy, and chemoorganoheterotrophy, similar to other large sulfur bacteria. The challenge has been to understand these activities in the context of the diversity of naturally occurring mixed populations of *Achromatium* spp. Studies of ^{14}C-labeled substrate uptake in Rydal Water, UK, have demonstrated that *Achromatium* cells from this community assimilated ^{14}C from labeled bicarbonate, acetate, and protein hydrolysate. ^{14}C-labeled glucose was not assimilated (Gray et al. 1999b). *Achromatium* spp. from another location in the UK (Hell Kettles), however, assimilated acetate (e.g. ❯ *Fig. 1.2d*) but did not assimilate bicarbonate in cell carbon, despite incorporating ^{14}C from bicarbonate into calcium carbonate inclusions. These carbon uptake patterns were corroborated by the detection of a homologue of the RuBisCO large subunit gene (*rbcL*) in DNA extracts from pure preparations of *Achromatium* cells from Rydal Water but not from Hell Kettles (Head et al. 2000b). Later studies of the Rydal Water community using combined microautoradiography and fluorescence in situ

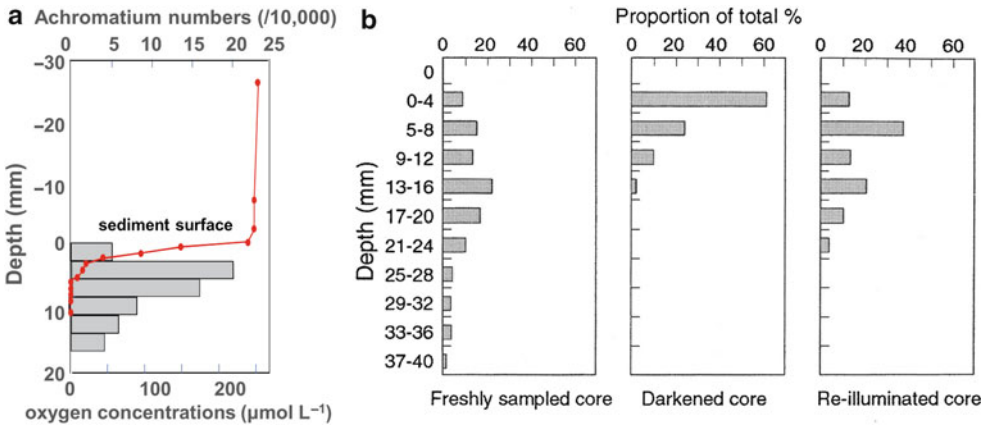

Fig. 1.5

The vertical redistribution of *Achromatium* populations. (a) Profiles of dissolved oxygen and *A. oxaliferum* cells in sediment cores (Head et al. 1996). (b) The redistribution of *Achromatium* in response to redox changes. In sediment cores incubated in the dark, the cells accumulate toward the sediment surface, and in re-illuminated cores, the cells migrate to greater depth. Illumination conditions of sediment cores have been shown to raise or lower the position of the oxic/anoxic boundary (Gray et al. 1997) (Reproduced from Head et al. 2000b with kind permission)

hybridization (Gray et al. 2000) showed that all individual *Achromatium* sp. identified (from clusters A and B) assimilated both ^{14}C-bicarbonate and ^{14}C-acetate, i.e., all are likely mixotrophs, whereas Hell Kettles *Achromatium* spp., regardless of the phylogenetic cluster to which they belong, are all likely chemoorganoheterotrophs. These data suggest that carbon metabolism is not correlated with any particular phylogenetic clade within the *Achromatium* radiation and that the sediment environment may play a role in the selection of *Achromatium* spp. with different modes of metabolism.

Respiration

The oxidation of reduced sulfur species to sulfate can be coupled to the reduction of oxygen, nitrate, and/or oxidized metals, e.g., Fe and Mn; however, *Achromatium* has generally been regarded as an obligate aerobe (La Rivière and Schmidt 1992). Evidence to support this obligate physiology comes from the presence of *Achromatium* in surface sediment layers and their tolerance to oxygen (as indicated by cell motility after purification from sediments). Recent studies have, however, refined our understanding of the respiratory capacity of *Achromatium* spp. For instance, depth profiles of oxygen concentration and *Achromatium* cell numbers in a freshwater sediment revealed that the population spanned the oxic-anoxic boundary in the top 3–4 cm of sediments (*Fig. 1.5*) and that some cells were present at depths without oxygen (Babenzien and Sass 1996; Gray et al. 1997). Furthermore, the distribution of different *Achromatium* spp. identified using FISH in relation to sediment redox profiles indicated that all coexisting populations were microaerophilic but adapted to different redox conditions with some capable of anaerobic metabolism (Gray et al. 1999a, *Fig. 1.6*). These conclusions are supported by artificial manipulation of redox conditions in sediment microcosms (Gray et al. 2004). Induced anoxia caused a wholesale decline in cell numbers relative to populations which were sustained in oxygenated controls; however, *Achromatium* sp. RY8 (subcluster B) decreased and *Achromatium* sp. RY5 (subcluster A) and RYKS (subcluster B) increased in relative representation over a 7-day incubation period. Critically, in the absence of oxygen but high levels of nitrate, the composition of the *Achromatium* community remained stable suggesting all the coexisting *Achromatium* spp. were obligate or facultative anaerobes that utilize nitrate. Some *Achromatium* spp. were clearly more sensitive to sediment redox conditions than others perhaps reflecting their respective affinities for nitrate. As with differences in carbon metabolism, differences in nitrate affinity are clearly not correlated with specific phylogenetic clusters of *Achromatium* spp. It is not yet known whether *Achromatium* are capable of reduction of nitrate to N_2 or reduction of nitrate to NH_3 as has been observed in the candidate genus "*Marithioploca*" in the *Beggiatoaceae* (Otte et al. 1999) and marine *Beggiatoa* (Sayama et al. 2005). Given the observed depth distribution of *Achromatium* spp. throughout the zone of sulfate reduction and the absence of nitrate storage vacuoles characteristic of nitrate-reducing *Beggiatoaceae*, it has been speculated that *Achromatium* species dwelling in anoxic sediments may be able to couple manganese and/or iron reduction to sulfur oxidation (Gray et al. 1997; Head et al. 2000b). Use of alternative electron acceptors such as these is tentatively supported by studies of anoxic microcosms without any addition of nitrate where it was still possible to detect all coexisting *Achromatium* spp. albeit at considerably reduced numbers after 13 days (Gray et al. 2004). Alternatively, to survive such conditions, *Achromatium* may utilize internally precipitated elemental sulfur as an electron acceptor. This respiratory versatility has been documented in

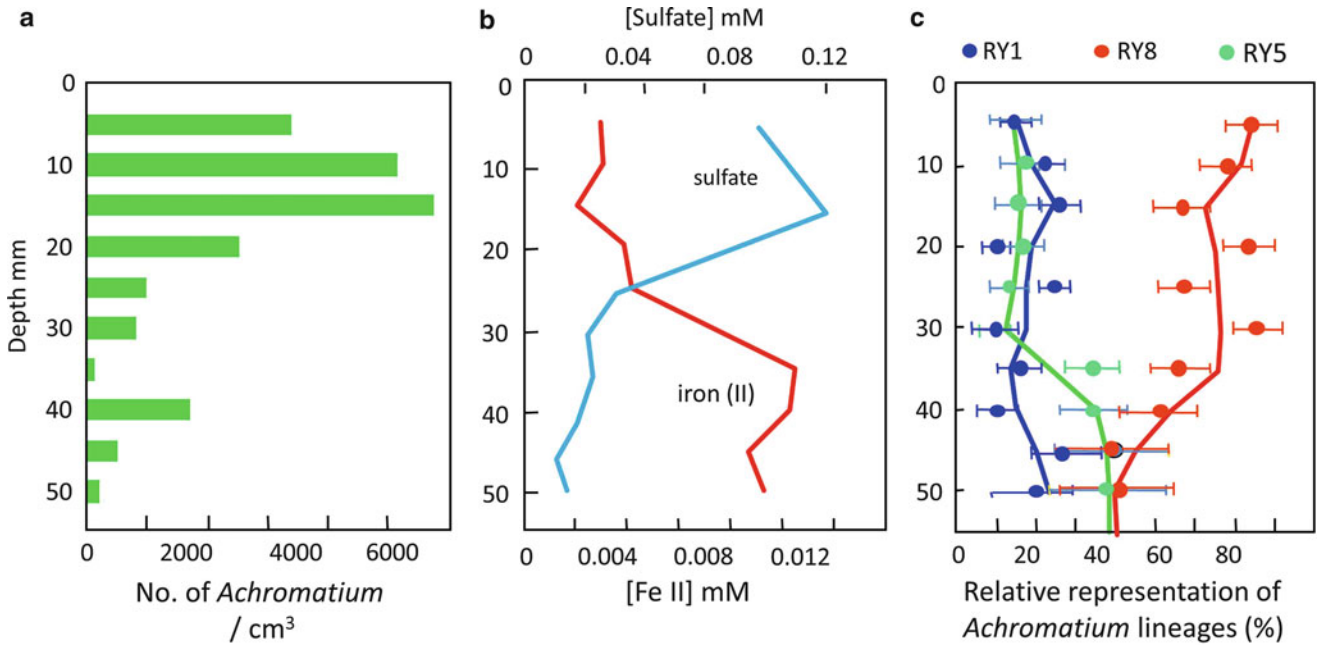

Fig. 1.6

Depth profiles of *Achromatium* cells in relation to redox-sensitive chemical species in Rydal Water. (a) Depth distribution of I cells. (b) Depth distribution of redox-sensitive chemical species (Fe^{2+} and SO_4^{2-}). (c) Relative abundance of three *Achromatium* lineages identified by FISH. The *trend* lines show a three-point moving average of the data, and error bars represent 95 % confidence intervals for counts of the *Achromatium* subpopulations (Adapted from Gray et al. 1999a with kind permission)

Beggiatoa alba strain B18LD which under anoxic conditions and in the presence of H_2 or endogenous carbon reserves can reduce elemental sulfur to sulfide (Schmidt et al. 1987).

Cell Division, Motility, and Cell Envelope Structure

Achromatium divide by constriction of the cell at the center of the long axis producing opposed indentations which progressively extend inward. In the final stage of division, cells are linked only by a thin tube of cell envelope (❯ *Fig. 1.2b*). Cell division takes approximately 24–48 hours (West and Griffiths 1913) and typically, in natural populations, >10% of cells are at different stages of this process (Head et al. 2000a). Motility is widely reported as a rolling, jerky motion. When cells are maintained in sediment suspensions on microscope slides and in counting chambers, *Achromatium* move in, seemingly, random alternating directions "reminiscent of a Mexican jumping bean" (Starr and Skerman 1965). Such a prosaic description certainly chimes with those who have spent hours observing these cells, and we have noticed a propensity to repeatedly attach, disengage, and reattach to the same and different sediment particles. Presumably, this type of motility and attachment occurs in situ but obviously with particles constrained within the more tightly packed sediment matrix. It seems clear that that movement cannot be entirely random and is likely driven by chemotaxis (Gray et al. 1997; Head et al. 2000b) which accounts for the vertical redistributions of the whole population observed in response to environmental perturbations (❯ *Fig. 1.5b*).

The cell envelope structure of *Achromatium* is fairly typical of Gram-negative bacteria (Head et al. 1996; de Boer et al. 1971; ❯ *Fig. 1.7a*), but the cell is surrounded by a slime (mucilaginous) capsule. Two cell types have been observed in scanning and transmission electron micrographs of "spray-frozen" *Achromatium* cells, namely, those that contain filamentous structures within a much thicker (1.5 μm) slime capsule (❯ *Fig. 1.7b, d*) and those that are without filaments (❯ *Fig. 1.7c*) and have a thinner (0.1 μm) slime capsule. Differences in the outer layer thickness of cells are also observable by conventional microscopy implying that smooth cell morphologies are not an artifact of preparation for SEM analysis. However, as yet no link between phylogeny based on 16S rRNA sequences and cell envelope characteristics has been identified (Head et al. 2000b). Originally the presence of filamentous structures was implicated in the motility of cells (de Boer et al. 1971) by their action within the slime layer, suggesting that they used a similar means of motility to gliding bacteria and explaining their inclusion with the non-fruiting gliding bacteria in the 1st edition of *Bergey's Manual of Systematic Microbiology* (La Rivière and Schmidt 1992, 1989). However, it seems likely that filament-free smooth cells must also be motile as the entire cell population in Rydal Water has been shown to actively redistribute in response to externally imposed changes in redox conditions (❯ *Fig. 1.6*; Head et al. 1996; Gray et al. 1997).

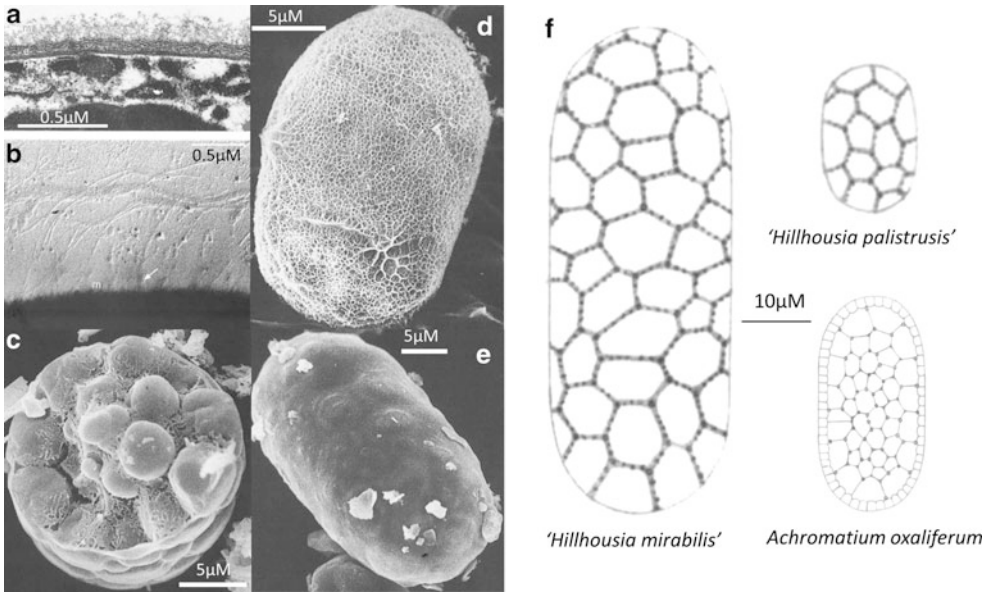

Fig. 1.7

Electron microscope images and drawings of *Achromatium*. (a) Thin section through cell surface showing Gram-negative membrane structure (*TEM*): *m* mucilage, *e* envelope, *p* plasmalemma. (b) Shadow-cast preparation of cell surface showing filaments (*f*) projecting through the mucilage layer (*m*) of a "rough"-surfaced cell. The *arrow* indicates dense inclusions in the mucilage. (c–e) Images of spray-frozen *Achromatium* cells (*SEM*). (c) Transverse section of a fractured cell showing internal arrangement of calcite inclusions. (d) Whole cell of the "smooth" surface type; cell inclusions are just visible below the surface. (e) Whole cell of the "rough" surface type. The pitted appearance of the mucilaginous cell covering is due to removal of small ice crystals during preparation (All images reproduced with kind permission from Head et al. 1996). (f). Line drawings of *Achromatium* cells depicted by West and Griffiths (1913) including their own drawings of *Hillhousia mirabilis* and *palustris* and a copy of Schewiakoff's drawing of *Achromatium oxaliferum* (Schewiakoff 1893) showing a differentiated peripheral region and central body (Reproduced with kind permission)

Recent data on cell envelope morphology may resolve another apparent contradiction in early descriptions of *Achromatium* species. In 1913 West and Griffiths, citing supporting observations by Virieux (1912) and Massart (1901), discriminated *Hillhousia mirabilis* and *Hillhousia palustris* from the previously described *Achromatium oxaliferum* (Schewiakoff 1893; *Fig. 1.7f*). The first somewhat spurious reason for their introduction of a new genus name was the almost certainly incorrect assumption by Schewiakoff that the large inclusions he observed were calcium oxalate (not calcium carbonate) and the smaller inclusions were chromatin grains thought to be involved in heredity, and not sulfur. Notwithstanding size, the more "profound" reason for a new genus name, as West and Griffiths attested, was a "differentiated peripheral zone" in *Achromatium oxaliferum* which was absent in *Hillhousia*. Regardless of this distinction, later authors considered these organisms as the same species (Bavendamm 1924 and Ellis 1932). Differences in descriptions could be attributed to variation in microscope quality, setup, and visual interpretation; however, with our current knowledge of *Achromatium* cell morphology, it now seems reasonable to speculate that *Achromatium* cells with a peripheral zone as described by Schewiakoff might be those with a thicker slime capsule. Indeed there is a close match between the dimensions of the peripheral layer (≅1.5 μm) as measured from the original Schewiakoff manuscript (*Fig. 1.7f*) and the width of the thick slime capsule measured by TEM (Head et al. 1996).

Calcium Carbonate Inclusions

Although extracellular precipitation of carbonates is a common consequence of bacterial metabolism, intracellular calcite precipitation is unique to *Achromatium*. With the recent taxonomic revision of the family *Achromatiaceae* whereby *Achromatium volutans* (van Niel 1948) has been reclassified based on 16S rRNA phylogeny as the genus "Candidatus *Thiophysa*" in the family *Beggiatoaceae* (Salman et al. 2011), the presence of intracellular calcium carbonate inclusions can also now be considered a ubiquitous, diagnostic feature of *Achromatium* spp. This phenotypic characteristic is, therefore, one of those rare occurrences where phenotype is entirely consistent with phylogeny. The inclusions were originally identified as being calcium carbonate by their dissolution on treatment with acids (e.g., West and Griffiths 1913). X-ray diffraction analysis of purified preparations of cells subsequently indicated that the calcium carbonate is most similar to the mineral calcite; however, the intensity ratios of D spacings (degrees 2θ) were not identical to those observed for crystalline standard materials indicating precipitation is likely under biological direction. SEM studies of

spray-frozen and fractured *Achromatium* cells have shown that their calcite inclusions have a highly regular arrangement around the periphery of the cell akin to "shaped stones in a building arch" with additional inclusions in the center (Head et al. 1996; *Fig. 1.7c*). Individual inclusions have been examined by TEM analysis of ultrathin sections which show a membrane around the inclusions and internal structures reminiscent of laminations and electron dense centers which are putative sites of crystal nucleation. Collectively inclusions make up a large but highly variable proportion of the cell (<30 % to >70 %) as determined by confocal scanning laser microscopy of randomly selected cells stained with Nile red and optically sectioned (Head et al. 2000b).

The precipitation of calcite as intracellular inclusions under biological control has led to speculation that *Achromatium* must have an active Ca^{2+} uptake system (Starr and Skerman 1965) presumably where membrane transport is catalyzed by Ca^{2+}-dependent ATPases (Head et al. 2000b). It has also been speculated that "calcium must accumulate in the cells against a very considerable gradient" (Starr and Skerman 1965). Reported dissolved calcium values for *Achromatium* habitats can, however, be quite high, i.e., 12.5–17.5 mM in saline springs (Lackey and Lackey 1961) or 0.25–1 mM in the freshwater Rydal Water (Head et al. 2000b). In this second location, however, it has been shown that there was a strong linear correlation between solid-phase calcium and *Achromatium* numbers in six sediment cores (R^2 0.71–0.98, $p < 0.001$–0.02) and vertical redistribution of *Achromatium* cells elicited very similar vertical changes in the solid-phase calcium distributions. These data suggest that bulk sediment calcium carbonate is almost entirely sequestered within *Achromatium* cells (Head et al. 2000b) at concentration levels at least an order of magnitude larger than those of dissolved calcium. This distribution of Ca is striking since it appears that calcite and other carbonate mineral phases are actually unsaturated in Rydal Water based on calcium speciation calculations performed using geochemical modeling (Parkhurst and Appelo 1999). The presence of calcifying *Achromatium minus* in the acidic Lake Fuchskuhle is even more striking given that carbonate dissolution is highly favored by the lower pH (pH 4.5) encountered in this environment (Glöckner et al. 1999). Calcium deficiency may, therefore, be a bar to high *Achromatium* abundance only in some soft water areas (La Rivière and Schmidt 1992). In such systems (e.g., Canadian Shield lakes or large parts of Scandinavia), it has recently been shown that Ca^{2+} concentrations below a threshold of 0.04 mM limit the growth of calcium-rich Daphnia species (Jeziorski et al. 2009).

The apparent unique character and ubiquitous occurrence of calcite inclusions within the family *Achromatiaceae* begs the question, what unique physiological challenge faced by these organisms necessitates this very specific intracellular process? Of all of the physical and behavioral adaptations displayed by giant sulfur bacteria, this one is probably the most enigmatic. Numerous hypotheses have been put forward over the last 100 years or so. With more recent information obtained from molecular and in situ studies, some older theories are now less plausible but others may be simultaneously true. At present no definitive proof is available to unequivocally accept or reject any theories (*Table 1.1*), but with the advent of genomic technologies and their application to *Achromatium* populations, it should soon be possible to gain a greater understanding of the genetic basis of calcification in *Achromatium*.

Isolation, Enrichment, and Maintenance Procedures

Currently, no pure cultures or enrichments of *Achromatiaceae* exist, and all biochemical, physiological, and molecular investigations have been performed on *Achromatium* cells purified from sediments or by in situ observation. West and Griffiths (1913) separated *Achromatium* cells from surrounding less dense sediment particles by tilting aliquots of sediments in a glass dish to separate less dense particles. Similarly we use sediment suspensions mixed with overlying water placed in a flat-bottomed glass beaker to a depth of a few mm. On briskly tilting the beaker (45°) from the vertical, a visible white line of cells (best observed against a black background) accumulates along the trailing edge of the water meniscus. By returning the beaker gently to the vertical and re-tilting, the white line can be progressively enriched; however, if abundances are low, the beaker can be turned horizontally (90°) and further tilting will concentrate cells into a patch more or less at the center of the beaker. By such a laborious approach cells can be obtained even from sparsely populated materials. De Boer et al. (1971) used a method suggested to them by C. B. van Niel whereby sediment suspensions (0.5 cm thick) were swirled in a tilted beaker that at the same time was rotated along the vertical axis. This process resulted in the accumulation of a narrow white band very close to that of the sand-particle fraction. It is recognized that such gravity-based extraction process will only work for calcite-filled cells but, based on an assumption of similar densities, different size classes should be equally enriched. Filter-sterilized habitat- or tap water should be used for additional purification steps to produce physically enriched cell suspensions free of other bacteria as sterile-deionized water causes cells to lyse, presumably as a result of the high osmotic potential of the cells.

Purified cells stop moving and loose calcium and sulfur grains fairly rapidly (West and Griffiths 1913; Starr and Skerman 1965); however, West and Griffiths reported that sulfur grains reappear on addition of H_2S and Starr and Skerman (1965) reported that cell activity was maintained on addition of 1,000 ppm calcium cysteinate. Nevertheless, natural *Achromatium* populations can only be maintained in the laboratory for longer periods (weeks to months) using sediment microcosms. Schewiakoff (1893) used a watch glass containing mud overlain with habitat water and, on exposure to sunlight, observed "energetic multiplication" and formation of white clumps (presumably on the surface) of 50–100 cells. La Rivière

Table 1.1
Evaluation of past and current theories regarding calcite deposition in *Achromatium*

Hypothesis proposed for calcite deposition by *Achromatium*	Support for or/and against hypothesis
Regulates acidity generated by H_2S oxidation (La Rivière and Schmidt 1992)	The oxidation of H_2S or HS^- is a proton-generating reaction; however in typical non-sulfidic, **near neutral** freshwater habitats typically inhabited by *Achromatium*, proton-generating reactions are demonstrably balanced by alkalinity and proton-consuming reactions, e.g., sulfate reduction
Precipitation and dissolution of calcite is used for buoyancy regulation in sediments (Babenzien 1992)	Calcite-filled cells are denser than calcite-free cells; however density is clearly not a bar to vertical positioning in the sediment since upward redistribution of *Achromatium* in response to redox change is accompanied by a very similar redistribution of solid-phase calcium (Head et al. 2000b). Calcite-filled cells are, however, unlikely to be able to migrate into the overlying waters
Calcite is an electron acceptor in carbonate respiration (Babenzien 1991)	*Achromatium* spp. are closely related to O_2 or NO_3^- reducing sulfur-oxidizing bacteria not hydrogenotrophic methanogenic Archaea or acetogens which oxidize hydrogen and use CO_2 as an electron acceptor. Further, sulfide oxidation coupled to CO_2 reduction is thermodynamically unfavorable in non-photosynthetic processes
Calcite inclusions maximize the ratio of cell surface area to cytoplasmic volume, reducing substrate uptake and transport limitation in such large cells (Head et al. 2000a)	Spherical cells are likely inefficient with respect to substrate uptake, and large vacuoles in other giant sulfur bacteria have been similarly proposed (Schulz and Jørgensen 2001) as a means of increasing surface area to cytoplasmic volume ratios. Such intracellular vacuoles are in fact principally of benefit to such organisms as a nitrate (electron acceptor) store to allow survival of cells rarely exposed to nitrate-rich overlying water. Presumably calcite inclusions likewise must have some other functional benefit unique to *Achromatium*
Calcite precipitation generates protons to balance the buildup of cytoplasmic hydroxide concentrations caused by conversion of HCO_3^- to CO_2 by the enzyme carbonic anhydrase to facilitate carbon fixation by the enzyme ribulose-1,5-carboxylase under low CO_2 conditions (Head et al. 1995)	Some Prymnesiophyceae (unicellular marine algae) deposit intracellular calcite for this purpose to compensate for the low availability of carbonic acid (H_2CO_3) compared to bicarbonate (HCO_3^-) in high pH marine settings (Borowitzka 1982). For instance, at pH 8 [HCO_3^-] exceeds [H_2CO_3] by two orders of magnitude. At near neutral to acidic pH (<6.5) where *Achromatium* often thrive, the [H_2CO_3] broadly equals or exceeds the concentration of HCO_3^-. Furthermore, microautoradiographic studies on carbon uptake in *Achromatium* populations from different geographical locations have demonstrated that some calcite-precipitating *Achromatium* communities do not utilize bicarbonate for biosynthesis (Gray et al. 1999b)
Calcite precipitation generates protons to balance the buildup of cytoplasmic hydroxide concentrations caused by generation and extracellular export of protons to the surface of acid-soluble sulfide minerals (see *Fig. 1.8*) to mobilize sulfide for subsequent oxidation (Gray 2006)	*Achromatium* are often abundant in sediments characterized by very low levels of free sulfide (Gray et al. 1997) where there is no upward diffusional flux of this substrate due to precipitation of mineral sulfides in the presence of reactive iron. In these sediments *Achromatium* are, therefore, necessarily distributed throughout the zone of sulfate reduction where they either compete with reactive iron for the uptake of available free sulfide prior to its precipitation or locally mobilize acid-soluble sulfide minerals. This explanation would be consistent with the observed attachment behavior of cells

and Schmidt (1992) suggested that this accumulation may be a result of chemotaxis. However, in other reports of sediment cores exposed to light (Gray et al. 1997; Head et al. 2000b), it was observed that *Achromatium* cells moved deeper into the sediment on exposure to illumination as a result of a measured downward movement in the oxycline (Gray et al. 1997). West and Griffiths (1913) placed mud overlain by tap water in a glass dish six inches in diameter and two inches deep which was stirred from time to time; in this way *Achromatium* survived for 9 months. De Boer et al. (1971) maintained natural populations for 10 months by placing the loosely packed mud from a pond in closed plastic bottles at 5 °C. The key point about all these reports is that the sediments were exposed to the air on the assumption that oxygen was required for population maintenance. We have had similar success in maintaining populations in serum bottles over weeks to months (Gray et al. 1997, 1999a, 2004, 2007). In these experiments, however, it has been possible to augment the population size by adding purified cells, observe uptake of radiolabeled substrates, and critically investigate the effect of an alternative electron acceptor (nitrate) which when supplied repeatedly was shown to maintain community size in the absence of atmospheric oxygen.

Ecology

Distribution and Habitat Variation

Achromatium populations have been reported in many parts of Europe (e.g., Babenzien 1991; Bavendamm 1924; Bersa 1920; de Boer et al. 1971; Gray et al. 1999a; Head et al. 1996; Massart 1901; Nadson 1913; Schewiakoff 1893; Skuja 1948; Virieux 1912; Warming 1875; West and Griffiths 1909, 1913). Populations have also been reported in the USA (Bourland, http://pinkava.asu.edu/starcentral/-microscope/portal.php?pagetitle=taxonfact-sheet&type=organism&taxon=Achromatium http://protist.i.hosei.ac.jp/pdb/images/Prokaryotes/Proteobacteria/Achromatium; Lackey and Lackey 1961; Nercessian et al. 2005), South Africa (West and Griffiths 1913), China (Song et al. 2012), and Japan (Tsukii, http://protist.i.hosei.ac.jp/pdb/images/Prokaryotes/Proteobacteria/Achromatium). This geographically wide but rather irregular distribution indicates that the genus *Achromatium* has a global but incompletely described distribution. Anecdotally, our colleague J. Gwyn Jones admitted to mistaking *Achromatium* cells for testate amoeba (Head et al. 2000b), and so perhaps, in other locations, *Achromatium* have simply not yet been recognized. At a local scale, *Achromatium* can be common but highly variable in abundance. For instance, in the English Lake district, we have observed sparse populations in sediment samples from Windermere, and Priest Pot (unpublished data) but in Rydal Water *Achromatium* can be present in relatively large numbers (10^4–10^5 cells cm^{-3}) and account for approximately 90 % of the bacterial biovolume (Head et al. 1996). Habitats which harbor *Achromatium* are diverse in character ranging from freshwater to brackish sediments encompassing pH values spanning those typically encountered in natural waters (4.5–8.5) (Babenzien 1991). With respect to more specific resource requirements it has been reported, as discussed above, that habitats have variable Ca^{2+} concentrations (Nadson and Visloukh 1923). Likewise there is also variation in sulfate levels. For instance Rydal Water in the English Lake district is characterized by sulfate concentrations of 15–168 nmol cm^{-3} whereas Hell Kettles pond located approximately 60 miles away contained 9,870–11,160 nmol cm^{-3} (Head et al. 2000b). As discussed above, in the relatively few geographical locations in which *Achromatium* diversity has been assessed it has been found that individual lakes harbor genetically distinct *Achromatium* populations but that 16S rRNA sequence phylogeny per se is not related to distribution (*Fig. 1.2*). This pattern suggests that habitat characteristics favor the proliferation of some *Achromatium* spp. but not all. The role of environmental selection in governing the distribution of *Achromatium* spp. has been experimentally tested by mixing two sediments found to harbor different *Achromatium* spp. (Gray et al. 2007). In these studies it appeared that an *Achromatium* community from one location (Rydal Water) persisted in sediment microcosms only when they were constructed predominantly from the native sediment and not when mixed with sediment from Hell Kettles.

The Ecological Niche of *Achromatium*

Detailed characterization of the chemistry of a few individual locations inhabited by *Achromatium* communities has elucidated some generic ecological niche characteristics. *Achromatium* reside within sedimentary habitats comprising of zones of sulfide production and more oxidizing overlying layers. These habitats do not, however, support the mats, veils, or streamers characteristic of other conspicuous sulfur bacteria positioned between opposed gradients of sulfide and oxygen. Neither do they appear to support sulfur bacteria with nitrate-accumulating storage vacuoles which rely on active or passive shuttling between spatially separated oxidized and sulfide-rich reduced zones (Gray and Head 2005). Instead a suggested feature of all *Achromatium*-bearing sediments is a weak sulfide generation capacity (La Rivière and Schmidt 1992). The low level of sulfide production imposes constraints on the buildup and flux of sulfide within the sediment. A weak sulfide generation capacity is certainly consistent with the assumption that *Achromatium* are physically prevented by cell density from moving either actively or passively into the water column (Babenzien 1991, 1992) and the observation that *Achromatium* are distributed throughout the top few cm of sediments. Here they presumably scavenge for scarce reduced sulfur sources. However, although sulfate reduction rates in two characterized *Achromatium* habitats were found to be low (Lake Stechlin 0.45–3.4 nmol cm^{-2} d^{-1} (Sass et al. 1997) and Rydal Water 27.43 ± 2.92 nmol cm^{-2} d^{-1} (Gray et al. 1997)), those measured in Hell Kettles were orders of magnitude higher (1824 ± 73 nmol cm^{-2} d^{-1}). These contrasting data suggest an additional explanation for the consistently low measured sulfide concentrations that were measured in all these sediments, i.e., ≤ 1 μM in zones containing the majority of *Achromatium* cells (Babenzien and Sass 1996; Gray et al. 1997; Head et al. 2000a, b).

It has more recently been suggested that a key feature of *Achromatium*-bearing sediments is efficient sulfide removal across the zone of sulfate reduction, e.g., by mineral precipitation (Gray et al. 1997; Gray 2006). High dissolved Fe^{2+} concentrations necessarily limit the maximum dissolved sulfide concentrations whereby saturation conditions necessary for the precipitation of iron sulfide mineral phases are a function of the multiplied concentrations of both components of these minerals. Low measured sulfide concentrations are, therefore, entirely consistent with the measured dissolved Fe^{2+} concentrations observed in Rydal Water (2.9–85.2 μM) and to an even greater extent Hell Kettles pond (31.3–299.12 μM) (Head et al. 2000b). These Fe^{2+} concentrations are also consistent with the documented accumulation of sulfide mineral phases in these sediments (Gray et al. 1997).

On the basis of geochemical data, it can be concluded that constraints on the availability of free sulfide in sediments are drivers of both the genetic diversification and evolution of phenotypic features of *Achromatium*: specifically formation of calcium carbonate inclusions and their putative role in sulfide mobilization (see *Table 1.1* and *Fig. 1.8*), their characteristic

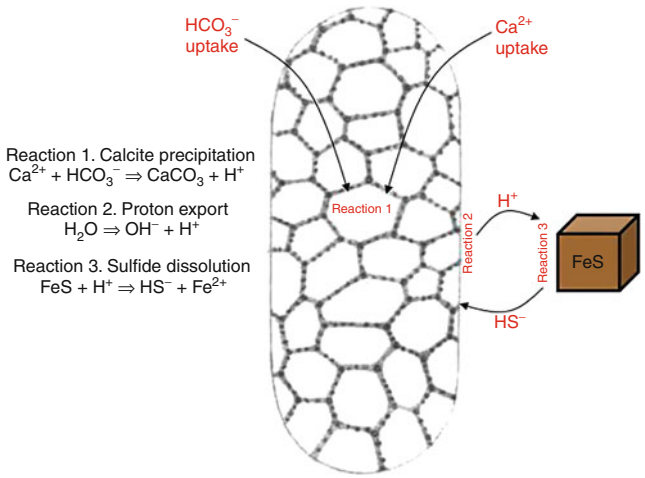

Fig. 1.8

Putative mechanism and reactions of intracellular calcification linked to sulfide mineral dissolution in *Achromatium*. Proton export from the cell membrane to the mineral surface results in the dissolution and uptake of HS^- but buildup of OH^- within the cell cytoplasm (Gray 2006). Calcite precipitation regenerates H^+ to neutralize OH^- in the cytoplasm

rolling jerky motility on mineral surfaces, migration in response to fine-scale changes in redox conditions within the sediment, their mixotrophic or chemolithoheterotrophic carbon metabolism which may provide this group of organisms with the metabolic flexibility to survive periods of scarce sulfide availability, their putatively facultative respiratory function and the coexistence of *Achromatium* spp. with different redox tolerances and hence vertical distributions, and their size differentiation which is a characteristic frequently associated with resource partitioning and niche differentiation in functionally similar but coexisting and competing species (Begon et al. 1996).

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2 The Family *Acidithiobacillaceae*

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Abstract
The *Acidithiobacillaceae* are one of the two families of the order *Acidithiobacillales*, containing a single genus, *Acidithiobacillus*, with five currently known species. All are obligately chemolithoautotrophic acidophiles, growing in the range pH 0.5–5.5; three are mesophiles, one is moderately psychrophilic, and one is a moderate thermophile. They oxidize elemental sulfur and its compounds to generate energy for carbon dioxide fixation and cell biosynthesis. Two species can also oxidize ferrous iron and sulfide minerals such as pyrite (FeS_2) for energy generation. They have relatively small genomes (ca. 3 Mb), which encode all the enzyme-proteins required for total biosynthesis from carbon dioxide and energy generation from the inorganic oxidations. The genomes exhibit high levels of gene acquisition by horizontal gene transfer. The family is currently assigned to the *Gammaproteobacteria*, but it is proposed to be a group separate from both the *Beta-* and *Gammaproteobacteria*, which justifies its recognition as a distinct Class of the *Proteobacteria*. Their mineral-degrading and sulfur-oxidation activities are commercially exploited worldwide to assist in the economic recovery of metals (e.g., copper, uranium, nickel, gold) from their low-grade ores and from mining wastes, by the process of bioleaching.

Taxonomy, Historical and Current

The *Acidithiobacillaceae* currently comprise Family I of the two families of Order II (the *Acidithiobacillales*) of the *Gammaproteobacteria*, as defined by Garrity et al. (2005a, b), using 16S rRNA gene sequences as the phylogenetic basis for classification (◉ *Fig. 2.1*). The type genus of the Order and Family is *Acidithiobacillus* Kelly and Wood 2000 (emend. Hallberg et al. 2010), with *Acidithiobacillus thiooxidans* as the type species (Waksman and Joffe 1922; Kelly and Wood 2000, 2005a). Four other species are currently recognized: *At. ferrooxidans, At. caldus, At. albertensis*, and *At. ferrivorans* (◉ *Table 2.1*; ◉ *Fig. 2.1*). The genus is of special interest because its species include some of the most extremely acidophilic *Bacteria* known, which tolerate extraordinarily high concentrations of some toxic metals. Their metabolism is of special interest, as they depend on energy generation from the oxidation of ferrous iron, sulfide minerals (such as pyrite, chalcopyrite, other metal sulfides), and inorganic sulfur compounds, to support autotrophic carbon dioxide fixation and biosynthesis. A consequence of the ability to degrade minerals is the release into solution of metals such as copper, nickel, and uranium. This is a property that has been exploited for the large-scale commercial recovery of these metals from their ores by "bioleaching," but can also result in environmental damage by the escape of acid and toxic metals into water and soil systems. These properties make them among the most interesting and important of the potentially commercially useful bacteria.

Their characteristic respiratory quinone is ubiquinone Q-8 (Hallberg and Lindström 1994; Hallberg et al. 2010; Katayama-Fujimura et al. 1982; Kelly and Wood 2005a; Robertson and Kuenen 2006). The principal non-hydroxylated fatty acids in *At. thiooxidans* are C19cyc (41–48 %), with C17cyc and C18:1 (6–18 %), while *At. ferrooxidans* has C18:1 in higher amounts (44 %), with C19cyc and C16:1 at 9–14 %; both contain 3-OH 14:0 at 9–15 % (Katayama-Fujimura et al. 1982). Both *At. thiooxidans* and *At. ferrooxidans* contain putrescine and spermidine as their principal polyamines, with spermidine being predominant: these are regarded as necessary for growth in their acidic habitats (Hamana 2000; Hamana and Matsuzaki 1990; Joshi et al. 2000).

The phylogenetic assignment of *Acidithiobacillus* species has been conjectural since the establishment of the proteobacterial classes based on their 16S rRNA signatures by Woese et al. (1984a, b, 1985). Lane et al. (1992) placed *At. thiooxidans* and *At. ferrooxidans* in the *Betaproteobacteria* but "very near the beta-gamma root." Later work reassigned these species to the *Gammaproteobacteria* and led to the creation of the genus

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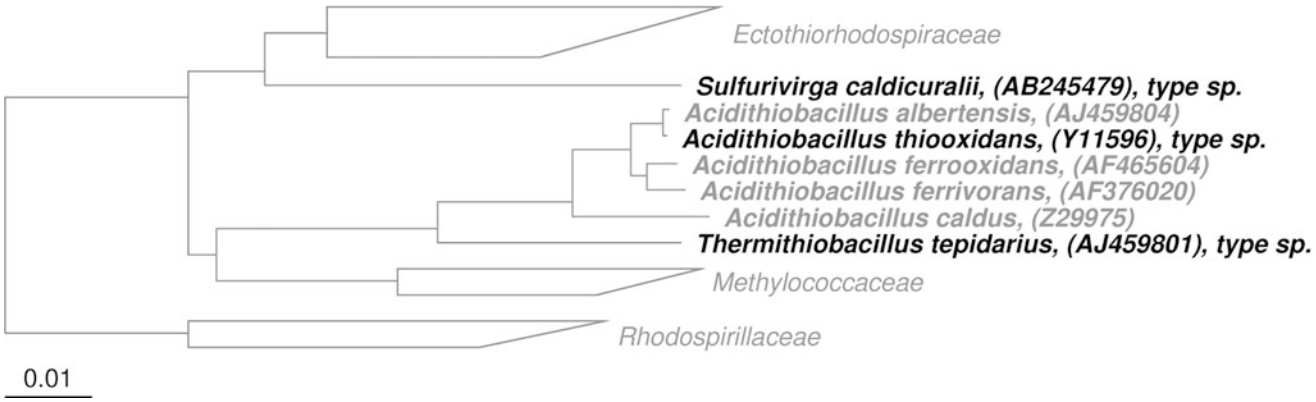

Fig. 2.1

Phylogenetic reconstruction of the family *Acidithiobacillaceae* based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes-Cantor correction. The sequence datasets and alignments were used according to the All-Species Living Tree Project (*LTP*) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high-quality-type strain sequences proportionally distributed among the different bacterial and archaeal phyla. In addition, a 40 % maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. Scale bar indicates estimated sequence divergence

Table 2.1

Type strains and isolation sources of the original isolates of the five species of *Acidithiobacillaceae*

Species	Culture collection number of type strains	Isolation source of original isolates	Primary publications for names and isolation methods
At. thiooxidans	ATCC 19377	Soil, sulfur, rock phosphate compost (USA)	Waksman and Joffe (1922)
			Lipman et al. (1921)
At. albertensis	ATCC 35403	Acid soil adjacent to a sulfur stockpile (Canada)	Bryant et al. (1983)
At. caldus (strain KU)	ATCC 51756	Coal spoil suspension (UK)	Hallberg and Lindström (1994)
			Marsh and Norris (1983)
At. ferrivorans (strain NO-37)	DSM 22755	Drainage from a copper mine spoil heap	Hallberg et al. (2010)
At. ferrooxidans	ATCC 23270	Acid drainage from a bituminous coal mine	Temple and Colmer (1951)
			Colmer et al. (1950)

Acidithiobacillus when the taxonomy of the genus *Thiobacillus* (previously containing examples of *Alpha-*, *Beta-*, and *Gamma-proteobacteria*) was rationalized (Kelly and Wood 2000). The availability of the complete genome of the type strain of *At. ferrooxidans*, as a representative of the genus, has enabled a resolution of this taxonomic uncertainty. The validity of the *Acidithiobacillales* (*Acidithiobacillus* and *Thermithiobacillus*; Kelly and Wood 2005a, b) as a gammaproteobacterial order was comprehensively reassessed using comparative multiprotein analysis of 356 protein families among 103 gammaproteobacterial genomes, representing all 14 Orders of the *Gammaproteobacteria* and five outgroup taxa from the *Alpha-*, *Beta-*, and *Zeta-proteobacteria* (Williams et al. 2010). That study used the genome of the type strain of *At. ferrooxidans* as the marker for the *Acidithiobacillales* and showed it (and *Mariprofundus*) to fall outside the group made up of the other 102 taxa confirmed as *Gammaproteobacteria*. This exclusion of *At. ferrooxidans* from the Class was also supported by a study of 124 genomes for the whole of the Domain Bacteria, confirming that *At. ferrooxidans* fell outside all of the classical proteobacterial groups (K. P. Williams, personal communication, 2012), including the *Zetaproteobacteria*, which was proposed as the sixth proteobacterial class, to accommodate the sole genus, *Mariprofundus* (http://www.bacterio.cict.fr/m/mariprofundus.html; McAllister et al. 2011). It is apparent that whole-genome comparisons among genera will lead to revision of the taxonomy of some taxa, and further new classes of *Proteobacteria* may be required to accommodate genera such as the *Acidithiobacillales*. Currently it is concurred that *Acidithiobacillus* should be regarded as a "sister" to the *Beta-* and *Gammaproteobacteria*, but not a member of either, and

arose after evolutionary divergence of the *Alphaproteobacteria*, but before the beta-gamma split (Williams et al. 2010). Consequently the Order seems sufficiently distinct to justify its reclassification as a novel Class of the *Proteobacteria* (as the *Acidithiobacillia*; Williams and Kelly 2013), as was justified for assignment of the *Mariprofundus* group to the *Zetaproteobacteria* (Emerson et al. 2007; McAllister et al. 2011).

Molecular Analyses

The phylogenetic relationships of the five species were delineated by their physiology, morphology, and 16S rRNA gene sequences (*Fig. 2.1*; *Tables 2.2* and *2.3*). Four of the five type species have been subjected to complete genome sequencing (*Table 2.4*). The genome of the fifth, *At. albertensis*, has not yet been sequenced, but is proposed to be carried out (D. S. Holmes, personal communication, 2012). All four genomes are relatively small (2.93–3.21 Mb), and differences between them include the number of plasmids present in strains of the different species and in the range of genomic GC (53–62 %) observed (*Table 2.4*). Non-type strains of *At. caldus* and *At. ferrooxidans* have also been sequenced (*Table 2.4*), each of which is very similar to that of the type strain. Unlike the type strain of *At. caldus*, strain SM-1 also contains a megaplasmid (252 kb, GC 57.2 %, comprising 255 genes and their encoded proteins) and three smaller plasmids (9.8, 14.1, and 29.7 kb, encoding 10, 13, and 28 proteins). The type strain of *At. ferrooxidans* contains no reported plasmids but other putative strains do: strain FC (from a South African mine) contains three, including pTF-FC2, which has been the subject of comparative work with plasmid pTC-F14 from *At. caldus* strain F (Dorrington and Rawlings 1989; Gardner et al. 2001; Loftie-Eaton and Rawlings 2012; Rawlings 2005; Van Zyl et al. 2003). An additional sequenced genome is that of *Acidithiobacillus* sp. strain GGI-221 (isolated from mine water in India), with a 3.17 Mb genome (GC = 58.6 %): (http://www.ncbi.nlm.nih.gov/genome/genomes/13770?details=on&). This contains three ribosomal RNA genes (one operon); its 16S rRNA gene shows 99.7 % identity to that of *At. ferrooxidans* and 97.8–97.9 % identity to *At. thiooxidans* and *At. ferrivorans*, indicating it to be a strain of *At. ferrooxidans* (authors' BLAST data).

Pseudogenes occur in the genomes of several species (*Table 2.4*), and large numbers have been reported in some strains to date (e.g., 192 in *At. ferrivorans*; *Table 2.4*; Liljeqvist et al. 2011a), but relatively little has been done to determine the relationship of these, if any, to possibly related functional genes. The available genomes have also shown that considerable diversity occurs in the number of core genes that are common to strains of the same species (which show 100 % 16S rRNA identity). Comparing pairs of strains of *At. thiooxidans*, *At. caldus*, and *At. ferrooxidans* showed that, on average, 81 % of genes were common to both strains, but this means that an average of 19 % of the core genes were not shared in each strain. The greatest difference reported

Table 2.2

16S rRNA gene sequence accession numbers, GC-content, and some morphological and physiological characteristics of the type strains of the *Acidithiobacillus* species

Character	*At. thiooxidans*	*At. albertensis*	*At. caldus*	*At. ferrivorans*	*At. ferrooxidans*
16S rRNA accession number	Y11596	AJ459804	Z29975	AF37020	AF465604
G + C of genomic DNA (mol%)	53.1[a]	61.5	61.4[b]	56.6[c]	58.8[d]
Complete genome sequence	+	–	+	+	+
Optimum temperature (°C)	28–30	28–30	45	27–32	30–35
Optimum pH	2.0–3.0	3.5–4.0	2.0–2.5	2.5	2.5
pH limits for growth	0.5–5.5	2.0–4.5	1.0–3.5	1.9–3.4	1.3–4.5
Motility	+	+[e]	+	+	–[f]
Growth substrates:					
Fe(II)	–	–	–	+	+
Sulfur (S^0)	+	+	+	+	+
Thiosulfate ($S_2O_3^{2-}$)	+	+	+	+	+
Tetrathionate ($S_4O_6^{2-}$)	+	+	+	+	+
Sulfide (HS^-)	+	nd	+	+	+
Sulfide minerals	–	–	–	+	+

[a]Valdés et al. (2011)
[b]From the complete genome sequence (http://www.ncbi.nlm.nih.gov/genome?term=acidithiobacillus%20caldus)
[c]From the complete genome sequence (http://www.ncbi.nlm.nih.gov/genome?term=acidithiobacillus%20ferrivorans)
[d]From the complete genome sequence (http://www.ncbi.nlm.nih.gov/genome?term=acidithiobacillus%20ferrooxidans)
[e]Possesses a tuft of polar flagella; other motile species have a single polar flagellum
[f]Hallberg et al. (2010), P. R. Norris, personal communication, 2012

was that 38 % of the genes in *At. thiooxidans* strain CLST were not present in the type strain (ATCC 19377), compared to 15 % of the type strain genes being absent from strain CLST (Cárdenas et al. 2012). The proportions of genes not shared between several strains of *At. caldus* and *At. ferrooxidans* were 9–19 %. One deduction from this is that the genetic diversity could indicate that considerable genome modification had resulted from horizontal gene transfer (HGT; D. S. Holmes, personal communication 2012).

The presence of genomic islands shown in several species is also indicative of the contribution of HGT to genomic diversity. To date, genomic islands have been identified in several strains of *At. ferrooxidans*. For example, several ORFs in the genome of strain ATCC 53993, but absent from that of the type strain, were located on a genomic island and encoded resistances to several metals (Orell et al. 2010); a cluster of 13 genes encoding Fe-Mo transporters was located in a genomic island of 69 genes in the type strain (ATCC 23270; Osorio et al. 2008). A 300 kb genomic island in the type strain genome, but absent from ATCC 53993, was an "actively excising integrative and conjugative element" which assisted in immunity to bacteriophage (Holmes et al. 2009; Cárdenas et al. 2012), while a 160 kb genomic island in ATCC 53993, but absent from the type strain, was related to copper resistance (Orellana and Jerez 2011; Cárdenas et al. 2012). The one genomic island of 300 kb in ATCC 23270 represents about 10 % of the total genomic DNA, so if several genomic islands are actually present in any strain, they could account for 10–20 % of the total DNA, presumably acquired by HGT, and indicate considerable genome plasticity.

It is noteworthy that while all the *Acidithiobacillus* genomes are relatively small (at about 3 Mb), they contain all the genes necessary to encode all the enzymes needed for the organisms to effect complete biosynthesis from carbon dioxide as well as those necessary for iron and sulfur compound oxidation and energy coupling. Complete gene complements to encode all the major metabolic pathways are present, with the notable exception of genes encoding 2-oxoglutarate dehydrogenase, thereby limiting the reactions of the Krebs' tricarboxylic acid cycle to a biosynthetic rather than energy-generating role,

Table 2.3

Phylogenetic relationships of the type species of *Acidithiobacillus*. Comparative 16S rRNA gene sequence identities (%) are shown

Species	1	2	3	4	5
1. *At. thiooxidans*	100	99.9	98.0	97.4	95.2
2. *At. albertensis*	99.9	100	98.1	97.5	95.3
3. *At. caldus*	98.0	98.1	100	98.1	95.6
4. *At. ferrivorans*	97.4	97.5	98.1	100	95.5
5. *At. ferrooxidans*	95.2	95.3	95.6	95.5	100

Table 2.4

Properties of the genomes of four species of *Acidithiobacillus* (Data from genome annotation reports on the NCBI genome database)

Species/strain	Characteristics						
	Reference sequence	Size (Mb)	GC content (mol %)	Ribosomal RNA genes[a]	Transfer RNA genes	Genes	Predicted proteins
At. thiooxidans	NZ_AFOH	3.02	53.1	5	45	3,103	3,053
ATCC 19377 (type species)	00000000.1						
At. caldus	NZ_ACVD	2.95	61.4	6	47	2,878	2,821
ATCC 51756 (type strain)	00000000.1						
At. caldus (strain SM-1)	NC_015850.1	2.93[b]	61.3	6	47	2,934	2,881
At. ferrivorans strain SS3	NC_015942.1	3.21	56.6	6	47	3,335[c]	3,093
DSM 22755 (type strain)							
At. ferrooxidans	NC_011761.1	2.98	58.8	6	81	3,303[d]	3,147
ATCC 23270 (type strain)							
At. ferrooxidans	NC_011206.1	2.89	58.9	6	46	2,959[e]	2,826
ATCC 53993							

[a]Each genome contains two 5S, 16S, 23S rRNA operons

[b]Also contains a megaplasmid (250 kb) and three smaller plasmids (10–30 kb) (http://www.ncbi.nlm.nih.gov/genome/genomes/1927?subset=plasmids&details=on&project_id=70791)

[c]Also contains 192 pseudogenes (http://metacyc.org/AFER743299/organism-summary?object=AFER743299)

[d]Also contains 64 pseudogenes (http://www.ncbi.nlm.nih.gov/genome/genomes/1014?details=on&project_id=57649)

[e]Also contains 77 pseudogenes (http://www.ncbi.nlm.nih.gov/genome/genomes/1014?details=on&project_id=58613)

a characteristic of many obligately chemolithoautotrophic bacteria (Valdés et al. 2008, 2009, 2011; Wood et al. 2004; You et al. 2011). Their genome size is comparable to those of some other obligate chemolithotrophs with similar metabolic abilities (e.g., *Thiomicrospira crunogena*, 2.43 Mb, and *Halothiobacillus neapolitanus*, 2.58 Mb), but much smaller than some facultative chemolithotrophs, including *Starkeya novella* (4.77), *Paracoccus denitrificans* (5.24 Mb), *Xanthobacter autotrophicus* (5.63 Mb), and *Cupriavidus necator* (formerly *Ralstonia eutropha* (7.42 Mb)), or of many heterotrophs, including *Klebsiella oxytoca* (5.97 Mb), *Streptomyces albus* (6.62 Mb), and *Pseudomonas fluorescens* (6.85 Mb). The *Acidithiobacillus* genomes thus represent examples of evolutionary genome size reduction with retention of a vast range of functional genes.

Phenotypic Analyses

The type species of the genus, *At. thiooxidans*, is an extreme acidophile, tolerating pH values approaching zero, with an optimum of pH 2–3. It is dependent on the oxidation of sulfur and its inorganic compounds for energy to drive carbon dioxide fixation by the Calvin-Benson-Bassham reductive pentose phosphate cycle. It is obligately chemolithoautotrophic, but like other sulfur chemolithoautotrophs is capable of assimilating some organic nutrients such as acetate and amino acids, but only at the expense of energy from sulfur compound oxidation. The other four validated species are also capable of sulfur-oxidation-dependent chemolithoautotrophic growth, but show differing pH and temperature requirements. All the species are small rods, in the size range 0.45 × 1.8 μm; on suitable media (e.g., with tetrathionate as growth substrate) they will form small, circular colonies 1–2 mm in diameter. All the species examined contain polyhedral bodies called carboxysomes, which consist mainly of the primary carbon dioxide-fixing enzyme, ribulose bisphosphate carboxylase/oxygenase.

At. caldus, At. ferrivorans, and *At. ferrooxidans* show optimal growth at around pH 2.5 and can grow at pH 1.0–1.9 (*Table 2.2*). The lower pH limit and optimum pH for *At. caldus* are higher, but unlike the other four species, which grow best at 25 or 30 °C, its optimum temperature for growth is 45 °C and it tolerates 52 °C (*Table 2.2*; Hallberg and Lindström 1994). *At. ferrivorans* is psychrotolerant and will grow at 5 °C, but its upper limit is about 30 °C. Two of the species, *At. ferrivorans* and *At. ferrooxidans*, are also capable of oxidizing ferrous iron (FeII) and sulfide minerals as their source of energy. The iron-oxidizing species can also oxidize sulfur anaerobically, using the reduction of ferric iron (FeIII → FeII) as the electron acceptor (Hallberg et al. 2010; Valdés et al. 2008). At least some species can also oxidize hydrogen aerobically.

All the type strains of the genus are motile except *At. ferrooxidans*. This species was originally described as motile, a putative property that was perpetuated through many reference publications (Kelly and Wood 2005a; Robertson and Kuenen 2006), but is in error. The motility observed in cultures of *At. ferrooxidans* is now believed to have been due to other iron-oxidizing or sulfur acidophiles (or heterotrophic commensals) that were motile, present in the supposedly pure cultures. The type strain has been definitively shown to be nonmotile (Hallberg et al. 2010; Valdés et al. 2008; P. R. Norris, personal communication, 2012) and that its genome lacks genes encoding flagella formation (Valdés et al. 2008). DiSpirito et al. (1982) examined numerous strains, all described as *Thiobacillus ferrooxidans*, but which exhibited single polar flagella or multiple peritrichous flagellation and eight strains with no flagella: only the last group is likely to have been *At. ferrooxidans*, consistent with there being multiple species of phylogenetic and genetic variants of iron-oxidizing bacteria (Amouric et al. 2011).

At. albertensis is motile, but unlike the other species which have a single polar flagellum, it has a tuft of unusually long polar flagella (Bryant et al. 1983). A seemingly identical strain (BY-05) was isolated from drainage from a copper mine (Gansu Province, China), with the tuft of polar flagella, and showing 99.9 % identity (1,438/1,439 aligned nucleotides) to the *At. albertensis* 16S rRNA gene sequence, as well as the same pH and temperature optima as the type strain (Xia et al. 2007). The variant flagellation could reflect acquisition of novel flagella genes by horizontal gene transfer (D. S. Holmes, personal communication 2012). Although the 16S rRNA genes of *At. albertensis* and *At. thiooxidans* show 99.9 % identity, they do show differences in GC-content of their DNA, pH tolerance, and flagellation, confirming their distinction as separate species (*Tables 2.2* and *2.3*).

Diversity of Strains Initially Identified as *Acidithiobacillus ferrooxidans*

Numerous environmental isolates, principally from mines and leaching operations, have been described as *At. ferrooxidans*, simply on the basis of being small Gram-negative rods that oxidized ferrous iron and sulfide minerals. This led to numerous incorrect attributions of isolates as *At. ferrooxidans*. This was recognized by Harrison (1982), who showed that 23 such strains, with GC values ranging from 55 % to 65 %, fell into seven DNA homology groups with only 0–52 % identity with each other (Kelly and Harrison 1989). These strains exhibited diverse physiological properties, including the supposed ability to grow heterotrophically. The latter observation led to the isolation of an acid-tolerant heterotroph, *Acidiphilium*, as a commensal in *At. ferrooxidans* cultures and in the mine drainage waters in which *At. ferrooxidans* occurred (Harrison 1981; Harrison et al. 1980). One of the homology groups was subsequently identified as a different genus, *Leptospirillum ferrooxidans* (Kelly and Harrison 1989), and one was the anomalous m-1 strain, discussed below. Use of DNA restriction profiles, DNA hybridization, and 16S rRNA gene sequencing showed varying degrees of taxonomic diversity among 25 physiologically similar isolates of *At. ferrooxidans* from worldwide locations (including Peru, the USA, Canada, India, Kazakhstan, Kyrgyzstan, Siberia). Most were "genomovars" of *At. ferrooxidans*, but six strains were highly divergent (Karavaiko et al. 2003). One molecular study illustrated just how genetically diverse strains isolated as

At. ferrooxidans and *At. thiooxidans* can be. Paulino et al. (2001) examined a total of 19 strains of putative *Acidithiobacillus* species, using ribotyping, *rep*-PCR, 16S rRNA sequencing, and DNA hybridization, and showed some of these to be of very low relatedness (e.g., numerous ribotypes, only 40 % DNA relatedness, low 16S rRNA similarities), indicative of possible novel species within *Acidithiobacillus*, or in one case of a strain of *At. caldus* that was incorrectly identified as *At. thiooxidans*. Current molecular methods have revealed novel species that might previously have been regarded as *At. ferrooxidans*: an example is *At. ferrivorans* (Hallberg et al. 2010). These methods have also confirmed that some supposed strains of *At. ferrooxidans* were in fact different, novel, species; such an anomalous strain was *At. ferrooxidans* strain m-1, which was included by Lane et al. (1992) as an example of *At. ferrooxidans* on a phylogenetic tree. Unlike the type strain of *At. ferrooxidans*, which they classified as a betaproteobacterium, strain m-1 was in the *Gammaproteobacteria*, but was also very close to the beta/gamma branch point. Subsequently, Hallberg et al. (2011) showed strain m-1 actually to belong to the family *Ectothiorhodospiraceae* and renamed it *Acidiferrobacter thiooxydans*. The corollary to the "lumping" of genera or species as examples of *At. ferrooxidans* was the creation of a novel genus and species, distinct from *At. ferrooxidans*, on the basis of apparent differences in their iron- and sulfur-oxidation abilities. These were *Ferrobacillus ferrooxidans* and *Ferrobacillus sulfooxidans*, which were reclassified by Kelly and Tuovinen (1972) as synonym strains of *At. ferrooxidans*. The type strain of *At. ferrooxidans* is the organism isolated and described by W. W. Leathen as *Ferrobacillus ferrooxidans* (Leathen et al. 1956; NCIB 8456^T; DSM 14882^T, ATCC 23270^T), as the original isolate of Temple and Colmer (1951) was not available from any culture collection (Kelly and Wood 2000).

Isolation, Enrichment, and Maintenance Procedures

Acidithiobacillus species can be enriched from environmental samples by culture in acidified liquid media (pH 1.5–4.5) containing sulfur or tetrathionate as growth substrate or ferrous sulfate for *At. ferrooxidans* or *At. ferrivorans*. Pure cultures can often be obtained by serial subculture in liquid culture, followed by single colony isolation on agar-, agarose-, or silicate-gelled media. Some strains (e.g., *At. ferrooxidans*) have proved difficult to culture and maintain on solid media and have been purified by most probable number dilution to extinction. This was discussed by Tuovinen and Kelly (1973). The most commonly used media are described below, with procedures for preparation, use, and preservation of cultures.

Waksman and Joffe (1922) medium for their original isolation of *At. thiooxidans*	
$(NH_4)_2SO_4$	2.00 g
K_2HPO_4	1.00 g
$MgSO_4$	0.50 g
KCl	0.50 g
$FeSO_4$	0.01 g
Sulfur	10.0 g
$Ca_3(PO_4)_2$	2.5 or 10.0 g
Distilled water	1 L

This was dispensed into 250 mL Erlenmeyer flasks in 100 mL amounts and sterilized in flowing steam on three successive days. Flasks of medium were inoculated with pure cultures or enrichment samples and incubated at 25 °C. Enrichment cultures (from acid-sulfur soil inocula) became turbid in 5 days (Waksman and Joffe 1922). The calcium phosphate acted to buffer the acid production from sulfur oxidation and released soluble phosphate; development was more rapid with only 2.5 g L^{-1}. The authors did not specify whether anhydrous or hydrated form of the magnesium and iron salts were used.

Bounds and Colmer (1972) medium for thiosulfate oxidizers	
The Bounds and Colmer salts solution contains:	
KH_2PO_4	3.00 g
$(NH_4)_2SO_4$	3.00 g
$MgSO_4{\cdot}7H_2O$	0.50 g
$CaCl_2{\cdot}2H_2O$	0.25 g
Distilled water	1 L
This can be supplemented (Tuovinen and Kelly 1974) with one of the following:	
$Na_2S_2O_3{\cdot}5H_20$	5.0 g
$K_2S_4O_6$	3.0 g
$K_2S_3O_6$	3.0 g
Elemental sulfur	5.0 g

Media with thiosulfate or tetrathionate can be sterilized at 121 °C for 15 min; sulfur medium at 110 °C for 5 min; the trithionate medium should be sterilized by membrane filtration. Solid medium can be made with suitable amounts of high-grade agar.

In a modification of this medium, Kelly and Wood (1998) prepared two stock solutions, which can be stored without sterilization:

KH_2PO_4	30 g
$(NH_4)_2SO_4$	30 g
$MgSO_4{\cdot}7H_2O$	5 g
Distilled water	1 L
$CaCl_2{\cdot}2H_2O$	2.5 g
Distilled water	1 L

A basal medium is prepared by mixing 100 mL of each solution with 800 mL distilled water and adding thiosulfate or tetrathionate as previously. Autoclave at 115 °C for 10 min, dispense 100 mL amounts into flasks, and add 0.5 mL of 0.1 % (w/v) $FeSO_4.7H_2O$ in 0.1 N HCl.

Tetrathionate Medium, pH 3.0

This medium was used for *Sulfolobus* and *Acidianus*, but is also satisfactory for *Acidithiobacillus* (Wood et al. 1987):

Basal salts solution:

$(NH_4)_2SO_4$	0.4 g
$MgSO_4{\cdot}7H_2O$	0.4 g
KCl	0.2 g
K_2HPO_4	0.2 g
$FeSO_4{\cdot}7H_2O$	0.01 g

Dissolve in 900 mL water adjusted to pH 3.0 with dilute sulfuric acid and autoclaved at 121 °C for 15 min.

After cooling, add 3 g $K_2S_4O_6$ dissolved in 100 mL water adjusted to pH 3.0 and presterilized by filtration through 0.2 µm pore-size filters (e.g., Sartorius "Minisart" units).

Media adjusted to pH 4.0 or to values below pH 3.0 can also be used.

Media for Growth on Ferrous Iron or Pyrite

The most widely used medium for the culture and maintenance of *At. ferrooxidans* has been the 9 K medium of Silvermann and Lundgren (1959), which is prepared in two parts:

A	
$(NH_4)_2SO_4$	3.0 g
K_2HPO_4	0.5 g
KCl	0.1 g
$MgSO_4{\cdot}7H_2O$	0.5 g
$Ca(NO_3)_2$	0.01 g
Distilled water	700 mL
5 M H_2SO_4	1 mL

B	
$FeSO_4{\cdot}7H_2O$	3 g
0.5 M H_2SO_4	10 mL
Distilled water	290 mL

Sterilize A by autoclaving and B by filtration and mix when cool. Tuovinen and Kelly (1973) described a modified medium, used at pH 1.3 (to which the type strain of *At. ferrooxidans* was adapted to grow), a pH value at which no ferric iron precipitated during growth, when pH rose to pH 1.7:

$(NH_4)_2SO_4$	0.4 g
K_2HPO_4	0.4 g
$MgSO_4{\cdot}7H_2O$	0.4 g
$FeSO_4{\cdot}7H_2O$	33.3 g
0.055 M H_2SO_4	1 L

In addition, media recipes can be found on the DSMZ website (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_MediumX.pdf – replacing X with the medium number shown below): a sulfur medium (pH 4.2) for *At. thiooxidans* (medium number 35); ferrous sulfate media (pH 1.4 or pH 2.0) for *At. ferrooxidans* (numbers 70 and 271); and media with thiosulfate (pH 4.4–4.7; number 71) or tetrathionate (number 72); a sulfur medium (pH 2.5) for *At. caldus* (number 150a); and a tetrathionate medium (pH 2.5) for *At. ferrivorans* (number 1234).

For culture of iron oxidizers, ferrous sulfate or sulfur compounds can be replaced with pulverized pyrite. Cultures on pyrite or ferrous iron or sulfur appear to remain viable at low temperature for very long periods. Live cultures on sulfur compounds require subculture at intervals of a few weeks or can be frozen by standard methods.

Ecology

Acidithiobacillus species occur in any acidic habitat in which sulfur or its reduced compounds are present or where oxidizable iron (as its sulfide minerals, e.g., pyrite, chalcopyrite, marcasite, pyrrhotite, greigite) is exposed. Such habitats can include acid soils and waters, sulfur springs, and corroding concrete, but the best known habitats are the drainage from coal mines, pyritic coal spoil dumps, mining wastes, and the leach dumps used for metal recovery. All the known species occur in such dump environments, with abundant numbers of *At. thiooxidans* and *At. ferrooxidans*. Newly exposed pyrite, such as that revealed in coal mining and any excavation of pyritic rock, can rapidly become a target for colonization by acidithiobacilli. A specific example is where the water beginning to fill a newly created reservoir, cut out of pyrite-containing bedrock, rapidly became acidic (pH 4 or below), with evidence of precipitated ferric iron. *At. ferrooxidans* was readily isolated from the water and was the obvious cause, but this problem self-cured as the newly exposed surfaces became weathered and pyrite oxidation ceased (D. P. Kelly, unpublished observations).

Commercial Application: Bioleaching of Metals from Their Minerals, Catalyzed by Iron- and Sulfur-Oxidizing Bacteria

"Bioleaching" is a microbially assisted process for the recovery of commercially valuable metals from mining wastes, still containing some unrecovered metal, and low-grade ores in which the metal content is too low for economic recovery by conventional extractive means. It has a very long history, for which the development of the process and the understanding of the role of the acidithiobacilli and other microorganisms is demonstrated by a vast number of research and review papers (e.g., Brierley 1982, 2009; Brierley et al. 1985; Cárdenas et al. 2012; Holmes et al. 2009; Johnson and

Hallberg 2003; Kelly 1976, 1985; Kelly et al. 1979; Rawlings and Kusano 1994; Rawlings and Johnson 2007a, b; Rohwerder et al. 2003; Sand et al. 2001; Tuovinen and Kelly 1972; Valdés et al. 2010). Mine workings and dumps of mining wastes produce acidic runoff water containing dissolved metals, such as copper, which can be recovered by various means. This phenomenon was probably exploited in antiquity, possibly as early as 1000 BCE (Brierley 1982), but reliable records of metal recovery from such liquors exist only from the sixteenth and seventeenth centuries (Kelly 1976). Large-scale development of the leaching of valuable metals began in Spain in the nineteenth century with the "dump leaching" of copper from its pyritic ores and was developed into a major recovery process for copper from mining waste and its low-grade ores in the USA and Chile in the twentieth century, particularly since 1980. The basic process of dump or heap leaching is simply the surface irrigation with acidified water of dumps of mining waste or heaps of crushed, pulverized low-grade ores. Metal is recovered from the drainage liquors, and the acid leach liquors are recycled back to the top of the dumps. This process is currently employed worldwide, with major heap-leaching operations in North and South America, Africa, Europe, and Russia. Among the largest dumps in use are those in Chile and the southern USA, with the largest probably being the Escondida mine (Chile), with a heap 5 × 2 km and 126 m in height, with a volume of about a billion (10^9) cubic meters (Valdés et al. 2008), containing 2–3 × 10^9 t of material. Worldwide, copper production from leaching processes amounts to 10–15 % of total copper mined (Kelly 1976, 1985; Tuovinen and Kelly 1972; Valdés et al. 2008), and the total value of metal recovery by heap leaching (and tank reactors for precious metal recovery) is in excess of US10^{10} (Paulino et al. 2001). Currently, copper and uranium are the principal metals recovered from low-grade ores by bioleaching, but it is also applied to the recovery of nickel (Qin et al. 2009) and the treatment of refractory ores containing silver and gold associated with pyrite and arsenopyrite (Logan et al. 2007; Olson et al. 2003; http://www.mintek.co.za/technical-divisions/biotechnology-bio/services-facilities/agitated-tank-bioleaching/).

It was only from the 1940s that the activities of bacteria were seen to be catalytic in the leaching of metals from their ores and for the production of acidity of bituminous coal mine drainage (Leathen et al. 1956), although Powell and Parr (1919) had suggested bacterial oxidation of pyrite in coal as a source of acid production. This led to the discovery of *At. ferrooxidans* (Colmer and Hinkle 1947; Colmer et al. 1950; Leathen et al. 1956; Temple and Colmer 1951), which was for many years regarded as the principal agent of degradation of pyrite (FeS_2, iron sulfide with a cubic crystal structure), and other sulfide minerals such as marcasite (orthorhombic FeS_2), chalcopyrite ($CuFeS_2$), and sphalerite ([Zn,Fe]S). The combined activities of *At. ferrooxidans* and *At. thiooxidans* can result in the leaching of copper from its ores and the degradation of pyrite: elemental sulfur arising from pyrite or sphalerite oxidation by *At. ferrooxidans* can form a coating on the mineral surface, which is removed by *At. thiooxidans*, thus accelerating mineral breakdown by preventing "passivation" of the mineral surface (Xia et al. 2008). Subsequent studies progressively showed that other iron- and sulfur-oxidizing acidophiles also occurred in bioleaching systems, including *Leptospirillum, Acidiphilium, Acidimicrobium, Ferromicrobium, Sulfobacillus*, and *Archaea*, such as *Sulfolobus, Acidianus*, and *Ferroplasma*, as well as acidophilic heterotrophs (Rawlings and Johnson 2007a, b; Wichlacz and Unz 1981), some of which are thermophiles and extreme thermophiles (Brierley and Lockwood 1977; Marsh and Norris 1983; Rawlings and Johnson 2007a). It is now recognized that the bioleaching process for metal sulfides is effected by an extremely diverse range of bacteria and *Archaea*, which are separated both spatially and temporally in their activities, in what has been described as a "symphony of microbial interactions" (Cárdenas et al. 2012), related to chemical and temperature gradients within heap leach systems, and involving consortial activity among different bacteria (Rohwerder et al. 2003; Valdés et al. 2010).

The process of bioleaching, catalyzed by *At. ferrooxidans* and other organisms, is twofold, described as a "contact mechanism," whereby the bacteria attach to the sulfide mineral surface and attack its structure directly, and "noncontact," in which mineral breakdown is brought about by ferric iron (Fe[III]) and sulfuric acid, produced by bacterial oxidation of Fe[II] and sulfur/sulfide (Rohwerder et al. 2003; Sand et al. 2001). There is observational evidence of direct attack by attached bacteria, which may be significant where "simple" sulfides such as ZnS and Cu_2S are oxidized, but in leach systems the principal reactions are probably the recycling of FeII/FeIII with chemical attack on the sulfide mineral structures. The bacterially catalyzed reactions are:

$$4FeSO_4 + 2H_2SO_4 + O_2 \rightarrow 2Fe_2(SO_4)_3 + 2H_2O$$

$$S_8 + 8H_2O + 12O_2 \rightarrow 8H_2SO_4$$

The oxidation of ferrous iron by *At. ferrooxidans* at pH 1.3–2.5 proceeds up to 10^6 times faster than solely by abiotic oxidation (Tuovinen and Kelly 1972). Reaction of ferric ions with minerals is purely chemical:

$$FeS_2 + Fe_2(SO_4)_3 \rightarrow 3FeSO_4 + 2[S]$$

$$CuFeS_2 + 2Fe_2(SO_4)_3 \rightarrow CuSO_4 + 5FeSO_4 + [S]$$

$$CuFeS_2 + 2Fe_2(SO_4)_3 + 2H_2O + 3O_2 \rightarrow CuSO_4 + 5FeSO_4 + 2H_2SO_4$$

$$Cu_2S + 2Fe_2(SO_4)_3 \rightarrow 2CuSO_4{}^+ \rightarrow 4FeSO_4 + [S]$$

Some direct mineral sulfide oxidation reactions have been suggested to be bacterial (see Tuovinen and Kelly 1972, for the earlier literature):

$$2FeS_2 + 2H_2O + 7O_2 \rightarrow 2FeSO_4 + 2H_2SO_4$$

$$2Cu_2S + 2H_2SO_4 + O_2 \rightarrow CuS + CuSO_4 + H_2O$$

$$CuFeS_2 + 2O_2 \rightarrow CuSO_4 + FeS$$

A separate important application of *At. ferrooxidans* and other acidophilic iron and sulfur oxidizers is the recovery of

uranium from its low-grade ores (Tuovinen and Bhatti 1999). This depends on the oxidation of the tetravalent uranium in UO_2 ores, such as uraninite, to soluble hexavalent uranium (U[IV] → U[VI]) by the action of ferric iron produced by oxidation of the pyrite associated with uraninite/pitchblende deposits, to produce leachate solutions containing uranyl sulfate:

$$UO_2 + Fe_2(SO_4)_3 \rightarrow UO_2SO_4 + 2FeSO_4$$

Bacterial reoxidation of Fe(II) to Fe(III) recycles the oxidant for uranium dissolution. Heap leaching of uranium ores is, or has been, practiced in many countries (including South Africa, the USA, Canada, Australia, Brazil, China, Ukraine, Mongolia, Zambia, and Namibia). The largest heap-leaching operation for uranium is that in Namibia, with a 4 km^2 site (http://www.miningweekly.com/article/africas-biggest-heap-leach-pad-project-shows-positive-volumes-2011-06-24). Uranium is also recovered by underground leaching of the walls of mines and of ores that have been fractured and pulverized in situ. Uranium is recovered from solution by passage over ion exchange resins. A problem can arise from the "poisoning" by polythionates in the leachates, which bind strongly to the resins. This can be counteracted by addition of sulfites or sulfur dioxide to the leachate or eluant (Yan 1983). This amelioration of the binding of polythionates can be attributed to the known degradation of polythionates by sulfite, predominantly to thiosulfate, depending on the polythionate sulfur-chain length (Kelly and Wood 1994).

Environmental Degradation and Contamination by Activities of *Acidithiobacillus*

A major problem arising from the natural and engineered activities of acidithiobacilli is the pollution of water (rivers, groundwater, potable water supplies) and soil with acid and toxic metals (including copper, nickel, arsenic, uranium, and radium) from the runoff from coal spoil heaps, mining wastes, old mine workings, and potentially from heap-leaching operations (Kelly 2010). These can be controlled or ameliorated by containment of runoff in storage dams for precipitation of metals and neutralization of sulfuric acid and by construction of bioleaching heaps with effective containment and basal liners to prevent loss of leachate to the surrounding environment. The latter is a well-developed technology that decreases loss of valuable metal and eluate, as well as decreasing pollution risk. Liljeqvist et al. (2011b) have demonstrated that process waters and effluents containing thiosulfate and tetrathionate from mining operations could be degraded in low-temperature bioreactors (6 °C), with psychrotolerant *At. ferrivorans* as the active organism, thereby reducing the environmental impact of the effluents. How to control the hazards of toxic mine tailings is a major environmental concern in the mining industry, but "a foolproof and safe way to get rid of these inconvenient tailings has not yet been found: dams can break or leak, water from pond storages can leak" (Fischer 2011). Worldwide, mine waste is a potentially serious problem, given the scale of abandoned and disused mines from which pollution may arise: estimates of the number of such abandoned mine sites in the USA alone are between 31,000 and 560,000 (Fischer 2011).

A "natural" problem is posed by the "microbially induced corrosion (MIC)" of concrete (Parker 1945). This involves several thiobacilli including *At. thiooxidans* and probably *At. ferrooxidans* (Parker 1947; Yamanaka et al. 2002). This was first reported as a microbial process due to the absorption of hydrogen sulfide from anoxic sewage into concrete sewer pipes, with its subsequent oxidation to sulfuric acid, leading to pipe collapse (Parker 1945, 1947; Kelly 2010). This problem can be progressively ameliorated by coating of pipe linings and replacing concrete pipes with corrosion-resistant plastic.

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3 The Family *Aeromonadaceae*

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Abstract
Aeromonadaceae, a family within the *Aeromonadales*, comprises the genera *Aeromonas*, *Tolumonas*, *Oceanimonas*, *Oceanisphaera*, and *Zobellella*. The family is distantly related to the families *Enterobacteriaceae*, *Pasteurellaceae*, *Succinivibrionaceae*, and *Vibrionaceae*. Members of the *Aeromonadaceae* share a number of major fatty acids and polar lipids that phenotypically define the family. They are strict aerobes or facultative anaerobes typically associated with aquatic environments. Strains of *Aeromonas* may also be recovered from foods, humans, and animals that have come into contact with water. Members of this genus may be opportunistic pathogens to humans and animals, in which they can cause a range of extraintestinal infections or diarrheal diseases. Information on the metabolism and ecology of *Tolumonas*, *Oceanimonas*, *Oceanisphaera*, and *Zobellella* is relatively scarce since most species of these genera were described on the basis of single strains.

Taxonomy, Historical and Current

Short Description of the Family

Aeromonadaceae (Ae. ro. mo. na. da'ce. ae. N.L. fem. n. *Aeromonas*, type genus of the family; suff.-*aceae*, ending to denote a family; N.L. fem. pl. n. *Aeromonadaceae*, the *Aeromonas* family). The description is an emended version of the description given in the second edition of *Bergey's Manual of Systematic Bacteriology* (Martin-Carnahan and Joseph 2005).

The *Aeromonadaceae* family is phylogenetically a member of the order *Aeromonadales* (Martin-Carnahan and Joseph 2005) within the phylum Gammaproteobacteria. According to the 16S rRNA gene tree of the All-Species Living Tree Project (Yarza et al. 2008, 2010) and J.P. Euzéby's List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.cict.fr/), the family contains the type genus *Aeromonas* (Stanier 1943), *Tolumonas* (Fischer-Romero et al. 1996), *Oceanimonas* (Brown et al. 2001), *Oceanisphaera* (Romanenko et al. 2003), and *Zobellella* (Lin and Shieh 2006). Cells are Gram-negative straight rods except for *Oceanisphaera* members which are coccoid or occur as short rods or rods. The family includes both motile and nonmotile members. Strict aerobic to facultatively anaerobic, producing acid from a variety of carbohydrates. Except for *Tolumonas* spp., all members of the *Aeromonadaceae* are oxidase positive and, if reported, catalase positive. Sodium chloride may or may not be required for growth. Members are halotolerant to moderately halophilic. Phosphatidylglycerol, diphosphatidylglycerol, and phosphatidylethanolamine are the major polar lipids. The major fatty acids include $C_{16:0}$, $C_{16:1\omega7c}$, and $C_{18:1\omega7c}$. If analyzed, ubiquinone Q-8 is the main respiratory quinone. The mol% G+C range of the DNA is 49–64 %.

Aeromonadaceae are typically found in aquatic environments. Depending on the species, these include freshwater, estuarine and coastal (brackish) water, seawater, ocean water, surface water, drinking water supplies and bottled water, polluted waters, wastewater effluent sludge, estuary mud and marine, and freshwater sediment and sand; aeromonads are also occasionally isolated from food, animals, and various clinical samples.

E. Rosenberg et al. (eds.), *The Prokaryotes – Gammaproteobacteria*, DOI 10.1007/978-3-642-38922-1_282,

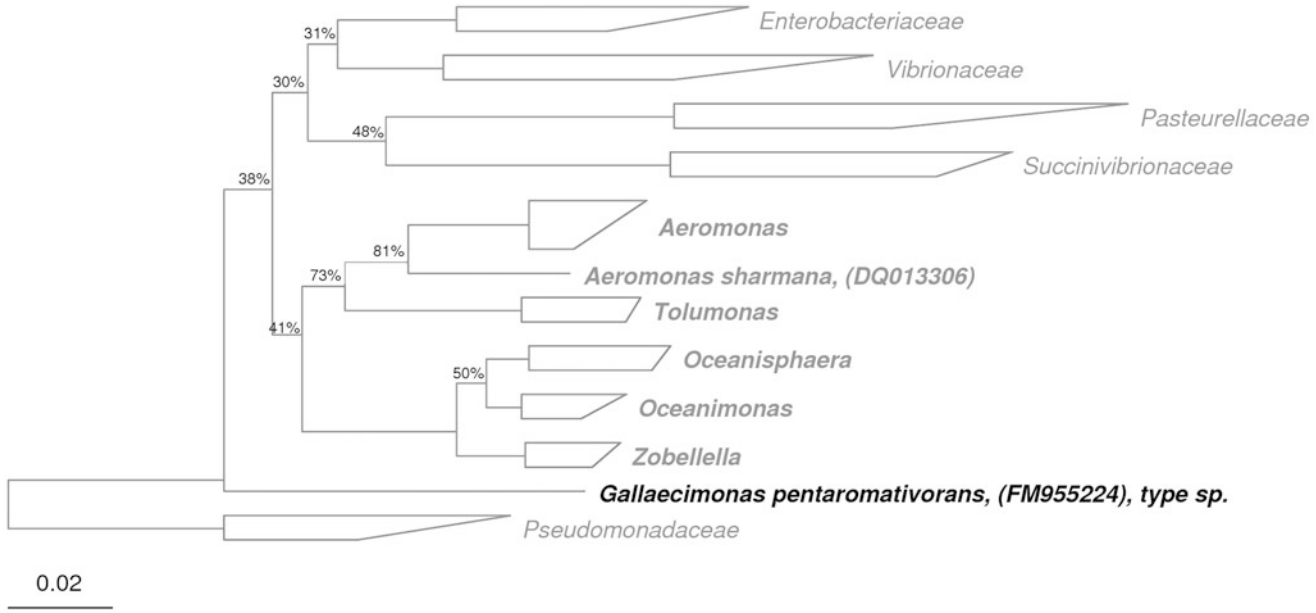

Fig. 3.1

Phylogenetic reconstruction of the family *Aeromonadaceae* and related families based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes–Cantor correction. 100 run bootstrapping was applied to retrieve a "most likely" topology since single runs returned variable topologies. Nodes with no value stand for 100 %. The sequence datasets and alignments were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). Scale bar indicates estimated sequence divergence

Phylogenetic Structure of the Family and Its Genera

According to the phylogenetic branching of the Gammaproteobacterial type strains in the 16S rRNA gene tree of the Living Tree Project (Yarza et al. 2010), the family is moderately related to the families *Enterobacteriaceae*, *Pasteurellaceae*, *Succinivibrionaceae*, and *Vibrionaceae* and to the species *Gallaecimonas pentaromativorans* (Rodriguez-Blanco et al. 2010) which is currently unassigned to a known family. A phylogenetically broad group representing the *Pseudomonadaceae* appears as a sister clade of these five families. The *Aeromonadaceae* family contains two major phylogenetic branches. The largest branch contains members of *Aeromonas* and *Tolumonas*, whereas a second one is more tightly organized around the genera *Oceanimonas*, *Oceanisphaera*, and *Zobellella* (*Fig. 3.1*). A phylogenetic tree detailing the species composition of each these genera is shown in *Fig. 3.2*.

In the first edition of *Bergey's Manual of Systematic Bacteriology*, the genus *Aeromonas* was allocated in the eubacterial family *Vibrionaceae* mainly based on phenotypic characters (Popoff 1984). However, a previous DNA hybridization study by Staley and Colwell (1973) had already indicated that the genomic relatedness between representative strains of *Aeromonas* and *Vibrio* was relatively low (i.e., <10 %), which evidenced the significant evolutionary distance between both taxonomic groups. From extensive molecular evidence (i.e., 16S rRNA catalogs, 5S rRNA sequences, and DNA–rRNA hybridization data), Colwell et al. (1986) concluded that the genus *Aeromonas* represents an evolutionary line that is sufficiently different from the *Vibrionaceae* and the *Enterobacteriaceae* to warrant its exclusion from these two families. Consequently, the authors proposed to allocate the genus *Aeromonas* in the new family *Aeromonadaceae* within rRNA superfamily I sensu De Ley (1992), a suggestion that was readily confirmed by subsequent rDNA sequencing (Martínez-Murcia et al. 1992a) and rRNA sequencing (Kita-Tsukamoto et al. 1993; Ruimy et al. 1994) studies.

Within the *Aeromonadaceae*, the genus *Aeromonas* is phylogenetically most closely linked to the genus *Tolumonas*. In the description of its type species *T. auensis*, the genus was phylogenetically placed in the γ subclass of the *Proteobacteria* without being assigned to any known family in that subclass (Fischer-Romero et al. 1996). Only with the description of the second species member *T. osonensis*, it was suggested on the basis of comparative 16S rRNA gene sequence analysis that this genus is phylogenetically situated in the *Aeromonadaceae* (Caldwell et al. 2011).

The taxonomic history of the genus *Oceanimonas* finds its origin in the reclassification of [*Pseudomonas*] *doudoroffii* DSM 7028^T (Baumann et al. 1983) by Brown et al. (2001). In the latter study, phylogenetic analysis demonstrated that [*Pseudomonas*]

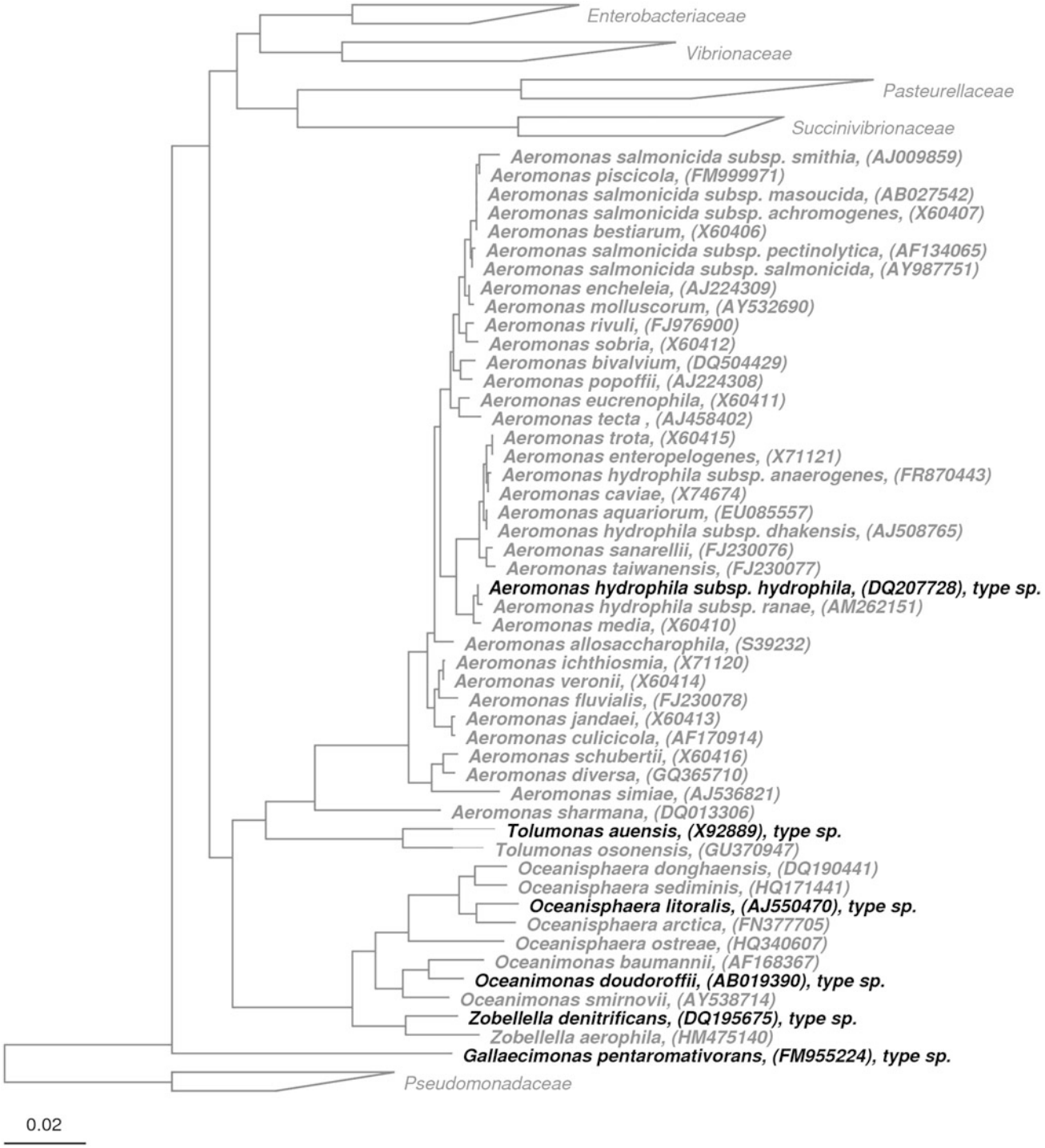

Fig. 3.2

Phylogenetic reconstruction of family *Aeromonadaceae* species members based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes–Cantor correction. Hundred run bootstrapping was applied to retrieve a ''most likely'' topology since single runs returned variable topologies. Nodes with no value stand for 100 %. The sequence datasets and alignments were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). Scale bar indicates estimated sequence divergence

doudoroffii should no longer be placed in the genus *Pseudomonas*. Previously, De Vos and colleagues (1989) had already reported this on the basis of RNA–DNA hybridizations and suggested that this species is a member of the genus *Aeromonas*. In contrast, Brown and co-workers found that *Tolumonas auensis* was the most closely related phylogenetic neighbor of [*Pseudomonas*] *doudoroffii*. Upon reevaluation of the phylogenetic relationship between [*Pseudomonas*] *doudoroffii* DSM7028^T and *A. hydrophila* ATCC7966^T, a 16S rDNA similarity of 91 ± 2 % did not indicate a close relationship between both strains (Brown et al. 2001). As phenotypic and chemotaxonomic data corroborated with this view, the latter authors proposed the reclassification of [*Pseudomonas*] *doudoroffii* DSM 7028^T into the genus *Oceanomonas* gen. nov. as *Oceanomonas doudoroffii* comb. nov. In the same study, also a second member of the new genus, *O. baumannii*, was proposed. The original spelling *Oceanomonas* (Brown et al. 2001) was later corrected to *Oceanimonas* by the List Editor of International Journal of Systematic and Evolutionary Microbiology (2001, 51, 269). By the time a third species member was described, *O. smirnovii* (Ivanova et al. 2005), the genus was still not assigned to a known family in the *Gammaproteobacteria*. The genus *Oceanimonas* was assigned to the *Aeromonadaceae* in *Bergey's Manual*, 2nd edition (Martin-Carnahan and Joseph 2005).

The genus *Oceanisphaera* was proposed to accommodate the halophilic bacterium KMM 3654^T which had *Oceanimonas doudoroffii* DSM 7028^T and *Oceanimonas baumannii* ATCC 700832^T (Brown et al. 2001) as the closest phylogenetic neighbors in almost-complete 16S rRNA gene sequence comparison (Romanenko et al. 2003). The authors further reported that *Tolumonas auensis* DSM 9187^T (Fischer-Romero et al. 1996) and members of the families *Vibrionaceae*, *Enterobacteriaceae*, and *Aeromonadaceae* were more distantly related (<92 % sequence similarity). Strain KMM 3654^T was proposed as the type strain of *Oceanisphaera litoralis* gen. nov., sp. nov. without being assigned to a known family. Also in subsequent descriptions of new *Oceanisphaera* species (Park et al. 2006; Choi et al. 2011; Shin et al. 2012; Srinivas et al. 2012), the genus was considered as a monophyletic clade most closely related to *Oceanimonas* spp. However, in none of these descriptions, *Oceanisphaera* was formally assigned to the *Aeromonadaceae*.

The genus *Zobellella* was created to harbor the denitrifying sediment isolates ZD1^T and ZT1^T (Lin and Shieh 2006). The closest relatives of both strains were species of *Oceanimonas* and *Oceanisphaera*, with overall 16S rRNA gene sequence similarities between the strains and members of these species in the range of 94.1–96.8 %. Still, Lin and Shieh (2006) concluded on the basis of % G+C contents, fatty acid and polar lipid composition, and 16S rRNA gene-based phylogeny that strains ZD1^T and ZT1^T were sufficiently divergent from the two aforementioned genera to be placed in a new genus within the *Alteromonas*-like *Gammaproteobacteria*. Finally, the two strains were assigned as the type strains of *Zobellella denitrificans* and *Z. taiwanensis*, respectively. In the subsequent description of *Z. aerophila* (Yi et al. 2011), the authors suggested that the genus *Zobellella* belongs to the order *Alteromonadales*. This in contrast to other sources, including the 16S rRNA gene tree of the Living Tree Project (Yarza et al. 2010) and J.P. Euzéby's List of Prokaryotic names with Standing in Nomenclature (http://www.bacterio.cict.fr/), which consider *Zobellella* a member of the *Aeromonadaceae* within the *Aeromonadales* order (*Figs. 3.1* and *3.2*). On the basis of 16S rRNA gene sequence tree-inferring methods, Yi et al. (2011) concluded that the genera *Zobellella*, *Oceanimonas*, and *Oceanisphaera* form a monophyletic clade with 100 % bootstrap support. Therefore, the authors considered the possibility to combine these three genera into a single genus in the future. A formal proposal in this regard has not yet been published.

Molecular Analyses

DNA–DNA Hybridization Studies

Since the late 1970s, taxonomic studies started to use DNA–DNA hybridizations to clarify the taxonomic relationships among the existing *Aeromonas* species. McInnes et al. (1979) concluded that the two main phenotypic groups in the genus *Aeromonas* corresponded to two legitimate genotypic groups: a diverse group of motile, mesophilic aeromonads and a more homogeneous group of nonmotile, psychrophilic aeromonads. Popoff and colleagues (1981) refined this view by unraveling the existence of at least seven DNA hybridization groups (HGs), i.e., three groups in *A. hydrophila* (formerly *A. hydrophila* biovar hydrophila), two groups in the newly proposed *A. caviae* (formerly *A. hydrophila* biovar anaerogenes), and two groups in *A. sobria*. This new classification was adopted by Popoff in the first edition of *Bergey's Manual of Systematic Bacteriology* (1984). However, Popoff pointed to the fact that the new HGs delineated in *A. hydrophila*, *A. caviae*, and *A. sobria* could not be established as new *Aeromonas* species since they could not be differentiated phenotypically within a given species. Based on the criteria outlined by Wayne and associates (1987), the definition of a HG (later also referred to as genomospecies or genomic species) implied that all constituting strains exhibit ≥70 % DNA relatedness with ≤5 % divergence between the related sequences. With respect to the chronological order of their first appearance in literature, the HGs originally delineated by Popoff and co-workers were referred to as HG1, HG2, HG3 (phenotypically resembling *A. hydrophila*), HG4, HG5, HG6 (phenotypically resembling *A. caviae*), HG7, and HG8 (phenotypically resembling *A. sobria*).

In the original *A. hydrophila* complex, HG1 corresponds to *A. hydrophila sensu stricto*. Later, two groups of phenotypically atypical *A. hydrophila* HG1 strains were proposed as subspecies of *A. hydrophila*, i.e., *A. hydrophila* subsp. *dhakensis* (Huys et al. 2002b) and *A. hydrophila* subsp. *ranae* (Huys et al. 2003). In these studies, an extended description was given of *A. hydrophila* subsp. *hydrophila* (Chester 1901) Stanier 1943. Martínez-Murcia et al. (2009) subsequently proposed to reclassify *A. hydrophila* subsp. *dhakensis* as a member of the later described species

Aeromonas aquariorum (Martínez-Murcia et al. 2008). However, according to a Note in J.P. Euzéby's List of Prokaryotic names with Standing in Nomenclature (http://www.bacterio.cict.fr/a/aeromonas.html), this reclassification proposal was not valid. With the description of *A. bestiarum* (Ali et al. 1996), formerly referred to as *A. hydrophila* HG2, a significant contribution was made to solve the taxonomic confusion between HG1 and HG2. Several years after the description of *A. bestiarum*, a group of HG2-like isolates was found to constitute a new species on the basis of DNA–DNA hybridization data for which the name *A. popoffii* was proposed (Huys et al. 1997b). Farmer III and colleagues (1986) showed that the type strain of the nonmotile species *A. salmonicida* was highly related in DNA–DNA hybridizations to the reference strain of the motile *A. hydrophila* strains in HG3. This led to the conclusion that the three subspecies of *A. salmonicida* (i.e., subsp. *salmonicida*, *achromogenes*, and *masoucida*) should be included in HG3, despite the striking phenotypic differences with the other, motile members of HG3. Later, two more subspecies were described, i.e., *A. salmonicida* subsp. *smithia* (Austin et al. 1989) and *A. salmonicida* subsp. *pectinolytica* (Pavan et al. 2000). It has been suggested that the motile strains in HG3 phenotypically resembling *A. hydrophila* might constitute another new subspecies in *A. salmonicida* (Altwegg et al. 1990).

In the original *A. caviae* complex, members of HG4 are considered as *A. caviae sensu stricto*. DNA–DNA hybridizations grouped the type strain of *A. media* (Allen et al. 1983) in HG5 together with representatives of *A. caviae* not belonging to HG4 (Hickman-Brenner et al. 1988b). Later, new DNA hybridization data suggested that HG5 may need to be split up in HG5A (phenotypically *A. caviae*) and HG5B (comprising strains that resemble *A. caviae* or *A. media*). The suggestions made by Altwegg et al. (1990) in considering HGs 5A and 5B as two subspecies of *A. media*, and their proposal to create two separate biogroups in HG5B, have not been validated to date. The third HG in the original *A. caviae* complex, HG6, was later assigned to *A. eucrenophila* (Schubert and Hegazi 1988). However, Huys et al. (1997b) reported phenotypic and DNA reassociation data showing that *A. eucrenophila* consisted of two subgroups. Subgroup I represented the taxonomic core of *A. eucrenophila*, whereas subgroup II was found to belong to the species *A. encheleia* which had already been previously described by Esteve et al. (1995a). On the basis of a DNA relatedness ranging from 83 % to 98 %, Huys et al. (1997b) suggested that also the two members of the very rare *Aeromonas* HG11 (i.e., the *A. veronii*-like clinical isolate ATCC 35941 (Hickman-Brenner et al. 1988a) and the environmental strain CDC 3136–78) should be included in *A. encheleia*. However, this contrasted with the study of Esteve and colleagues (1995a) who reported DNA homology values between *A. encheleia* and HG11 strain ATCC 35941 not higher than 37 %. Phylogenetic analysis by Martínez-Murcia (1999) later revealed that the two HG11 strains exhibited a species-specific sequence including unique nucleotides at two positions. In 2008, Demarta and colleagues performed a polyphasic study including DNA–DNA hybridizations to determine the taxonomic position of five "*A. eucrenophila*-like" strains (Demarta et al. 2004) and assigned them to the new species *A. tecta*.

In the *A. sobria* complex as first described by Popoff et al. (1981), HG7 originally harbored only two strains. The other member of this complex, HG8, was found to exhibit high levels of DNA relatedness to the type strain of *A. veronii* HG10 (Hickman-Brenner et al. 1987). However, because strains of HG8 differed in a number of phenotypic properties from HG10 including the lack of ODC production, HG8 was considered a biogroup of *A. veronii* for which the name *A. veronii* biovar sobria was proposed. Accordingly, HG10 was referred to as *A. veronii* biovar veronii (Joseph et al. 1991).

In the years following the proposal of Popoff et al. (1981) to group *Aeromonas* strains in HGs, several new ones were described. In 1985, Fanning and associates discovered a third HG in *A. sobria*, i.e., HG9 (Fanning et al. 1985), that had not been recognized before by Popoff et al. (1981). Subsequently, Carnahan and colleagues (1991c) proposed the name *A. jandaei* for the relatively rare *Aeromonas* HG9. Based on DNA–DNA hybridizations, Hickman-Brenner et al. (1988a) proposed *A. schubertii* for a group of human isolates formerly referred to as CDC Enteric Group 501 (Hickman-Brenner et al. 1988b). In the genotypic classification scheme of the genus *Aeromonas*, the new species was added as HG12 (Altwegg et al. 1990). In an Addendum in Proof, Hickman-Brenner et al. (1988a) reported on the existence of two *A. schubertii*-like isolates from human origin that probably had diverged somewhat from this species. These two strains were placed in *Aeromonas* Group 501. Phylogenetic data have supported the notion that *Aeromonas* Group 501 may represent a new *Aeromonas* species closely related to *A. schubertii* (Martínez-Murcia 1999). In 2010, the two strains of *Aeromonas* Group 501 were assigned to the newly proposed species *A. diversa* (Miñana-Galbis et al. 2010a).

Schubert and colleagues (1990a, b) proposed two new *Aeromonas* species, i.e., *A. ichthiosmia* and *A. enteropelogenes*. Their status as new species was later questioned on the basis of phylogenetic evidence by Collins et al. (1993), who suggested that *A. ichthiosmia* and *A. enteropelogenes* were in fact identical to *A. veronii* and *A. trota*, respectively. The latter species was described by Carnahan and colleagues (1991b) only a couple of months following the publication of the *A. enteropelogenes* proposal (Schubert et al. 1990a). In a later study, Huys et al. (2001b) reported DNA–DNA hybridization data showing that the type strains of *A. veronii* and *A. ichthiosmia* exhibited a genomic relatedness of 84–96 %. Because *A. veronii* (Hickman-Brenner et al. 1987) was the first validated of the two species, it was proposed to reject the species name *A. ichthiosmia* as a junior synonym of *A. veronii* (Huys et al. 2001b). Likewise, Huys et al. (2002a) concluded that *A. trota* and *A. enteropelogenes* should be considered synonyms on the basis of 86–98 % DNA homology between their respective type strains. A request for an opinion was proposed to decide whether the later validated but most cited species name *A. trota* (Carnahan et al. 1991b) can obtain priority over the first proposed but rarely referred name of *A. enteropelogenes* (Schubert et al. 1990a).

The description of the species *A. allosaccharophila* (Martínez-Murcia et al. 1992b) was the first species proposal in *Aeromonas* to be based on 16S rRNA gene sequence analysis and not on DNA–DNA hybridization data. Whereas Esteve and associates (1995b) later demonstrated in a comparative DNA–DNA hybridization study that *A. allosaccharophila* represented a distinct genomic species, DNA homology data reported by Huys et al. (2001b) showed that the type strains of *A. allosaccharophila* and *A. veronii* were 78–82 % related. Based on additional DNA–DNA hybridizations and *dnaJ* gene sequence analysis, Nhung et al. (2007) suggested that *A. allosaccharophila* is a later heterotypic synonym of *A. veronii*.

Triggered by the increased use of molecular methods such as amplified fragment length polymorphism (AFLP) fingerprinting and especially sequence analysis of one or several housekeeping genes (see MLSA) to recognize potentially novel taxa, *Aeromonas* taxonomy has witnessed an explosion of new species proposals since 2004. In addition to phenotypic, genotypic, and/or phylogenetic evidence, all of these new descriptions incorporated DNA–DNA hybridizations to confirm the species status of the new genus members. In this way, species proposals were made for *A. molluscorum* (Miñana-Galbis et al. 2004), *A. simiae* (Harf-Monteil et al. 2004), *A. bivalvium* (Miñana-Galbis et al. 2007), *A. aquariorum* (Martínez-Murcia et al. 2008), *A. tecta* (Demarta et al. 2008), *A. piscicola* (Beaz-Hidalgo et al. 2009), *A. diversa* (Miñana-Galbis et al. 2010a), *A. fluvialis* (Alperi et al. 2010b), *A. taiwanensis* (Alperi et al. 2010a), *A. sanarellii* (Alperi et al. 2010a), and *A. rivuli* (Figueras et al. 2011). An exception to this rule was the proposal of *A. sharmana* (Saha and Chakrabarti 2006), which did not include DNA–DNA hybridization values. However, soon after its description, several workers argued that *A. sharmana* does not belong to the genus *Aeromonas* (Saavedra et al. 2006; Martínez-Murcia et al. 2007). Likewise, the species status of *A. culicicola* (Pidiyar et al. 2002) has been the subject of taxonomic discussions. Based on new DNA–DNA hybridization data, Huys et al. (2005) proposed that this species is a later subjective synonym of *A. veronii* Hickman-Brenner et al. 1988. Further support to include *A. culicicola* in *A. veronii* was provided by gene sequence analysis of *rpoD* (Saavedra et al. 2006) and *dnaJ* (Nhung et al. 2007) and additional DNA–DNA hybridizations (Nhung et al. 2007).

Martínez-Murcia and colleagues (1992a) were the first to report a remarkable lack of congruence between DNA–DNA hybridizations and 16S rRNA gene sequence similarities for several *Aeromonas* species. As a result of the very high level of 16S rRNA gene similarity (98–100 %) found between *Aeromonas* type strains, several cases the phylogenetic interrelationships of the corresponding species completely disagreed with previously published DNA–DNA hybridization results. For instance, the 16S rRNA sequences of the type strains of *A. caviae* (HG4) and *A. trota* (HG13) differed by only one nucleotide (i.e., 99.9 % sequence similarity) (Martínez-Murcia et al. 1992a), whereas Carnahan et al. (1991b) determined that the DNA relatedness between these two strains was as low as 30 %. A similar phenomenon was observed with *Aeromonas* HG2 and HG3 and HG1 and *A. media*, respectively. In contrast, reference strains of *A. veronii* HG8 and HG10 showed identical 16S rDNA sequences in agreement with the very high genotypic similarity between these two taxa (Hickman-Brenner et al. 1987). Additional discrepancies between 16S rDNA sequence analysis and DNA–DNA hybridization results have been reported on the taxonomic position of *Aeromonas* HG11 (Huys et al. 1997b; Martínez-Murcia 1999) and for the discrimination between *A. salmonicida* and *A. bestiarum* (Martínez-Murcia et al. 2005). Evidence for the occurrence of rare recombination events (Sneath 1993) and intragenomic heterogeneity (Morandi et al. 2005) in the 16S rRNA gene sequences of some *Aeromonas* species suggested that this gene should be used with caution for use in *Aeromonas* phylogeny and identification. On the same note, there are multiple examples of inconsistencies between different sets of DNA–DNA hybridization data published by different authors using the same reference strains (Esteve et al. 1995a, b; Huys et al. 1997b, 2001b, 2002a; Martínez-Murcia 1999; Martínez-Murcia et al. 2005).

The current species composition of the genus *Aeromonas* is phylogenetically reconstructed in a neighbor-joining 16S rRNA sequence tree shown in *Fig. 3.2*.

For the other genera of the *Aeromonadaceae* for which the current taxonomic structure is much less complex compared to *Aeromonas*, DNA–DNA hybridizations were performed as a part of the new species descriptions and usually only included the type strains of the neighboring taxa. For further details on these DNA–DNA hybridization studies, the reader is referred to the original descriptions of species within the genera *Tolumonas*, *Oceanimonas*, *Oceanisphaera*, and *Zobellella*.

MLSA

In the genus *Aeromonas*, *gyrB* (encoding the B subunit of DNA gyrase, a type II DNA topoisomerase) and *rpoD* (encoding the s70 factor, one of the sigma factors that confers promoter-specific transcription initiation on RNA polymerase) were the first housekeeping genes to be used as alternative phylogenetic markers for the 16S rRNA gene (Yáñez et al. 2003; Soler et al. 2004). Subsequently, one or both of these markers have been used in support of many new species descriptions such as *A. aquariorum* (Martínez-Murcia et al. 2008), *A. tecta* (Demarta et al. 2008), *A. piscicola* (Beaz-Hidalgo et al. 2009), *A. diversa* (Miñana-Galbis et al. 2010a), *A. rivuli* (Figueras et al. 2011), *A. fluvialis* (Alperi et al. 2010b), *A. taiwanensis* (Alperi et al. 2010a), and *A. sanarellii* (Alperi et al. 2010a) and to reassess the position of species with unclear or controversial phylogenetic affiliations (Saavedra et al. 2006). In the slipstream of *gyrB* and *rpoD*, a series of other housekeeping genes have been proposed for phylogenetic analysis and identification of aeromonads. In combination with the 16S rRNA and *gyrB* genes, Küpfer et al. (2006) introduced *rpoB* (encoding the β subunit of DNA-dependent RNA polymerase) to clarify the taxonomy and the phylogenetic relationships of *Aeromonas* strains. As a stand-alone approach, partial *rpoB* gene sequence analysis was later validated for identification of environmental

Aeromonas isolates (Lamy et al. 2010). Other single housekeeping gene approaches that have been proposed for sequence-based differentiation of *Aeromonas* species include the genes *dnaJ* (encoding heat-shock protein 40; Nhung et al. 2007), *recA* (encoding the RecA protein which has important functions in homologous DNA recombination, DNA damage repair, and induction of the SOS response; Sepe et al. 2008), *cpn60* (encoding the type I chaperonin Cpn60; Miñana-Galbis et al. 2009), and *mdh* (encoding malate dehydrogenase; Farfán et al. 2010). Martínez-Murcia et al. (2011) argued that phylogenetic analysis of a single gene could be inappropriate for species classification in genera where recombination may be relatively frequent, as is likely to be the case in *Aeromonas* (Sneath 1993; Morandi et al. 2005). Therefore, the authors introduced a multilocus phylogenetic analysis (MLPA) scheme based on concatenated sequences of seven housekeeping gene fragments (*gyrB*, *rpoD*, *recA*, *dnaJ*, *gyrA*, *dnaX*, and *atpD*) which spans a total of 4,705 bp. The MLPA approach allowed phylogenetic delineation of all currently described *Aeromonas* species with uniformly high bootstrap values (Martínez-Murcia et al. 2011).

In addition to their use in a strictly phylogenetic context, housekeeping genes have also been applied for a number of other purposes in studies on aeromonads. A combination of sequences derived from the genes *cpn60*, *dnaJ*, *gyrB*, and *rpoD* was used to verify the identity of strain CIP 57.50 (= ATCC 7965 = NCTC 7812), an important taxonomic reference in the genus *Aeromonas* (Miñana-Galbis et al. 2010b). In combination with phenotypic characterization, this multigene approach resulted in the reclassification of *A. hydrophila* CIP 57.50 as *A. salmonicida* CIP 57.50. Martino et al. (2011) developed an MLST scheme based on concatenated sequences of *gyrB*, *groL*, *gltA*, *metG*, *ppsA*, and *recA* for strain typing. This scheme was further developed for implementation as a web-based MLST sequence database (http://pubmlst.org/aeromonas) specific for the genus *Aeromonas* and may have further applications as a tool for outbreak traceability, host range diffusion, and ecological studies. As an alternative to traditional HPLC-based methods, a selection of genes (*cpn60*, *dnaJ*, *gyrB*, *rpoB*, and *rpoD*) has been used to estimate the G+C content of *Aeromonas* species (Lorén et al. 2010).

DNA Patterns

The evaluation of Amplified Fragment Length Polymorphism (AFLP) analysis as a DNA fingerprinting method in bacterial taxonomy included *Aeromonas* as one of the first test cases (Janssen et al. 1996). Initially based on autoradiography and later on fluorescence as means of detection, AFLP proved to be a highly discriminating technique in the genotypic classification of *Aeromonas* species (Huys et al. 1996a, Huys and Swings 1999). In addition, AFLP fingerprinting was also used to study the position of species with unclear taxonomic status, including *A. eucrenophila*, *A. encheleia*, *Aeromonas* HG11, *A. ichthiosmia*, *A. enteropelogenes*, *A. allosaccharophila*, and *A. culicicola* (Huys et al. 1996a, 1997b, 2001b; Miñana-Galbis et al. 2004) and to delineate new *Aeromonas* taxa such as *A. popoffii* (Huys et al. 1997c), *A. hydrophila* subsp. *dhakensis* (Huys et al. 2002b), *A. hydrophila* subsp. *ranae* (Huys et al. 2003), *A. molluscorum* (Miñana-Galbis et al. 2004), *A. bivalvium* (Miñana-Galbis et al. 2007), *A. tecta* (Demarta et al. 2004, 2008), and *A. diversa* (Miñana-Galbis et al. 2010a). Although in many cases AFLP grouping was in agreement with results from DNA–DNA hybridization, exceptions have been reported and discussed (Esteve 1997; Huys et al. 1997a; Martínez-Murcia 1999). In combination with user-generated local databases, AFLP has been used in various studies to determine the HG identity of aquatic (Huys et al. 1996b, 2000, 2001a), sewage (Rahman et al. 2007), clinical (Kühn et al. 1997a), and fish (Rahman et al. 2002) isolates of *Aeromonas*. In addition to its taxonomic potential, AFLP has also been used for strain typing of aeromonads in ecological studies. For instance, AFLP fingerprinting was used to study the survival of a genetically marked *A. hydrophila* strain in freshwaters and nutrient-poor waters (Kersters et al. 1996), to assess the clonal relationship among a set of *A. hydrophila* HG3 strains collected from a Swedish drinking well during a 4-year study (Kühn et al. 1997c), and to determine the genetic variability among atypical *A. salmonicida* isolates from marine fishes (Lund et al. 2002).

Randomly amplified polymorphic DNA (RAPD) analysis and pulsed-field gel electrophoresis (PFGE) have been used for strain typing rather than for taxonomic purposes in *Aeromonas* (Oakey et al. 1996b). RAPD fingerprinting with randomly designed PCR primers was employed for the genetic differentiation of *A. salmonicida* and *A. hydrophila* (Miyata et al. 1995), the clonal identification of *A. hydrophila* from nosocomial infections (Talon et al. 1998), and analysis of the genotypic relatedness among strains of *A. salmonicida* (Inglis et al. 1996; Kwon et al. 1997). RAPD has also been used for strain typing together with other fingerprinting techniques such as ERIC-PCR (Davin-Regli et al. 1998; Szczuka and Kaznowski 2004; Aguilera-Arreola et al. 2005), serotyping (Sinha et al. 2004), ribotyping, and plasmid profiling (Hänninen et al. 1995; Austin et al. 1998). PFGE fingerprinting of macro-restriction DNA fragments has been used for strain differentiation among *A. hydrophila* from hospital-acquired infections (Talon et al. 1996), *A. salmonicida* isolates (Hänninen and Hirvelä-Koski 1999; Garcia et al. 2000), motile *Aeromonas* spp. from Italian ready-to-eat foods (Villari et al. 2000), and *Aeromonas* spp. from natural mineral waters (Villari et al. 2003). Other applications of PFGE include a study of the genetic stability of *Aeromonas* isolates after passage through a host (Livesley et al. 1999) or the construction of a physical map of the chromosome of *A. hydrophila* (Dodd and Pemberton 1998) and *A. salmonicida* (Umelo and Trust 1998). A large study on 227 isolates from different geographical locations in the United States used PFGE in combination with colony blots to compare the virulence signatures of aquatic and clinical *Aeromonas* isolates (Khajanchi et al. 2010).

Plasmid profiling is perhaps the most primitive DNA fingerprinting method for typing individual *Aeromonas* strains in ecological and epidemiological studies. This method has been used to differentiate between typical and atypical *A. salmonicida*

strains (Belland and Trust 1989) and has proven to be a useful epidemiological marker for *A. salmonicida* associated with furunculosis (Hänninen and Hirvelä-Koski 1997; Pedersen et al. 1996; Sorum et al. 2000). Clearly, plasmid profiling cannot be considered a universal typing as strains lacking any plasmids actually remain untypable.

Other less frequently used DNA patterning methods in *Aeromonas* include restriction endonuclease analysis (Altwegg et al. 1988), RFLP of 16S–23S rDNA intergenic regions (Martínez-Murcia et al. 2000; Soler et al. 2003; Laganowska and Kaznowski 2004), ERIC-PCR and REP-PCR (Soler et al. 2003; Figueras et al. 2006; Nováková et al. 2009), BOX-PCR (Tacao et al. 2005a; Singh et al. 2010), PCR-based typing analysis with primers targeting four different structural genes (Hoie et al. 1999), and *gyrB*-based DGGE profiling (Tacao et al. 2005b). Recently, Roger and colleagues (2012) explored the possibilities of a 16S rRNA multi-operon fingerprinting approach in the genus *Aeromonas*. This approach relied on the combination of PFGE, to assess rrn operon number and distribution across the chromosome, and PCR-temporal temperature gel electrophoresis (TTGE), to assess rrs V3 region heterogeneity.

Ribotyping

Martinetti Lucchini and Altwegg (1992) were among the first to determine that ribotyping patterns correlated with taxonomic groups in *Aeromonas* when a 567 bp restriction fragment of the 16S rRNA operon was used as probe. Discrimination among 12 *Aeromonas* HGs was possible by visual comparison of banding patterns. Despite its value for research purposes, however, this rather laborious ribotyping methodology was not suitable for implementation in diagnostic laboratories (Altwegg 1993). Hänninen and Siitonen (1995) used ribotyping for identification of aquatic, food, and human *Aeromonas* isolates at the genomic species (HG) level. Subsequently, ribotyping was also used to support the species descriptions of *A. bestiarum* (Ali et al. 1996) and *A. popoffii* (Huys et al. 1997c) and to determine the taxonomic positions of *A. eucrenophila*, *A. encheleia*, and *Aeromonas* HG11 in a polyphasic study (Huys et al. 1997b). Nsabimana and colleagues (2000) used the ribotyping technique together with biochemical tests to assess the taxonomic diversity of aeromonads in activated sludge.

Ribotyping using the entire rRNA operon as a probe was also applied for epidemiological typing of clinical *Aeromonas* isolates (Moyer et al. 1992b). Likewise, Demarta and associates (2000) used this method to investigate epidemiological relationships between *Aeromonas* strains isolated from symptomatic children and household environments. Ribotyping also proved very useful to study the genetic diversity among atypical isolates of *A. salmonicida* from North European aquaculture environments (Hänninen et al. 1995; Pedersen et al. 1996; Hänninen and Hirvelä-Koski 1997; Austin et al. 1998).

To circumvent the often laborious probing step in ribotyping, Borrell et al. (1997) described RFLP analysis of PCR-amplified 16S rRNA genes as an alternative method to discriminate the type and reference strains of all known *Aeromonas* species. The use of the endonucleases *Alu*I and *Mbo*I allowed differentiation among most species pairs except among *A. salmonicida*, *A. bestiarum*, and *Aeromonas* HG11, which required the enzyme combination *Nar*I and *Hae*II for separation. Subsequently, Graf (1999a) used an extended collection of 62 reference strains to further examine the validity of 16S rRNA RFLP-PCR as a taxonomic tool in *Aeromonas*. Importantly, this study showed that *A. veronii* biovar sobria strains may produce diverse RFLP-PCR patterns. Graf (1999a) argued that this could lead to possible misidentifications and recommended supplementary biochemical tests in such cases. The method originally described by Borrell and colleagues (1997) was later extended with the endonucleases *Alw*NI and *Pst*I (Figueras et al. 2000) and used to support the taxonomic delineation of *A. fluvialis* (Alperi et al. 2010b).

MALDI-TOF

The first major exploration of the matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) method in the genus *Aeromonas* indicated the presence of three common mass ions among all 17 species tested (Donohue et al. 2006). These signatures, with an average m/z of 6,301, 12,160 or 12,254, and 13,450, may have potential as genus-specific biomarkers in unknown samples. Also at species level, the MALDI-MS spectra were found to yield sufficient resolving power to effectively discriminate between *Aeromonas* species (Donohue et al. 2006). In a follow-up study (Donohue et al. 2007), the authors extended their strain panel to construct an identification library which was tested in two blind groups. In group 1, it was demonstrated that all unknown water isolates with a typical biochemical profile were correctly identified with the MALDI-MS database. In group 2, which harbored strains with atypical phenotypic profiles, 18 out of 27 isolates were assigned to the correct species. In the same study, it was also shown that cultures of the same *Aeromonas* reference strain obtained from different collections yielded MALDI-MS profiles that were highly comparable. Benagli et al. (2012) constructed a MALDI-TOF MS database from 92 genotypically well-characterized *Aeromonas* strains. The taxonomic resolution achieved with this database at species level was reported to be comparable to that offered by the housekeeping gene *gyrB*. Next, the database was evaluated for the identification of 741 clinical and environmental *Aeromonas* isolates, of which 93 % could be correctly identified at species level. The remaining 7 % of unidentified isolates primarily belonged to more recently described and rarely isolated species not yet covered by the MALDI-TOF MS database. Others have shown that, compared to *rpoB* gene sequencing, MALDI-TOF MS reaches >90 % species-level accuracy for the identification of taxonomic reference strains and unknown environmental and clinical isolates (Lamy et al. 2011). In recent years, MALDI-TOF MS profiling has been used in support of the description of novel *Aeromonas* species such as *A. aquariorum* (Martínez-Murcia et al. 2008) and *A. piscicola*

Table 3.1
General features of published *Aeromonadaceae* genomes

Species	Strain	DDBJ/EMBL/ GenBank Acc No	Chromosome size (bp)	Plasmid(s)	GC content (%)	rRNA operons	tRNAs	References
A. aquariorum	AAK1	BAFL01000001 to BAFL01000036; AP012343	4,763,532	4,161 bp	61.7			Wu et al. 2012
A. caviae	Ae398	CACP01000001 to CACP01000149	4,339,218	>30 kb	61.4			Beatson et al. 2011
A. hydrophila	ATCC 7966^T	CP000462	4,744,448		61.5	10	128	Seshadri et al. 2006
A. media	WS	ALJZ00000000	4,317,138		61.4	3	54	Chai et al. 2012
A. salmonicida subsp. *salmonicida*	A449	NC_009348 (chr); NC_009439 (pAsa4); NC_009350 (pAsa5)	4,702,402	pAsa4 (166,749 bp), pAsa5 (155,098 bp), and 3 other plasmids	58.5	9	110	Reith et al. 2008
A. salmonicida	01-B526	AGVO00000000	4.75 Mb	pAsa5 (155 kb), pAsa1 (5,424 bp), pAsa2 (5,247 bp), and pAsa3 (5,616 bp)	58.5			Charrette et al. 2012
A. veronii	B565	CP002607	4,551,783		58.7	10	102	Li et al. 2011
Aeromonas sp.	159	ALOT00000000	4,470,895		59.3	1	46	Chan et al. 2012
Oceanimonas sp.	GK1	CP003171 (chr); CP003172 (plasmid 1); CP003173 (plasmid 2)	3,514,537	8,462 bp and 4,245 bp	61.1			Yeganeh et al. 2012

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(Beaz-Hidalgo et al. 2009). Collectively, aforementioned studies suggest that the MALDI-TOF MS system is currently one of the most accurate and rapid methods for chemotaxonomic identification of aeromonads.

Genome Features

So far, nine complete genome sequences of *Aeromonadaceae* members were effectively published (*Table 3.1*). In the genus *Aeromonas*, one genome each was released for the species *A. hydrophila* (Seshadri et al. 2006), *A. caviae* (Beatson et al. 2011), *A. veronii* (Li et al. 2011), *A. media* (Chai et al. 2012), and *A. aquariorum* (Wu et al. 2012), whereas two genomes were so far published of *A. salmonicida* strains (Reith et al. 2008; Charette et al. 2012). In addition, one *Aeromonas* genome with unknown species identity was released (Chan et al. 2012). Noteworthy, new insights from these studies are currently limited as many of the recently published *Aeromonas* genomes are draft versions or have not been analyzed and compared in great depth. As far as the other genera in the family are concerned, only one genome of *Oceanimonas* sp. (Yeganeh et al. 2012) is currently available.

In 2006, the first published *Aeromonas* genome was released for the type strain of the type species, *A. hydrophila* ATCC 7966^T (Seshadri et al. 2006). The 4.74 Mb genome of this emerging pathogen revealed considerable potential to survive and proliferate in both aquatic and host environments. The detection of a large array of putative virulence factors, including functions for adhesion, toxin production, iron acquisition, and antibiotic resistance, may reflect the organism's ability to infect a wide range of hosts. Strikingly, two recognized virulence factors (a type III secretion system and a lateral flagellum) commonly found in other *A. hydrophila* strains were not identified in ATCC 7966^T. The genome clearly revealed the broad metabolic capabilities of this strain including dissimilatory sulfate reduction and resistance mechanisms against toxic compounds encountered in polluted waters (such as thiopurine reductase, arsenate reductase, and phosphonate degradation enzymes). The absence of transposases, resolvases, or insertion sequence elements indicated a general lack of mobile genetic elements in this strain and thus provides little evidence of genome fluidity to explain the renowned ubiquity and free-living lifestyle of this organism. In contrast, several of such elements as well as plasmids have been reported in the genomes of other *Aeromonas* spp. (Reith et al. 2008; Beatson et al. 2011; Charette et al. 2012). The future availability of additional genome sequences of *A. hydrophila* strains will help to investigate if the observed lack of mobile elements in ATCC 7966^T is a strain-specific or a species-specific phenomenon.

The first complete genome sequence of *A. salmonicida* subsp. *salmonicida* A449, the causative agent of furunculosis in salmonid fish, was determined to provide a better understanding of the virulence factors used by this pathogen to infect fish (Reith et al. 2008). Strain A449, originally isolated from a brown trout, was found to contain a 4.70 Mb chromosome with a large inversion which may reorientate the relative position of the origin of replication for some genes and thus have an impact on the regulation of their expression. The strain also contained two large plasmids of 166.7 kb (pAsa4) and 155.1 kb (pAsa5) as well as three smaller plasmids. Plasmid pAsa4 harbored a Tn*21* composite transposon containing mercury resistance genes and an In2 integron encoding genes for resistance to streptomycin/spectinomycin, quaternary ammonia compounds, sulfonamides, and chloramphenicol. A large number of genes encoding potential virulence factors were found, including functions for secretion, adhesion, toxin production, iron acquisition, antibiotic resistance, and quorum sensing. However, many of these potential virulence genes appeared to be pseudogenes containing insertion sequences, frameshifts, or in-frame stop codons. A number of the pseudogenes identified in *A. salmonicida* A449 were also investigated in other *Aeromonas* strains and species. While nearly all the pseudogenes tested are present in *A. salmonicida* subsp. *salmonicida* strains, only about 25 % were found in other *A. salmonicida* subspecies, and none were detected in other *Aeromonas* species. Reith and colleagues (2008) suggested that these genome-wide rearrangements may be a consequence of the specific adaptation of this bacterium to salmonid fish hosts. Triggered by the fact that strain A449 was not virulent in the alternative host model *Dictyostelium discoideum* amoeba (Daher et al. 2011), Charette et al. (2012) determined the genomic sequence of the true virulent *A. salmonicida* strain 01-B526 isolated from an infected brook trout and displaying high virulence against both fish and amoeba. However, the authors did not specify the subspecies identity of this strain. The genome sequencing project revealed that it contained a 4.75 Mb chromosome, which is 50 kb bigger than the one of strain A449, the large plasmid pAsa5 (155 kb), and three smaller plasmids pAsa1, pAsa2, and pAsa3. One additional plasmid known to occur in strain 01-B526 was missed in the genome sequence, possibly due to the very high homology of a big part of this plasmid with pAsa3 and the presence of one IS also found elsewhere in the genome.

The only genome so far released of *A. veronii*, an opportunistic pathogen which may cause wound infections, diarrhea, or septicemia in immunocompromised patients but is also the causative agent of bacterial hemorrhagic septicemia in fish, was determined for *A. veronii* strain B565 (Li et al. 2011). This particular strain was isolated from aquaculture pond sediment and may be used to control fungal or *Myxozoa*-related diseases due to its ability to produce chitinase. Noteworthy, the authors did not indicate to which of the two biovars currently recognized in *A. veronii* (i.e., sobria and veronii) strain B565 belonged to. The 4.55 Mb genome of this strain was found to contain 5 genes encoding chitinase, all of which were also found in the genomes of *A. hydrophila* ATCC 7966^T (Seshadri et al. 2006) and *A. salmonicida* subsp. *salmonicida* A449 (Reith et al. 2008). Compared to the latter two genomes, however, strain B565 encodes fewer putative virulence factors which included hemolysins, an RTX protein, an adhesion factor, flagella, and mannose-sensitive hemagglutinin. According to Li et al. (2011), this may indicate that some aeromonads use a stepwise process for the acquisition of their virulence traits.

Together with *A. hydrophila* and *A. veronii*, *A. caviae* is the most commonly encountered *Aeromonas* species in human infections. The single *A. caviae* genome sequence currently available was determined for strain Ae398 (Beatson et al. 2011). This strain was isolated as the sole pathogen from a stool specimen of a child with profuse diarrhea and was assigned to the species *A. caviae* by conventional biochemical tests. The 4.43 Mb genome of strain Ae398 harbors various mobile genetic elements including at least one conjugative plasmid of >30 kb and five different IS element types. It also contains an ~33 kb putative prophage bound by 55 bp repeats at the tRNA–Leu attachment site. Furthermore, the genome of strain Ae398 also encodes various putative virulence factors, including a type II secretion system, an RTX toxin, and polar flagella.

Strains of *A. media* are characterized by the production of a soluble brown pigment. The pigment produced by *A. media* strain WS, isolated from lake water and identified by phenotypic characterization and 16S rDNA sequencing, was shown to be melanin (Wan et al. 2009). Triggered by the photoprotective properties of this compound, the genome sequence of strain WS was determined (Chai et al. 2012). Compared to the genomes of *A. hydrophila* ATCC 7966^T, *A. salmonicida* A449, and *A. veronii* B565, the 4.32 Mb genome of *A. media* strains WS contains 659 unique genes. Most of these are transposases and hypothetical proteins with unknown functions, which may indicate its potential for gene exchange. Conversion of tyrosine by bacterial tyrosinase is thought to initiate one of the major pathways for melanin production (Wan et al. 2009), but no predicted proteins for tyrosinase were found in the genome of strain WS. However, multiple other genes potentially involved in melanin formation, such as genes encoding phenylalanine 4-monooxygenase and pterin-4-alpha-carbinolamine dehydratase were identified.

Strains of *A. aquariorum* have so far been reported in aquatic environments, ornamental fish, and clinical samples, which suggests a ubiquitous lifestyle. The single genome sequence available for this species was determined for strain AAK1 (Wu et al. 2012), a blood isolate from a cirrhotic patient which was taxonomically characterized using *rpoD* and *gyrB* gene sequencing. The 4.81 Mb genome of strain AAK1, which also includes a small (4.16 kb) plasmid, contains multiple genes encoding putative virulence factors. Many of these involve mechanisms for adhesion, toxin production, and iron acquisition that have also been found in other *Aeromonas* species such as *A. hydrophila* (Seshadri et al. 2006) and *A. salmonicida* (Reith et al. 2008) in addition to a quorum-sensing regulon activator (AhyR). Interestingly, quorum-sensing functions (i.e., homologues of

luxI and *luxR*) have also been found in the 4.47 Mb genome of unidentified *Aeromonas* strain 159 isolated from patient stool (Chan et al. 2012).

In the genus *Oceanimonas*, one genome sequence has been determined for *Oceanimonas* sp. strain GK1 isolated from wetland (Yeganeh et al. 2012). This strain contained a 3.51 Mb chromosome and two plasmids of 8.4 and 4.2 kb, together containing 111 structural RNAs and 2.516 nonhypothetical protein and 711 hypothetical protein-coding sequences. The production of large amounts of poly-β-hydroxybutyrate (PHB) by strain GK1 could be linked to the presence of β-ketoacyl-coenzyme A (CoA) thiolase-, acetoacetyl-CoA reductase-, and PHB synthase-encoding genes of the three-step PHB synthesis pathway. Further investigation of this genome sequence identified genes encoding functions for osmoregulation, including components of the Trk system for potassium uptake, the choline and betaine uptake system, and the betaine biosynthesis system. The presence of heavy metal and toxic compound resistance protein-coding genes, including cobalt, zinc, cadmium, arsenic, and chromium compound resistance genes, suggests the genetic potential of the halotolerant *Oceanimonas* sp. strain GK1 to adapt to multiple extreme conditions.

Phenotypic Analyses

The main features of members of the *Aeromonadaceae* are listed in *Tables 3.2*, *3.3*, *3.4*, *3.5*, and *3.6*.

Aeromonas Stanier 1943, 213[AL]

Ae.ro.mo'nas. Gr. n. *aer aeros*, air, gas; Gr. fem. n. *monas* unit, monad; N.L. fem. n. Ae*romonas*, gas(−producing) monad.

The members of the genus *Aeromonas* are straight rods with rounded ends, but sometimes can appear as coccobacilli or with filamentous forms. They are 0.3–1.0 μm in diameter and 1.0–3.5 μm in length and can occur singly, in pairs, or even in short chains. Most species are motile by a single, polar flagellum of 1.7 μm wavelength, peritrichous flagella may be formed on solid media in young cultures, and lateral flagella occur in some species. Depending on the strain or species, aeromonads can grow over a wide temperature range (0–45 °C). Mesophilic strains grow between 10 °C and 42 °C (Hänninen 1994), with a temperature optimum range of 22–37 °C. The psychrophilic strains among the *A. salmonicida* subspecies grow at temperatures generally ranging from 2 °C to 30 °C. Clinical isolates are generally incubated at 37 °C, whereas environmental isolates are commonly recovered at 22–30 °C, which compromises interlaboratory comparisons of biochemical reactions. There are some mesophilic strains that appear to be biochemically more active at 22 °C than at 37 °C, or cases of some biochemical tests having quite different results at the two different temperatures for the same strain (Ali et al. 1996). Aeromonads are generally resistant to 150 μg of the vibriostatic agent 2,4 diamino-6,7-diisopropylpteridine (0/129). The mol% G+C range of the DNA is 57–63 %. The type species of the genus is *Aeromonas hydrophila* (Chester 1901) Stanier 1943 (Approved Lists 1980).

Traditionally, the genus *Aeromonas* has been divided into two major groups of species, i.e., motile versus nonmotile species and mesophilic versus psychrophilic species. Whereas this approach is still used throughout literature, atypical strains have been reported in both divisions. For instance, *Aeromonas salmonicida* is generally considered to be a nonmotile species, but strains of this species harboring flagellin genes have been reported (Umelo and Trust 1997). Likewise, *A. salmonicida* is usually referred to as a psychrophilic species not able to grow above 30 °C, whereas some strains may actually grow at 37 °C (Austin 1993).

On standard laboratory media, colonies of motile mesophilic aeromonads are 1–3 mm in diameter, smooth, circumscribed, circular, convex, translucent, and greyish white to buff with a sometimes buttery consistency after 24–48 h incubation at 35 °C. Older colonies can develop a greenish hue, similarly seen with certain *Vibrio* species, and a somewhat strong odor. There can be variation in colony size in some instances, either on original isolation or after subculture. Strains of the species *A. media* can produce a brown diffusible pigment on trypticase soy agar (TSA) (Allen et al. 1983). Colonies of the psychrophilic, nonmotile *A. salmonicida* species are pinpoint in size after 18–24 h at 20–22 °C but after 4 days incubation are circular, convex, entire, friable, and 1–2 mm in diameter (Griffin et al. 1953). Several of the *A. salmonicida* subspecies produce a brown, diffusible pigment after 5 days, especially on media containing tyrosine (Bernoth and Artzt 1989). The presence of conspicuous encapsulation results in the appearance of glistening to mucoid colonies.

Aeromonads are facultative anaerobes that ferment D-glucose to acid or acid with gas and are oxidase and catalase positive, reduce nitrate to nitrites, and are enzymatically very active. Also reported is the production of amylase, DNase, chitinase, elastase, esterases, peptidases, arylamidases, and other hydrolytic enzymes (Hsu et al. 1981; Waltman et al. 1982; Janda 1985; Carnahan et al. 1988; Hänninen 1994). In addition, other enzymes such as adenylyl cyclase 2 (Sismeiro et al. 1998) and collagenase (Yang et al. 2011) have been sporadically reported in *Aeromonas* strains. There is evidence that *Aeromonas* may exhibit anaerobic respiration and dissimilatory metal reduction (Knight and Blakemore 1998). In addition to glucose, both motile and nonmotile aeromonads may assimilate a range of other carbohydrates (Popoff 1984; Arduino et al. 1988; Renaud et al. 1988; Carnahan et al. 1989; Kuijper et al. 1989; Altwegg et al. 1990; Kämpfer and Altwegg 1992; Abbott et al. 1992, 2003; Carnahan and Joseph 1993; Hänninen and Siitonen 1995; Noterdaeme et al. 1996; Oakey et al. 1996a). In their comprehensive study, Abbott et al. (1992) identified a number of common biochemical features among members of HGs 1–15 (excluding nonmotile *A. salmonicida* subspecies of HG3) other than the aforementioned production of acid from glucose, oxidase and catalase activity, and reduction of nitrate. These included production of acid from D-trehalose; failure to utilize malonate or mucate as the sole carbon source; inability to

Table 3.2

Morphological and chemotaxonomic characteristics of genera of the *Aeromonadaceae*

Characteristic	*Aeromonas*	*Tolumonas*	*Oceanimonas*	*Oceanisphaera*	*Zobellella*
Morphology	Straight rods	Straight rods	Straight rods	Coccoid, short rods, or rods	Straight rods
Motility	+ or −	−	+	+ or −	+
Metabolism	Facultative anaerobic	Aerobic and anaerobic	Respiratory	(Facultative) Aerobic	Facultative anaerobes or strict aerobes
Major polar lipids	PG, PE	PG, PE	PG, PE, DPG	PG, PE, DPG	PG, PE
Major fatty acids	$C_{16:0}$, $C_{16:1\omega7c}$, SF 7 ($C_{18:1\omega7c}$, $C_{18:1\omega9t}$, and/or $C_{18:1\omega12t}$)	$C_{16:1\omega7c}$, $C_{16:0}$, $C_{18:1\omega7c}$	$C_{16:1\omega7c}$, $C_{16:0}$, $C_{18:1\omega7c}$	$C_{16:1\omega7c}$, $C_{16:0}$, $C_{18:1\omega7c}$	SF 3 ($C_{16:1\ \omega6c}$ and/or $C_{16:\ 1\omega7c}$), SF 8 ($C_{18:1\omega6c}$ and/or $C_{18:1\omega7c}$), $C_{16:0}$
Main quinone(s)	Q-8	Q-8, MK-8	ND	Q-8	ND
Mol% G+C	57–63	49–52	54–55.6	56.1–60.1	59–64

Abbreviations for polar lipids: *PG* phosphatidylglycerol, *DPG* diphosphatidylglycerol, *PE* phosphatidylethanolamine
ND, no data reported
SF, summed feature, i.e., a group of two or three fatty acids which could not be separated by gas–liquid chromatography using the given instrument used (e.g., the MIDI system)

Table 3.3

Comparison of selected differentiating characteristics of *Tolumonas* species (modified from Caldwell et al. 2011)

Characteristic	*T. auensis* TA 4^T	*T. osonensis* OCF 7^T
Toluene production from phenylalanine or phenylacetate[a]	+	−
Phenol production from tyrosine[a]	+	−
Utilization of		
Arabinose	+	−
Fumarate	−	+
Lactose	−	+
Pyruvate	−	+
Hydrolysis of urea	−	+
Lactate production from glucose	−	+

[a]Tests performed in the presence of another carbon source

ferment adonitol, dulcitol, erythritol, inositol, and D-xylose; and growth in nutrient broth containing 0 and 3 % NaCl. For several other phenotypic properties, test results were nearly always positive (98–99 %) and included motility, β-galactosidase activity, and resistance to O/129. In contrast, less than 2 % of the strains were positive for urea hydrolysis, pectin degradation, or acid production from arabitol, D-raffinose, or D-amygdalin. Variable test results were obtained for production of acetyl methyl carbinol, indole, phenylpyruvic acid, acetate, citrate, and ascorbate utilization; elaboration of elastase, hemolysin, staphylysin, deoxyribonuclease, and corn oil lipase; esculin and gelatin hydrolysis; growth in KCN broth; ornithine and lysine decarboxylase, arginine dihydrolase, and pyrazinamidase activities; H_2S production in gelatin–cysteine–thiosulfate medium; pigment production at 25 °C; gluconate oxidation; susceptibility to cephalothin and ampicillin; and acid production from arabinose, cellobiose, glycerol, lactose, α-methylglucoside, maltose, mannitol, mannose, melibiose, rhamnose, salicin, and sorbitol fermentation. In a follow-up study, Abbott et al. (2003) revisited their previous findings in a phenotypic investigation of 193 strains encompassing 14 genomic species of *Aeromonas*. They also detected that some strains may display a number of properties which are unusual or aberrant in the genus *Aeromonas*, including susceptibility to the vibriostatic agent, fermentation of *m*-inositol and D-xylose, hydrolysis of urea, and the lack of cytochrome oxidase activity.

In combination with other tests, nine discriminatory features selected from the study of Abbott et al. (1992) were included in a diagnostic scheme to assign clinical *Aeromonas* isolates to the most common species (Carnahan et al. 1991a; Furuwatari et al. 1994). However, given that this scheme relies on test results obtained at 35–37 °C, it may not be reliable for the identification of environmental isolates that usually display much lower optimal growth temperatures. Noteworthy, many

Table 3.4

Comparison of selected differentiating characteristics of *Oceanimonas* species (modified from Ivanova et al. 2005)

Characteristic	*O. smirnovii* 31–13^T	*O. doudoroffii* DSM 7028^T	*O. baumannii* GB6^T
Number of flagella	2–5	1–3	1–4
Pigment	Melanin like	ND	None
Reduction of nitrate to nitrite	+	–	–
Tolerance to NaCl (%)	12	5	7
Utilization of			
D-Glucose	+	–	–
Mannitol	+	–	–
Maltose	+	–	–
Gluconate	+	–	–
Caprate	–	+	+
Sucrose	+	–	–
Galactose	–	–	+
L-Lysine	+	ND	–
Glycerol	–	–	+

Data compiled from Baumann et al. (1972), (1983), Brown et al. (2001), and Ivanova et al. (2005)

rapid miniaturized biochemical identification systems fail to accurately recognize motile *Aeromonas* species belonging to rarer clinical species (Hickman-Brenner et al. 1987; Janda 1991; Carnahan et al. 1991a; Janda and Abbott 1998; Abbott et al. 1992, 1998). Also for typical and nontypical nonmotile *A. salmonicida* strains, extensive biochemical studies including large numbers of isolates have been published (Griffin et al. 1953; Smith 1963; Schubert 1974; Popoff 1984; Austin et al. 1989; Dalsgaard et al. 1994; Hänninen et al. 1995). However, several discrepancies for key reactions previously published as useful for separating the various subspecies have been identified (Millership 1996), possibly resulting from differences in the number of strains under study and variations between test parameters such as temperature and time of incubation. In addition, several studies have reported atypical strains of *A. salmonicida* (Hänninen et al. 1995; Hänninen and Hirvelä-Koski 1997; Austin et al. 1998; Wiklund and Dalsgaard 1998) which are taxonomically difficult to place in one of the subspecies of this taxon. This situation is further complicated by a lack of standardization of methods both within and between laboratories for phenotypic identification of *A. salmonicida* (Dalsgaard et al. 1998).

Hemolysin production is variable both among and between *Aeromonas* species. In addition, differences can occur in the presence and/or the type of hemolysis displayed depending on the type of red cells used (Brenden and Janda 1987). Among the more commonly isolated species, *A. hydrophila* and *A. veronii* are strongly β-hemolytic on sheep blood agar, but also less commonly isolated species may exhibit this type of hemolysis. β-hemolysis can be displayed as a broad zone, a double zone of partial hemolysis, or a narrow zone of β-hemolysis just under the colony edge. Most *A. caviae* isolates are partially hemolytic, displaying α-hemolysis, but can also display narrow zones of β-hemolysis (Carnahan and Joseph 1993).

Tolumonas Fischer-Romero, Tindall, and Jüttner 1996

N.L. n. *toluolum*, toluol (German for toluene); L. fem. n. *monas*, monad unit; N.L. fem. n. *Tolumonas*, toluene-producing unit.

General features as described here are based on the original genus description (Fischer-Romero et al. 1996) and a subsequent emended description (Caldwell et al. 2011). Members of the genus *Tolumonas* are nonmotile and occur as straight rods (2.5–3.6 × 0.9–1.2 μm) singly and in pairs. Organisms grow under oxic and anoxic conditions. Colonies of *T. auensis* observed on agar plates and agar deeps were 0.3–0.5 mm in diameter, lens shaped, and white. Anaerobic cultures are catalase and oxidase negative, whereas aerobic cultures are catalase positive and oxidase negative. Growth occurs between 12 °C and 37 °C, with an optimum at 22 °C. The pH in which growth was observed ranges from 5.5 to 8.5, with optimal growth occurring between pH 7.0 and 8.0. The mol% G+C range of the DNA is 49–52 %. The type species of the genus is *Tolumonas auensis* Fischer-Romero et al. 1996.

The major fermentation products in peptone–yeast extract–glucose cultures are ethanol, acetic acid, and formic acid. Indole is not formed. End products from glucose are acetate, ethanol, formate, and/or lactate. In the presence of air (oxic conditions), the following organic compounds are catabolized: L-arabinose, D-fructose, D-galactose, and D-glucose.

Additional species-specific characteristics can be found in the descriptions of *T. auensis* (Fischer-Romero et al. 1996) and *T. osonensis* (Caldwell et al. 2011), albeit sometimes tested using different methods and test conditions. Differentiating phenotypic properties for the two currently described *Tolumonas* species are displayed in *Table 3.3*.

Table 3.5

Comparison of selected differentiating characteristics of *Oceanisphaera* species (Modified from Shin et al. 2012)

Characteristic	*O. litoralis* KMM 3654^T	*O. donghaensis*	*O. ostreae* T-w6^T	*O. sediminis*	*O. arctica* V1-41^T
Salinity range (%, w/v)	0.5–10.0	0.5–8.0	0–10.0	0–12.0	0–3.0
Motility	+	+	+	+	–
Assimilation of					
Citrate	+	v	–	ND	+
Malate	+	w	+	ND	ND
D-Arabitol	w	–	ND	–	–
Maltose	w	–	ND	–	–
3-Methyl glucose	+	+	ND	+	w
Methyl α-D-glucoside	+	+	ND	w	w
Methyl β-D-glucoside	+	+	ND	w	w
Methyl α-D-mannoside	–	–	ND	–	+
Palatinose	–	–	ND	–	w
Sedoheptulosan	w	–	ND	–	+
Lactamide	w	+	ND	w	w
D-Lactic acid methyl ester	w	w	ND	–	–
L-Lactic acid	w	–	ND	–	+
D-Malic acid	w	–	ND	–	–
L-Malic acid	w	+	ND	+	+
Succinic acid monomethyl ester	w	w	ND	–	w
Pyruvic acid	–	w	+	–	+
Succinic acid	–	–	+	–	+
L-Alaninamide	w	w	ND	+	+
D-Alanine	–	+	ND	–	+
L-Alanyl glycine	–	+	ND	+	–
L-Glutamic acid	w	+	ND	+	+
Glycyl L-glutamic acid	–	w	ND	–	–
L-Serine	+	+	ND	–	+
2,3-Butanediol	–	–	ND	w	w
Glycerol	–	+	ND	+	w
Adenosine	–	–	ND	+	+
Acid production from					
D-Ribose	+	w	ND	+	+
D-Fructose	+	–	–	–	w
Enzyme activity					
Esterase lipase (C8)	–	w	–	w	–
Valine arylamidase	w	–	–	w	w
Acid phosphatase	w	w	ND	w	+
Naphthol-AS-BI-phosphohydrolase	w	w	–	w	+
β-Glucuronidase	+	–	–	–	–
Hydrolysis of					
Urea	+	–	+	–	+
Gelatin	–	–	+	–	–

Data of carbon source assimilation, acid production, and enzyme activity for *O. litoralis* KMM 3654^T, *O. donghaensis*, *O. sediminis*, and *O. arctica* V1-41^T were determined by Shin et al. (2012) using GN2MicroPlate (Biolog), API 50CHB, and API ZYM or API 20NE test strips (bioMérieux). Other data for these species were retrieved from the original species descriptions. Data for *O. ostreae* T-w6^T were from Choi et al. (2011)

ND no data reported, *w* weakly positive

Table 3.6

Comparison of selected differentiating characteristics of *Zobellella* species (modified from Yi et al. 2011)

Characteristic	*Z. denitrificans* ZD1^T	*Z. taiwanensis* ZT1^T	*Z. aerophila* JC2671^T
Growth in 0 % NaCl	+	+	–
Anaerobic growth on marine agar	w	w	–
Facultative anaerobic growth with KNO_3	+	+	–
Reduction of nitrate to nitrite	–	–	+
Reduction of nitrate to nitrogen	+	+	–
Fermentation of glucose	+	+	–
Decomposition of Tween 20	–	–	+
Decomposition of gelatin	–	–	V
Arginine dihydrolase	–	–	+
Lysine decarboxylase	–	–	+
Ornithine decarboxylase	–	–	+
Urease	–	–	+
API ZYM enzyme activities			
Valine arylamidase	w	w	–
Naphthol-AS-BI-phosphohydrolase	w	w	–
Acid phosphatase	+	w	–
α-Glucosidase	+	w	–
Fermentation in API 50CH of			
Glycerol	+	–	+
Erythritol	+	–	–
D-Galactose	+	–	–
Salicin	+	–	–
Melibiose	+	–	–
Trehalose	+	–	–
Melezitose	+	–	–
Raffinose	+	–	–
Turanose	+	–	–
Potassium gluconate	+	–	–
D-Ribose	+	+	–
D-Glucose	+	+	–
Maltose	+	+	–
Methyl α-D-glucopyranoside	+	+	–
Sucrose	+	+	–
Starch	+	+	–
D-Adonitol	–	+	–
Xylitol	–	+	–
L-Arabitol	–	+	–

Data were obtained from Yi et al. 2011
w weakly positive, *v* variable

Oceanimonas Brown, Sutcliffe, and Cummings 2001, 71^{VP}

Oceanimonas (O.ce.a.ni.mo'nas. L. n. *oceanus* ocean; L. fem. n. *monas* monad, unit; N.L. fem. n. *Oceanimonas* ocean monad). Note: The original spelling *Oceanomonas* (Brown et al. 2001) was corrected to *Oceanimonas* by the List Editor of International Journal of Systematic and Evolutionary Microbiology (2001, 51, 269).

General features as described here are based on the original genus description (Brown et al. 2001) and a subsequent emended description (Ivanova et al. 2005). Members of the

genus *Oceanimonas* are straight rods, 0.7–1.2 × 1.5–2.5 µm, and motile by one to four polar flagella (Brown et al. 2001; Ivanova et al. 2005). On Marine Agar 2216 (Difco), colonies of *O. smirnovii* are 1–3 mm in diameter, convex, regular, butyrous, and light creamy. They are obligately respiratory with molecular oxygen as the terminal electron acceptor and oxidase positive. *O. doudoroffii* and *O. baumannii* have an absolute requirement for Na^+ for growth (Brown et al. 2001). In contrast, *O. smirnovii* does not have this obligate requirement (Ivanova et al. 2005). Growth occurs between 10 °C and 45 °C, with an optimum at 25–30 °C. The mol% G+C range of the DNA is 54–55.6 %. The type species of the genus is *O. doudoroffii* corrig (Baumann et al. 1972) Brown et al. 2001.

All members of *Oceanimonas* have a chemoorganotrophic lifestyle and are able to utilize malate, citrate, ethanol, betaine, sarcosine, sodium succinate, L-proline, and L-glutamate. All members are negative for amylase, lipase, gelatinase, agarase, arginine dihydrolase, and utilization of D-arabinose, mannose, adipate, phenylacetate, and L-valine. *O. doudoroffii* has been shown to have at least three different lyases which can cleave dimethylsulfoniopropionate, an abundant compatible solute made by different classes of marine phytoplankton (Curson et al. 2012). *O. doudoroffii* and *O. baumannii* can degrade phenol.

Additional species-specific characteristics can be found in the original species descriptions (Brown et al. 2001; Ivanova et al. 2005), albeit sometimes tested using different methods and test conditions. Differentiating phenotypic properties for the three currently described *Oceanimonas* species are displayed in ❯ *Table 3.4*.

Oceanisphaera Romanenko, Schumann, Zhukova, Rohde, Mikhailov, and Stackebrandt 2003

Oceanisphaera (O.ce.a.ni.sphae'ra. L. masc. n. *oceanus* ocean; L. fem. n. *sphaera* ball, globe, sphere; N.L. fem. n. *Oceanisphaera* oceanic sphere).

General features as described here are based on the original genus description (Romanenko et al. 2003) and subsequent descriptions of new *Oceanisphaera* species (Park et al. 2006; Choi et al. 2011; Shin et al. 2012; Srinivas et al. 2012). Members of this genus are coccoid (0.5–1.2 µm) or occur as short rods or rods (0.5–1.0 × 1.2–5.0 µm). On Marine Agar 2216 (Difco), strains produce smooth, shining, slightly beige to light greyish-yellow colonies mostly with regular edges that are 1–5 mm in diameter after 2–3 days incubation at 25 °C. Diffusion of a yellowish pigment into the medium was observed for *O. litoralis* KMM 3654^T. They are motile by means of a single polar flagellum except for *O. arctica* which is nonmotile. *Oceanisphaera* include aerobes with a chemoorganotrophic lifestyle. They have an absolute requirement for sodium ions which varies between species and are considered moderately halophilic. Members are oxidase and catalase positive. Growth occurs between 4 °C and 42 °C, with an optimum at 25–30 °C. The pH range in which growth was observed is very broad, from pH 5.0 to 11.0, with optimal growth occurring between pH 7.0 and 8.0. The mol% G+C range of the DNA is 56.1–60.1 %. The type species of the genus is *O. litoralis* Romanenko et al. 2003.

All members are positive for nitrate reduction. They do not or only weakly hydrolyze aesculin. They do not produce acid from glucose, sucrose, maltose, arabinose, and galactose. Members of *O. donghaensis* have manganese-oxidizing capacity (Park et al. 2006).

Additional species-specific characteristics can be found in the original species descriptions (Romanenko et al. 2003; Park et al. 2006; Choi et al. 2011; Shin et al. 2012; Srinivas et al. 2012), albeit sometimes tested using different methods and test conditions. Differentiating phenotypic properties for the currently described *Oceanisphaera* species are displayed in ❯ *Table 3.5*.

Zobellella Lin and Shieh 2006

Zobellella (Zo.bell.el'la. N.L. dim. ending -ella; N.L. fem. n. *Zobellella* named after C. E. ZoBell, a pioneer marine microbiologist).

General features as described here are based on the original genus description (Lin and Shieh 2006) and the emended description of Yi et al. (2011). Members of *Zobellella* occur as straight rods (1.0–6.9 × 0.5–1.1 µm). They are motile by means of a single, polar flagellum. The organisms require NaCl for growth (*Z. aerophila*) or for growth stimulation (*Z. denitrificans* and *Z. taiwanensis*). Colonies of *Z. denitrificans* and *Z. taiwanensis* produced on polypeptone–yeast extract (PY) plate medium are circular, off-white, and nonluminescent. Colonies of *Z. aerophila* on Marine Agar 2216 (Difco) at 30 °C are cream colored, convex, and circular with entire margins. Growth occurs in 0–14 % NaCl, with optimal growth in 1–3 % NaCl. They are facultative anaerobes or strict aerobes, capable of both respiratory and fermentative metabolism. Oxidase and catalase positive. Growth occurs between 5 °C and 45 °C, with an optimum at 30–35 °C, but no growth is observed at 4 or 50 °C. The organisms are able to grow in a pH range from 5.0 to 10.5, with optimal growth occurring between pH 7.0 and 8.0. The mol% G+C range of the DNA is 59–64 %. The type species of the genus is *Zobellella denitrificans* Lin and Shieh 2006.

All members reduce nitrate to nitrite (*Z. aerophila*) or nitrogen (*Z. denitrificans* and *Z. taiwanensis*). *Zobellella* strains are able to ferment D-mannitol and myo-inositol with production of acid, but no gas. They cannot ferment D-arabinose, L-arabinose, lactose, xylose, or dulcitol. Negative for agarase, amylase, and lipase. H_2S is not produced from thiosulfate, and indole is not produced from tryptophan. Alkaline phosphatase and leucine arylamidase activities were detected in API ZYM tests.

Additional species-specific characteristics can be found in the original species descriptions (Lin and Shieh 2006; Yi et al. 2011), albeit sometimes tested using different methods and test conditions. Differentiating phenotypic properties for the currently described *Zobellella* species are displayed in ❯ *Table 3.6*.

Isolation, Enrichment, and Maintenance Procedures

A variety of primary plating media has been proposed for the isolation of *Aeromonas* species from aquatic, clinical, fish, and food samples (von Graevenitz and Bucher 1983; Joseph et al. 1988; Farmer III et al. 1992; Moyer 1996). Below, a concise overview is given of the most commonly used formulations and protocols per sample type.

For isolation of aeromonads from environmental samples, dextrin fuchsin sulfite (DFS) in conjunction with membrane filtration for enumeration of aeromonads from unpolluted waters has long been recommended (Schubert 1967, 1987). The DFS-based approach was followed by a selective and differential Rimler Shotts (RS) agar, later modified into MRSM (Shotts and Rimler 1973; Seidler et al. 1980). Rippey and Cabelli (1979) formulated the m*Aeromonas* (mA) medium containing trehalose, ampicillin, and ethanol for use with a membrane filter. In a comparative study including 11 media for isolation of aeromonads from polluted waters, mA was reported as the preferred medium followed by DNase–toluidine blue–ampicillin agar (DNTA), MacConkey Tween 80 (MACT), and starch bile (SB) agar (Arcos et al. 1988). The usefulness of mA was later also confirmed in other studies (Poffe and Op de Beeck 1991; Bernagozzi et al. 1994). According to Moyer (1996), ampicillin dextrin agar (ADA), mA, starch ampicillin (SA), Pril xylose ampicillin agar (PXA), and starch–glutamate–ampicillin–penicillin-based medium (SGAP-10) appear to be the most widely used plating media. Multiple studies have evaluated different media and sample processing methods for isolation of aeromonads from drinking water sources (Burke et al. 1984a, b; Millership and Chattopadhyay 1985; Cunliffe and Adcock 1989; Havelaar et al. 1990; Moyer et al. 1992a). A study comparing ADA, xylose ampicillin agar (XAA), *Aeromonas* agar (Difco), and Ryan's *Aeromonas* Agar found the latter medium to be best performing (Holmes and Sartory 1993). CromoCen® AGN agar, on which *Aeromonas* forms colonies with light green, greenish, and salmon pigments with or without a surrounding wide transparent zone (halo), is a chromogenic medium which showed satisfactory specificity and sensitivity for the recovery of aeromonads from various samples (Aguilera-Arreola et al. 2012).

Recovery of *Aeromonas* from sterile body sites is achievable on primary media such as MacConkey or blood agar. The latter allows the simultaneous detection of hemolysis, oxidase, and indole production, as well as the presence of more than one *Aeromonas* species (Janda et al. 1984). On the other hand, it has to be kept in mind that fecal isolates of lactose-fermenting *A. caviae*-like strains on MacConkey agar may be overlooked for further identification. The majority of *Aeromonas* species can be recovered from fecal specimens on Yersinia Selective Agar (YSA, Difco) (Alonso et al. 1996), a modified version of CIN (Cefsulodin–Irgasan–Novobiocin) Agar which is normally used for the selection of *Yersinia enterocolitica* (Altorfer et al. 1985). This way, YSA allows simultaneous detection of *Aeromonas* and *Yersinia* from fecal matter at 25 °C for 28 h. Although it is common practice to supplement blood agar with varying concentrations of ampicillin (10–30 mg/l) for inhibition of normal enteric microbiota (Mishra et al. 1987; Kelly et al. 1988), ampicillin-susceptible strains of clinically significant *Aeromonas* species may be missed. In fact, most strains of *A. trota* and several strains of *A. caviae* are susceptible to 10 mg/l (Carnahan et al. 1991b; Kilpatrick et al. 1987; Singh and Sanyal 1994). An international multilaboratory study to establish the optimal culture media, incubation time, and incubation temperature found that enrichment in alkaline peptone water (APW) in combination with blood agar containing 10 mg/l ampicillin exhibited the highest recovery rate (Moyer et al. 1991). In addition, also Ryan's *Aeromonas* Medium Base (Oxoid) with an Ampicillin Selective Supplement can also be used for isolation of clinical species. APW adjusted to pH 8.6–9.8 is commonly used for enrichment of aeromonads from stool samples (Millership et al. 1983; Moulsdale 1983; Moyer et al. 1991; von Graevenitz and Bucher 1983). The basic formulation contains 1 % peptone in water at pH 8.6, but some workers increased the pH to 9.8 and added ampicillin (10–40 mg/l) and/or 0.5 % desoxycholate to increase the medium's selectivity (Khardori and Fainstein 1988). Between studies, there is little consensus on the choice of the incubation temperature (25 °C or 37 °C) for APW enrichments (Millership and Chattopadhyay 1984; Price and Hunt 1986). Enrichment with APW has been shown to increase the number of *A. caviae* isolates, suggesting that APW may be detecting not bona fide pathogens, but merely transient colonizers (Moyer et al. 1991).

Several studies have compared media for isolation of aeromonads from foods, all of which with slightly different recommendations for the best medium to use (Fricker and Tompsett 1989; Gobat and Jemmi 1995). Media recommended for the detection of motile aeromonads from food samples include starch–ampicillin agar (SA), blood agar containing 30 mg/l ampicillin, bile salts Irgasan brilliant green agar (BIBG), Ryan's *Aeromonas* Agar (Oxoid, Basingstoke, England), and APW (Palumbo et al. 1985a; Moyer 1996). For the isolation of *Aeromonas* from oysters, trypticase soy ampicillin broth (TSAB 30 mg/l) and MacConkey agar have been recommended (Abeyta et al. 1986). Food samples may be placed in enrichment broths to detect low numbers of aeromonads and/or to resuscitate cell injured or stressed cells. The most frequently used broths for enrichment and enumeration of aeromonads from foods by the most probable number (MPN) technique are trypticase soy ampicillin (30 mg/l) broth (TSAB) and APW with or without ampicillin.

A. salmonicida strains can be isolated on trypticase soy agar (TSA) at 22–25 °C for 48 h, mostly with the presence of brown pigmentation. The recovery of typical and atypical *A. salmonicida* may increase when an enrichment procedure with tryptone soy broth (TSB) is used (Austin and Adams 1996). Strains of *A. salmonicida* do not display optimal growth on enteric agars and TCBS (thiosulfate citrate bile salts sucrose), the medium that is generally used for the isolation of *Vibrio* species. For isolation of aeromonads from piscine sources, RS medium and SGAP-10C (Jenkins and Taylor 1995) are generally recommended.

Maintenance of *Aeromonas* stock cultures can be achieved by lyophilization or cryopreservation in TSB with 15 % glycerol at −20 °C for a few months or with 20 % glycerol at −70 °C for several years or in serum inositol broth (consisting of 25 g of inositol dissolved in 50 ml of distilled water, filter sterilized, and aseptically added to 450 ml of sterile calf serum) at −70 °C for long-term storage. Working cultures of *Aeromonas* spp. can be maintained for short-term storage on standard laboratory media that do not contain fermentable carbohydrates at 5 °C (Moyer 1996). Marine isolates of *Aeromonas* may be maintained on a medium containing 0.5 % peptone, 0.1 % yeast extract, 2.4 % NaCl, 0.7 % Mg_2SO_4, 0.075 % KCL, and 1.5 % agar (Effendi and Austin 1991).

As for members of the rare genera *Tolumonas*, *Oceanimonas*, *Oceanisphaera*, and *Zobellella*, it has to be remarked that details of isolation, enrichment, and maintenance procedures are restricted to the information described in the original species proposals, in many cases based on single-strain studies.

Members of the genus *Tolumonas* were isolated on minimal media following anoxic or anaerobic enrichment. The basal toluene production medium (TP medium), a modified medium described by Eichler and Pfennig (1988), was used for enrichment, isolation, and cultivation of *T. auensis* TA 4^T (Fischer-Romero et al. 1996). The latter authors reduced the concentrations of sodium sulfide and glucose in TP medium to 5 mM and 1 g/l, respectively, added yeast extract (10 mg/l) and phenylalanine (0.4 mM), and adjusted the pH to 7.2. Using screw-capped bottles, this medium was inoculated with anoxic sediment sample and incubated at 22 °C in the dark without shaking. When the toluene concentration in the bottles was equal to the concentration of the added phenylalanine, aliquots were transferred to fresh TP medium, and this was repeated until morphological features of the bacterial population became stable. Aliquots of the resulting liquid culture were used to isolate toluene-producing bacteria by the agar deep dilution method (Widdel and Pfennig 1984). The toluene-producing isolate was routinely cultured by plating on solid TP medium prepared from double-strength TP medium with the addition of 2 % agar. *T. osonensis* OCF 7^T was isolated following incubation of a sediment sample in anaerobic enrichment medium (pH 7.3) based on mineral salts and trace metal solutions, vitamins, TES, yeast extract (Difco), and $NaHCO_3$, with addition of cysteine sulfide (Tanner 2007) as reducing agent and fructose (0.5 %) as substrate (Caldwell et al. 2011). Subsequent dilutions of the bacterial enrichments and isolation were performed using agar roll tubes (Hungate 1969). The isolate was propagated on the same medium with an increased yeast extract concentration (0.5 g/l). Specific maintenance procedures were not reported for *Tolumonas* spp.

Strains of *Oceanimonas* were isolated using quite diverse medium formulations. Members of *O. doudoroffii* were first isolated on an agar medium containing allantoin, benzoate, creatine, caprylate, or D-aminovalerate after enrichment in a liquid medium with the same composition (Baumann et al. 1972). *O. baumannii* GB6^T was isolated following enrichment in a liquid salts medium containing phenol as primary carbon source and 5 % NaCl at 25 °C for 7 days and inoculation on a solid medium with the same composition (Brown et al. 2001). *O. smirnovii* 31-13^T was isolated by direct plating of seawater onto Marine Agar 2216 (Difco) or medium B based on peptone, casein hydrolysate, yeast extract, glucose, and 50 % (v/v) natural seawater and incubation at 22–25 °C for 5–10 days (Ivanova et al. 2005).

Maintenance of *O. doudoroffii* strains was achieved on Marine Agar 2216 (Difco) with monthly transfer (Baumann et al. 1972). The type strain of *O. smirnovii* was stored at −80 °C in Marine Broth 2216 (Difco) supplemented with 20 % (v/v) of glycerol.

Strains of *Oceanisphaera* were mostly isolated on Marine Agar 2216 (Difco) without prior enrichment. *O. litoralis* KMM 3654^T was isolated by serially diluting a sand sample in sterile seawater and spreading an aliquot of each dilution on Marine Agar 2216 (Difco) followed by incubation at 28 °C for 7 days (Romanenko et al. 2003). Strains of *O. donghaensis* were isolated by diluting a sediment sample in filtered seawater and spreading aliquots of each dilution on an artificial marine agar medium (Stein et al. 2001) containing 1 mM $MnCl_2$ at 17 °C for 2 weeks (Park et al. 2006). *O. ostreae* T-w6^T and strains of *O. sediminis* were isolated by means of the standard dilution plating technique on Marine Agar 2216 (Difco) at 25 °C (Choi et al. 2011; Shin et al. 2012). *O. arctica* V1-41^T was isolated by suspending a sediment sample in saline (0.9 %, w/v NaCl) and plating serial dilutions of this suspension in saline on Zobell marine agar (Zobell 1941) at 4 °C for 15 days (Srinivas et al. 2012).

Maintenance of *O. litoralis* KMM 3654^T was achieved by storage at −80 °C in liquid medium supplemented with 30 % (v/v) glycerol (Romanenko et al. 2003). The strain was routinely grown on Marine Agar 2216 or TSA or in Marine Broth 2216 (all from Difco). Isolates of *O. donghaensis* were stored as glycerol suspensions (20 %, w/v) at −70 °C (Park et al. 2006). *O. ostreae* T-w6^T, *O. sediminis*, and *O. arctica* V1-41^T were routinely grown on Marine Agar 2216 at 25–30 °C (Choi et al. 2011; Shin et al. 2012; Srinivas et al. 2012).

The first two species described in the genus *Zobellella* were isolated using an enrichment cultivation method (Lin and Shieh 2006). For isolation of *Z. denitrificans* ZD1^T and *Z. taiwanensis* ZT1^T, sediment samples were vigorously shaken in 95 ml sterile NaCl–MOPSO buffer (20 g NaCl and 0.45 g 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (MOPSO) in 1 l deionized water, pH 7.0). The shaken solutions were decimally diluted with the same buffer, after which 1 ml of each dilution was transferred to a rimless tube (16 mm × 10 cm) containing 5 ml polypeptone–yeast extract–nitrate broth medium (Lin and Shieh 2006) in which an inverted Durham insert for gas accumulation had been placed. Following aerobic enrichment at 25 °C in the dark for 3–7 days, visually turbid cultures that produced gas were streaked on polypeptone–yeast extract (PY) agar medium (Lin and Shieh 2006) and further purified. *Z. aerophila* JC2671^T was isolated using a standard dilution plating method on Marine Agar 2216 (Yi et al. 2011).

For maintenance, *Z. denitrificans* ZD1^T and *Z. taiwanensis* ZT1^T were inoculated in PY stab medium and stored at 25 °C (Lin and Shieh 2006). *Z. aerophila* JC2671^T was maintained in a glycerol suspension (20 %, w/v) at −80 °C (Yi et al. 2011).

Ecology

The natural habitats of mesophilic aeromonads include various aquatic environments such as freshwater, estuarine (brackish) water, surface water (especially recreational), drinking water supplies (including treated, well water, and bottled water), and polluted waters (Hazen et al. 1978; Seidler et al. 1980; Rippey and Cabelli 1980, 1985; Kaper et al. 1981; LeChevallier et al. 1982; Van der Kooj 1988; Araujo et al. 1989; Alonso et al. 1994; Holmes et al. 1996; Pablos et al. 2009; Figueira et al. 2011). They have also been isolated from wastewater effluent sludge and sewage (Schubert 1975; Monfort and Baleux 1991; Rahman et al. 2007). Although mesophilic aeromonads are not considered to be typical marine organisms, they may occur in estuarine and coastal environments where seas and oceans interface with freshwaters (Hazen et al. 1978). They do not commonly occur in groundwaters, which are usually poor in nutrients (Havelaar et al. 1990). In nutrient-rich waters, however, mesophilic *Aeromonas* spp. can reach high numbers. Burke et al. (1984a, b) reported a seasonal distribution in both temperate freshwater lakes and chlorinated drinking water, usually with highest numbers during the summer months.

Due to their omnipresence in surface waters used for the production of drinking water, *Aeromonas* have gained interest as indicators of chlorine resistance, disinfection efficacy, regrowth potential, and biofilm development during both production and distribution of potable water (Holmes and Niccolls 1995; Holmes et al. 1996; Kühn et al. 1997a, b; Sisti et al. 1998; Massa et al. 1999; Chamorey et al. 1999). As a result, public health authorities in the Netherlands proposed guidelines for maximum levels of *Aeromonas* in drinking water. These levels were set at 20 CFU/100 ml for water leaving the production plant and 200 CFU/100 ml for drinking water in distribution (Van der Kooj 1988). Aeromonads may also form biofilms in exhumed pipes (LeChevallier et al. 1987; Holmes and Niccolls 1995), and a part of this population may still be present after disinfection with 1 mg/l chlorine. This suggests that planktonic cells may be relatively susceptible to disinfection, but that populations associated with the biofilm may survive high chlorine dosing. It has been demonstrated that quorum-sensing mechanisms may trigger the development of *A. hydrophila* biofilms. Using continuous-flow chamber experiments, it was shown that biofilm formation by *A. hydrophila* strain AH-1 N is regulated by an *N*-acylhomoserine lactone (AHL)-dependent quorum-sensing system (Lynch et al. 2002). In natural mineral waters, it has been suggested that *Aeromonas* spp. may develop localized biofilms with no exogenous contamination of the aquifer (Villari et al. 2003).

Aeromonas spp. may infect a range of aquatic animals including fishes, reptiles, and amphibians but may also cause infections in humans and other animals following exposure to aquatic environments (Austin and Adams 1996; Gosling 1996; Janda and Abbott 1996, 1998). In general, human infections are associated with *Aeromonas* species and HGs other than those isolated from drinking water and environmental sources. Still, putative virulence factors have been reported in both populations (Holmberg et al. 1986; Millership et al. 1986; Cahill 1990; Havelaar et al. 1992; Kirov et al. 1994; Hänninen 1994, 1995; Holmes and Niccolls 1995; Kühn et al. 1997a, b). In this context, some workers have stressed the need for more studies to compare the taxonomic distribution of aeromonads in aquatic environments versus those associated with human gastroenteritis (Holmes et al. 1996; Joseph 1996; Khajanchi et al. 2010).

Several *Aeromonas* species have been isolated from a range of food sources, including raw chicken (Kirov et al. 1990; Akan et al. 1998), milk (Santos et al. 1996; Eneroth et al. 1998), cheese (Santos et al. 1996), ground meats (Okrend et al. 1987; Singh 1997), seafood (Tsai and Chen 1996; Pianetti et al. 1997; Lipp and Rose 1997; Di Pinto et al. 2012), poultry eggs (Yadav and Verma 1998), fish, fish eggs and shrimp (Hänninen et al. 1997), sausages (Fontes et al. 2012), pigs and swine slaughter plants (Palumbo and Yu 1999; Fontes et al. 2010, 2011), and vegetables (Callister and Agger 1987; Pedroso et al. 1997; McMahon and Wilson 2001; Xanthopoulos et al. 2010; Elhariry 2011). Most probably, the occurrence of aeromonads in foods reflects contact of these food matrices with untreated or processed water. A series of factors may determine the survival and outgrowth of *Aeromonas* spp. in food products such as temperature, water activity, pH, atmosphere, NaCl, nitrite, and essential oils of spices (Stecchini et al. 1993; Palumbo et al. 1985b, 1996). Although raw meats and fresh grocery produce are a possible source for *Aeromonas* to be ingested by humans (Kirov 1993; Palumbo 1996), a conclusive link between the consumption of *Aeromonas*-containing food and diarrheal disease has not yet been established. Still, also in food isolates of *Aeromonas*, a range of putative virulence factors have been detected (Palumbo 1996; Pin et al. 1997; Pedroso et al. 1997). Possibly, the lack of clear cases of *Aeromonas*-associated food poisoning may be related to the fact that expression of the known virulence factors is temperature dependent and/or that a series of unknown virulence factors are involved in the disease process (Daily et al. 1981; Palumbo 1993).

Members of *A. salmonicida* inhabit natural waters and have been isolated from aquaculture ponds where salmonid fish is produced. This psychrophilic species is considered an important cause of furunculosis in various species of fishes (Austin and Adams 1996; Wiklund and Dalsgaard 1998). Strains are more difficult to isolate from the surrounding aquatic environment than from the kidney of the infected fish. This cryptic character may be associated with the recovery of cell wall-deficient forms or so-called L forms (McIntosh and Austin 1990).

A few *Aeromonas* species are known to occur as endosymbionts in medicinal leeches. It is well documented that *A. veronii* biovar sobria is the main culturable bacterium in the digestive tract of the medicinal leech *Hirudo medicinalis* (Graf 1999b), and that this may give rise to *Aeromonas* infections in plastic surgery patients (Whitaker et al. 2011). In culture-independent studies, it was later shown that *A. veronii* biovar sobria constitutes a two-member bacterial community in *H. medicinalis* together with an uncultured novel member of the *Rikenellaceae* (Worthen et al. 2006). In the North American medicinal leech *Macrobdella decora*, *A. jandaei* was found as the dominant

culturable symbiont in leeches from a broad geographic area (Siddall et al. 2007). In the digestive tract microbiota of *Hirudo orientalis*, a European medicinal leech, isolates of both *A. veronii* and *A. jandaei* were recovered together with uncultured *Rikenella*-like bacteria (Laufer et al. 2008). Collectively, these findings suggest the possibility of a species-specific habitat of *Aeromonas* species in leeches.

As for members of the rare genera *Tolumonas*, *Oceanimonas*, *Oceanisphaera*, and *Zobellella*, it has to be remarked that insights in their natural habitats and general ecology are very limited and almost exclusively based on the minimal information of sample origins given in the original species proposals. Moreover, in many cases these species descriptions concern single-strain studies.

Strains of *Tolumonas* spp. have so far been exclusively isolated from anoxic freshwater sediments. *T. auensis* TA 4^T was recovered from anoxic sediment obtained from the shallow eutrophic freshwater Lake Au, a separate part of Lake Zürich in Switzerland (Fischer-Romero et al. 1996). *T. osonensis* OCF 7^T originates from an anoxic sediment sample collected from the freshwater Oso Creek, Corpus Christi, Texas, USA (Caldwell et al. 2011).

Members of the genus *Oceanimonas* seem to be associated with aquatic environments, preferably of marine origin. Strains of *O. doudoroffii* were first isolated from surface water of the Pacific Ocean, whereas *O. baumannii* GB6^T was isolated from estuary mud (Brown et al. 2001). The type strain of *O. smirnovii* 31-13^T was isolated from seawater of the Black Sea (Ivanova et al. 2005).

Oceanisphaera strains have so far primarily been isolated from marine sediments. Habitats where members of this genus have been found include bottom sand from the coastal seawater area of Peter the Great Bay, Sea of Japan (Russia), for *O. litoralis* KMM 3654^T (Romanenko et al. 2003), sediment of the East Sea (Korea) for *O. donghaensis* (Park et al. 2006), sediment from a cage-cultured ark clam farm in Gangjin Bay (Korea) for *O. sediminis* (Shin et al. 2012), and sediment from the Kongsfjorden fjord (Spitsbergen) for *O. arctica* V1-41^T (Srinivas et al. 2012). In contrast, *O. ostreae* T-w6^T was not isolated from sediments but from seawater of an oyster farm on the southern coast of Korea (Choi et al. 2011).

All currently known members of *Zobellella* appear to be associated with sediment or sand. *Z. denitrificans* ZD1^T and *Z. taiwanensis* ZT1^T were recovered from sediment samples collected from estuarine mangrove ecosystems in Taiwan (Lin and Shieh 2006). *Z. aerophila* JC2671^T was isolated from seashore sand in Dokdo, Korea (Yi et al. 2011).

Pathogenicity, Clinical Relevance

Historically, *Aeromonas* has long been considered as an opportunistic organism notorious for infections in immunocompromised individuals. However, improved isolation and identification strategies have somewhat changed that view and now also provide evidence for the role of certain *Aeromonas* species as a causal agent of extraintestinal diseases and for a strong statistic association with gastrointestinal infections (as reviewed by Janda 1991; Janda and Abbott 1996, 1998, 2010; Joseph 1996; Figueras 2005; Parker and Shaw 2011).

Isolation rates have ranged from <1 % to >60 % in diarrheic populations in various geographic locations. The first well-documented extraintestinal infection concerns a case of septicemia in a menstruating female after swimming in Jamaican waters. The isolate was initially identified as *Vibrio jamaicensis* but was later shown to be a member of the genus *Aeromonas* (Hill et al. 1954; Caselitz 1955). Since, a large variety of extraintestinal infections including wound infections, septicemia, meningitis, ophthalmitis, surgical site infections, and infections associated with leech therapy have been attributed to *Aeromonas* (Altwegg 1985; Altwegg and Geiss 1989; Janda 1991; Janda and Abbott 1996, 1998; Graf 1999a; Tena et al. 2009; Lamy et al. 2009). Due to the lack of significantly large outbreaks and the unsuccessful outcome of one human volunteer study (Morgan et al. 1985), there is still no definite proof to recognize *Aeromonas* as an etiologic agent of gastrointestinal disease (Morgan and Wood 1988; von Graevenitz 2007). Especially in pediatric populations, however, there is considerable circumstantial evidence based on sporadic cases and small outbreaks (Joseph 1996; Janda and Abbott 1998). Current insights indicate that the *Aeromonas* taxa most frequently isolated from human clinical specimens are members of *A. hydrophila* HG1, *A. veronii* biovar sobria HG8, *A. caviae* HG4, *A. jandaei* HG9, *Aeromonas schubertii*, and *Aeromonas trota* (Altwegg et al. 1990; Carnahan et al. 1991c; Janda 1991; Carnahan and Joseph 1993; Janda and Abbott 1998). In patients undergoing plastic surgery, *A. veronii* biovar sobria is well recognized as an infectious agent (Graf 1999b; Whitaker et al. 2011). By comparing the genotypes and virulence signatures of aquatic and clinical isolates, Khajanchi et al. (2010) claimed to have found a first suggestive evidence of successful colonization and infection by particular strains of certain *Aeromonas* species after transmission from water to humans.

Also in various animal species, *Aeromonas* has been associated with a range of infections. Most notably, such cases have been frequently reported in poikilothermic animals including amphibians, reptiles, fish, and shrimps (Marcus 1971, 1981; Shotts et al. 1972; Brackee et al. 1992; Burns et al. 1996; Gosling 1996; Schilliger 1997; Dierckens et al. 1998). One of the first animal infections attributed to *Aeromonas* was the so-called red-leg disease in frogs (Sanarelli 1891). Also in birds and domestic animals, aeromonads have been associated with various infections such as pneumonia, peritonitis, and various localized infections (Shane et al. 1984; Shane and Gifford 1985; Gray 1984; Gosling 1996). In fish, both motile and nonmotile species of *Aeromonas* were recognized as causal agents of hemorrhagic disease, ulcerative disease, furunculosis, red sore disease, and septicemia (Joseph and Carnahan 1994; Austin and Adams 1996).

As reviewed by several authors (Cahill 1990; Janda 1991; Gosling 1996), a broad spectrum of putative virulence factors has been reported for *Aeromonas* spp. including cytotoxic and cytotonic toxins, proteases, hemolysins, lipases, adhesins,

agglutinins, pili, enterotoxins, various enzymes, and outer membrane arrays. In this context, it is important to note that taxonomic characterization of *Aeromonas* isolates in older studies was not always in accordance with present-day criteria. As a consequence, it is difficult to link specific virulence factors to all aeromonads, a particular species or a particular genotype. Below, a concise overview of known *Aeromonas* virulence factors is given.

Filamentous structures of aeromonads with virulence potential include short, rigid and long, and wavy types of pili as well as polar and lateral flagella. A type IV pilus gene cluster (*tap a*, *b*, *c*, *d*) encoding a 17 kDa pilus has been identified. *Tap d* encodes a type IV leader peptidase/methyltransferase which, in addition to processing Tap pilin, is responsible for the extracellular secretion of aerolysin and other enzymes via the type II secretion pathway (Strom and Lory 1993). Although the widespread conservation of the *tap* gene cluster among most *Aeromonas* spp. suggests that it has an important function, Tap pili were not found important for adhesion to enterocytes or as colonization factors in animal models with Tap pilin mutants (Barnett and Kirov 1999). In addition, a long, wavy type IV pilus, designated Bfp (bundle-forming pilus), has been characterized. These pili have a molecular mass of 19–23 kDa, occur singly or in bundles, are closer to type IVA than type IVB, and have a close homology to the MSHA of *V. cholerae*. There are several lines of evidence suggesting that this pilus type triggers adherence to enterocytes, i.e., observations made with EM and immuno-electron microscopy, reduction of adhesion capacity of the organism upon removal of the pili, adhesion to Henle 407 cells, and blocking experiments with purified pili or antibody to Bfp (Kirov and Sanderson 1995, 1996; Barnett et al. 1997).

Genetic characterization of the polar (Pof) and lateral (Laf) flagella of *Aeromonas* spp. provides evidence that they play a role as an adhesin and as an accessory colonization factor at the cell surface, respectively (Kirov et al. 2002; Gavin et al. 2002, 2003). The Laf purified from an *A. caviae* strain showed considerable homology to the Laf of *Vibrio parahaemolyticus*. Lateral flagella are optimally expressed when bacteria are grown on solid media for <8 h at 37 °C.

The toxins described for *A. hydrophila* include enterotoxic (i.e., aerolysin), cytotoxic (i.e., Act), and cytotonic toxins (i.e., Alt and Ast) (Chopra et al. 1996; Ferguson et al. 1997; Xu et al. 1998; Buckley and Howard 1999; Chopra and Houston 1999a, b). A multiplex PCR method for the detection of the Act, Alt, and Ast toxins in a single assay was described by Bin Kingombe et al. (2010). There is evidence to suggest that the genetic makeup and biological function of these toxins may vary substantially between strains and species. For instance, differences in restriction maps and divergent flanking sequences have been reported when comparing the Act toxin of *A. hydrophila* strain SSU with the aerolysin toxin of *A. trota* strain AH 2 (Chakraborty et al. 1987) and that of *A. bestiarum* (formerly *A. hydrophila* HG2) AH-65 (Buckley et al. 1981). Possibly, this may be due to different host cell receptors, and variability in the role of selected amino acid residues in post-cleavage activity of the pro-toxin and eventual folding. Other studies have reported that Act may increase levels of pro-inflammatory cytokines, which could mediate inflammation and tissue damage during *Aeromonas* infections and of prostaglandin PG-1 or PG-2 activity. Furthermore, it has been shown that the use of PG inhibitors such as NS 398 and Celebrex eliminated activity or reduced the time of production of PG including INOS and $SsPLA_2$ (GrpV) levels in CHO cells (Chopra and Houston 1999a, b). Through marker exchange mutagenesis and mice studies, it was found that aerolysin AH2 from *A. trota* (formerly *A. hydrophila/A. sobria*) is an important virulence feature (Chakraborty et al. 1987) that is first released as a proaerolysin. This toxin is inactive until proteolytical cleavage and removal of a C-terminal fragment of approximately 40 amino acids. For the aerolysin of *A. bestiarum* strain AH 65, a type II secretion pathway has been described. Two operons, *exeAB* and *exeC-N*, form the central components of this type II secretion pathway and appear to form an inner membrane complex that may function as an energy-dependent gating of the port (Howard et al. 1993, 1996).

Various enzymes have been described as potential virulence factors in *Aeromonas*. Some strains may produce a lipase, i.e., glycerophospholipid–cholesterol acyltransferase (GCAT), which can produce cholesteryl esters and can act as a phospholipase by digesting plasma membranes (Buckley 1983). Two distinct types of proteases have been characterized from aeromonads including metallo- and serine proteases (Rodriguez et al. 1992). These enzymes probably protect the organism in various environmental niches, including humans, by inducing tissue destruction and assist in the degradation of substrates for catabolic metabolism. Other enzymes potentially involved in *Aeromonas* virulence include amylase, chitinase, elastase, lecithinase, and nucleases (Gosling 1996).

The presence of an extracellular polysaccharide capsule has been characterized for strains of *A. salmonicida* and *A. hydrophila* serogroups O:11 and O:34 (Garrote et al. 1992; Martinez et al. 1995). It has been suggested that the capsule is a virulence factor that assists in complement resistance and/or in adherence to and invasion of fish cell lines (Merino et al. 1996a, b, 1997a, b). Much better documented are the so-called S-layers, which are paracrystalline structures composed of identical protein subunits translocated across the cytoplasmic membrane, periplasm, and outer membrane to the cell surface. S-layers are assembled and tethered to the cell via an interaction with the O-polysaccharide side chains of the lipopolysaccharide, through which they have the ability to bind host factors such as fibronectin, laminin, and vitronectin and can provide resistance to serum killing and protease digestion. S-layer mutants have an altered ability to produce disease (Noonan and Trust 1997). The S-layer was first described in *A. salmonicida*, for which it was shown that strains with the S-layer could cause furunculosis in fish, while those without S-layers were avirulent. The significance of the S-layer in mesophilic aeromonads such as *Aeromonas hydrophila* and *Aeromonas veronii* biovar sobria appears to be less significant (Janda et al. 1987; Paula et al. 1988; Kokka et al. 1990, 1991a, b).

Aeromonads may produce several types of siderophores. At least two different mechanisms for iron acquisition have been reported for *A. salmonicida* (Chart and Trust 1983). Mesophilic

aeromonads, on the other hand, may produce the amonabactin siderophore (i.e., a four peptide-based bis-catecholate siderophore) or an enterobactin-like siderophore. Amonabactins are bis-catecholates with the backbone composed of either tri- or tetrapeptides (Telford and Raymond 1997). Under iron-limiting conditions, *Aeromonas* spp. display significantly increased siderophore production. In general, clinical strains are more active in siderophore production compared to environmental strains (Naidu and Yadav 1997).

In general, the majority of motile *Aeromonas* strains are resistant to penicillin, ampicillin, carbenicillin, and ticarcillin and susceptible to second- and third-generation cephalosporins, aminoglycosides, carbapenems, chloramphenicol, tetracyclines, trimethoprim–sulfamethoxazole, and the quinolones (Koehler and Ashdown 1993; Janda and Abbott 1998; Altwegg 1999; Kämpfer et al. 1999; Huys et al. 2000, 2001a; Aravena-Roman et al. 2012). Phenotypic resistance to several β-lactam antibiotics has been linked to the production of inducible chromosomal β-lactamases of which at least three types have been described (Bakken et al. 1988; Iaconis and Sanders 1990; Morita et al. 1994; Walsh et al. 1997). Regional differences in antimicrobial resistance profiles of *Aeromonas* spp. have been reported. Compared to isolates from Australia and the United States, antimicrobial resistance to tetracycline, trimethoprim–sulfamethoxazole, some extended-spectrum cephalosporins, and aminoglycosides appeared to increase among clinical *Aeromonas* isolates in Taiwan (Ko et al. 1996). In a study of the spectrum of extraintestinal diseases associated with aeromonads in tropical Queensland, Australia, it was reported that in nine cases the empirical antibiotic regimen prescribed was not adequate to treat the *Aeromonas* infection (Kelly et al. 1993). This suggests that species identification of clinical *Aeromonas* isolates may be an important prerequisite to select the most appropriate antimicrobial therapy. Even if the therapy is effective for the patient, possible development of resistant isolates should not be neglected. One study reported the recovery of both imipenem-susceptible and imipenem-resistant *A. veronii* biovar sobria isolates with different morphologies and antimicrobial susceptibilities from bile samples of a patient with cholangitis (Sánchez-Céspedes et al. 2009). These isolates belonged to the same clone, and the imipenem-resistant isolates displayed overexpression of the *imiS* gene, encoding a chromosomal carbapenemase.

A number of specific phenotypic resistances have been identified as potential diagnostic markers. For instance, resistance to cephalothin (a first generation cephalosporin) can be a potential marker for clinical *A. veronii* biovar sobria isolates (Janda and Motyl 1985). A critical MIC concentration of colistin (4 μg/ml) could be a marker for *Aeromonas jandaei* (Carnahan et al. 1991c). The most well-documented example is the species-specific susceptibility to ampicillin of *Aeromonas trota*, and also *A. caviae* harbors a significant number of ampicillin-susceptible strains (Kilpatrick et al. 1987; Carnahan et al. 1991b). Consequently, the use of ampicillin in selective media for enrichment and isolation may lead to an underestimation of these species. For the more rarely isolated clinical *Aeromonas* species, more subtle differences in antimicrobial resistance patterns have been reported between species. Overman and Janda (1999) reported that *A. veronii* biovar veronii and *A. schubertii* exhibited markedly increased resistance to tobramycin, a general resistance to imipenem of *A. veronii* biovar veronii and *A. jandaei*, and a decreased susceptibility of *A. schubertii* and *A. trota* to cefoxitin compared to broad-spectrum cephalosporins.

Also in isolates of *A. salmonicida*, at least three β-lactamases have been reported (Hayes et al. 1994). Multidrug-resistant isolates of this species have been frequently reported. In a study on farmed Atlantic salmon in Scotland, a substantial part of the *A. salmonicida* isolates exhibited phenotypic resistance to amoxicillin, oxolinic acid, oxytetracycline, and potentiated sulfonamide (Grant and Laidler 1993). Dalsgaard et al. (1994) conducted a large survey on 130 *A. salmonicida* isolates from farmed salmonid fish isolated in Denmark, Norway, Scotland, Canada, and the United States. Antibiograms revealed increased resistance to oxytetracycline and quinolones and the presence of multi-resistant strains in several countries.

Several studies have revealed that *A. salmonicida* (Aoki et al. 1971; Toranzo et al. 1983) and motile aeromonads (Chang and Bolton 1987) may harbor one or more resistance plasmids, e.g., conferring resistance to streptomycin, chloramphenicol, tetracycline, and sulfathiazole. More specific studies in fish farms and eel ponds have revealed antibiotic resistance encoded on 20–30 Mda plasmids, which may potentially affect the efficiency of antimicrobial compounds used in aquaculture (Aoki 1988). In fact, transfer of oxytetracycline resistance from *A. salmonicida* to *Escherichia coli* has been demonstrated through broth conjugation experiments (Adams et al. 1998). In another study (Chaudhury et al. 1996), single or multiple resistance was identified in 107 clinical and environmental isolates encompassing seven *Aeromonas* species, with the highest incidence for resistance to β-lactam antibiotics other than cefotaxime. Of these, 35 isolates harbored transferable resistance plasmids encoding resistance to ampicillin, cephalexin, cefoxitin, erythromycin, and furazolidone, either alone or in combination. One of the first complete sequences of a conjugative resistance plasmid isolated from a motile *Aeromonas* strain was determined by Rhodes and colleagues (2004). The 84,748 bp plasmid in question, pFBAOT6, was previously detected in an *A. caviae* HG5 isolate recovered from hospital effluent in the United Kingdom (Rhodes et al. 2000) and harbored 'a' *tet*(A) gene conferring resistance to several tetracyclines. Sequence comparisons indicated that pFBAOT6 is an IncU type plasmid belonging to a group of related plasmids with global ubiquity and presence in both clinical and nonclinical environments. McIntosh et al. (2008) reported the first plasmid-mediated florfenicol-resistant *A. salmonicida* in North America as well as the first plasmid-associated AmpC β-lactamase sequence in aeromonads. Each of the multidrug-resistant *A. salmonicida* isolates carried the same plasmid which was related to the IncA/C plasmid pSN254 previously found in *Salmonella enterica*.

So far, no pathogenic character has been reported for members of the genera *Tolumonas*, *Oceanimonas*, *Oceanisphaera*, and *Zobellella*. Antibiotic susceptibility data for these organisms were reported in the original species descriptions.

Application

No applications in agriculture, food industry, enzyme and antibiotic production, bioremediation, or other industrial processes are known for members of the *Aeromonadaceae*.

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4 The Family *Alcanivoraceae*

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Abstract
The family *Alcanivoracaceae* comprises of species capable of degradation of petroleum-derived compounds as their main carbon source. These are Gram-negative, aerobic, rod-shaped bacteria commonly isolated in marine waters and sediments worldwide. The family includes the type genus *Alcanivorax* and the genus *Kangiella*. *A. borkumensis* is the type species, an almost exclusively hydrocarbonoclastic bacteria, which dominates marine environments suffering from oil contamination through its ability of nutrient scavenging, oligotrophic growth, and biofilm formation. Five species have the complete genome sequenced, revealing the molecular basis of the features that provide these microbes a competitive advantage in oil-polluted environments. The capability of this family of microbes to biodegrade oil is of great importance given the amount of petroleum and petroleum-derived compounds released in the ocean every day by means of seeping from natural oil fields and the spill from petroleum extraction, transport, and refining activities. Some aromatic compound-degrading members have considerable potential for biodegradation of organic waste material and bioremediation of polluted environments.

Taxonomy, Historical, and Current

The *Alcanivoracaceae* family is part of the gammaproteobacterial order *Oceanospirillales*. Phylogenetic trees based on 16S rRNA gene sequences revealed that the *Alcanivoracaceae* family forms a deep evolutionary lineage of descent within the *γ-Proteobacteria*.

Genus *Alcanivorax* Yakimov, Golyshin, Lang, Moore, Abraham, Lündsdorf and Timmis 1998, SK2^T

Al'.ca.ni.vo'.rax. N.L. masc. n. *alcanum*, alkane, aliphatic hydrocarbon; L. adj. *vorax*, voracious, gluttonous; N.L. masc. *Alcanivorax*, alkane-devouring.

The mol% G+C of the DNA varies between 53 and 66.

The first member of the *Alcanivoracaceae* family described was *Alcanivorax borkumensis*, isolated from enriched cultures for biosurfactant-producing bacteria from seawater/sediment samples collected near the Isle of Borkum (North Sea) using Mihagol-S ($C_{14\text{-}15}$–*n*-alkane) as the principal carbon source (Yakimov et al. 1998). Phylogenetic analysis based on 16S sequence showed this species as a member of the γ subclass of the *Proteobacteria*, distant related to known hydrocarbon-degrading genus *Marinobacter*, *Acinetobacter*, and *Arthrobacter*.

The genus *Alcanivorax* consists of seven species: the above mentioned *A. borkumensis*, *A. balearicus* (Rivas et al. 2007), *A. dieselolei* (Liu 2005), *A. hongdengensis* (Wu et al. 2009), *A. pacificus* (Lai et al. 2011), *A. venustensis*, and *A. jadensis* (Fernandez-Martinez 2003). *A. jadensis* was first described as comprising a new genus, *Fundibacter* (Bruns and Berthe-Corti 1999); however, this species shares 97.2 % sequence similarity in the 16S rRNA with *A. borkumensis*, in addition to many phenotypic characters, and the differences were within the mean differences characteristic for a single and well-defined genus (García-Martinez et al. 1999). Based on these evidences, *Fundibacter jadensis* was then renamed *Alcanivorax jadensis* (García-Martínez and Rodríguez-Valera 2000; Fernandez-Martinez 2003). The description of another species, *A. indicus*, was not formally published until the time of writing.

Members of the *Alcanivorax* genus, especially *A. borkumensis*, present a very characteristic growth with biofilm formation in the interface between water and oil (Yakimov et al. 1998). This growth pattern facilitates the oil emulsification through

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extracellular biosurfactants (Yakimov et al. 1998). *A. borkumensis* becomes predominant in crude-oil-containing seawater bacterial community when nitrogen and phosphorus nutrients are supplemented (Kasai et al. 2001, 2002), where it can comprise up to 80–90 % of the oil-degrading microbial community (Harayama et al. 1999). This is due to its ability to degrade branched alkanes (pristane and phytane), in contrast to other oil-degrading bacteria such as *Acinetobacter venetianus*, which is limited to the use of non-branched alkanes (Hara et al. 2003). The dominance of *Alcanivorax* members in beach sands impacted by oil spill and oil spill mesocosms is of great ecological importance as these organisms are microbial indicators and model hydrocarbon degraders (Chikere et al. 2011; Kostka et al. 2011).

Genus *Kangiella* Yoon, Oh and Park 2004, SW-125T

Kan.gi.ella. N.L. dim. fem. n. *Kangiella* named to honor Professor Kook Hee Kang, a Korean microbiologist, for his contribution to microbial research.

The mol% G+C of the DNA varies between 43.7 and 48.9.

Members of the *Kangiella* genus are heterotrophic marine bacteria, commonly found in marine sediments. First members were isolated from tidal flat sediments in Yellow sea, Korea, and were classified as *K. koreensis*, the type species, and *K. aquimarina* (Yoon 2004). These are Gram-negative, nonmotile, nonspore-forming, rod-shaped, and catalase- and oxidase-positive organisms. The genus comprises eight species: the type-species *K. koreensis* (Yoon 2004), *K. aquimarina* (Yoon 2004), *K. japonica* (Romanenko et al. 2010), *K. taiwanensis* (Jean et al. 2012), *K. marina* (Jean et al. 2012), *K. spongicola* (Ahn et al. 2011), *K. geojedonensis* (Yoon et al. 2012), and *K. sediminilitoris* (Lee et al. 2013). Phenotypic characteristics as ubiquinone-9 as the predominant respiratory lipoquinone and fatty acids profiles differentiate this genus from other *Gammaproteobacteria* genus including *Alcanivorax*.

Molecular Analyses

Phylogeny

Phylogenetic analysis based on 16S and *gyrB* gene shows the monophyly of the *Alcanivorax* genus (Lai et al. 2011). However, the position of the family *Alcanivoracaceae* within the *Gammaproteobacteria* 16S rRNA trees has been controversial. First studies point the genus *Alcanivorax* branching deep within the *Gammaproteobacteria*, just distantly related to *Marinomonas*, *Oceanospirillum*, and *Halomonas* (Yakimov et al. 1998), while more recent trees show it as a sister group of the genus *Halomonas* (Lai et al. 2011). As well, the genus *Kangiella* was first described not forming a clade with *Alcanivorax* and, more recently, with closest phylogenetic relationship with *Saccharospirillum* and *Reinekia* (Yoon 2004; Jean et al. 2012). However, current phylogenetic analysis shows the monophyly of the *Alcanivoracaceae* family, including *Alcanivorax* and *Kangiella* genera, and its close relationship to *Pseudomonadaceae* family (*Fig. 4.1*).

Genome Analysis

In 2006 *A. borkumensis* was the first hydrocarbonoclastic bacteria to have its genome sequenced, revealing high adaptation to oil consumption, with a paucity of mobile genetic elements and energy generation-related genes but a variety of genes which allow for a wide range of hydrocarbon substrates and high efficiency in oil degradation (Schneiker et al. 2006). The genome of *A. borkumensis* is a single circular chromosome that contains 3,120,143 base pairs. Biological roles were assigned to 2,241 of the 2,755 predicted coding sequences. The remaining 514 coding sequences comprise 316 conserved hypothetical and 198 of unknown function.

A. borkumensis presents two important genomic islands, with a different proportion of CG content, which encode for cellular surface polysaccharides biosynthesis and for alkane degradation. This can suggest a horizontal gene transfer (HGT) origin of the ability to use oil as carbon source, although the presence of few mobile elements suggests that HGT did not play a significant role in *A. borkumensis* genome. The *alk* SB_1GHJ operon that encodes for hydrocarbon catabolism by alkane hydroxylases shares >80 % similarity with alkane degradation components in *Pseudomonas putida*. Another possible pathway for alkane degradation involves rubredoxin reductases.

The high specialization of *A. borkumensis* for alkane degradation is reflected by the presence of two copies of the *alk* B gene that encodes an alkane hydroxylase. This feature is rare in the marine environment as revealed by comparison to sequenced marine genomes and metagenomes. Furthermore, the presence of three cytochromes, ferredoxin, transcription factors, and other genes, in an operon-like organization seems to be linked to the broad range of hydrocarbons catabolized by *A. borkumensis*. Two cytochromes are homologous to an *Acinetobacter* isolate cytochrome responsible for terminal oxidation of alkanes. The gene clusters responsible to hydrocarbon assimilation are located in two genomic islands that seem to be acquired from *Yersinia* lineage ancestor (Reva et al. 2008).

A. borkumensis genome harbors many glycosyltransferases, *pil*, translocon, and Type II secretion system genes, all of them related to biofilm formation in the oil–water interface of oil droplets. Moreover, a huge number of permeases, high affinity ABC-type, and many other transporters found in its genome can be responsible to the efficient scavenging of nutrients in marine oligotrophic environments. The absorption of nitrogen and phosphorous can be done alternatively by more than one transporter, as well as oligo-elements like zinc, molybdate, and magnesium, allowing this bacteria to thrive in the typical carbon/nutrients imbalance of oil-contaminated marine environments.

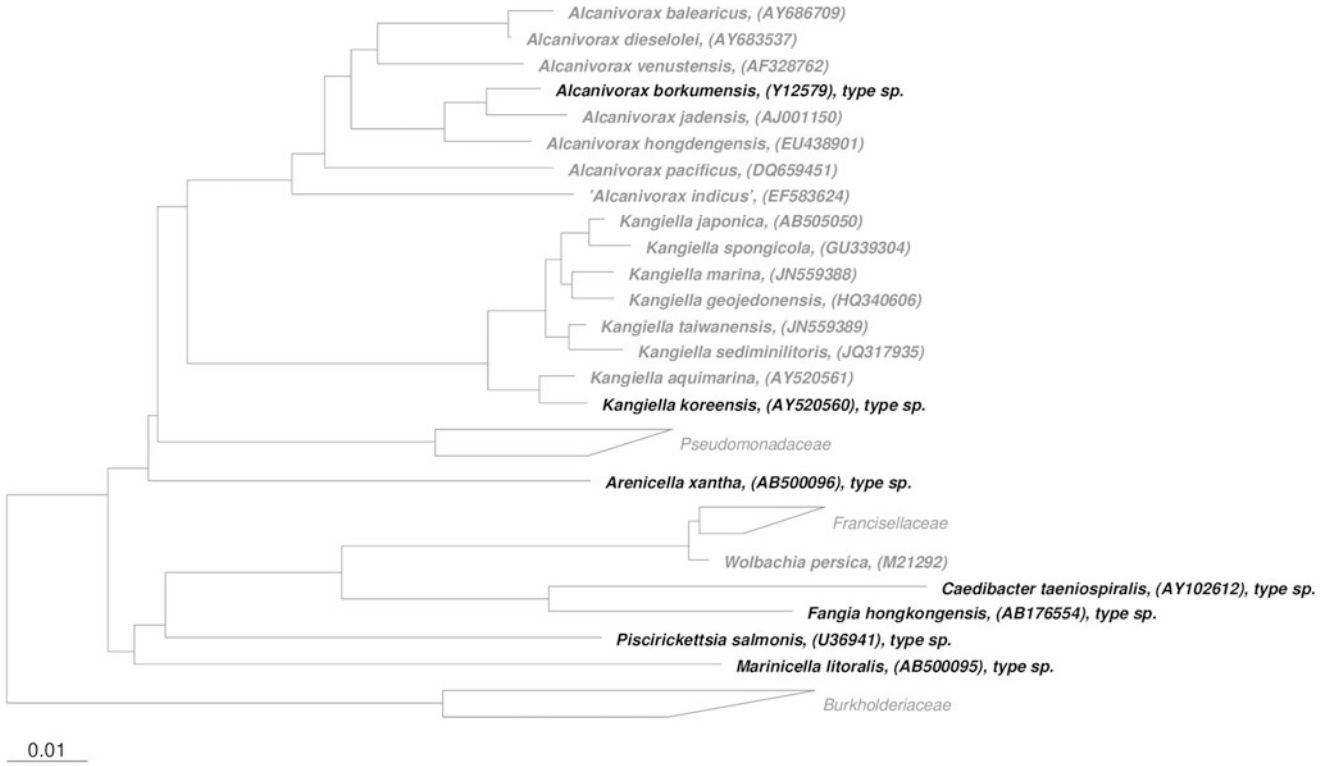

Fig. 4.1

Phylogenetic reconstruction of the family *Alcanivoracaceae* based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes-Cantor correction. The sequence dataset and alignment were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high-quality-type strain sequences proportionally distributed among the different bacterial and archaeal phyla. In addition, a 40 % maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. Scale bar indicates estimated sequence divergence

Table 4.1

Genomic features of *Alcanivoracaceae* species with sequenced genome

	A. borkumensis SK2	*A. dieselolei* B5T	*A. hongdengensis* A-11-3 T	*A. pacificus* W11-5 T	*K. koreensis* SW-125 T
CG content (%)	54.7	61.63	60.68	62.62	43.69
Length (bp)	3,120,143	4,928,223	3,664,876	4,137,438	2,852,073
Coding intensity (%)	87.4	89.13	89.1	3,762	90.64
Total of genes	2,803	4,417	3,459	3,806	2,695
Protein-coding genes	2,755	4,369	3,416	3,762	2,647
rRNA operons	3	2	3	1	2
***AlkB* genes**	2	3	4	1	n.d.

A. dieselolei, *A. hongdengensis*, and *A. pacificus* also had their genomes sequenced (Lai et al. 2012; Lai and Shao 2012a, b) (*Table 4.1*). *A. dieselolei* strain B5^T presents single circular chromosome 4,928,223 bp in length and an average GC content of 61.63 %. There are a total of 4,417 putative open reading frames (with an average size of 994 bp) (Lai et al. 2012). The genome also shows a high adaptation for alkane degradation, with three integral-membrane alkane monooxygenases (AlkB) genes and three cytochrome P450 and three flavin-binding family monooxygenases (AlmA). Long-chain alkane degradation in *A. dieselolei* was described as dependent of *AlmA* expression (Liu et al. 2011).

A. hongdengensis genome is 3,664,876 bp in length and has an average GC content of 60.68 %. In respect of alkane degradation, 10 alkane monooxygenase genes are found in the genome sequence, including four *alkB* genes, three

cytochrome P450 genes, and three *almA* genes. Moreover, two new *alkB* genes and two new *almA* genes were found by genome sequencing in addition to those previously detected (Lai and Shao 2012b). *A. pacificus* strain W11-5^T genome is 4,137,438 bp long and has an average GC content of 62.62 %. The genome contains 3,762 candidate protein-encoding genes (with an average size of 964 bp). Four *alkB*, four P450 cytochrome and four *almA* genes are encoded by its genome (Lai and Shao 2012a).

Within the *Kangiella* genus only one species had its genome sequenced, *Kangiella koreensis* strain SW 125^T (Han et al. 2009). The circular chromosome is 2,852,073 bp long and shows 43.69 % GC content. Most of the protein-coding genes have a putative function (71.7 %), and the remaining are hypothetical proteins.

16S DNA Sequences

The search for environmental *Alcanivoracaceae* 16S DNA sequences in GenBank accessed by culture-independent methods as DGGE and 16S clone libraries provides information about the broad environmental distribution of this family. There are sequences found in Mediterranean Sea, French sediment (Païssé et al. 2010), Atlantic Ocean seawater, seawater reverse osmosis membranes, Arctic deep-sea water, deep-sea hydrothermal vents, and oil-contaminated beach microcosm (Roling et al. 2002) and associated to the marine brown algae *Saccharina japonica* (Balakirev et al. 2012) and to a deep-sea octocoral.

Proteomic Analysis

The capacity to degrade an exceptionally broad range of hydrocarbons by *A. borkumensis* was investigated by proteomic analysis, which revealed the differences in hexadecane-grown and pyruvate-grown cells (Sabirova et al. 2006). Most of the differences account for membrane-bound proteins, including three enzyme systems able to convert alkanes via terminal oxidation to fatty acids: the known AlkB1 system and two new alkane hydroxylating systems. Other membrane proteins, such as P450 cytochrome monooxygenase, a putative flavin-binding monooxygenase, and cytoplasmatic enzymes mediating β-oxidation of fatty acids, also participate in the alkane usage. Moreover, cytoplasmatic enzymes related to glyoxylate bypass and gluconeogenesis are active in alkane-grown cells, providing key metabolic intermediates to a central metabolism based on fatty acids (Sabirova et al. 2006). Lipoprotein-releasing system (Lol) is also upregulated in alkane-grown cells, what may be related to surfactant release. All these features shown by proteomic study allow the ecological success of this bacterium in oil-contaminated environments.

Phenotypic Analyses

A. borkumensis is nonmotile, rod shaped, with no flagella, catalase and oxidase positive, and nonspore forming. The cell size can vary according to the medium, from 2.0 to 3.0 μm in length and 0.4–0.7 μm in diameter when growing in pyruvate-supplemented medium to 1.0–1.5 μm in length when growing in alkane medium (Fig. 4.2a). It produces a new class of cell-bound and extracellular surface-active glucose lipids with surfactant activity (Fig. 4.2b). *A. borkumensis* is capable of nitrate reduction to nitrite in facultative anaerobic conditions and is moderately halophilic, with optimum growth between 3 % and 10 % NaCl and 25 °C and 30 °C. It cannot use sugar or amino acid as carbon sources, and alkanes are mainly degraded via β-oxidation (Dutta and Harayama 2001). *A. borkumensis* SK2 is able to degrade a large range of alkanes up to C32 and branched aliphatic, as well as isoprenoid hydrocarbons (e.g., phytane), alkylarenes, and alkylcycloalkanes. Phenotypic features of other species in *Alcanivorax* genus are listed in Table 4.2.

K. koreensis are rods of 0.2–0.5 × 1.5–4.5 μm in size. The colonies are smooth, raised, circular to irregular, light-yellowish-brown in color, and 2.0–3.0 mm diameter after seven-day incubation at 30 °C in marine agar 2216 (MA) (Difco). Growth under anaerobic conditions occurs on MA supplemented with nitrate. Strain SW-125T hydrolyses casein, tyrosine, Tween 20, Tween 40, and Tween 60, but not hypoxanthine or xanthine. Furthermore, H_2S is not produced, and nitrate is not reduced under aerobic conditions, but oxidased to nitrogen gas under anaerobic conditions. Acid is not produced from the following sugars: adonitol, L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, D-mannose, D-melezitose, melibiose, *myo*-inositol, D-raffinose, L-rhamnose, D-ribose, D-sorbitol, sucrose, D-trehalose, or D-xylose. Unfortunately, a list of carbon sources from which acid is produced is not delivered. When assayed with the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, and naphthol-AS-BI-phosphohydrolase are present, but lipase (C14), cystine arylamidase, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase are absent. Phenotypic features of other species in *Kangiella* genus are listed in Table 4.3.

Isolation, Enrichment, and Maintenance Procedures

The first described member of the family, *A. borkumensis*, was isolated using enrichment culture techniques with *n*-alkanes (Merck) as sole carbon source. The isolate was cultivated aerobically in synthetic seawater medium 1 (SM1) containing 1–1 distilled water: 23 g NaCl, 0.75 g KCl, 1.47 g $CaCl_2.2H_2O$, 5.08 g $MgCl_2.6H_2O$, 6–16 g $MgSO_4.7H_2O$, 0.89 g

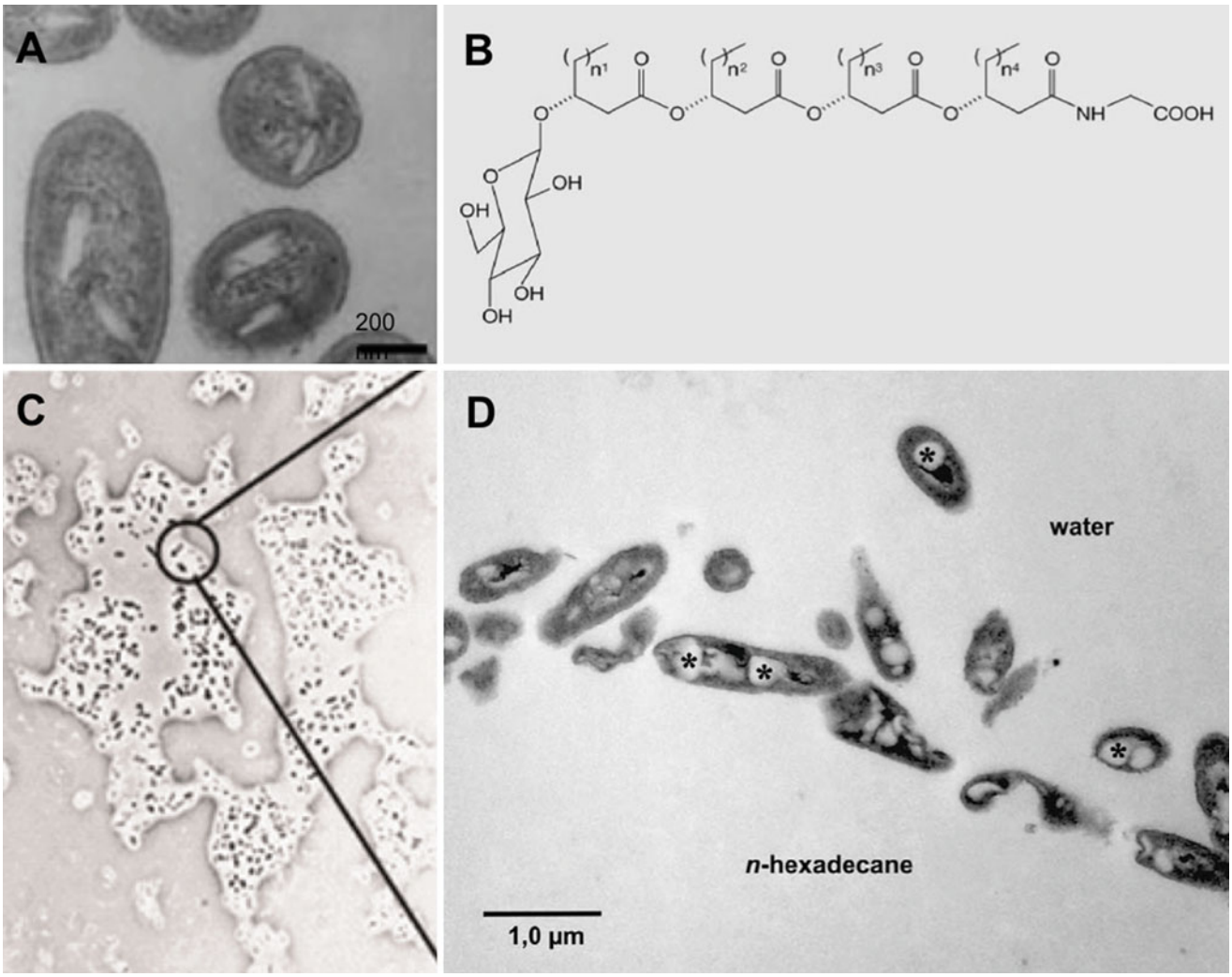

Fig. 4.2

Cell structure and surfactant properties of *Alcanivorax borkumensis*. (a) Transmission Electron Microscopy of cells grown in media supplemented with hexadecane (Cappello and Yakimov 2010); (b) glucose lipid surfactant produced by *A. borkumensis* (Golyshin et al. 2003); (c) oil droplets emulsified by surfactants in *A. borkumensis* media (Cappello and Yakimov 2010); and (d) *A. borkumensis* biofilm formed at water-hexadecane interface (Golyshin et al. 2003)

$Na_2HPO_4.2H_2O$, 5 g $NaNO_3$, and 0.03 g $FeSO_4.7H_2O$. SM1 was supplemented with 3 % Mihagol-S (mixture of C14,15-*n*-alkanes) or with 10 g pyruvate L^{-1} and all major cations and anions present in concentrations greater than 1 mg L^{-1} in seawater. Nitrogen was provided in the form of $NaNO_3$ and phosphorous in the form of Na_2HPO_4. Bacto agar (Difco) (15 g L^{-1}) can be added to the first solution for the preparation of solid media. The optimal temperature is between 25 °C and 30 °C and optimum NaCl concentration between 3 % and 10 %.

Alcanivorax members can also be enriched in microcosm supplemented with crude oil, tetradecane, and naphthalene (Yakimov et al. 2005).

Kangiella strains SW-125 T and SW-154 T were isolated by the dilution plating technique on marine agar 2216 (MA) (Difco) at 25 °C and grow optimally at 30–37 °C (Yoon 2004). These strains require peptone for growth. The conditions of growth in dependence of pH were determined in marine broth 2216 (MB, Difco). The optimal pH is 7.0–8.0. Growth is still possible at pH 5.5, but not at pH 5.0. Growth at various NaCl concentrations (1–15 %) was investigated in MB or trypticase soy broth (TSB, Difco). The optimal growth occurs in the presence of 2–3 % NaCl (MB); growth still occurs in the presence of 12 % NaCl (MB), but not without NaCl (TSB) or in the presence of more than 13 % NaCl (MB).

Ecology

Since the first description of *A. borkumensis* from the North Sea in 1998, it has been detected in many marine and coastal habitats

Table 4.2
Phenotypic features of *Alcanivorax* species

	A. borkumensis	*A. balearicus*	*A. dieselolei*	*A. hongdengensis*	*A. pacificus*	*A. venustensis*	*A. jadensis*
Catalase	+	+	+	–	+	+	–
Oxidase	w	w	w	–	+	+	+
Motility	–	+	+	–	–	+	–
Ionic requirement	Complex	–	Na+	Na+	Na+	Complex	Na+
Growth in 17 % NaCl	–	–	w	–	–	+	–
Growth at 42 °C	–	–	+	+	+	+	+
Growth at 45 °C	–	–	+	–	–	+	+
Nitrate reduction	–	w	–	–	+	–	–
D-Glucose fermentation	–	–	–	+	w	–	–
Urease, β-glucosidase, D-glucose, L-arabinose, *N*-acetylglucosamine	–	–	–	–	+	–	–
Gelatin hydrolysis	+	–	–	+	–	–	–
Capric acid, phenylacetic acid	–	+	+	–	–	–	–
Adipic acid	–	+	+	–	w	+	–
Malic acid	–	–	+	–	+	–	–
Trisodium citrate	–	+	+	–	–	–	–
Acid phosphatase	+	+	+	w	w	+	+
Alkaline phosphatase	+	+	+	–	w	w	+

All strains are negative for denitrification, indole production, arginine dihydrolase, and β-galactosidase activities and for the utilization of D-mannose, D-mannitol, maltose, and potassium gluconate. All strains were positive for esterase (C4), esterase lipase (C8), lipase (C14), and leucine aminopeptidase; weakly positive for valine aminopeptidase; and negative for cystine aminopeptidase, *N*-acetyl-β-glucosaminidase, trypsin, α-chymotrypsin, α-fucosidase, α-glucosidase, α-mannosidase, and β-glucuronidase
+ Positive, *W* weakly positive, – negative

worldwide including the Mediterranean Sea, the Pacific Ocean, the Japanese and Chinese Seas, and the Arctic Sea (Harayama et al. 1999; Kasai et al. 2001; Syutsubo et al. 2001; Golyshin et al. 2003; Yakimov et al. 2005). Other species of this genus were also isolated from many different marine environments: *A. pacificus* from deep-sea sediment of the Pacific Ocean (Lai et al. 2011); *A. balearicus* from subterraneous saline Lake Myrtle, Spain (Rivas et al. 2007); *A. dieselolei* from surface water of the Bohai Sea (Liu 2005); *A. jadensis* from intertidal sediment of German coast (Bruns and Berthe-Corti 1999); *A. hongdengensis* from surface seawater of Malacca and Singapore (Wu et al. 2009); and *A. venustensis* from seawater of Mediterranean Sea (Fernandez-Martinez 2003).

Members of the *Kangiella* genus have been isolated from sediments of Sea of Japan, Russia (Romanenko et al. 2010), coastal seawater from Taiwan (Jean et al. 2012), marine sponge *Chondrilla nucula* tissue (Ahn et al. 2011), seawater from Korea (Yoon et al. 2012), and tidal flat sediment from South Korea (Lee et al. 2013). *Alcanivorax* species became the dominant group in oil spill mesocosms not only from seawater but also from mud flat sediments, where it carries aerobic hydrocarbon degradation (Coulon et al. 2012).

Pathogenicity and Clinical Relevance

There are no reports about pathogenicity or clinical relevance of the *Alcanivoracaceae* family. All the *Alcanivorax* species are sensitive to (mg per disc) polymyxin B (30) but resistant to clindamycin (2), furazolidone (15), lincomycin (2), metronidazole (5), oxacillin (1), and vancomycin (30). *K. Koreensis* strain SW-125 T was found to be susceptible to polymyxin (50 U), streptomycin (50 μg), penicillin (20 U), chloramphenicol (50 μg), ampicillin (10 μg), cephalothin (30 μg), and erythromycin (15 μg) and to be resistant to novobiocin (5 μg) and tetracycline (30 μg).

Application

Bioremediation

Petroleum is one of the most important sources of energy. The extraction, transport, and storage of this fuel represent great potential for oil spills, which can impact marine environments. Once shed in the sea, crude oil is subjected to weathering and the degradation by microbial activity

Table 4.3
Phenotypic features of *Kangiella* species

	K. koreensis	*K. spongicola*	*K. japonica*	*K. aquimarina*	*K. geojedonensis*	*K. taiwanensis*	*K. marina*	*K. sediminilitoris*
Growth at 4 °C	+	–	+	–	–	–	–	–
Growth at 45 °C	–	–	+	+	–	–	–	–
Anaerobic growth	+	+	–	+	+	+	+	+
Nitrate reduction	+	+	–	+	–	+	+	–
NaCl tolerance (%)	13	15	13	13	15	12	8	13
Gelatin hydrolysis	+	–	v	+	+	+	+	–
Casein hydrolysis	+	+	+	+	+	–	+	+
Tween 80	+	+	+	+	+	+	–	+
D-glucose	–	–	+	–	–	–	–	–
Naphthol-AS-BI-phosphatase	+	+	–	+	+	–	–	w
Esterase (C4)	+	–	+	+	+	+	+	+
Lipase (C14)	+	–	–	+	–	–	–	
α -Chymotrypsin	–	–	–	–	–	+	w	w
Acid phosphatase	–	+	–	+	n.d.	–	–	–
Alkaline phosphatase	+	+	–	+	+	–	+	+

All species of the genus *Kangiella* are Gram-negative-staining, nonmotile rods. All strains were positive for oxidase and catalase activities and for hydrolysis of tyrosine (nor determined for *K. taiwanensis* and *K. marina*) and Tween 20. All strains were negative for hydrolysis of starch, aesculin, and ferric citrate
+ Positive, – negative, *V* variable, *W* weak, *n.d.* not determined

(Readman et al. 1992). The predominance of *Alcanivorax* members in oil-contaminated seawater is evidence of its role in oil degradation, and this group can account for more than 90 % of the microbial community in seawater cultures containing crude oil (Harayama et al. 1999; Kasai et al. 2002). In fact, bacteria of this genus produce glucose lipids with surfactant action, turning oil slick in many oil droplets (*Fig. 4.2b*) (Abraham et al. 1998). Bacterial cells then grow in the droplet surface forming a biofilm (*Fig. 4.2c, d*) (Yakimov et al. 1998). As the growth of these bacteria in the marine environment can be limited by nitrogen and phosphorous availability, the enrichment of the oil-impacted areas could be helpful to stimulate microbial activity (Atlas 2007). This strategy was first attempted in 1989 after the spill from the Exxon Valdez, in which fertilizers were applied to the polluted beaches. After 2–3 weeks, beaches that had been treated with fertilizers had become significantly cleaner than those in the control area (Pritchard and Charles 1991). Mesocosm experiments corroborate the high abundance of *Alcanivorax*-related bacteria and their role in alkane degradation (Cappello et al. 2007), (Singh et al. 2011). The enrichment of oil-contaminated beach mesocosm with inorganic nutrients shows enhancement of hydrocarbon degradation, even with low concentrations of nitrogen and phosphorous added (Roling et al. 2002).

Eco-engineering, the strategy of stimulating a desired metabolic activity, such as hydrocarbon degradation, within the indigenous community instead of inoculating or genetically engineering allochthonous species, is an ongoing alternative for oil spill treatment (Lorenzo 2006). This approach is especially applicable to the use of *Alcanivorax borkumensis* for oil removal as this bacterium grows exclusively on alkanes, rejecting sugars or amino acids, and puts this species as a reference for further exploration of bioremediation as an oil-removal cleanup technology.

Biocatalysis

A. borkumensis exhibits a biotechnological potential that goes well beyond its application to marine oil degradation. Its genome encodes a number of proteins putatively involved in metabolic reactions of potential biotechnological interest, including eight hydrolases of the haloacid dehydrogenase/epoxide family, two determinants for dienelactone hydrolase, three for deacetylases, 36 for various cytochrome proteins, and 30 for oxidoreductases (Schneiker et al. 2006). The genome also includes 11 esterases of potential biotechnological application, two of them were purified and functionally characterized, exhibiting enzymatic activity up to two orders of magnitude higher than typical esterases, and have a wide substrate profile, remarkable enantioselectivity, and chemical resistance, which underscores their potential for the resolution of chiral mixtures in biocatalysis. The AlkB2 recombinant alkane hydroxylase, which performs hydroxylation of different linear and branched alkanes, has been cloned and expressed in *Escherichia coli* (Miri et al. 2009). This activity is of biotechnological interest because chemical hydroxylation of hydrocarbons is a challenge reaction as the C-H bonds are nonpolar and activation of this bond in the Ziegler process requires high energy and metal catalysts (Azapagic et al. 2003). The mechanism of lipid synthesis and export in *A. borkumensis* was explored as a possible way to improve secretion of lipophilic

products into the culture medium rather than its intracellular accumulation, which could significantly reduce the costs of product recovery (Manilla-Perez et al. 2010a, b).

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5 The Family *Alteromonadaceae*

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Abstract
The family *Alteromonadaceae* collects a diverse set of gammaproteobacteria, mostly marine in origin and requiring sodium to grow. The type genus *Alteromonas* was among the first marine bacteria described. They have large cells that grow quite fast with minimal nutritional requirements, and although all are obligate aerobic heterotrophs, they display a diverse set of potential substrates and extensive degradative properties. They have large genomes that contain several degradative genes and sometimes secondary metabolites. Ecologically they are often associated to nutrient-rich environments such as particulate material, marine snow, or marine animals. They have relatively high optimal growth temperatures for marine bacteria except for those isolated from cold environments such as *Glaciecola*. *Saccharophagus* has been shown to utilize a large set of sugars and polysaccharides as carbon and energy source. Members of *Marinobacter* have been described as hydrocarbon degrading, although this characteristic is spread among other genera of the family. Some are agarolytic (*Agarivorans, Aliagarivorans*). Some strains of *Alteromonas macleodii* have the most oxygen-resistant hydrogenase described to date although this species cannot grow on hydrogen as energy source and does not fix CO_2.

Taxonomy, Historical and Current

Alteromonadaceae (Al.te.ro.mo.na.da´ce.ae. N.L. fem. n. *Alteromonas* type genus of the family; *-aceae* ending to denote a family; N.L. fem. pl. n. *Alteromonadaceae*, the *Alteromonas* family).

Species of the genera of the family *Alteromonadaceae* are Gram-negative, rod-shaped bacteria. Motile. Do not form endospores or microcysts. Chemoorganotrophs. Oxygen or nitrate can be used as the electron acceptors. Aerobic or facultatively anaerobic. Arginine dihydrolase is absent. Require Na^+ ions for growth. In most species, the major isoprenoid quinone is Q8. The major fatty acids are 16: 0, 16: 1ω7, and 16: 1ω7. Members of the family have been isolated from coastal, open, and deep-sea waters and invertebrates from marine environments. The family is a member of the "Gammaproteobacteria" with the following nucleotide sequence characteristics: 304 (A), 734 (A), 736 (T), 770 (T), and 809 (A). The family comprises the type genus *Alteromonas* and the genera *Aestuariibacter, Agarivorans, Aliagarivorans, Alishewanella, Bowmanella, Glaciecola, Haliea, Marinimicrobium, Marinobacter, Marinobacterium, Melitea, Microbulbifer, Saccharophagus*, and *Salinimonas*.

The genus *Alteromonas* was created by Baumann et al. (1972). *Alteromonas* gen. nov. included nonspore-forming, Gram-negative, straight or curved, rods which were motile by means of unsheathed, polar flagella. None accumulated PHB as an intracellular reserve product. The moles % GC content of the DNA ranged from 43 to 48. All were chemoorganotrophic, strict aerobes which were unable to denitrify, luminesce, fix molecular nitrogen, or grow with molecular hydrogen as the sole source of energy and carbon dioxide as the sole source of carbon. None had a constitutive arginine dihydrolase. All strains were capable of growth on a minimal medium containing artificial seawater with D-glucose as the sole source of carbon and energy and ammonia as the sole source of nitrogen. Sodium ion but no organic growth factors were required for growth. Most strains were able to utilize a variety of organic compounds as sole sources of carbon and energy, including carbohydrates, monocarboxylic fatty acids, and amino acids. Some species might produce an extracellular amylase, lipase, gelatinase, or chitinase. Two species utilized *m*-hydroxybenzoate, *p*-hydroxybenzoate, and quinate. These compounds were degraded by means of an m cleavage of protocatechuate. None of the strains hydrolyzed agar or utilized cellulose, formate, C5-C, lO-dicarboxylic acids, benzoate, or *n*-hexadecane. The strains which comprised this genus were common inhabitants of the open-sea and coastal waters. *A. macleodii* was designated as the type species (Baumann et al. 1972).

Originally the genus included five species: *Alteromonas macleodii*, *A. haloplanktis*, *A. marinopraesens* (reclassified as *A. haloplanktis* (Reichelt and Baumann 1973), *A. communis*,

E. Rosenberg et al. (eds.), *The Prokaryotes – Gammaproteobacteria*, DOI 10.1007/978-3-642-38922-1_233,

and *A. vaga* (Gauthier and Breittmayer 1992). Later, a number of other species were described and rRNA-DNA hybridization experiments carried out by Van Landschoot and De Ley (1983) revealed the genetic heterogeneity of the genus *Alteromonas* and identified four distinct clusters: (a) the *A. macleodii* cluster; (b) the *A. haloplanktis* cluster, which included the majority of *Alteromonas* species and one species from the genus *Pseudomonas*, *Pseudomonas piscicida* ((Bein 1954); (Buck et al. 1963)); (c) the *A. putrefaciens* and *A. hanedai* cluster (Jensen et al. 1980); and (d) the *A. vaga* and *A. communis* cluster, which was classified as a new genus, *Marinomonas* (Gauthier and Breittmayer 1992).

Three species, *Alteromonas putrefaciens, Alteromonas hanedai*, and *Alteromonas colwelliana*, were subsequently reassigned to the new genus *Shewanella* on the basis of the results of a 5S rRNA sequence analysis (MacDonell and Colwell 1985). By the early 1990s, the genus *Alteromonas* had been supplemented with several novel species, *Alteromonas denitrificans* (Enger et al. 1987), *A. atlantica*, *A. carrageenovora* (Akagawa-Matsushita et al. 1992), *A. tetraodonis* (Simidu et al. 1990), *A. rava* (Kodama et al. 1993), *A. fuliginea, A. distincta*, and *A. elyakovii* (Romanenko et al. 1994, 1995); (Ivanova et al. 1996).

Gauthier et al. (1995) analyzed 17 strains by 16S rRNA gene sequencing and separated the species *Alteromonas macleodii* from other species of the genus, and they included isolates from the genera *Alteromonas, Shewanella, Vibrio, and Pseudomonas* in the new genus *Pseudoalteromonas*. The revised genus *Alteromonas* contained only one species, *A. macleodii*, while the new genus *Pseudoalteromonas* was proposed to include the remaining species.

In 2001, Ivanova and Mikhailov (2001) suggested to combine the genera *Alteromonas, Pseudoalteromonas, Idiomarina*, and *Colwellia* into the new family, *Alteromonadaceae* fam. nov., on the basis of phenotypic, genotypic, and phylogenetic characteristics, with the type genus *Alteromonas*. However, 3 years later Ivanova et al. (2004) established the phylogenetic relationships among marine *Alteromonas*-like bacteria of the genera *Alteromonas, Pseudoalteromonas, Glaciecola, Thalassomonas, Colwellia, Idiomarina, Oceanimonas, Oceanisphaera, Shewanella, Moritella, Ferrimonas, Psychromonas*, and several other genera of the class γ-Proteobacteria. Based on these phylogenetic analyses of 16S rRNA gene sequences and despite their close phenetic similarity, bacteria of the genera *Alteromonas, Pseudoalteromonas, Glaciecola, Thalassomonas, Colwellia*, and *Idiomarina* did not form a clade and these authors limited the family *Alteromonadaceae* to the genus *Alteromonas* and *Glaciecola*. After this classification, different genera have been isolated or reclassified and added to this family, for example, genera such as *Marinobacter, Marinobacterium*, and *Microbulbifer* that historically had been classified with *Oceanospirillum*, as incertae sedis within the γ-subdivision of the Proteobacteria. The new Family *Alteromonadaceae* included lesser-known Gram-negative, marine, and/or halophilic aerobes and facultative anaerobes of the class γ-Proteobacteria. Presently, the family *Alteromonadaceae* accommodate an assemblage of marine Gram-negative bacteria that form a phylogenetic clade in the order *Alteromonadales*, together with the families *Colwelliaceae*, Ferrimonadaceae, Idiomarinaceae, Moritellaceae, Pseudoalteromonadaceae, Psychromonadaceae, and Shewanellaceae. Members of the *Alteromonadaceae* include the genera *Aestuariibacter, Agarivorans, Aliagarivorans, Alishewanella, Alteromonas, Bowmanella, Glaciecola, Haliea, Marinimicrobium, Marinobacter, Marinobacterium, Melitea, Microbulbifer, Saccharophagus*, and *Salinimonas* (Table 5.1). A phylogenetic reconstruction within the family *Alteromonadaceae* is shown in Fig. 5.1.

Molecular Analyses

The real abundance and distribution of *A. macleodii* started only to be underpinned when PCR-amplified 16S rDNA genes were sequenced directly from marine samples. *A. macleodii* closely related 16S rRNA genes were detected in Mediterranean waters from the deep-chlorophyll maximum (ca. 50 m deep) and from deeper waters (400 m deep) when the large-size fraction (>2 μm) was analyzed (Acinas et al. 1999). rDNA sequences closely associated to *A. macleodii* were also recovered as an important part of the total biomass from confinement and mesocosm experiments in Mediterranean bacterioplankton (Pukall et al. 1999; Schafer et al. 2000). Later studies by rRNA internal transcribed spacer (ITS) sequencing and by hybridization of DNA samples from several marine samples from around the world (Garcia-Martinez et al. 2002) indicated that *A. macleodii* is found as a significant fraction of the bacterial population associated to particles or aggregates (2–5 μm filters) in temperate or tropical waters with average temperatures above 10 °C. This temperature limitation precluded the presence of this microbe in any deepwater samples with the exception of the Mediterranean, where the deepwater mass never gets below 12 °C. Analysis of isolates from the deep Mediterranean revealed differences in ITS and housekeeping genes that suggested the presence of a "Deep Ecotype" (DE) in the Mediterranean (Ivars-Martinez et al. 2008a). A more extense MLSA work confirmed the previous findings (Ivars-Martinez et al. 2008b).

Recently, there has been a rapid development of technologies to facilitate high-throughput sequence analysis of DNA. These advances have revolutionized the field of genomics allowing to reduce the cost of whole genome sequencing. For this reason the number of genomes available has increased. General features of the genome of some strains belonging to *Alteromonadaceae* are shown in Table 5.2.

Ivars-Martinez et al. (2008b) compared two isolates of the type strain *Alteromonas macleodii*. The genome of one *A. macleodii* Deep Ecotype (AltDE) isolate obtained from 1,000 m deep in the South Adriatic was determined and compared with the draft genome of the type strain *A. macleodii* ATCC 27126^T (AltATCC, a surface isolate from Hawaii). Comparative genomics of both strains show that they represent very divergent lineages and could be considered borderline members of the same species (ANI 81.25 %). The genome of AltDE was assembled into a single replicon of 4.4 Mbp. *A. macleodii* strain AltATCC was pyrosequenced and automatically assembled into

Table 5.1
Type strains of species in the family *Alteromonadaceae*

Type strain of the genus	Biological interest	Isolation source	Isolation place	References
Aestuariibacter salexigens	–	Sediment sample of the getbol	Ganghwa Island, Korea	Yi et al. (2004)
Agarivorans albus	Agarolytic	Internal organs of Mollusca	Coast of the Kanto area in Japan	Kurahashi and Yokota (2004)
Aliagarivorans marinus	Agarolytic	Seawater	Shallow coastal region of An-Ping Harbour, Tainan, Taiwan	Dar Jean et al. (2009)
Alishewanella fetalis	–	Autopsy	Human fetus	Vogel et al. (2000)
Alteromonas macleodii ATCC27126^T	–	Seawater	Hawaiian archipelago	Baunmann et al. (1972)
Bowmanella denitrificans	Denitrifying	Seawater	Shallow coastal region of An-Ping Harbour, Tainan, Taiwan,	Dar Jean et al. (2006)
Glaciecola punicea	–	Sea-ice diatom assemblages	Antarctica	Bowman et al. (1998)
Haliea salexigens	–	Seawater	Northwestern Mediterranean Sea	Urios et al. (2008b)
Marinimicrobium koreense	–	Tidal flat sediment	South Sea, Korea	Lim et al. (2006)
Marinobacter hydrocarbonoclasticus	Extremely halotolerant Hydrocarbon degrading	Seawater	Gulf of Fos (French Mediterranean coast) near a petroleum refinery	Gauthier et al. (1992)
Marinobacterium georgiense	–	Marine pulp mill effluent enrichment cultures	Augusta, Georgia	González et al. (1997)
Melitea salexigens	–	Seawater	Northwestern Mediterranean Sea	Urios et al. (2008a)
Microbulbifer hydrolyticus	–	Marine pulp mill effluent enrichment cultures	Augusta, Georgia	González et al. (1997)
Saccharophagus degradans	Carbohydrate degrading	Salt marsh grass (*Spartina alterniflora*)	Chesapeake Bay, USA	Ekborg et al. (2005)
Salinimonas chungwhensis	–	Solar saltern	Yellow Sea in Korea	Jeon et al. (2005)

716 contigs, with an estimated genome size of 4.6 Mbp. Both organisms' genomes are quite similar in structure, in size, and also in the GC content (44.9 % AltDE and 44.6 % AltATCC). Among the 4102 ORFs found in AltDE, 2696 had orthologs in AltATCC.

Thirteen genomic islands (GI) were detected in AltDE. These genomic differences indicate that AltDE is probably better suited to microaerophilic conditions (hydrogenases, nitrate reductase, and microaerophilic respiratory chains) and for the degradation of recalcitrant compounds such as urea and were reminiscent of life even more committed to the particle-associated lifestyle. Three of these GI are related, or reflect, interactions with phages and another contains clustered regularly interspaced short palindromic repeats (CRISPRs) and also CRISPR-associated (CAS) genes. CRISPR is thought to function as an antiphage defense system against infection (Horvath and Barrangou 2010).

Alteromonas sp. strain SN2 is another member of the genus *Alteromonas* in which the genome has been sequenced. *Alteromonas* sp. strain SN2 was isolated from a sea-tidal flat, and it was shown to be responsible for the in situ degradation of polycyclic aromatic hydrocarbons (PAHs) in crude oil-contaminated marine sediment (Jin et al. 2011). Strain SN2 has a larger genome (4.97 Mbp) than the other *Alteromonas* strains with a GC content of 43.5 % and no plasmids. The genome contains 4,355 predicted protein-coding sequences, 64 tRNA genes, 5 complete rRNA loci, and 8 noncoding RNAs.

The ANI values between strains SN2 and AltDE and AltATCC were 74.03 % and 70.8 %, which indicates that the three *Alteromonas* strains represent members of different species within the genus *Alteromonas*. Fifteen putative genomic islands were identified from the genome sequence; the largest genomic island (with 60 protein-coding genes) harbored a PAH-degrading

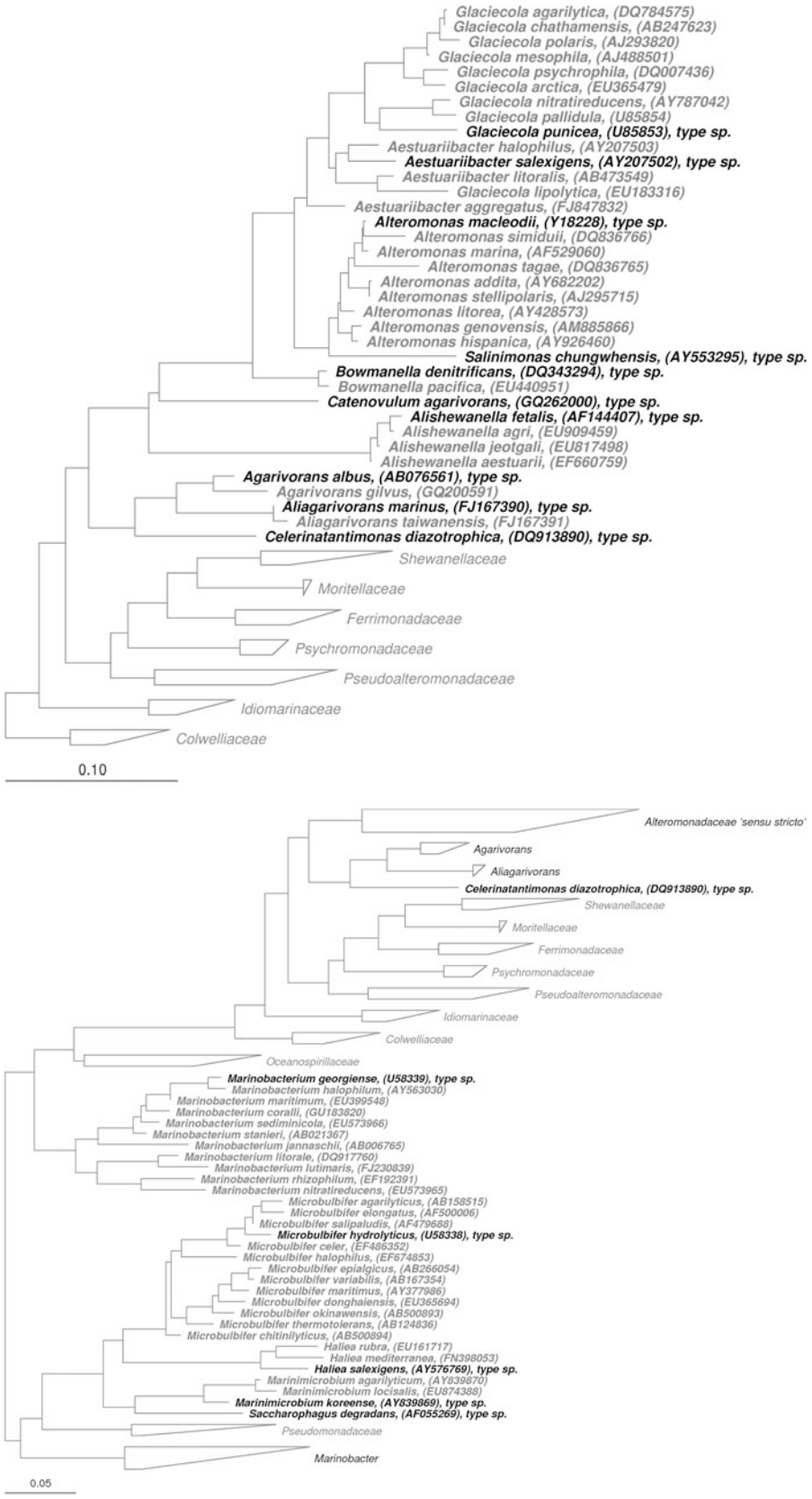

Fig. 5.1
(continued)

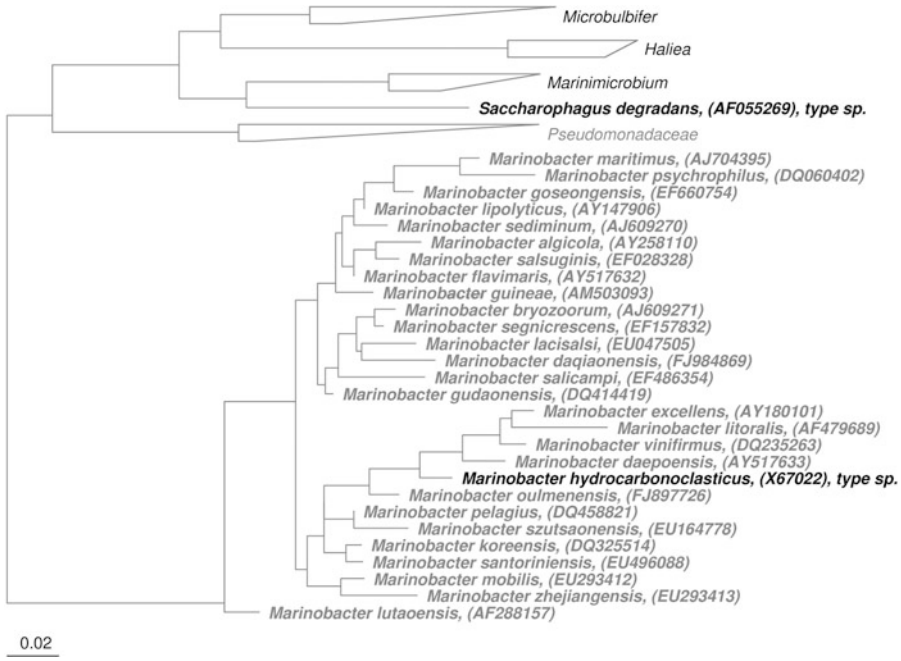

Fig. 5.1

Phylogenetic reconstruction of the family *Alteromonadaceae* based on 16S rRNA and created using the maximum likelihood algorithm RAxML (Stamatakis 2006). The sequence datasets and alignments were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). Representative sequences from closely related taxa were used as outgroups. In addition, a 40 % maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. Scale bar indicates estimated sequence divergence

gene cluster, which was expressed during PAH biodegradation (Math et al. 2012). The presence of phage remnant-like transcription regulator AlpA in GI-2, GI-9, and GI-12 suggested active phage invasion into the genome of strain SN2.

In the genus *Glaciecola* there are 10 recognized species. Phylogenetic analysis showed that the 10 species appeared in two lineages. Lineage I includes *Glaciecola punicea* (the type species), *G. pallidula*, and *G. nitratireducens* and lineage II includes the seven other species (Zhang et al. 2011).

The genome of the type strain *G. punicea* ACAM611T contained 2,883 predicted protein-coding sequences and 45 tRNA genes, with a GC content of 43.10 % (Qin et al. 2012). The *G. punicea* ACAM611T genome size was 3.08 Mbp and contains many fewer genes related to motility and signal transduction function than the genomes of *Glaciecola* sp. 4H-3-7_YE-5 and *G. nitratireducens* FR1064T, which is consistent with the observation that the strain does not have flagella. *G. nitratireducens* strain FR1064T is another strain from *Glaciecola* lineage I sequence. It was isolated from seawater off Jeju Island, Republic of Korea (Baik et al. 2006). The *G. nitratireducens* FR1064T genome was contained in one chromosome with a total size of 4.13 Mbp, which equal to the type strain is smaller than those of the two sequenced lineage II strains, *G. mesophila* (5.14 Mbp) and *Glaciecola* sp. 4H-3-7_YE-5 (5.39 Mbp). The GC content was 42.3 %. The genome had 3.654 predicted protein-coding sequences, four rRNA operons, and 54 tRNA genes (Bian et al. 2011). The lineage II strain *Glaciecola* sp. 4H-3-7_YE-5 was isolated from marine subseafloor sediments (31.4 m below the seafloor) collected at a water depth of 755 m at Suruga Bay (Japan) after enrichment on cellulose, xylan, and chitin as sole carbon sources. The sequenced genome of *Glaciecola* sp. 4H-3-7_YE-5 is contained within one large chromosome (5.05 Mbp) and one plasmid (pGLAAG01, 341,282 bp). The complete genome had a total GC content of 45 % and 4.548 predicted protein-coding sequences (Klippel et al. 2011). Yuan et al. (2011) sequenced the *Glaciecola mesophila* strain genome, isolated from internal liquor of a specimen of the ascidian *Halocynthia aurantium* from Troitsa Bay, Sea of Japan, Russia (Romanenko et al. 2003). The genome consisted of a circular chromosome of 5.14 Mbp in length, 4,528 predicted protein-coding sequences, 59 tRNA, and 15 rRNA genes. The GC content of the assembled sequence was 44.6 %.

Weiner et al. (2008) analyzed the complete genome sequence of the marine bacterium *Saccharophagus degradans* to understand the potential role of prokaryotes in marine carbon transformation. The genome was a single circular chromosome of 5.1 Mbp and had 4.008 predicted protein-coding sequences. The genome also included 50 tRNAs and GC content was 45.8 %. 180 open reading frames that code for carbohydrases were identified including the first characterized marine cellulose system. Some of these open reading frames coding for complex polysaccharide depolymerases were clustered, including

Table 5.2
General features of the genomes

	Alishewanella jeotgali	*A. macleodii* ATCC27128	*A. macleodii* Deep Ecotype	*Alteromonas* SN2	*Glaciecola punicea*	*Glaciecola nitratireducens*	*Glaciecola* 4H-3-7+YE-5	*Marinobacter adherens*	*Marinobacter manganoxydans*	*Marinobacterium stanieri* S30	*Saccharophagus degradans*
Size (bp)	3 844 563	4 607 844	4 412 285	4 972 148	3 076 861	3 333 334	5 052 309	4 421 911	4 549 590	4 370 691	5 057 531
% GC	50.7	44.6	44.9	43.5	43.1	42.3	44.1	56.9	57.3	55.9	45.8
Contigs	65	716	1	1	35	16	1	1	88	74	1
Total ORFs	3,669	4,444	4,102	4,355	2,883	3,654	4,547	4 355	4,171	4,007	4,008
rRNA operons	6	5	5	5	–	4	–	3	–	–	2
tRNAs	64	48	40	64	45	54	–	51	51	25	41
Hypothetical proteins	–	1 588	1 045	954	–	–	–	391	–	–	704
Function assigned	–	3 319	3 057	3 401	–	–	–	3 363	3 128	–	2 994
Plasmids	–	–	–	–	–	–	1 (341 kbp)	2(187 and 42 kbp)	–	–	–

amylases, arabinoxylosidases, pectinases, and alginases. The analysis identified uniquely degrading at least 10 different complex polysaccharides, including agar, chitin, alginic acid, cellulose, β-glucan, laminarin, pectin, pullulan, starch, and xylan, representing an extraordinary range of catabolic capability.

Marinobacter adhaerens HP15 was isolated from marine particles sampled in the German Wadden Sea. This organism is interesting due to its capability to specifically attach in vitro to the surface of the diatom *Thalassiosira weissflogii*, inducing exopolymer and aggregate formation and thus generating marine snow particles (Gärdes et al. 2010). *M. adhaerens* HP15 possesses three replicons; the chromosome had 4.4 Mbp and coded for 4,180 predicted protein-coding sequences, 51 tRNAs, and three rRNA operons, while the two circular plasmids were ~187 kb and ~42 kb in size and contained 178 and 52 predicted protein-coding sequences, respectively. The genome had a 56.9 % GC content. Wang et al. (2012) report the draft genome from another species of the genus, *Marinobacter manganoxydans* MnI7-9, isolated from a deep-sea hydrothermal vent in the Indian Ocean. The genome was assembled into 88 contigs with a total size of 4.5 Mbp and a GC content of 57.3 %. A total of 4,171 predicted protein-coding sequences and 51 tRNAs were annotated. 2,562 proteins have orthologs with *M. adhaerens* HP15. Many genes involved in nickel, mercury, copper, chromate, arsenic, zinc, cobalt, and cadmium resistance were found and explain the high tolerance of *M. manganoxydans* MnI7-9 to many metal(loid)s.

Two species of the genus *Alishewanella* have been sequenced and show differences with respect to genome size, plasmid presence, and protein abundance. *Alishewanella agri* BL06T was isolated from landfill soil in Pohang (South Korea) and genome size was 3.50 Mbp with a GC content of 50.6 %, 3,223 predicted protein-coding sequences, 68 tRNA genes, and 3 rRNA genes and might have a 74-kb plasmid (Kim et al. 2012). The presence of two genes that code for proteins involved in pectin degradation (pectin methylesterase and pectate lyase) showed the ability to degrade this heteropolysaccharide present in the cell wall of plants. The other strain sequenced, *Alishewanella jeotgali* MS1T, was isolated from a traditional Korean fermented seafood, gajami sikhae (jeotgal). The genome size was bigger than that of *Alishewanella agri* BL06T; however, it did not have a plasmid. The *A. jeotgali* MS1T genome size was 3.84 Mbp with a GC content of 50.66 %, 3,669 predicted protein-coding sequences, 64 tRNA genes, and 6 rRNA genes. The strain has 2 extracellular proteases which the authors suggest that they may play an important role in the fermentation environment of food containing fish flesh (Jung et al. 2012).

Phenotypic Analyses

The main features of the genera included in the *Alteromonadaceae* are listed in ❯ *Table 5.3*.

The genus *Aestuariibacter*, which belongs to the family *Alteromonadaceae*, was proposed by Yi et al. (2004) to appoint two species which were isolated from a "getbol" sediment sample collected from Ganghwa Island, Korea. These two species named as *Aestuariibacter halophilus* and *Aestuariibacter salexigens* were strictly aerobic, chemoheterotrophic, salt-requiring, mesophilic, neutrophilic, and nonspore-forming rods and motile by means of single polar flagella. There are only two more species with validly published names in the genus *Aestuariibacter*: *Aestuariibacter aggregates* which was isolated from seawater of the Yellow Sea off China (Wang et al. 2010) and *A. litoralis* which came from a sandy sediment sample collected offshore in the Sea of Japan (Tanaka et al. 2010).

Aestuariibacter (Aes.tu.ar.i.i.bac´ter. L. neut. n. *aestuarium*-i tidal flat; N.L. masc. n. *bacter* rod; N.L. masc. n. *Aestuariibacter*, rod-shaped bacterium from tidal flat).

Gram negative and oxidase and catalase positive. Strictly aerobic, chemoheterotrophic, halophilic, mesophilic, and neutrophilic. Cells are rod shaped and motile with a polar flagellum. Abundant growth occurs on MA, CSY-3, and SMM media. Spores are not formed. Major isoprenoid quinone is Q8. Predominant cellular fatty acids are C16: 0, C18: 1ω7*c*, and a mixture of C16: 1ω7*c* and iso-C15: 0 2-OH. DNA G+C content is 48–54 mol%. The type species is *Aestuariibacter salexigens*.

Description of *Aestuariibacter salexigens* sp. nov.

Aestuariibacter salexigens (sa.lex´i.gens. L. n. *sal salis* salt, seawater; L. v. *exigo* to demand; N.L. part. adj. *salexigens*, seawater demanding). Cells are approximately 1.0–1.8 mm long and 0.4–0.6 mm wide. Optimal growth is observed at 35 °C, pH 7–8, and 2–6 % artificial sea salts. Colonies are circular, raised, entirely margined, brittle, rough, opaque and white on MA, and hard to emulsify. Viability is lost rapidly after 7 days on MA at 30 °C. Reduces nitrate to nitrite. Decomposes DNA, aesculin, gelatin, starch, and Tween 80, but not agar, alginate, casein, cellulose, chitin, or egg yolk. Does not produce arginine dihydrolase, β-galactosidase, fluorescein, H2S, indole, polyhydroxybutyrate, or urease. Does not ferment carbohydrates. Produces alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin, and naphthol-AS-BI-phosphohydrolase, but not lipase (C14), valine arylamidase, cystine arylamidase, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, b-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-b-glucosaminidase, α-mannosidase, or α-fucosidase. Utilizes acetate, glycine, inulin, L-arginine, L-ornithine, and *N*-acetylglucosamine as sole carbon sources. D-Galactose, D-ribose, D-sorbitol, ethanol, L-lysine, and succinate are utilized weakly. Does not utilize acetamide, adenine, benzoate, citrate, D-cellobiose, D-fructose, D-glucose, D-mannose, D-raffinose, D-salicin, D-xylose, inositol, 2-propanol, lactose, L-cysteine, L-rhamnose, sucrose, salicylate, or thiamin. DNA G+C content is 48 mol%.

The genus *Agarivorans*, belonging to the family *Alteromonadaceae*, was first proposed by Kurahashi and Yokota (2004). Six bacterial strains were isolated from healthy marine organisms that were collected from the coast of the Kanto area in Japan. Phylogenetic analysis based on 16S rDNA sequence similarity showed that the six isolates formed a separate cluster in the γ-*Proteobacteria* and were related to the genera *Alteromonas* and *Glaciecola*. One particular characteristic of the isolates was

Table 5.3

Differential characteristics of marine gammaproteobacteria family *Alteromonadaceae*

Characteristic	*Alteromonadaceae*						
	Aestuariibacter	*Agarivorans*	*Aliagarivorans*	*Alishewanella*	*Alteromonas*	*Bowmanella*	*Glaciecola*
Pigmentation	None	White	Off-white	None	Cream	Off-white	Pink-red
Flagellum	Single polar	Single polar	Single polar	Nonmotile	Single polar	Single polar	Single polar
Morphology	Rod	Rod	Rod	Rod	Rod	Rods	Rods
Catalase	+	+	+	+	+	–	+
Oxidase	+	+	–	+	+	–	+
Nitrate reduction	+	+	+	+	–	+	–
Metabolism	Aerobic	Aerobic	Facultative anaerobic	Facultative anaerobic	Aerobic	Facultative anaerobic	Aerobic
Sampling environment	Marine sediment	Internal organs of Mollusca	Seawater	Autopsy of a human fetus	Seawater	Seawater	Sea Ice
Halotolerance (% NaCl)	1–8	<10	2–4	Up to 8	1–12	0–10	1–6
Growth at							
4 °C	–	nd	–	–	–	–	+
37 °C	+	+	+	+	+	+	–
42 °C	–	nd	–	+	+	–	–
Hydrolysis of							
Chitin	–	nd	–	nd	–	nd	–
Agar	–	+	+	nd	–	–	+
Starch	+	nd	–	nd	+	+	+
Gelatin	+	–	+	+	+	+	–
Utilization							
D-Glucose	–	+	+	–	+	+	+
D-Fructose	–	+	–	nd	+	+	–
D-Mannose	–	-	–	–	nd	+	–
L-Rhamnose	–	nd	–	nd	nd	–	–
Sucrose	–	-	–	nd	+	+	–
Cellobiose	–	nd	+	nd	+	+	–
Lactose	–	nd	–	nd	+	+	–
Glycerol	nd	nd	–	nd	nd	nd	+
Major fatty acids	C16:0, C18:1 ω7c	C16:0, C16:1 ω7c	C18:1 ω7c	C17:1B C16:1 *cis*9	C16:0, C16:1 ω7c	C16 : 0, C18 : 1ω7c	C16:0, C16:1 ω7c
GC content (mol%)	48	49.5	52.9	51	45-46	50	40

Characteristic	*Alteromonadaceae*						
	Marinimicrobium	*Marinobacter*	*Marinobacterium*	*Melitea*	*Microbulbifer*	*Saccharophagus*	*Salinimonas*
Pigmentation	Cream	Cream	None	Cream	None	Cream	Cream
Flagellum	Single polar	Single polar	Single polar	Single polar	None	Single polar	Single polar
Morphology	Short rod	Rod	Rod	Rod	Rod	Pleomorphic rod	Short rod
Catalase	–	+	+	+	+	+	+
Oxidase	–	+	+	+	+	+	+
Nitrate reduction	–	–	–	nd	–	+	–

Table 5.3 (continued)

Characteristic	*Alteromonadaceae*						
	Marinimicrobium	*Marinobacter*	*Marinobacterium*	*Melitea*	*Microbulbifer*	*Saccharophagus*	*Salinimonas*
Metabolism	Aerobic	Facultative anaerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic
Sampling environment	Tidal flat seawater	Dinoflagellates	Seawater	Seawater	Seawater	Salt marsh grass	Solar saltern
Halotolerance (% NaCl)	0–15	1–12	0.01–2	0.7–7	0.1–1	1–10	2–15
Growth at							
4 °C	–	–	+	–	–	+	–
37 °C	+	+	+	+	+	+	+
42 °C	+	–	–	–	–	–	+
Hydrolysis of							
Chitin		nd	–	nd	+	+	nd
Agar	–	+	–	nd	–	+	nd
Starch	+	+	–	nd	–	+	+
Gelatin	–	–	–	nd	+	–	+
Utilization							
D-Glucose	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	–	nd	–
D-Mannose	+	–	+	+	–	nd	–
L-Rhamnose	nd	–	–	–	–	nd	–
Sucrose	+	–	–	+	–	nd	–
Cellobiose	nd	–	+	+	+	nd	nd
Lactose	+	nd	–	+	–	nd	–
Glycerol	–	+	+	–	–	nd	–
Major fatty acids	C16:0, C19:0 ω8c	C16:0, C16:1 ω7c	C16:0	Iso-17:1ω9c	C15:0, C17:1 ω9c	Iso C16:0	C16:0, C18:1 ω7c
GC content (mol%)	57	55	54.9	57	57.6	45.8	48

+ positive, – negative, *nd* no data available

the ability to hydrolyze agar. The name *Agarivorans albus* gen. nov., sp. nov., was proposed for the six isolates. Actually, *Agarivorans gilvus* represented a novel agarase-producing, non-endospore-forming marine bacterium isolated from a fresh seaweed sample collected from the coast of Weihai (China) species within the genus *Agarivorans* (Du et al. 2011).

Agarivorans (A.ga.ri.vo´rans. N.L. neut. n. *agarum* agar; L. part. adj. *vorans* devouring, destroying; N.L. adj. *Agarivorans*, agar devouring).

Gram negative, strictly aerobic, and agar hydrolyzing. Cells are rod shaped and approximately 1.5–1.9 × 0.7–0.9 μm in size. Spores are not formed; motile by single polar flagella. No growth occurs without NaCl. Mesophilic. Hydrolysis of agar is observed. Major quinone is ubiquinone-8. Predominant cellular fatty acids are saturated and monounsaturated, straight-chain fatty acids.

Description of *Agarivorans albus* sp. nov.

Agarivorans albus (al´bus. L. adj. *albus* white). In addition to the characteristics that define the genus, the following characteristics are observed. Grows on marine agar 2216 and forms colonies with a smooth surface that are thin, circular in shape, and white in color. Major fatty acids are C16: 1ω7*c*, C16: 0, C18: 1ω7*c*, and C18: 1ω6*c*. Isolated from gastrointestinal tracts and attached internal organs of Mollusca. The type strain, MKT 106 T (=IAM 14998 T = LMG 21761 T), was isolated from the marine mollusc *Omphalius pfeifferi pfeifferi*. DNA G+C content of the type strain is 49.5 mol%.

The genus *Aliagarivorans* was proposed by Jean et al. (2009) to include two agarolytic strains of Gram-negative, heterotrophic, facultatively anaerobic, marine bacteria. Two species were proposed, designated *Aliagarivorans marinus* and *Aliagarivorans taiwanensis*, obtained from a seawater sample collected in the shallow coastal region of An-Ping Harbour, Tainan, Taiwan.

Description of *Aliagarivorans* gen. nov. *Aliagarivorans* (A.li.a.ga.ri.vo9rans. L. pronoun *alius* other, another; N.L. n. *Agarivorans* a name of a bacterial genus; N.L. masc. n. *Aliagarivorans*, the other *Agarivorans*).

Members are heterotrophic, Gram-negative rods belonging to the *Gammaproteobacteria*. Cells grown in broth cultures are

motile by means of a single polar flagellum. Facultative anaerobes capable of both respiratory and fermentative metabolism. Mesophilic, halophilic, and agarolytic. Oxidase negative. Catalase positive. Major isoprenoid quinone is Q-8. Major cellular fatty acids include C18 : 1v7c, summed feature 3 (C16 : 1v7c and/or iso-C15 : 0 2-OH), and C16 : 0. The DNA G+C content is approximately 52–53 mol%. The type species is *Aliagarivorans marinus*.

Description of *Aliagarivorans marinus* sp. nov.

Aliagarivorans marinus (ma.ri9nus. L. masc. adj. marinus of the sea, marine). This species has the following characteristics in addition to those given for the genus. Cells during late exponential to early stationary phase of growth in broth cultures are straight rods, approximately 1.4–2.0 μm long and 1.0–1.2 μm wide. Colonies produced on marine agar and PY plate medium at 30 °C for 4–7 days are circular (approx. 1–2 mm in diameter), off-white, opaque, convex, nonluminescent, and agarolytic; agarolytic activity is indicated by the development of softening and depressions, but not by liquefaction of the agar surrounding the colonies. Swarming does not occur. Able to ferment D-glucose, D-arabinose (weakly), L-arabinose, cellobiose, D-fructose (weakly), D-galactose, D-lactose, maltose, D-mannose, melibiose, melezitose, raffinose (weakly), sucrose (weakly), trehalose, D-xylose, dulcitol, myo-inositol (weakly fermentative), D-mannitol, and D-sorbitol (weakly) with production of acid, but no gas. Unable to ferment D-ribose. Nitrate is reduced to nitrite, but not further to N_2O or N_2. Poly-b-hydroxybutyrate is accumulated as an intracellular reserve product. Indole is not produced from tryptophan. Growth occurs between 15 and 37 °C, with optimum growth at 25–30 °C; no growth occurs at 4–10 or 40–42 °C. Sodium ions are required for growth; growth occurs at NaCl levels of 1–7 % (w/v), with optimum growth at 2–3 %, and no growth occurs at 0 or 8–10 % NaCl. Able to grow over the pH range 7–9, but not at pH 5–6. Not hemolytic. Agar, alginate, and gelatin are hydrolyzed, but aesculin, casein, cellulose, chitin, DNA, lecithin, starch, Tween 80, and urea are not. Arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase are absent. The following constitutive enzyme activities are detected in API ZYM tests: leucine arylamidase, esterase (C4), b-galactosidase and naphthol-AS-BI-phosphohydrolase. Able to grow on the following compounds as sole carbon and energy sources: L-arabinose, cellobiose, D-galactose, D-glucose, maltose, D-mannitol, acetate, fumarate, b-hydroxybutyrate, pyruvate, L-alanine, L-aspartate, L-glutamate and L-glutamine. Unable to grow on the following compounds as sole carbon and energy sources: D-arabinose, D-fructose, D-lactose, D-mannose, melibiose, raffinose, L-rhamnose, D-ribose, sucrose, D-xylose, trehalose, salicin, dulcitol, glycerol, myoinositol, D-sorbitol, citrate, lactate, malate, succinate, tartaric acid, L-arginine, L-citrulline, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-ornithine, L-phenylalanine, L-serine, L-threonine, L-tyrosine, L-tryptophan, and L-valine.

The genus *Alishewanella* is one of the major branches of the family *Alteromonadaceae* and was first proposed by Vogel et al. (2000) to accommodate *Alishewanella fetalis*, isolated from an autopsy of a human fetus in 1992. Currently, the genus *Alishewanella* contains 6 species, which have been isolated from different habitats such as human fetus (Vogel et al. 2000), tidal flat sediment (Roh et al. 2009), fermented food (Kim et al. 2009), landfill soil (Kim et al. 2010), Qurugol Lake located in Azarbayian region (Tarhriz et al. 2011), and traditional Korean fermented seafood, gajami sikhae (jeotgal) (Jung et al. 2012). The fact that organisms in this genus have been isolated from various sources implies that *Alishewanella* species may have great adaptability to diverse environments and unique genetic requirements for each habitat. ➲ *Table 5.4* indicates the phenotypic properties of *Alishewanella* species.

Alishewanella (A.li.she.wa.nel´la. L. pronoun. *Alius* the other; M.L. fem. n. *Alishewanella*, the other *Shewanella*).

Cells are Gram-negative, nonmotile rods. Facultatively anaerobic and uses TMAO, nitrate, thiosulfate, and nitrite, but not sulfite or ferric iron, as electron acceptors. Oxidase and catalase positive. Halotolerant and requires NaCl for growth. Glucose is not catabolized with production of acid. Hydrolyzes gelatin and aesculin but is unable to produce indole, urease, *b*-galactosidase, arginine dihydrolase, and H_2S. Does not grow at temperatures of 20 °C or less. The DNA G+C content is 51 mol%. The type species is *Alishewanella fetalis*.

Description of *Alishewanella fetalis* sp. nov.

Alishewanella fetalis (fe.ta´lis. L. adj. *fetalis* pertaining to the fetus, from which the organism was isolated). Cells are Gram-negative, nonmotile rods with a length of 2 μm and a width of 0.5 ± 1.0 μm. They occur typically as single cell. Growth occurs between 25 and 42 °C with an optimum at 37 °C. Halotolerant, requires NaCl for growth, and withstands NaCl concentration up to 8 % but does not grow at 10 % NaCl. Oxidase and catalase positive and unable to ferment carbohydrates (glucose, maltose, ribose, and arabinose). Hydrolyzes gelatin and aesculin but is unable to produce indole, urease, *b*-galactosidase, and arginine dihydrolase. Facultatively anaerobic and uses TMAO, nitrate, thiosulfate, and nitrite but not sulfite or ferric iron, as electron acceptors. The DNA G+C content is 51 mol%. Isolated at an autopsy of a human fetus in 1992.

The genus *Alteromonas* was isolated and named by Baumann et al. (1972) (emended description by Gauthier et al. 1995 and later Van Trappen et al. 2004a and Yi et al. 2004) and originally contained a phylogenetically and phenotypically heterogeneous group of Gram-negative, heterotrophic, marine bacteria, motile by a single polar flagellum. There were at least 21 validly described species within the genus *Alteromonas*. However, the majority of these species have been reclassified in other genera, namely, *Marinomonas*, *Pseudoalteromonas*, and *Shewanella* (Coyne et al. 1989; Gauthier et al. 1995; Ivanova et al. 2000, 2001; MacDonell and Colwell 1985; Sawabe et al. 2000; van Landschoot and De Ley 1983). Nowadays *Alteromonas* comprises only ten valid species: *A. macleodii* (Baumann et al. 1972, 1984; Gauthier et al. 1995; Yi et al. 2004), *A. marina* (Yoon et al. 2003b), *A. stellipolaris* (Van Trappen et al. 2004a), *A. litorea* (Yoon et al. 2004c), *A. hispanica* (Martínez-Checa et al. 2005), *A. addita* (Ivanova et al. 2005), *A. simiduii*, *A. tagae* (Chiu et al. 2007), *A. genovensis* (Vandecandelaere et al. 2008), and *A. halophila* (Chen et al. 2009b).

Table 5.4
Phenotypic properties of *Alishewanella* species (Table modified from Kim et al. 2010)

Characteristic	*A. agri*	*A. fetalis*	*A. aestuarii*	*A. jeotgali*
Motility	–	–	+	+
Growth at				
4 °C	–	–	–	(+)
10 °C	+	–	–	+
Temperature range (°C)	10–44	25–42	18–44	4–40
Growth in				
0 % NaCl	+	–	+	–
6 % NaCl	+	+	–	–
8 % NaCl	–	+	–	–
NaCl concentration range (%)	0–6	0–15	0–5	1–2
Hydrolysis of aesculin	+	+	–	+
Assimilation of				
d-Glucose	+	–	–	+
Malate	–	+	–	–
Glycerol	–	+	–	–
d-Fructose	–	+	+	–
Inositol	–	+	–	–
d-Mannitol	–	+	+	–
Aesculin	+	–	–	+
Trehalose	–	–	–	+
Raffinose	–	–	+	–
Glycogen	–	–	–	+
DNA G+C content (mol%)	54.8	51.0	49.5	53.6
Isolation source	Soil	Human fetus	Tidal flat sediment	Fermented food

+ positive, (+) weakly positive, – negative

Alteromonas (*Al.te.ro.mo´nas.* L. adj. *altera* another; Gr. n. *monas* a unit, monad; M.L fem. n. *Alteromonas*, another monad)

Gram-negative, nonspore-forming straight rods that are 0.7–1 μm in diameter and 2–3 μm long. Motile by means of a single unsheathed polar flagellum. Not luminescent and not pigmented. Strictly aerobic. Chemoorganotroph with respiratory but not fermentative metabolism. Oxidase positive and catalase negative. Growth occurs at 20–35 °C but not at 4 °C. No constitutive arginine dihydrolase system. Does not accumulate poly-*P*-hydroxybutyrate from the monomer *P*-hydroxybutyrate. Requires a seawater base for growth, but not organic growth factors. The G+C content of the DNA is 44–47 mol%.

Description of *Alteromonas macleodii* sp. nov.

Strains belonging to this species are straight rods which are motile by means of a single polar flagellum originating from one pole. None of the strains accumulates *P*-hydroxybutyrate as an intracellular reserve product or utilize aromatic compounds. The GC content in the DNA of five representative strains is 45.6 + 0.8 mol%. All or a majority of the strains have an extracellular amylase, gelatinase, and lipase and are able to utilize cellobiose, melibiose, lactose, and salicin. A distinctive property of this species is its inability to utilize tricarboxylic acid cycle intermediates such as succinate, fumarate, citrate, a-ketoglutarate, and aconitate. Positive for catalase reaction. Major isoprenoid quinone is ubiquinone-8. Positive for β-galactosidase, aesculinase, and lecithinase. Negative for urease, caseinase, and cellulase. Does not produce indole. Utilizes D-raffinose, D-ribose, L-arginine, and succinate as sole carbon sources, but not D-sorbitol or thiamin. Produces alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase, but not lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, or α-fucosidase.

The genus *Bowmanella* was proposed by (Jean et al. 2006) to accommodate a heterotrophic, nonfermentative, denitrifying isolate, designated *Bowmanella denitrificans*, obtained from a seawater sample collected in the shallow coastal region of

An-Ping Harbour, Tainan, Taiwan. The other species of this genus is *Bowmanella pacifica* which was isolated from a pyrene-degrading consortium, enriched from the sediment of the Pacific Ocean (Lai et al. 2009).

Bowmanella (Bow.man.el´la. L. dim ending *-ella*; N.L. fem. n. *Bowmanella* named after John P. Bowman, to honor his work in marine microbiology).

Members are Gram-negative rods belonging to the class *Gammaproteobacteria*. Cells grown in broth cultures are motile by means of a single, polar flagellum. Chemoorganotrophs capable of respiratory but not fermentative metabolism. Mesophilic, growing well at 25–35 °C; no growth occurs at 4 or 45 °C. NaCl stimulates growth but is not an absolute requirement. Oxidase and catalase positive. Major polar lipids are phosphatidylethanolamine and phosphatidylglycerol. Isoprenoid quinones consist of Q-8 (most abundant), Q-9, and Q-10. Major cellular fatty acids are C16: 0, C18: 1ω7*c*, and summed feature 3 (C16: 1ω7*c* and/or C15: 0 iso 2-OH). The DNA G+C content of the type strain of the type species is 50 mol%. The type species is *Bowmanella denitrificans*.

Description of *Bowmanella denitrificans* sp. nov.

Bowmanella denitrificans (de.ni.tri´fi.cans. N.L. v. *denitrifico* to denitrify; N.L. part. adj. *denitrificans*, denitrifying). Cells during late exponential to early stationary phase of growth in broth cultures are polar flagellated, curved rods, approximately 3.7–5.3 µm long by 1.2–1.6 µm wide. Colonies produced on agar plates are circular, off-white in color, and nonluminescent. Swarming does not occur. Growth occurs at temperatures between 10 and 40 °C, with an optimum at 30–35 °C. Growth occurs at pH values in the range 6–10, with an optimum at pH 8. Growth occurs at NaCl levels of 0–10 %, with an optimum at 1–3 %; no growth occurs at 12 % NaCl. Unable to ferment D-glucose, D-arabinose, L-arabinose, D-cellobiose, D-galactose, D-mannose, sucrose, D-trehalose, D-xylose, dulcitol, inositol, or D-mannitol. Molecular oxygen is a universal electron acceptor, while anaerobic growth can be achieved by carrying out denitrification with NO_3^- or N_2O as the terminal electron acceptor. Indole is produced from tryptophan. H_2S is not produced from thiosulfate. Aesculin, casein, DNA, gelatin, lecithin, starch, Tween 80, and urea are hydrolyzed, but agar is not. Arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase activities are not detected. D-Cellobiose, D-fructose, D-glucose, D-galactose, D-lactose, D-mannose, sucrose, D-trehalose, D-xylose, acetate, and β-hydroxybutyrate can be utilized as sole carbon and energy sources, but D-arabinose, L-arabinose, adonitol, dulcitol, inositol, D-mannitol, D-melezitose, D-melibiose, D-sorbitol, and L-lactate cannot.

The genus *Glaciecola* was originally created to describe aerobic, psychrophilic, halophilic bacteria and initially comprised two species, isolated from sea-ice diatom assemblage samples collected from coastal areas of eastern Antarctica, *Glaciecola punicea* and *Glaciecola pallidula* (Bowman et al. 1998). In recent years, seven new species of this genus have been isolated from marine environments: *G. mesophila* (Romanenko et al. 2003) from marine invertebrates; *G. polaris* (Van Trappen et al. 2004b) from Arctic Ocean seawater; *G. nitratireducens* (Baik et al. 2006) from coastal surface seawater collected off Jeju Island, Republic of Korea; *G. chathamensis* (Matsuyama et al. 2006) from Pacific Ocean floor sediment; *G. psychrophila* (Zhang et al. 2006) and *G. agarilytica* (Yong et al. 2007) from East Sea sediments; *G. lipolytica* from coastal surface seawater near Tianjin City, China (Chen et al. 2009a); and *G. arctica* from Arctic marine sediment (Zhang et al. 2011). Phylogenetic analysis showed that these species appeared in two lineages, with lineage I including *G. punicea* (the type species), *G. pallidula*, and *G. nitratireducens* and lineage II including the other species. ❯ *Table 5.5* indicates the phenotypic properties of *Glaciecola* species.

Glaciecola (Gla.ci.e'co.la. L. fem. n. *glacies* ice; L. gen. n. *incola* an inhabitant; M.L. fem. n. *Glaciecola*, inhabitant of ice).

Cells are slender with a rodlike shape. Cells may be slightly curved, curved, or spiral. Gram-negative, motile, non-sporulating, strictly aerobic chemoheterotroph. Psychrophilic and requiring seawater for growth. Slightly halophilic. Oxidase and catalase positive. Major fatty acids are hexadecanoic acid (16:1ω7*c*), hexadecanoic acid (16 : 0), and octadecanoic acid (18: 1ω7*c*). DNA G+C content is 40–46 mol% (determined from thermal denaturation curves, Tm). Member of the gamma subclass of the *Proteobacteria*. Antarctic sea-ice diatom assemblages are the only known habitat. Type species is *Glaciecolii punicea*.

Description of *Glaciecola punicea* sp. nov.

Glaciecola punicea (pu.nice'a. L. fem. adj. *punicea* pink-red, referring to the species pigmentation). Same as genus description plus the following. Temperature optimum is about 15 °C and temperature maximum is 20–25 °C. Some strains require yeast extract for growth and growth is stimulated by vitamin growth factors. Oxidative acid production from carbohydrates is weak and delayed and may be formed from D-glucose, D-galactose, D-melibiose, and glycerol. Some strains slowly degrade uric acid, aesculin, or L-tyrosine. The enzymes α-galactosidase, β-galactosidase, β-galactosidase-6-phosphate, and alkaline phosphatase are formed. Utilizes the following substrates as carbon and energy sources: succinate, L-malate, fumarate, oxaloacetate, and L-proline. Some strains can utilize a-glycerophosphate. Colonies have a bright pink-red pigment and are circular and convex in shape, with an entire edge and possessing a butyrous consistency. DNA G+C content is 44–46 mol%.

The genus *Haliea* was described to accommodate a marine strain isolated from seawater of the Mediterranean Sea named as *Haliea salexigens* (Urios et al. 2008b). Recently two other species has been isolated. *H. rubra* (Urios et al. 2009) isolated from the same habitat as *H. salexigens* and *H. mediterranea* that was isolated from seawater from Castellón, Spain (Lucena et al. 2010).

Haliea (Ha´lie.a. N.L. fem. n. *Haliea* named after Halie, a sea nymph in Greek mythology, referring to the marine source of the first strain).

Motile Gram-negative rods. The major fatty acids are 17: 1ω8*c*, 16:1ω7*c*, 18:1ω7*c*, and 17: 0. The ubiquinone is Q-8 and the polar lipids are diphosphatidylglycerol and phosphatidylglycerol. Phylogenetically affiliated with the class

Table 5.5

Phenotypic properties of *Glaciecola* species (Table modified from Chen et al. 2009a)

Characteristic	*Glaciecola lipolytica*	*Glaciecola mesophila*	*Glaciecola chathamensis*	*Glaciecola polaris*	*Glaciecola agarilytica*	*Glaciecola punicea*	*Glaciecola pallidula*	*Glaciecola nitratireducens*	*Glaciecola psychrophila*
Flagellation	Single polar	Single polar	Single polar	Single polar	Single polar	nd	nd	nd	nd
Pigmentation	–	–	–	–	–	Pink-red	Pale pink	–	–
Growth temperature range (°C)	4–36	7–35	4–30	5–30	7–30	−2 to 25	−2 to 20	15–30	4–15
Growth in 10 % NaCl	–	–	+	+	–	–	–	–	–
Oxidase	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Hydrolysis of									
Gelatin	+	–	–	–	nd	–	–	+	nd
Aesculin	+	w	+	+	+	v (+)	–	–	–
Starch	+	+	nd	+	+	–	v (+)	nd	+
Agar	–	+	–	–	+	–	–	–	–
Tween 80	+	+	+	+	nd	v (–)	+	nd	+
Utilization of									
L-Arabinose	+	–	–	–	nd	–	–	+	–
D-Glucose	+	+	+	+	–	–	–	–	–
Maltose	+	+	+	+	–	–	–	+	+
D-Galactose	+	v (+)	+	+	+	–	–	nd	+
Cellobiose	+	v (+)	–	+	+	–	–	nd	–
D-Fructose	+	v (+)	+	+	+	–	–	nd	–
D-Mannose	+	v (w)	+	+	w	–	–	+	–
D-Mannitol	+	+	+	+	+	–	–	–	–
Glycerol	+	–	–	–	nd	–	+	nd	–
N-Acetylglucosamine	+	–	–	–	nd	–	–	+	–
Glycogen	+	+	nd	+	–	–	+	nd	nd
DNA G+C content (mol%)	40.8	44.8	44.8	44	45	44–46	40	44	42.9

All species were positive for motility, sodium ion requirement for growth, oxidase and catalase, and growth in 2–6 % (w/v) NaCl

+ positive, – negative, *v* variable between strains, with the reaction for the type strain in parentheses, *w* weak or delayed, *nd* no data available

Gammaproteobacteria within the family *Alteromonadaceae*. The type species is *Haliea salexigens*.

Description of *Haliea salexigens* sp. nov.

Haliea salexigens (sa.le´xi.gens. L. n. sal, salis salt, seawater; L. v. exigo to demand; N.L. part. adj. salexigens, seawater demanding). Displays the following properties in addition to those given in the genus description. Produces cream colonies on marine agar 2216. Cells are 1.6 ± 0.3 μm long and 0.5 ± 0.2 μm wide, with single polar flagella. The G+C content of the type strain is 61 mol%. Growth occurs at 10–37 °C (optimum, 25–30 °C), at pH 5.0–9.0 (optimum, pH 8.0), and at salinities in the range 7–70 g NaCl l^{-1} (optimum, 42 g l^{-1}). Positive reactions with Biolog GN2 plates are obtained for Tweens 40 and 80, pyruvic acid methyl ester, succinic acid methyl ester, β-hydroxybutyric acid, α-ketovaleric acid, succinic acid, glutamic acid, glycyl L-glutamic acid, and glycerol. Positive API ZYM reactions are obtained for the following enzyme activities: alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase. Oxidase and catalase positive. The major fatty acids also include 15: 1*ω6c* and 15: 0.

The genus *Marinimicrobium* was created by Lim et al. (2006) with the description of two species, *Marinimicrobium koreense* and *Marinimicrobium agarilyticum*. Phylogenetic analyses based on 16S rRNA gene sequences showed that the genus *Marinimicrobium* falls within the class Gammaproteobacteria (Lim et al. 2006). Moller et al. (2010) isolated a new species from hypersaline surface sediment of the southern arm of Great Salt Lake (Utah, USA), *Marinimicrobium haloxylanilyticum*.

Marinimicrobium (Ma.ri.ni.mi.cro´bi.um. L. adj. *Marinus* of the sea; N.L. neut. n. *microbium* microbe; N.L. neut. n. *Marinimicrobium*, microbe living in the sea).

Strictly aerobic, chemoheterotrophic, and moderately halotolerant. Colonies are creamy, smooth, glistening, and circular/slightly irregular. Cells are Gram-negative, nonspore-forming, short rods, approximately 0.5–0.8 μm wide and 0.9–1.5 μm long. Nitrate is not reduced to nitrite. Cells are motile by means of a flagellum. Catalase negative. The predominant isoprenoid quinone is Q-8. The major fatty acids are C16: 0, C19: 0 cyclo *ω*8c, and summed feature 3 (C16: 1*ω7c* and/or iso C15: 0 2-OH). The G+C content of the genomic DNA is 57–58 mol% (HPLC). Phylogenetically, the genus belongs to the class *Gammaproteobacteria*. The type species is *Marinimicrobium koreense*.

Description of *Marinimicrobium koreense* sp. nov.

Marinimicrobium koreense (ko.re.en´se. N.L. neut. adj. *koreense*, pertaining to Korea). Growth of cells occurs at salinities in the range 0–15 %(w/v) NaCl (optimum 1–3 % w/v). Oxidase negative. Grows at between 10 and 45 °C (optimum 35–40 °C) and from pH 6.0 to 10.5 (optimum pH 7.0–7.5). API ZYM kit gives positive results for alkaline phosphatase, esterase (C4), esterase lipase, leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, α-glucosidase, and *N*-acetyl-β-glucosaminidase and negative results for lipase, cystine arylamidase, trypsin, a-chymotrypsin, acid phosphatase, β-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase, and α-fucosidase. Aesculin, starch, and Tween 20 are hydrolyzed. Casein, gelatin, L-tyrosine, xanthine, and Tween 80 are not hydrolyzed. Acids are produced from D-glucose, D-fructose, D-ribose, D-xylose, a-D-lactose, maltose, D-trehalose, L-arabinose, D-melibiose, D-mannose, and sucrose, but not from D-mannitol, adonitol, raffinose, glycerol, or inositol. Shows no agarolytic activity. Contains large amounts of phosphatidylethanolamine and diphosphatidylglycerol and small amounts of two unknown phospholipids (PL1, PL2) as the polar lipids. The DNA G+C content is 57 mol%.

The genus *Marinobacter*, a member of the class *Gammaproteobacteria*, was first proposed by Gauthier et al. (1992). The type species of the genus *Marinobacter hydrocarbonoclasticus* was isolated from sediments collected in the Gulf of Fos (French Mediterranean coast) at the mouth of a petroleum refinery outlet chronically polluted by hydrocarbons (Gauthier et al. 1992). The genus comprised 25 species with validly published names isolated from diverse environments, including *M. aquaeolei* (Huu et al. 1999), *M. litoralis* (Yoon et al. 2003d), *M. lipolyticus* (Martín et al. 2003), *M. excellens* (Gorshkova et al. 2003), *M. lutaoensis* (Shieh et al. 2003), *M. flavimaris* and *M. daepoensis* (Yoon et al. 2004b), *M. bryozoorum* and *M. sediminum* (Romanenko et al. 2005), *M. maritimus* (Shivaji et al. 2005), *M. algicola* (Green et al. 2006), *M. vinifirmus* (Liebgott et al. 2006), *M. koreensis* (Lim et al. 2006), *M. gudaonensis* (Gu et al. 2007), *M. salsuginis* (Antunes et al. 2007), *M. segnicrescens* (Guo et al. 2007), *M. salicampi* (Yoon et al. 2007b), *M. pelagius* (Xu et al. 2008), *M. guineae* (Montes et al. 2008), *M. psychrophilus* (Zhang et al. 2008), *M. mobilis* and *M. zhejiangensis* (Huo et al. 2008), *M. goseongensis* (Roh et al. 2008), *M. szutsaonensis* (Wang et al. 2009b), *M. santoriniensis* (Handley et al. 2009), *M. lacisalsi* (Aguilera et al. 2009), and *M. zhanjiangensis* (Zhuang et al. 2009). Marinobacter species have been isolated from various marine environments, including hydrothermal vents, sea ice, sulfide-rich sediments, and oil-contaminated environments.

Marinobacter (Ma.ri'no.bac. ter. L. adj. *marinus*, of the sea; M. L. n. *bacter*, masc. equivalent of Gr. neut. n. *bacterion*, rod or staff; M. L. masc. n. *Marinobacter*, rod of the sea).

Gram negative, rod shaped, nonspore forming, 0.3–0.6 μm by 1.4–3.0 μm in size, and motile by means of a single polar flagellum. 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. The type species is *Marinobacter hydrocarbonoclasticus* strain ATCC 49840.

Description of *Marinobacter hydrocarbonoclasticus* sp. nov.

Marinobacter hydrocarbonoclasticus (hy'dro.car.bo.no.clas'ti.cus M. L. part. adj. hydrocarbonoclastic, hydrocarbon dismantling). Cells are rod shaped (2–3 μm long and 0.3–0.6 μm wide in logarithmic growth phase) and harbor numerous surface blebs

when grown on eicosane in mineral medium. Cells are Gram negative, nonspore forming, and motile by means of a single unsheathed polar flagellum in media containing 0.2–1 M NaCl. They are unflagellated in media with a lower or higher NaCl concentration. Colonies on agar media are white when young and pinky beige after 48 h of incubation. The cells are able to grow at temperatures ranging from 10 °C to 45 °C (mesotrophic), with optimal growth at 32 °C. They exhibit extreme halotolerance and can grow in NaCl concentrations ranging from 0.08 M to 3.5 M. They have an absolute requirement for sodium ion. Aerobic, with a nonfermentative metabolism, they can grow anaerobically with nitrate or on succinate, citrate, or acetate but not on glucose. They are able to denitrify, with N_2 production. They exhibit oxidase, cytochrome oxidase, catalase, tweenase, and lecithinase activities. They grow on acetate, butyrate, caproate, succinate, fumarate, adipate, DL-lactate, and citrate as sole carbon sources but not on carbohydrates and amino acids (except L-proline and L-glutamate). They degrade a large variety of aliphatic or aromatic hydrocarbons and produce a nondialyzable bioemulsifier when grown on hydrocarbons.

The genus *Marinobacterium* was proposed by González et al. (1997) with the description of a single species isolated from marine sediments, *Marinobacterium georgiense*. Subsequently, *Oceanospirillum jannaschii* and *Pseudomonas stanieri* were reclassified into the genus as *Marinobacterium jannaschii* and *Marinobacterium stanieri* (Satomi et al. 2002; Euzéby and Tindall 2004). Three further species in the genus, *M. halophilum* (Chang et al. 2007), *M. litorale* (Kim et al. 2007), and *M. rhizophilum* (Kim et al. 2008), were described recently. Finally, Huo et al. (2009) using a polyphasic approach isolated two more strains from marine sediment of the East China Sea, *M. nitratireducens* and *M. sediminicola*.

Marinobacterium (Ma.ri.no.bac.te'ri.um. L. adj. *marinus*, of the sea; Gr. neut. n. *bakterion*, a small rod; L. neut. n. *Marinobacterium*, marine rod).

Cells are rod shaped and Gram negative and have numerous vesicles on their surfaces. Strict aerobe. Oxidase and catalase positive. Grows on sugars, fatty acids, aromatic compounds, and amino acids. Requires sea salt-based medium for growth. The type species is *Marinobacterium georgiense*.

Description of *Marinobacterium georgiense* sp. nov.

Marinobacterium georgiense (ge.or.gi.en'se. L. adj. *georgiense*, from Georgia, the place where the organism was isolated). Cells of type strain KW-40 are rod shaped (1.6–2.3 μm long and 0.5–0.7 μm wide in exponential phase growth in glucose medium) and have numerous vesicles on their surfaces. Has a polar flagellum, fimbriae, and a typical Slayer. Cells are Gram negative and motile. Colonies on marine agar 2216 are translucent. The temperature range for growth is 4–41 °C, and optimal growth occurs at 37 °C. The NaCl range for growth is 0.01–2 M, and optimal growth occurs at NaCl concentrations between 0.1 and 0.5 M. The optimal pH is 7.5, and the pH range is 5.5–9.5. It is strict aerobe, does not denitrify, and does not accumulate polyhydroxybutyrate. The sole carbon sources utilized include some carbohydrates and amino acids as well as aromatic compounds, such as phenol, benzoate, and *p*-hydroxybenzoate, and lignin-related compounds, such as *p*-coumarate, cinnamate, ferulate, and vanillate. Methanol and formate are utilized, but methylamine is not utilized. The alcohols utilized include ethanol, 1-propanol, 2-propanol, and L-butanol. Exhibits oxidase, catalase, and Tweenase activities. Growth factors are not required, although a complex vitamin solution or yeast extract stimulates growth. The G+C content of the DNA is 54.9 mol%.

The genus *Melitea* was created by Urios et al. (2008a) for the description of one species, *Melitea salexigens*. This aerobic, Gram-negative bacterial strain was isolated from waters in the coastal northwestern Mediterranean Sea.

Melitea (Me.li´te.a. N.L. fem. n. *Melitea* named after Melite, a nymph of the sea in Greek mythology, referring to the marine origin).

Cells are motile, Gram-negative rods. The major fatty acids are 17 : 1ω8c, 17 : 0, 18 : 1ω7c, 16:1ω7c, 15 : 0, 11 : 0 3-OH, iso-11 : 0 3-OH, 16 : 0, iso-15 : 0, 9 : 0, 10 : 0 3-OH, iso-17 : 0, and iso-13 : 0. The ubiquinone is Q-8 and the polar lipids are diphosphatidylglycerol and phosphatidylglycerol. Phylogenetically affiliated to the class *Gammaproteobacteria*. The type species is *Melitea salexigens*.

Description of *Melitea salexigens* sp. nov.

Melitea salexigens (sa.le´xi.gens. L. n. *sal, salis* salt, seawater; L. v. *exigo* to demand; N.L. part. adj. *salexigens*, seawater demanding). Displays the following properties in addition to those described for the genus. Cream colonies are formed on MA medium. Cells are 1.8 ± 0.2 μm long and 0.7 ± 0.1 μm wide with single polar flagella. The DNA G+C content of the type strain is 57 mol%. Growth occurs at 15–37 °C (optimally at 30 °C), at pH 6.0–10.0 (optimally at pH 8.0), and at salinities in the range 7–70 g NaCl l^{-1} (optimally at 42 g l^{-1}). Positive reactions with Biolog GN2 plates are obtained for D-fructose, D-glucose, maltose, D-mannose, D-psicose, raffinose, sucrose, trehalose, turanose, α-ketoglutaric acid, α-ketovaleric acid, succinamic acid, L-glutamic acid, and L-serine. Positive API ZYM reactions for enzyme activities are obtained for alkaline phosphatase, leucine arylamidase, acid phosphatase, and naphthol-ASBI-phosphohydrolase. Oxidase and catalase positive.

The genus *Microbulbifer* was originally proposed by González et al. (1997) for a biopolymer-decomposing marine gammaproteobacterium, *Microbulbifer hydrolyticus*. Since then, *M. salipaludis* (Yoon et al. 2003c), *M. maritimus* (Yoon et al. 2004a), *M. elongatus* (Yoon et al. 2003), *M. celer* (Yoon et al. 2007a), *M. agarilyticus* and *M. thermotolerans* (Miyazaki et al. 2008), *M. variabilis* and *M. epialgicus* (Nishijima et al. 2009), *M. donghaiensis* (Wang et al. 2009a), and *M. chitinilyticus* and *M. okinawensis* (Baba et al. 2011) have been described. Chemical markers for this genus include the presence of iso- 15: 0 and iso-17: 1ω9c as major fatty acids and Q-8 as the major ubiquinone (Yoon et al. 2004a). Microbulbifer species have been isolated from various marine environments, including salt marshes, intertidal sediments, and coastal waters.

Microbulbifer (Mi.cro. bul'bi.fer. Gr. adj. *micro*, small; L. m. n. *bulbus*, onion, bulb; L. suff. *-fer*, carrying, bearing; L. m. n. *Microbulbifer*, small bearer of bulbs).

Cells are rod shaped, Gram negative, strictly aerobic, and oxidase and catalase positive. The cell envelope has numerous surface vesicles derived from the outer membrane. Grows on sugars, fatty acids, and amino acids. Requires sea salt-based medium for growth. The type species is *Microbulbifer hydrolyticus*.

Description of *Microbulbifer hydrolyticus* sp. nov.

Microbulbifer hydrolyticus (hy.dro.ly'ti.cus. Gr. n. *hydor*, water; Gr. adj. *lytikos*, dissolving, splitting; M. L. adj. *hydrolyticus*, splitting with [by] water, referring to the hydrolytic activity of the bacterium). The cells of type strain are rod shaped (1.1–1.7 μm long and 0.3–0.5 μm wide in exponential phase growth in glucose medium) and have numerous vesicles on their surfaces. Cells are Gram negative and nonmotile. Colonies on marine agar 2216 are cream colored. The temperature range for growth is 10–41 °C, and optimal growth occurs at 37 °C. The NaCl range for growth is 0.1–1 M, and optimal growth occurs at NaCl concentrations between 0.1 and 0.5 M. The optimal pH is 7.5, and the pH range is 6.5–8.5. The organism is a strict aerobe and does not denitrify or accumulate polyhydroxybutyrate. It utilizes a limited number of carbohydrates and is able to grow only on glucose, xylose, *N*-acetyl-D-glucosamine, and cellobiose. Amino acids and some aromatic compounds, such as vanillate and ferulate, are used. Exhibits oxidase, catalase, cellulase, xylanase, chitinase, gelatinase, amylase, and Tweenase activities. Growth factors are not required, although a complex vitamin solution or a low concentration of yeast extract is stimulatory. Capsules are produced in liquid medium. The G+C content of the DNA as determined by high-performance liquid chromatography is 57.7 mol%.

In 1988, Andrykovitch and Marx (1988) isolated the bacteria involved in the degradation of a salt marsh grass, *Spartina alterniflora*, found in the lower Chesapeake Bay, USA. One of these was designated strain 2-40T. Based upon phenotypic characteristics, it was placed in the order *Alteromonadales* of the *Gammaproteobacteria*, in the family *Alteromonadaceae*. Later, based on sequence analysis of the 16S rRNA gene, its distinctive cellular morphology, and its complex polysaccharides-degradative capabilities, 2-40T was designated as *Microbulbifer degradans*. The analysis of the completed genome of *Microbulbifer degradans* 2–40 led to it being renamed to *Saccharophagus degradans* 2–40 and placed into its own genus *Saccharophagus* (Ekborg et al. 2005).

Saccharophagus (Sac´cha.ro.pha9gus. Gr. n. *saccharon* sugar; N.L. masc. n. *phagus* from Gr. masc. n. *phagos* glutton; N.L. masc. n. *Saccharophagus*, sugar devourer).

Gram negative, motile, heterotrophic, pleomorphic, rod shaped, aerobic, catalase positive, and oxidase positive. Numerous cell surface blebs and vesicles are produced. Degrades numerous complex polysaccharides. Requires sea salts for growth. The type species is *Saccharophagus degradans*.

Description of *Saccharophagus degradans* sp. nov.

Saccharophagus degradans (de.gra´dans L. part. adj. *degradans*, bringing back into the former order, used to refer to the ability of the type strain to degrade several complex carbohydrates). Cells are pleomorphic rods, averaging 1.5–3.0 μm long and 0.5 μm wide during the exponential phase of growth in glucose. In media containing complex polysaccharides as sole carbon sources, cells can be pleomorphic and produce surface protuberances and vesicles. Cells form coils and filaments when grown at high salinity. Colonies are cream in color and then turn black upon eumelanin production. Colonies rapidly pit agar plates. Capable of utilizing the following complex carbohydrates as sole carbon sources: agar, alginate, chitin, cellulose, fucoidan, laminarin, pectin, pullulan, starch, and xylan. Hydrolyzes tyrosine. Temperature range for growth is 4–37 °C with an optimum of 30 °C. Optimum pH for growth is 7.5 with a range of 4.5–10. Requires sea salts for growth in the range 1–10 % with an optimum of 3.5 %. Secretes proteases. The G+C content of the type strain is 45.8 mol% as determined by genomic sequencing.

The genus *Salinimonas* was first created by Jeon et al. (2005) with the description of *Salinimonas chungwhensis*, which was isolated from a solar saltern in the Chungwha area on the Yellow Sea in Korea. In 2012, novel polysaccharide-degrading microorganisms from a tidal flat sediment on the southern coast in Korea were isolated. One of these isolates, designated DPSR-4 T, which shows degradative activities against several polysaccharides was designated as *Salinimonas lutimaris* (Yoon et al. 2012).

Salinimonas (Sa.li.ni.mo´nas. L. fem. pl. n. *salinae* salterns, salt-works; L. fem. n. *monas* unit, monad; N.L. fem. n. *Salinimonas*, monad from salterns).

Cells are Gram-negative, nonspore-forming short rods. Oxidase and catalase positive. Strictly aerobic, chemoheterotrophic, and moderately halophilic. Cells are motile with a polar flagellum. No growth occurs without NaCl or in the presence of more than 15 % (w/v) NaCl. Major isoprenoid quinone is Q-8. DNA G+C content is 48 mol% (HPLC). Predominant cellular fatty acids are C16: 0, C18: 1ω7c, and a mixture of C16: 1ω7c/iso-C15: 0 2-OH. Phylogenetically, the genus belongs to the family *Alteromonadaceae*. The type species is *Salinimonas chungwhensis*.

Description of *Salinimonas chungwhensis* sp. nov.

Salinimonas chungwhensis (chung.when´sis. N.L. fem. adj. *chungwhensis*, belonging to Chungwha, where the type strain was isolated). Cells are approximately 0.8–1.0 μm wide and 1.2–1.5 μm long. Colonies are creamy and circular/slightly irregular on MA. Optimal growth is observed at 30–35 °C, pH 7–8, and 2–5 % (w/v) NaCl. Aesculin, casein, hypoxanthine, gelatin, starch, Tween 80, L-tyrosine, and urea are hydrolyzed. Hydrolysis of xanthine is not observed. Nitrate is not reduced to nitrite. Acid is produced from D-glucose, D-ribose, D-xylose, maltose, D-trehalose, and L-arabinose, but not from D-fructose, D-mannitol, a-D-lactose, L-rhamnose, glycerol, adonitol, D-raffinose, arbutin, D-salicin, D-melibiose, and D-mannose. Produces alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase, but not lipase (C14), cystine arylamidase, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Valine arylamidase and a-chymotrypsin activities are weak. Major isoprenoid quinone is Q-8. DNA G+C content is 48 mol% (HPLC).

Isolation, Enrichment, and Maintenance Procedures

Isolation and Enrichment

Aestuariibacter was isolated from a sediment sample of the "getbol" of Ganghwa Island, Korea. The sample was diluted with sterilized artificial seawater (ASW; Lyman and Fleming (1940)), spread onto a plate that contained marine agar 2216 and MR2A (R2A supplemented with artificial sea salts) and incubated at 25 °C for 3 weeks. Strains were cultured routinely on marine agar 2216 at 30 °C; optimal growth occurred at pH 7–8 and 2–3 % sea salts (Yi et al. 2004).

Agarivorans was isolated from healthy marine organisms that were collected from the coast of the Kanto area in Japan. The collected marine creatures were washed several times with sterile sea water. Excised gastrointestinal tracts and attached internal organs were homogenized and diluted serially to a ratio of 1: 10 in sterile sea water. Aliquots (0.1 ml each) of the dilution were spread onto marine agar 2216. Plates were then incubated at 23 °C for 1 week (Kurahashi and Yokota 2004).

Aliagarivorans was isolated from seawater samples collected in the shallow coastal region of An-Ping Harbour, Tainan, Taiwan. Seawater samples were collected from the shallow coastal regions of this harbor in the morning at low tide and diluted with sterile NaCl-Tris buffer. Aliquots (0.1 ml each) were spread onto polypeptone-yeast extract plate medium. The plates were incubated at 25 °C in the dark for 7 days under aerobic conditions. Individual colonies that appeared to be agarolytic were picked off and purified by successive streaking on polypeptone-yeast extract plates. Maintenance of the isolates was kept at 20 °C under aerobic conditions (Dar Jean et al. 2009).

Alishewanella was isolated from an autopsy of a human fetus in Uppsala, Sweden (Vogel et al. 2000). This strain was grown in veal infusion broth, in brain-heart infusion broth, on Long and Hammer's medium (Van Spreekens 1974), and on iron agar (Gram et al. 1987) at 37 °C.

Alteromonas can be isolated from seawater. Membrane filters having a pore diameter of 0.22 or 0.45 μm are used to filter the seawater samples. Filters are placed on marine agar containing 0.2 % (w/v) lactose. Plates are maintaining at 25 °C. After days of inoculation colonies are transferred and purified on the same medium. Seawater or NaCl are necessary for growth. Good aeration and keeping in the dark is recommended (Gauthier and Breittmayer 1992).

Bowmanella was isolated from seawater samples collected in the shallow coastal region of An-Ping Harbour, Tainan, Taiwan. Seawater samples were collected from the shallow coastal regions of this harbor in the morning at low tide and diluted with sterile NaCl-Tris buffer. Aliquots (1 mL each) were transferred to a rimless tube containing polypeptone/yeast extract/nitrate broth medium into which an inverted Durham tube had been placed. This medium was used for the enrichment cultivation of denitrifying bacteria in the seawater. Tubes were incubated aerobically at 25 °C in the dark for 3–7 days. Cultures that developed visible turbidity and produced gas were streaked on polypeptone/yeast extract plate medium. Individual colonies appearing on each plate were picked off and purified by successive streaking on polypeptone/yeast extract plates at 25 °C (Dar Jean et al. 2006).

Glaciecola have been obtained from algal assemblages present within coastally attached sea ice. Ice samples are melted in seawater at 4 °C to avoid hypotomic shock to the bacteria. The sample is maintained during 1–2 days at 2 °C in marine agar 2216 liquid media then is plated into marine agar 2216. The unusual pigments produced by *Glaciecola* strains make them distinctive on primary isolation plates. Strains can be stored as active cultures on Marine 2216 agar plates at 2 °C for at least 12 months. Antifungal agent should be added to prevent contamination (Bowman et al. 1998).

Haliea was isolated from the surface of coastal waters in the northwestern Mediterranean Sea. Samples of the surface microlayer of seawater in the bay of Banyuls-sur-Mer were collected by submerging a metal screen (Agogué et al. 2004). Subsamples were spread on marine agar 2216 plates and incubated at 25 °C for 2 weeks. Colonies were picked and purified after three subcultures (Urios et al. 2008b).

Marinimicrobium was isolated from tidal flat sediment of the South Sea in Korea (the Korea Strait). For isolation, sediment sample was collected from the surface of tidal flat sediment and diluted serially in saline solution (10 % w/v). The diluted soil samples were spread on marine agar 2216 with the addition of 8 % (w/v) NaCl and incubated for 2 days at 30 °C (Lim et al. 2006).

Marinobacterium and *Microbulbifer* were obtained from an enrichment community growing on the high-molecular-weight fraction of a black liquor sample from Federal Paper Board Company Inc., Augusta, Ga. Isolates were obtained from single colonies restreaked on YTSS medium (4 g of yeast extract, 2.5 g of tryptone, 20 g of sea salts, 18 g of agar, and 1 L of distilled water) several times following the third transfer of the pulp mill waste enrichment culture (González et al. 1997).

Melitea was isolated from the surface microlayer waters in the coastal northwestern Mediterranean Sea by submerging a metal screen (Agogué et al. 2004). Subsamples were spread on marine agar 2216 plates and incubated at 25 °C for 2 weeks. Colonies were picked and purified after three subcultures (Urios et al. 2004).

Saccharophagus was first isolated from decaying salt marsh cordgrass *Spartina alterniflora* found in the Chesapeake Bay, USA. *S. degradans* was isolated by pressing partially decomposed *S. alterniflora* into 1 % peptone-half-strength-seawater agar plates. Optimal growth occurred at pH 7.5 with an optimum of 30 °C. Sea salt (optimum 3.5 % w/v) and carbohydrates as carbon source were needed for the growth of the bacteria (Ekborg et al. 2005).

Salinimonas was isolated from a solar saltern in the Chungwha area on the Yellow Sea in Korea. Soil samples were serially diluted with 1 % (w/v) saline solution and spread on marine agar 2216 with the addition of 5 % (w/v) NaCl and incubated for 2 days at 35 °C. The isolate was routinely grown aerobically on marine agar 2216 for 2 days at 35 °C.

Maintenance

Strains can be stored for a long-term mediated cryopreservation and stored in liquid nitrogen using glycerol at −80 °C. Lyophilization can also be used. Type strain *Alteromonas* can be maintained for days preferred at 15 °C and darkness on marine agar plate because at 4 °C survival seems to be poor.

Ecology

Bacteria of this group, with the exception of the type strain of the genus *Alishewanella*, because of their NaCl requirement, are widespread inhabitants of marine environments and have been isolated from diverse marine sources as seawater, sea ice, internal organs of marine animals, solar salterns, or salt marsh grass (*Table 5.1*.)

The type strain of *A. macleodii* was isolated from coastal waters of Oahu (Hawaii) in one of the first large-scale studies of aerobic marine bacterial isolates in 1972 (Baumann et al. 1972). Studies carried out by Garcia-Martinez et al. (2002) using rRNA internal transcribed spacer (ITS) sequencing and hybridization of DNA samples collected from several marine samples from around the world revealed that *A. macleodii* cells represent a significant fraction of the bacterial population associated with particles or aggregates (2–5 μm filters) in temperate or tropical waters with average temperatures above 10 °C. This temperature limitation precluded the presence of this microbe in any deepwater samples with the exception of the Mediterranean, where the deepwater mass never gets below 12 °C.

Several studies support the nature of *A. macleodii* as a typical *r* strategist that can bloom to very high cell densities in the marine environment under different circumstances. The numbers of the microbe in most open-ocean metagenomes are relatively small, although very persistent in warm and temperate water masses (Martin-Cuadrado et al. 2007; Ivars-Martinez et al. 2008a). The clear association to large cell and particulate fractions in filtration protocols is quite clear in metagenomic datasets. However, there are reports of unusually large fractions of this microbe in some metagenomes of the deep Mediterranean and related habitats (Quaiser et al. 2011; Smedile et al. 2012). Besides, several reports indicate very large increments in the contributions of this microbe to marine communities confined in mesocosms and other situations where the conditions change. In addition to the previously mentioned mesoscosm experiments (Schafer et al. 2000), some microcosm experiments have been carried out to monitor the changes in transcript populations, in a water sample from 75 m deep in the central Pacific gyre (near Hawaiian Ocean Time-Series, HOT station) (McCarren et al. 2010); (Shi et al. 2012). In one experiment water was amended with dissolved organic matter (DOM) concentrated from the same environment, and in a second experiment water from a deeper sample (700 m) was added to the surface one to simulate the fertilization that happens when an upwelling or water mixing event takes place. The *A. macleodii* population increased in both experiments, from undetectable to ca. 10 % of the population during the study period of 25 h. This data illustrate by a totally different methodology the relevance of this microbe as an *r* strategist that blooms under conditions of sudden increase in the availability of resources (probably DOM released or exudated by the phytoplankton) (Zemb et al. 2010; Romera-Castillo et al. 2011; Tada et al. 2011).

A. macleodii is a remarkably euryhaline and eurythermal bacterium, and its relatively large and complex genome reflects such an adaptable phenotype. All the available data indicate that *A. macleodii* is a typical *r* strategist (investing most energy in multiplying fast) that takes advantage of its relatively large cell and genome size to exploit intensively localized (in time or space) nutrient-rich micro-niches, such as when a bloom of phytoplankton occurs or when a nutrient-rich particle becomes available. Its reliance on fast growth to compete efficiently might help explain its absence in waters where water temperature is low and prevents rapid growth. Obviously the large cells required to carry these large genomes cannot compete in normal conditions with the streamlined *K*-strategist of the *Candidatus* Pelagibacter ubique type in the diluted purely planktonic lifestyle. However, whenever a concentrated pool of organic matter is available, they can multiply more rapidly than any other heterotrophic prokaryote and reach high population densities. One trait that is missing in the *Alteromonadaceae* is the potential to use light as energy source, by rhodopsin- or bacteriochlorophyll-dependent mechanisms, an extremely common feature in marine prokaryotes (Newton et al. 2010). On the other hand, they seem to be highly specialized in the use of polymers and their degradation products (Varbanets et al. 2011).

Comparison analysis between *A. macleodii* strains isolated from the deep-sea (400–3,500 m) and surface waters showed marked differences in cell size and composition of fatty acids (Klochko et al. 2012). Deep-sea strains were two times longer ($2.1 \pm 0.2 \times 0.7 \pm 0.1$ μm) than strains from surface water ($1.1 \pm 0.1 \times 0.6 \pm 0.1$ μm) (*Fig. 5.2*). Based on fatty acid composition, strains are easily divided into these two separate groups.

Application

Marine bacteria represent the largest unexploited biotechnological resource on the planet. There is an enormous biological and genetic diversity in the world's ocean because of the adaptation of marine bacteria to the harsh environment. These bacteria can be a potential source of new bioactive compounds for industrial, agricultural, environmental, pharmaceutical, and medical uses.

Agar is a complex polysaccharide extracted from marine red algae. This marine polysaccharide is widely used as a gelling agent for microbiological culture media and as an ingredient in the food industry. Agar is degraded by agarases using two different pathways: α-agarases cleave the α-1,3 linkage of agarose (Young et al. 1978) and β-agarases cleave the β-1,4 linkage on agarose (Duckworth and Turvey 1969). Some agarases have been purified and characterized. Leon et al. (1992) identified and

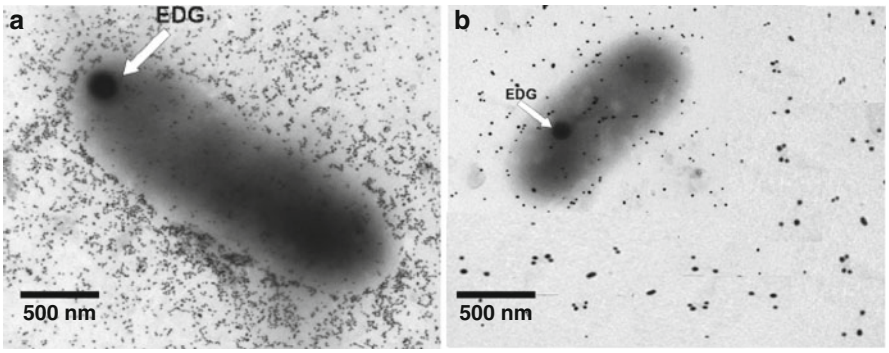

Fig. 5.2
Electron micrographs of cells of *A. macleodii* strains marked with lectin-gold complexes (modified from Klochko et al. 2012). (a) Adriatic-1 (deep strain), (b) D7 (surface strain). *EDG* electron dense granule

purified an extracellular agarase from *Alteromonas* sp. strain C-1 with an optimum pH of about 6.5 and 52 kDa of molecular weight. In 2006, Wang et al. (2006) identified a novel extracellular β-agarase from the marine *Alteromonas* sp. SY37-12. This agarase with an optimum pH of about 7.0 and 39.5 KDa of molecular weight caused agar softening around the colonies grown on solid agar. Gene of another β-agarase was cloned from a newly isolated marine bacterium, *Agarivorans* sp. LQ48 (Long et al. 2010). It encodes a protein with a molecular weight of 51.2 kDa and a wide range of pH stability (3.0–11.0) that could make this agarase interesting to industrial applications (food, cosmetics, etc.). Recently it has been found that the marine bacteria *Microbulbifer maritimus* produced an extracellular agarase which causes agar liquefaction around the colonies. The purified protein exhibited maximal activity at pH 7.5 and a molecular weight of 75.2 kDa (Vijayaraghavan and Rajendran 2012).

Alginate lyases, also known as alginases or alginate depolymerases, catalyze the degradation of alginate by a β-elimination of the glycosidic bond. Alginate is a linear polymer consisting of β-D-mannuronic and α-L-guluronic acid residues and is found in great abundance as part of the cell wall and intracellular material in the brown seaweeds (Wong et al. 2000). *Alteromonas* sp. strain H-4, isolated from *Laminaria* fronds, produces at least five different extra- and intracellular alginate lyases and utilized alginate as its sole carbon source; however, several of them have the capability of heterogeneous substrate specificities (Sawabe et al. 1997, 1998). From the marine bacterium *Alteromonas* sp. strain no. 272 isolated from sea mud in Omura Bay (Iwamoto et al. 2001), another alginate lyase was purified and characterized. Recently, Wakabayashi et al. (2012) found a novel alginate lyase gene from the bacterium *Microbulbifer* strain 6532A. The strain was capable of rapidly degrading Wakame (*Undaria pinnatifida*) thallus fragments and both alginate and cellulose in the culture medium.

Cellulases are other economically important enzymes for industry. Cellulases could have applications in the treatment of agricultural waste and help overcome current challenges in biofuel production. Within the *Alteromonadaceae*, the marine bacterium *Marinimicrobium* sp. LS-A18 showed extracellular production of novel halotolerant and thermostable carboxymethyl cellulase with a maximum activity obtained at 55 °C and pH 7.0 in the absence of NaCl (Zhao et al. 2012). This marine bacterium, isolated from a marine solar saltern near the Yellow Sea in China, was also the first report from a microorganism with extracellular inulinolytic activity (Li et al. 2012) and showed optimal activity at pH values ranging from 3.5 to 7.0.

Chitin is an insoluble linear β-1,4-linked polymer of *N*-acetylglucosamine (GlcNAc). This polysaccharide is an important carbon and nitrogen source for marine organisms found in the exoskeletons of crustaceans (Gooday 1990). *Alteromonas* sp. strain O-7 secretes four chitinases (ChiA, ChiB, ChiC, and ChiD) in the presence of chitin. Orikoshi et al. (2005) investigated the role of these four chitinases in the chitin degradation. Among the four, ChiA was produced at the highest level and was the most active enzyme against powdered chitin. Although, synergistic effects combining the four chitinases, increased hydrolytic activity suggested that *Alteromonas* sp. strain O-7 produces multiple chitinases for the efficient degradation of chitin in the natural environment.

It's remarkable the case of *Saccharophagus degradans*. This aerobic γ-proteobacterium of the *Alteromonadaceae* was first isolated from decaying salt marsh cordgrass *Spartina alterniflora* found in the Chesapeake Bay (Ekborg et al. 2005). *S. degradans* is able to degrade at least 10 different complex polysaccharides (CP) including agar, alginate, cellulose, chitin, β-glucan, laminarin, pectin, pullulan, starch, and xylan (Ekborg et al. 2006). It is surprising and unusual in its ability to utilize CPs of algal, higher plant, fungal, and animal origin as sole carbon and energy sources. For these characteristics, *S. degradans* is being studied as a powerful bioremediation tool and may help with the increased problem of this type of waste products.

Within the *Alteromonadaceae* there are other species that have demonstrated applications in biotechnology. For example, *Alishewanella* sp. strain KMK6, who was able to decolorize and degrade different azo dyes and displayed high dye degradation ability and tolerance (Kolekar and Kodam 2012). *Alteromonas*

sp. SN2, isolated from hydrocarbon-contaminated sea-tidal flat sediment, has been shown to play an important role in the biodegradation of polycyclic aromatic hydrocarbons (Math et al. 2012). Suzuki et al. (2012) isolated two *Haliea* sp. strains (ETY-M and ETY-NAG) from seawater around Japan and demonstrated that these strains had the ability to degrade gaseous hydrocarbons. Vargas et al. (2011) characterized a [NiFe] hydrogenase (HynSL) from the marine bacterium *A. macleodii* AltDE and showed an unusual stability in the presence of oxygen and high temperature. Furthermore, HynSL from AltDE was expressed in *E. coli* and was active (Weyman et al. 2011). These features make such hydrogenases potential candidates for biotechnological applications as an accessory for H_2 production technologies, an important clean alternative energy source.

Exopolysaccharides (EPS) are high-molecular-weight polymers that are composed of sugar residues and are secreted outside of their cell walls to resist adverse and extreme environmental conditions (Sutherland 2001). At the same time, this EPS has great biotechnological interest because of their potential applications to the industry as emulsifying, viscosifying, suspending, and chelating agents (Vandamme et al. 2002). For example, *Alteromonas* sp. strain 1545 produces acidic EPS with thickening properties (Talmont et al. 1991). EPS obtained from a heterotrophic mesophilic aerobic bacterium isolated from a deep-sea hydrothermal vent, *Alteromonas infernus*, has anticoagulant activity similar to heparin and therefore is interesting for the treatment of some diseases (Guezennec et al. 1998). *Alteromonas* sp. strain 1644 produces EPS with high affinity towards heavy metals decreasing metal toxicity produced in the environment (Bozzi et al. 1996).

Many marine bacteria have been shown to produce secondary metabolites that play antibacterial properties (Zheng et al. 2005). The marine bacteria *Alteromonas rava* sp. nov. SANK 73390, isolated from a culture broth, was found to produce a new antimicrobial acting against Gram-positive and Gram-negative bacteria named Thiomarinol (Gao and Hall 2005). Riquelme et al. (1997) noticed that *Alteromonas haloplanktis* had inhibitory activity against pathogenic *Vibrio* sp. and *A. hydrophila*. *Microbulbifer* sp. L4-n2 is another marine bacteria belonging to the *Alteromonadaceae*. This isolate from the temperate calcareous marine sponge *Leuconia nivea* produced novel parabens with antibacterial activity against Gram-positive reference bacteria *Staphylococcus aureus* as well as against marine *Bacillus* sp. and *Planococcus* sp. isolates derived from the sponge, indicating a possible ecological role as chemical mediators (Quévrain et al. 2009).

The genus *Alteromonas* was found commonly associated with marine sponges. Shigemori et al. (1992) isolated an *Alteromonas* sp. associated with the marine sponge *Halichondria okadai* that was responsible for the production of a well-known lactam alteramide A. Alteramide A is a tetracyclic alkaloid compound with cytotoxic and antimicrobial properties. Recently, a new algicidal strain was isolated from a harmful alga bloom (Korea) and identified as *Alteromonas* sp. based on 16S sequencing (Cho 2012). Analysis of four different compounds from this algicidal strain provided the first report of quinolone and pyrone derivatives isolated from the genus *Alteromonas*. Another direct algicidal activity has been reported from the strain *Alteromonas* sp. A14 isolated from the southern coast of Korea (Lee et al. 2008).

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6 The Family *Beggiatoaceae*

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Abstract

The family *Beggiatoaceae* contains a wide range of morphologically conspicuous, aerobic, or nitrate-dependent sulfide-oxidizing bacteria that span the range from obligate sulfur-based chemolithoautotrophy to heterotrophic growth supplemented by sulfur oxidation. The *Beggiatoaceae* are the model organisms for the concept of chemolithotrophy, developed by Sergei Winogradsky during his postgraduate studies using natural populations of filamentous freshwater *Beggiatoaceae* collected in sulfur springs. Since the metabolism of the *Beggiatoaceae* requires access to reduced sulfur species and oxidants such as oxygen or nitrate, these bacteria thrive in microbial mats, surficial sediments, and sediment–water interfaces where these electron donors and acceptors coexist and can be intercepted for microbial energy generation before gradual abiotic sulfide oxidation sets in. All *Beggiatoaceae* have the ability to oxidize sulfide to elemental sulfur that is stored as intracellular sulfur globules, which make the cells highly refractory and conspicuous with the unaided eye and under the microscope. This characteristic, together with the absence of photosynthetic pigments, has led to their traditional designation as members of the "colorless sulfur bacteria," in contrast to the photosynthetic purple and green sulfur bacteria or the cyanobacteria. The white, yellow, or occasionally orange color of the *Beggiatoaceae*, their frequently filamentous or chain-like morphology, their growth pattern in flocs and mats on sediment surfaces, and their large cell size and capacity for storing several different compounds intracellularly have made these organisms fascinating research targets. Extensive microscopic and morphological surveys have focused on these bacteria since the late nineteenth and early twentieth century. To a surprising extent, early microscopic and morphological observations on large, morphologically conspicuous sulfur bacteria can be reintegrated into the emerging molecular and phenotypic taxonomy of the *Beggiatoaceae* today.

Taxonomy, Historical and Current

The family *Beggiatoaceae* represents one of the major mutually exclusive phylogenetic lineages of the morphologically conspicuous sulfur bacteria (Bavendamm 1924) within the *Gammaproteobacteria*. The *Beggiatoaceae* have undergone major expansions and revisions in the recent past: The genera *Beggiatoa* and *Thioploca* were recognized as phylogenetically intertwined (Teske et al. 1999) and in need of taxonomic revision that better reflects their natural evolutionary relationships in relation to each other and to *Thiomargarita* (Jørgensen et al. 2005; Teske and Nelson 2006). To accommodate the emerging natural diversity among these bacteria after substantive revision based on 16S rRNA and its sequences, cell morphology, and physiology, the family *Beggiatoaceae* retains the currently recognized genera *Beggiatoa*, *Thioploca*, and *Thiomargarita* in revised form and also includes the recently proposed genus-level Candidatus groups Maribeggiatoa, Marithioploca, Marithrix, Isobeggiatoa, Parabeggiatoa, Allobeggiatoa, Halobeggiatoa, and Thiopilula, the revived candidate genus *Thiophysa*, and some distinct phylogenetic lineages that for now remain unnamed (Salman et al. 2011; Hinck et al. 2011; Grünke et al. 2012). The *Beggiatoaceae* do not include the filamentous, heterotrophic freshwater bacterium *Vitreoscilla*, a betaproteobacterium that does not form intracellular sulfur globules (Strohl et al. 1986).

Recently, the combined family name *Thiotrichaceae* was introduced to include the genera *Beggiatoa*, *Thioploca*, *Thiomargarita*, *Thiothrix*, *Leucothrix*, *Achromatium*, *Thiobacterium*, and *Thiospira* (Garrity et al. 2005). However, this polyphyletic assemblage comprises physiologically and phylogenetically divergent bacteria, including the type genera (*Beggiatoa*, *Leucothrix*, and *Achromatium*) of the validly published families *Beggiatoaceae*, *Leucotrichaceae*, and *Achromatiaceae*. The genera *Beggiatoa*, *Thioploca* and *Thiomargarita* form a monophyletic lineage within the *Gammaproteobacteria* (Ahmad et al. 2006; Jørgensen et al. 2005); the genera *Thiothrix* and *Leucothrix* form the second (Howarth et al. 1999); the genus *Achromatium* constitutes the third of these lineages (Head et al. 1996); *Thiobacterium* is not yet phylogenetically assigned (Grünke et al. 2010). This phylogenetic framework based on 16S rRNA sequences is remarkably consistent with the validly published families *Beggiatoaceae* (Leadbetter 1974; Strohl 1989), *Leucotrichaceae* (Brock 1974), and *Achromatiaceae* (Van Niel 1948) that were based on distinct cell morphology and physiological characteristics and hold up well in the light of modern molecular taxonomy. Since each family is monophyletic, they provide a robust, natural phylogenetic framework that can accommodate future updates and novel taxa and should be retained.

This chapter provides an overview of the family *Beggiatoaceae* that synthesizes published taxonomic descriptions, physiology based on pure cultures and field samples, morphology and size of filaments, cell clusters and single cells, and 16S rRNA gene sequences obtained from pure cultures and single cells (Fig. 6.1).

Beggiatoaceae Migula 1894.

Beg.gia.to.a'ce.ae. N.L. fem.n. *Beggiatoa* type genus of the family; *-aceae* ending to denote a family; N.L. fem. pl. n. *Beggiatoaceae* the *Beggiatoa* family.

Type genus: *Beggiatoa* (Trevisan 1842).

Beg.gia.to'a. M.L. fem.n. *Beggiatoa* named for F.S. Beggiatoa, a physician of Vicenza.

Molecular Analyses

Molecular analyses have uncovered wide phylogenetic diversity within the family *Beggiatoaceae* and delineated the *Beggiatoaceae* from other families of morphologically conspicuous sulfur bacteria. Early on, *Beggiatoa alba* and *Thiothrix nivea* were recognized as distinct lineages of the Gammaproteobacteria, initially by reverse transcription sequencing of extracted 5S rRNA (Stahl et al. 1987) and 16S rRNA (Lane et al. 1992) and later by sequencing of PCR-amplified and cloned 16S rRNA genes (Teske et al. 1995). Since then, 16S rRNA gene sequencing of individual filaments or single cells has played a crucial role in defining mutually exclusive monophyletic

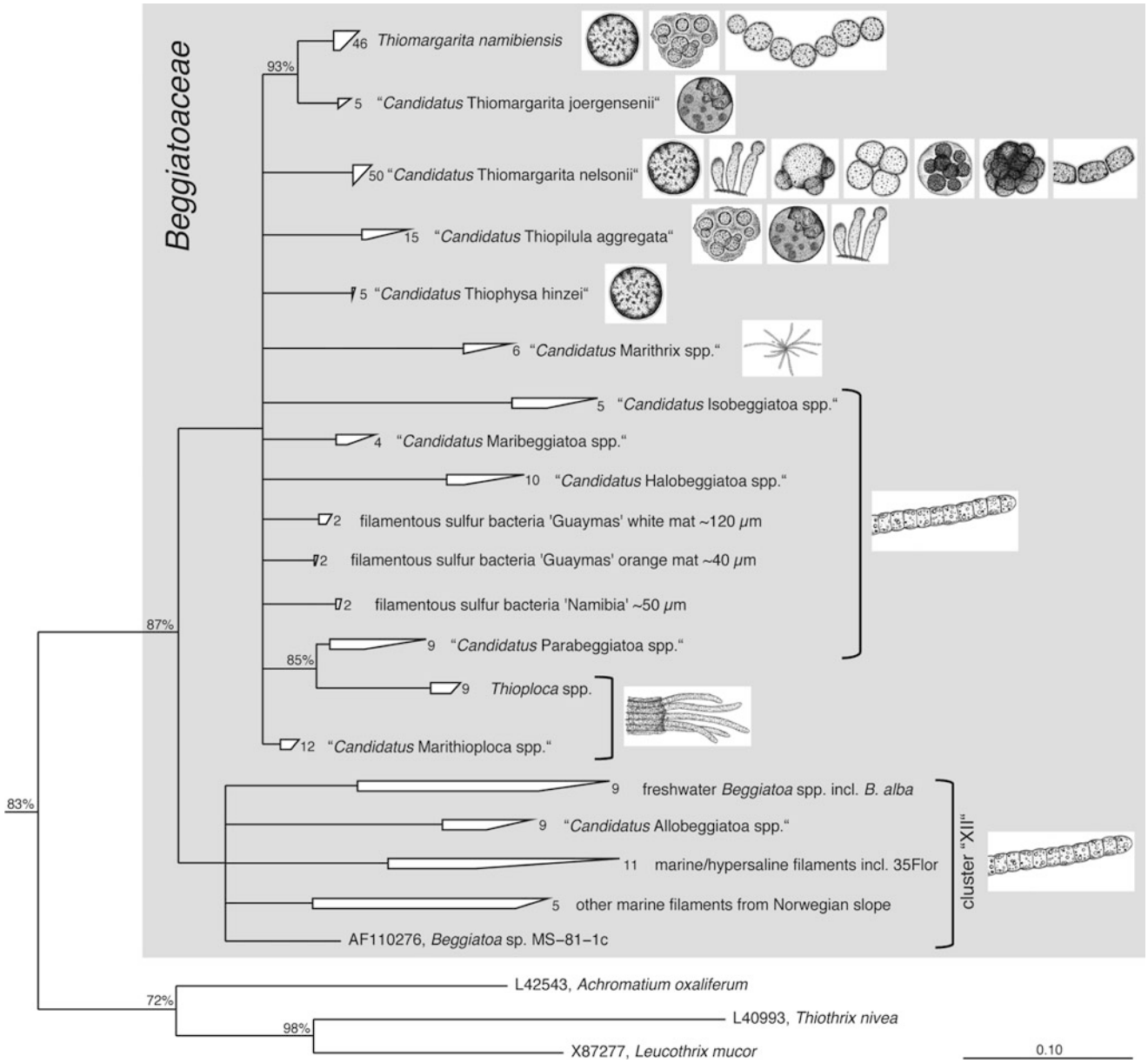

Fig. 6.1

Phylogenetic tree of Beggiatoaceae 16S rRNA gene sequences*. The phylogeny was inferred based on *E. coli* positions 279 to 1290, using maximum likelihood and 100 bootstrap runs. Nodes with less than 60 % bootstrap support were collapsed into polytomies. The family *Beggiatoaceae* separates into distinct phylogenetic groups: ''Cluster XII'' contains several lineages of filamentous sulfur bacteria, including the type species *Beggiatoa alba* (Salman et al. 2011). Subgroups within ''Cluster XII'' might need reclassification in the future. The top part of the tree with clusters I–XI contains filamentous and nonfilamentous large sulfur bacteria of various cell morphologies and arrangements. The 16S rRNA phylogeny shows that morphology is not a monophyletic feature within the *Beggiatoaceae

phylogenetic lineages that form the basis for several proposed candidate genera and species. Since these taxa are mostly uncultured and therefore incompletely described, no type strains can be given; listed instead are the currently known 16S rRNA gene sequences, morphological and physiological characteristics, and the environmental origin of natural samples and specimens.

A fundamental caveat for the study of natural *Beggiatoaceae* samples has to be kept in mind: Some key physiological characteristics (for example, intracellular nitrate accumulation) cannot be identified from the same filament or cell that is used for sequence identification; instead, morphologically indistinguishable filaments or cells from the same sampling site are used for parallel phylogenetic identification, as well as phenotypic and physiological characterization. Therefore, genotype/morphotype matches are to some extent inferred, especially for newly defined taxa with a small sample base. Repeated and consistent identification of natural populations in different settings and locations will solidify the evolving taxonomy of the *Beggiatoaceae*.

Genus *Beggiatoa*

In contrast to all other genera and candidate lineages of the *Beggiatoaceae*, the genus *Beggiatoa* has cultured representatives and a well-characterized type species, *Beggiatoa alba* strain B18LD, isolated from freshwater sediments near Baton Rouge, Louisiana, USA (Fig. 6.2c) (Mezzino et al. 1984). *Beggiatoa alba* is a deeply branching member of the *Beggiatoaceae* in 16S rRNA phylogenies (Ahmad et al. 2006); its phylogenetic position near the root of the *Beggiatoaceae*, among multiple lineages of freshwater and marine *Beggiatoaceae*, was recently confirmed in a comprehensive reanalysis of all known members of this group (Salman et al. 2011). *Beggiatoa alba* B18LD (AF110274; Strohl et al. 1981a) forms a monophyletic cluster with the closely related freshwater strains, *Beggiatoa alba* B15LD (L40944, Strohl and Larkin 1978a; Strohl et al. 1982), *Beggiatoa* sp. OH-75-2a (Fig. 6.2a) (AF110273; Nelson and Castenholz 1981a, b), and *Beggiatoa* sp. D-401 and D-402 (AY583995 and AY583996; Grabovich et al. 1998, 2001). The molecular mass of the *Beggiatoa alba* genome has been determined by CoT analysis as 2.02×10^9, which corresponds to 3.03×10^6 base pairs, similar to *E. coli* (Genthner et al. 1985). The G+C content for *Beggiatoa alba* strains B18LD, B15LB, and B25RD ranged from 40 to 42.7 mol%. The phenotypically

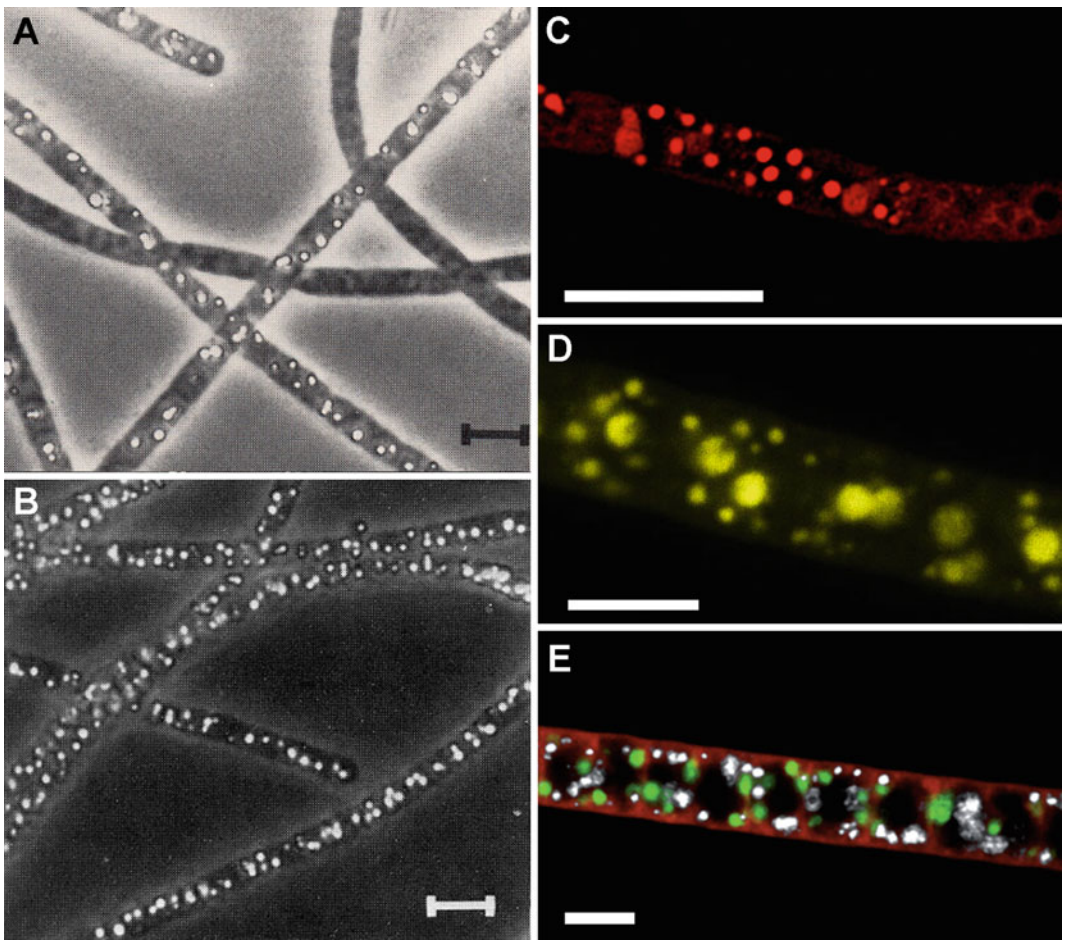

Fig. 6.2

Filaments affiliated with "Cluster XII." **(a)** ***Beggiatoa*** **sp. strain OH-75-2a, light micrograph showing bright white spots representing elemental sulfur and poly-β-hydroxyalkanoate inclusions. Scale bar 5 μm. (b)** ***Beggiatoa*** **sp. strain MS-81-1c, phase contrast micrograph likewise showing bright white inclusions bodies. Scale bar 5 μm. (c)** ***Beggiatoa alba*** **strain B18LD stained with Nile Red reveals membrane structures and inclusions of poly-β-hydroxyalkanoates. Scale bar 5 μm. (d)** ***Beggiatoa*** **sp. strain 35Flor stained with DAPI reveals polyphosphate inclusions at an emission wavelength of 525 nm (instead of 460 nm usually used for the specific detection of DNA). Scale bar 5 μm. (e) "*** ***Candidatus*** **Allobeggiatoa sp." filaments stained with Nile Red (*** ***red*** **) show location of membrane structures, and SYBR Green (*** ***green*** **) stains DNA nucleoids. White spots represent sulfur inclusions, and the void compartments in the center of each cell are the aqueous vacuoles, in which nitrate can be stored. Scale is 5 μm (Photos (a) and (b) Doug Nelson, University at California at Davis; Photo (c) Verena Salman, University of North Carolina at Chapel Hill; Photo (d) adapted from Brock et al. 2012; (e) adapted from Hinck et al. 2011)**

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 to 12.8 × 10^6 or 18.9–19.7 kb with no described function (Minges et al. 1983). Independent analyses (Nelson, unpublished) determined the following mol G+C values: B18LD (37.1 %), B25RD (35.5 %), and OH-75-2a (38.5 %). *Beggiatoa alba* B18LD contains the genes for the linear C_1-oxidation pathway of alpha-, beta-, and gammaproteobacterial methylotrophic bacteria (Jewell et al. 2008). *Beggiatoa alba* B15LD (DSM 1416) also contains the soxB gene, shared with a wide range of sulfur-oxidizing *Proteobacteria* and *Chlorobia* (Genbank accession number EF618583) (Meyer et al. 2007), and the chaperonin-60 gene (Genbank accession number JF745935). Genome sequencing of type strain *Beggiatoa alba* B18LD has been completed at the Joint Genome Institute (JGI Project ID 16466). Pending additional analyses, only the *Beggiatoa alba* cluster may constitute the phylogenetically validated genus *Beggiatoa* in the strict sense.

Beggiatoa alba **(Vaucher 1803) Trevisan 1842**

Al'ba. L. adj. *albus*, white.

B. alba grows chemoorganotrophically and aerobically, with a preference for microaerobic conditions. When grown in the presence of reduced sulfur sources, sulfur is deposited in inclusions surrounded by the cytoplasmic membrane. Anaerobic cell maintenance with sulfur as electron acceptor is possible. Necridia and hormogonia can be formed. Colonies on agar may appear as circuitously curled filaments. The filaments of *B. alba* are about 1.5–4 µm in diameter and may vary with growth conditions. Cells are usually 3.0–9.0 µm long, with filament lengths averaging 60–120 µm.

The neotype strain, B18LD, was isolated from an enrichment obtained from a rice paddy in Lacassine, Louisiana, USA. This strain is described in detail by Mezzino et al. (1984). The well-characterized strains OH-75-2a and B15LD should be considered strains of *B. alba*.

Type strain: LSU B18LD, ATCC 33555.

Genbank accession numbers of 16S rRNA gene sequence: AF110274.

Other morphologically and physiologically similar isolates and enrichments of filamentous sulfur bacteria constitute sister lineages to *Beggiatoa alba* radiating near the base of the *Beggiatoaceae*; these form separate 16S rRNA branches and cannot be subsumed under the *Beggiatoa alba* lineage (Ahmad et al. 2006). These lineages were termed "cluster XII" (*Fig. 6.1*), understood as a temporary designation until more taxonomic work establishes several well-defined groups (Salman et al. 2011). Several of these "cluster XII" organisms appear in the literature under the genus name *Beggiatoa*, but they are overdue for updated formal description and taxonomic revision, as suggested previously based on 16S rRNA sequences (Salman et al. 2011) and also by heterogeneous G+C content of genomic DNA (Mezzino et al. 1984). This taxonomic revision has started with the recently proposed Candidatus genus-level group "Allobeggiatoa" (Hinck et al. 2011). Two additional groups of *Beggiatoaceae* within "cluster XII" require taxonomic revisions (*Fig. 6.1*):

1. Filamentous *Beggiatoa*-like bacteria from freshwater habitats include not only the *B. alba* strains B15LD and B18LD (L40994 and AF11024, Strohl and Larkin 1978a; Strohl et al. 1981, 1982), but also the pure culture strains *Beggiatoa* sp. OH-75-2a (AF110273, Ahmad et al. 2006), *Beggiatoa* sp. AA5A (Genbank No. AF110275, Ahmad et al. 2006), *Beggiatoa* sp. D-401 and D-402 (AY583995 and AY583996, Grabovich et al. 1998, 2001), *Beggiatoa* sp. 1401–13 (L40997; Pringsheim 1964), *Beggiatoa* sp. LPN from a sewage outlet (EU015402, Kamp et al. 2006), and thin (5–7 µm diameter) *Beggiatoa* filaments naturally enriched in a cave stream for which a 16S rRNA FISH probe has been designed (DQ133935; Macalady et al. 2006, 2008). The older literature contains several studies of *Beggiatoa* strains that grew preferentially under heterotrophic conditions or with organic carbon amendments to chemoautotrophic media (Faust and Wolfe 1961; Scotten and Stokes 1962; Morita and Stave 1963; Burton et al. 1966; Kowallik and Pringsheim 1966; Pringsheim 1967); these strains might have their taxonomic home in "Group XII" as well. A 16S rRNA gene sequencing survey and further characterization of those strains that might have survived in culture collections (for example, Schlösser 1982) are overdue. A sequence-based study should also reexamine the taxonomic borders between heterotrophic *Beggiatoa* spp. and morphologically similar, filamentous *Vitreoscilla* spp. that share the same freshwater benthic habitat; the genus *Vitreoscilla* differs from *Beggiatoa* by not forming sulfur globules (Strohl et al. 1986).
2. A second group consists of uncultured marine morphotypes from hypersaline lagoons (GU117706 and GU117707; de Albuquerque et al. 2010), several phylotypes from the Håkon Mosby mud volcano in the Arctic Ocean (FR847882 to FR847887; Grünke et al. 2012), the cultured autotrophic marine strain MS-81-6 (AF110277) from Sippewissett salt marsh near Woods Hole, MA (Nelson et al. 1982; no longer available in culture), brackish-water filaments enriched from sediments off southeast India (HM598303, JN588607, JN674459; Saravanakumar et al. 2012), and the cultured marine strain 35Flor (FR717278) originating from corals infected with black band disease. The members of this marine cluster have filament diameters of ca. 2–7 µm (compiled in Brock et al. 2012). Near the root of this cluster branches the autotrophic marine strain MS-81-1c, also isolated from Sippewissett salt marsh (AF110276; Nelson et al. 1982) but no longer available in culture (*Fig. 6.2b*). Currently, strain 35Flor is the only marine *Beggiatoa* strain that is available in culture (coculture with a *Pseudovibrio* sp.); it has been studied extensively for its polyphosphate

inclusions (Fig. 6.2d) (Brock and Schulz-Vogt 2011; Brock et al. 2012) and anaerobic sulfur respiration (Schwedt et al. 2012).

Genus *Thioploca*. Lauterborn 1907

Thi.o.plo'ca Gr. neut. n. *thein* (latin transliteration thium) sulfur; Gr. fem.n. *ploke* a braid, a twist; M.L. fem. n. *Thioploca* sulfur braid. The genus *Thioploca* includes thin filaments occurring in sheathed bundles that inhabit freshwater and brackish-water surficial sediments and decaying plant material (Fig. 6.3g). The type species of the genus *Thioploca*, *T. schmidlei* from Lake Constance, Germany (Lauterborn 1907), has been observed recently in Lake Baikal, Russia (Zemskaya et al. 2009), but it is not represented by 16S rRNA gene sequences. The second described species *T. ingrica* is morphologically similar to *T. schmidlei*, but has a smaller filament diameter (Wislouch 1912; Maier 1984). *T. ingrica* is represented by a tight cluster of mutually similar 16S rRNA gene sequences (AF452892; AY115530; AB263619; FR690997; FR690998; EU718069-71; L40998; AB699673 to AB699684) that were obtained from filaments in temperate freshwater lakes of Japan and Germany (Kojima et al. 2003, 2006), from Lake Baikal (DQ338566; Zemskaya et al. 2009), from brackish fjords in Denmark (Høgslund et al. 2010; Salman et al. 2011), and from a shallow tropical lake in Cambodia (Nemoto et al. 2012). A specific 16S rRNA FISH probe for this cluster has validated the 16S rRNA sequencing results for environmental filaments (Kojima et al. 2003). The microbial epibionts inhabiting the sheaths produced by *T. ingrica* have been analyzed by 16S rRNA gene sequencing and FISH, yielding predominantly *Chloroflexi* phylotypes (Kojima et al. 2006; Nemoto et al. 2011). Intergenic spacer region and partial 23S rRNA gene sequences (AB699673 to AB699684) allow for a fine-scale resolution of the genus *Thioploca*; the tropical *Thioploca* phylotypes diverge from their temperate lake counterparts (Nemoto et al. 2012).

Thioploca schmidlei. Lauterborn 1907

schmid'le.i. M.L. gen.n., *schmidlei* of Schmidle.

Identified from sediments of freshwater and brackish-water localities in Europe and from Lake Baikal, Russia. Originally found in Lake Constance, southern Germany. Multicellular filaments, diameter 5–9 µm, constant width over the entire length of the filament, forming bundles, gliding motility.

Type strain: none isolated.

Thioploca ingrica. Maier 1984

In'gri.ca. M.L. adj. ingrica pertaining to Ingria, ancient district of St. Petersburg, Russia.

Identified from sediments of freshwater and brackish-water localities in central Europe, from Lake Erie, USA; from Lake Biwa, Japan; and Lake Tonle Sap, Cambodia. Multicellular filaments, constant width over the entire length of the filament, diameter 2–4.5 µm, forming bundles; gliding motility.

Type strain: none isolated.

Genbank accession number of 16S rRNA gene sequence: L40998.

Taxonomic note: The genus *Thioploca* is not represented by pure cultures; its type species *T. schmidlei* is only rarely found, and its sole described species, *T. ingrica*, remains uncultured and incompletely characterized. Such a combination is usually characteristic of a Candidatus group; at present, *Thioploca* retains its status as a validly described genus due to historical precedent.

"*Candidatus* Marithioploca". Salman et al. 2011

This group of uncultured, filamentous, sheath-forming sulfide-oxidizing bacteria (Fig. 6.3a–f) was originally included in the genus *Thioploca* and contained the large marine *Thioploca* species *T. araucae and T. chileae* (Maier and Gallardo 1984b). Since it constitutes a monophyletic 16S rRNA gene lineage distinct from freshwater *Thioploca* (Teske et al. 1995, 1999) and also shows substantial physiological differences, the marine and freshwater *Thioploca* sp. were separated into two taxonomic groups. The smaller brackish and freshwater representatives are retained as the genus *Thioploca sensu stricto*, and the large marine strains constitute the *Candidatus* taxon "Marithioploca" (Salman et al. 2011). FISH hybridization experiments with group-specific 16S rRNA probes have validated the 16S rRNA sequences obtained from size-sorted and cleaned filaments (Teske et al. 1995, 1999). The "Marithioploca" group forms two separate, yet mutually closely related subclusters. One subcluster contains the original published partial sequence of *Thioploca araucae* (L41043; Teske et al. 1995), the near-complete sequence of a large, single, marine *Beggiatoa*-like filament from the Bay of Concepción (AF035956; Teske et al. 1999), and several sequences from bundled and single filaments collected offshore Concepción, Chile (FR690987 to FR690993; Salman et al. 2011). The filament diameter range within this group is largely congruent with the range given in the original description of *T. araucae* (30–43 µm; Maier and Gallardo 1984b). The second subcluster contains the original partial sequences for *T. chileae* (L40999; Teske et al. 1999) and three other sequences originating again from bundled and single filaments (FR690994 to FR690996; Salman et al. 2011) that are congruent with the published size range for *T. chileae* (12–20 µm; Maier and Gallardo 1984b). 16S rRNA gene sequence identities among the two clusters are as high as 98.3–99.5 %, which could argue against a taxonomic separation. On the other hand, the two species show not only nonoverlapping filament diameter distributions but also distinct environmental distributions and habitat preferences (Schulz et al. 1996, 2000). Based on mutually consistent ecophysiological, morphological, and molecular differences, the two species are retained.

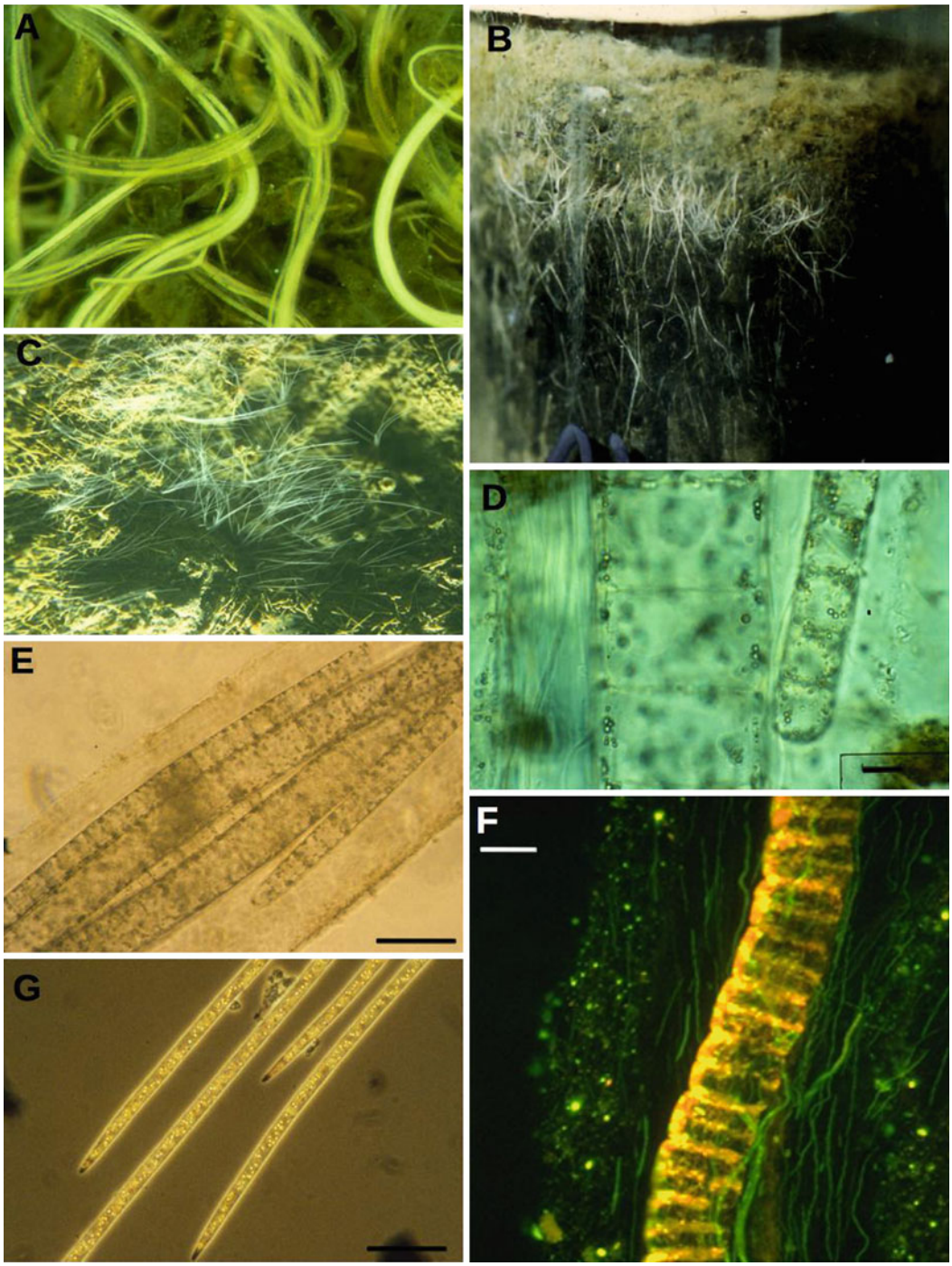

Fig. 6.3
''*Candidatus* Marithioploca'' *and Thioploca*. (a) Washed bundles of ''*Candidatus* Marithioploca'' from the continental shelf of Chile. The filaments appear white due to their internal sulfur content; bundles of filaments are surrounded by transparent sheaths. (b) Sediment core with reducing marine sediment, embedded vertically oriented ''Marithioploca'' filament bundles, and olive-green phytoplankton debris on top. (c) Individual filaments of ''Marithioploca'' emerging from their buried sheaths to take up nitrate from the overlying seawater (Hüttel et al. 1996). (d) Adjacent filaments of ''Marithioploca araucae'' (*left*) and ''Marithioploca chileae'' (*right*). Note the vacuole space taking up the cell interior and the sulfur globules within the peripheral cytoplasm. Scale bar = 10 μm. (e) Filaments of ''Marithioploca araucae'' (*left*) and ''Marithioploca chileae'' in a shared sheath. Scale bar = 50 μm. (f) Filament of ''Marithioploca araucae'' surrounded with filamentous bacterial epibionts (members of the sulfate-reducing genus *Desulfonema*; Fukui et al. 1999). Scale bar = 25 μm. (g) Filament tips of *Thioploca ingrica* from a brackish fjord (Randersfjord, Denmark). Scale bar = 20 μm (Photos (a–c) Markus Hűttel, Florida State University; (d) Jan Kűver, Institute for Material testing, Bremen; (e, g) Andreas Teske, University of North Carolina at Chapel Hill; (f) Manabu Fukui, Hokkaido University, Sapporo, Japan)

"*Marithioploca araucae*". Salman et al. 2011

Ma.ri.thi.o.plo'ca. L. gen. n. *maris* of the sea; N.L. fem. n. *Thioploca* a genus name; N.L. fem. n. *Marithioploca* the *Thioploca* of the sea, the truly marine *Thioploca*; T. araucae (Maier and Gallardo 1984b), Approved Lists 1980; a.rau'ca.e. N.L. fem. adj. *araucae* of Arauco in Central Chile.

Identified from oxygen-poor upwelling area offshore Concepción, Chile. Multicellular filaments either free-living filaments or bundled by a common mucous sheath; filament diameter 25–43 μm; constant width over the entire length of the filament; gliding motility; vacuolated; ability to store nitrate; sulfur inclusions, marine.

Genbank accession number of 16S rRNA gene sequence: L41043; FR690987 to FR690993.

"*Marithioploca chileae*". This Publication

Ma.ri.thi.o.plo'ca. L. gen. n. *maris* of the sea; N.L. fem. n. *Thioploca* a genus name; N.L. fem. n. *Marithioploca* the *Thioploca* of the sea, the truly marine *Thioploca*; T. chileae (Maier and Gallardo 1984b), Approved Lists 1980; chi'le.ae. N.L. gen.n. *chileae* of Chile.

Identified from oxygen-poor upwelling area offshore Concepción, Chile. Multicellular filaments either free-living filaments or bundled by a common mucous sheath; filament diameter 12–20 μm; constant width over the entire length of the filament; gliding motility; vacuolated; ability to store nitrate; sulfur inclusions, marine.

Genbank accession number of 16S rRNA gene sequence: L40999, FR690994, FR690995, FR690996.

Taxonomic note: The near-identical 16S rRNA gene sequences of filaments growing as sheathed filament bundles and those thriving as single, free-living filaments affiliating with this taxon show that the morphological (sheath-based) distinction of the genera *Beggiatoa* and *Thioploca* was phylogenetically shallow (Teske et al. 1999; Salman et al. 2011).

Genus *Thiomargarita*. Schulz et al. 1999

The first discovery of nonfilamentous, very large, vacuolated, nitrate-accumulating, and spherical cells in highly reducing marine sediments offshore Namibia led to the description of the new genus *Thiomargarita* based on a distinct morphology (◉ *Fig. 6.4a*) and 16S rRNA gene sequence (AF129012) (Schulz et al. 1999). *Thiomargarita* is among the largest known bacteria by volume; observed cell diameters diverge widely, and current observations indicate a range of 16–750 μm (Salman et al. 2011). Large *Thiomargarita* cells are discussed as an alternate explanation for late proterozoic microfossils that are commonly regarded as eukaryotic blastocytes (Bailey et al. 2007).

The chain-forming *Thiomargarita* specimens of the original description have been supplemented by 16S rRNA gene sequencing of numerous unicellular (◉ *Fig. 6.4b*) or aggregate-forming (◉ *Fig. 6.4e*) *Thiomargarita* cells from Namibia, Chile, and Costa Rica (FN811663; FR690879 to FR690921); these phylotypes cluster together and form the species *Thiomargarita namibiensis* (Salman et al. 2011). Two additional *Thiomargarita* species are proposed as species-level Candidate taxa. *Candidatus* "Thiomargarita joergensenii" forms a distinct clade of 16S rRNA gene sequences (FR690922 to FR690925) and shows a homogenous morphology: multiple spherical cells are inhabiting an intact centric diatom frustule that has no openings or passages for fully grown cells, suggesting an initial colonization of the frustule by substantially smaller daughter cells (Salman et al. 2011).

This morphology/lifestyle is also found in the candidatus taxon "Thiopilula." Cells belonging to the candidate species "Thiomargarita nelsonii" occur in a wide range of morphologies (◉ *Fig. 6.4c, d*), i.e., spherical unicells; cylindrical cells in chains; symmetrically arranged, fourfold-divided aggregates; aggregates of several tens of cells; attached budding cells (◉ *Fig. 6.4f*); and nonattached, extremely large spherical cells with budding spherical caps. This species is also represented by a distinct 16S rRNA gene lineage (FR690926 to FR690967, FN811658, FN811659, FN811661, FN811662, HF954103, HF954105, HF954106, HF954108-110, HF954113) (Salman et al. 2011; Bailey et al. 2011; Salman et al. 2013). Specific PCR primers for the genus *Thiomargarita* were developed for specific amplification of 16S rRNA genes from contamination-prone single cells (Bailey et al. 2011; Salman et al. 2011).

Members of the genus *Thiomargarita* contain up to four self-splicing introns within their 16S rRNA genes; they enlarge the 16S rRNA genes considerably (up to app. 3.5 kb) and interfere with PCR amplification of 16S rRNA genes (Salman et al. 2012). One or two introns are also found within the 16S rRNA genes of the candidate genera "Marithioploca," "Thiopilula," and "Thiophysa." The introns occur in specific, conserved positions within the 16S rRNA gene (*E. coli* positions 795, 1078, 1396, and 1495) and often encode genes for intron-encoded homing endonuclease proteins. Intron persistence within the large, vacuolated sulfide-oxidizing bacteria might be connected to their suggested degree of polyploidy (Salman et al. 2012); an unusually high amount of nucleoids is documented for cells of *Thiomargarita namibiensis* (Schulz 2006). A few intron-encoded endonucleases would be sufficient to jump-start endonuclease-catalyzed spread of intron sequences throughout the polyploid genome (Salman et al. 2012).

Thiomargarita namibiensis Schulz et al. 1999

Thi'o.mar.ga.ri'ta Gr. neut. n. *theion* (Latin transliteration *thium*), sulfur; L. n. *margarita* pearl; N.L. fem. n. *Thiomargarita* sulfur pearl; na.mi.bi.en'sis. M.L. gen. n. *namibiensis* of Namibia.

Spherical cells, occurring unicellular, in chains or in aggregates; single cells occasionally motile by slow jerky rolling; vacuolated; can store nitrate in vacuole (up to 800 mM); sulfur

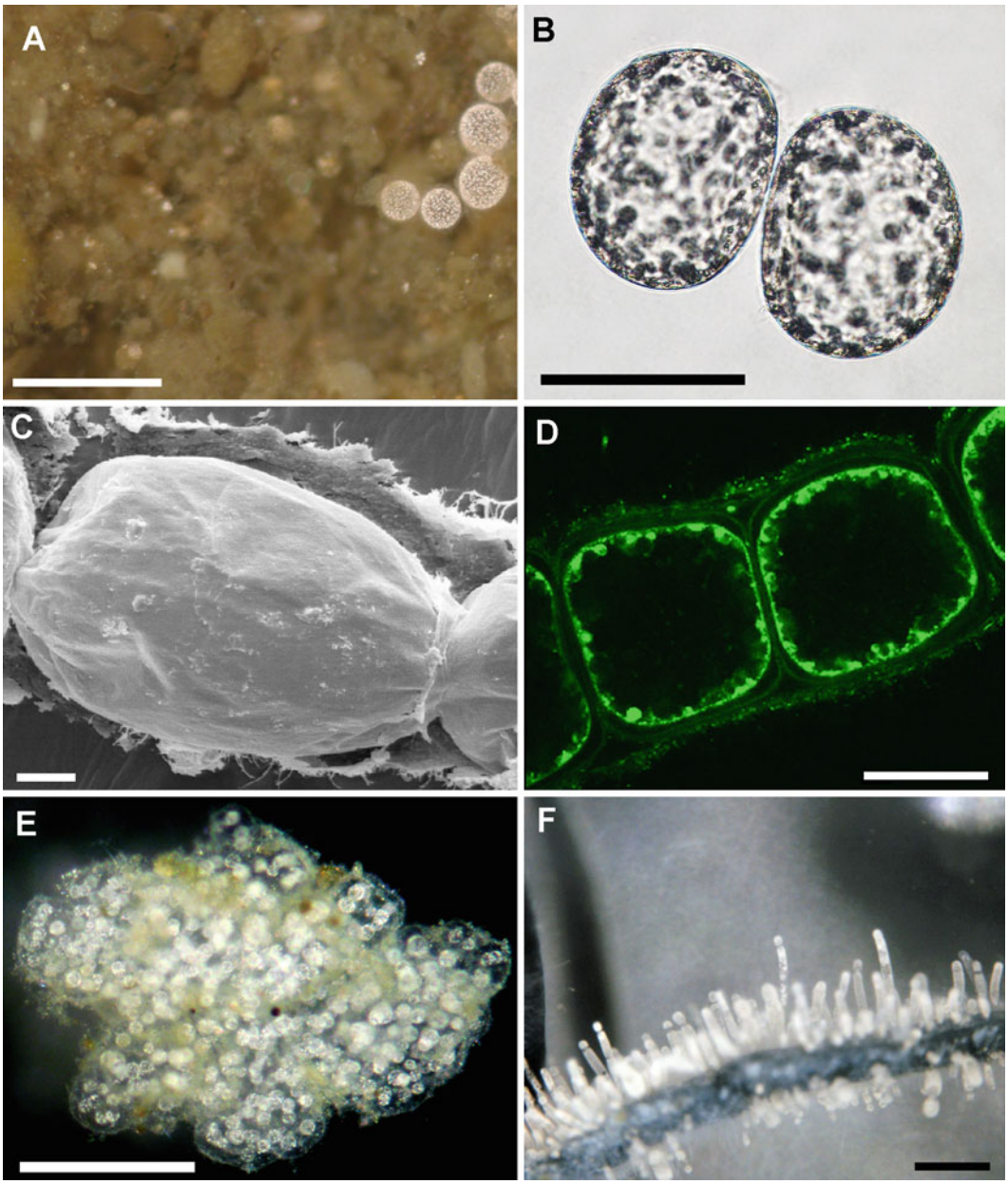

Fig. 6.4

***Marine nonfilamentous sulfur bacteria*.** (**a**) Namibian sediment is a liquid decaying diatomaceous ooze that contains predominantly nonfilamentous, chain-forming giant sulfur bacteria of the genus *Thiomargarita*. Scale bar 0.5 mm. (**b**) Microscopic image of two cells showing their elemental sulfur inclusions as black drop-like spots surrounding a large void internal vacuole—"in-focus" are the inclusions of the outermost rim of the cells, and they appear "out-of-focus" in the center as they are actually located above and below the focal plane. Scale bar 100 μm. (**c**) After removing the outer sheath of a "*Candidatus* Thiomargarita nelsonii" cells, the outer cell envelope can be observed with scanning electron microscopy. Scale 20 μm. (**d**) FITC staining of a "*Candidatus* Thiomargarita nelsonii" chain reveals the thin cytoplasmic rim at the periphery of each cell and leaves the inside (vacuole) unstained. The mucus and epibionts living thereon are stained as well. Scale is 50 μm. (**e**) Nonfilamentous sulfur bacteria like these collected off Namibia produce a mucous sheath that holds numerous spherical cells in a large aggregate. Scale is 0.5 mm. (**f**) Some unicellular sulfur bacteria are capable to produce a holdfast structure to attach themselves to solid surface and proliferate by forming small spherical buds at the apical ends. These cells were collected at the Costa Rica margin. Scale is 1 mm (Photos (**a**–**c**) Verena Salman, UNC Chapel Hill; (**d**) adapted from Salman et al. 2011; (**e**) Verena Salman University of North Carolina at Chapel Hill; (**f**) Jake Bailey, University of Minnesota)

inclusions; in sediments off Namibia, Chile, and Costa Rica and at mud volcano off Egypt; marine.

Genbank accession numbers of 16S rRNA gene sequence: FR690879-FR690921, FN811663, HF954102, HF954104.

"*Candidatus* Thiomargarita joergensenii" Salman et al. 2011

Thi.o.mar.ga.ri'ta. Gr. neut. n. *theion* (Latin transliteration *thium*), sulfur; L. n. *margarita* pearl; N.L. fem. n. *Thiomargarita* sulfur pearl; joer.gen.se'ni.i. N.L. gen. n., *joergensenii* of Jørgensen, named in honor of Bo Barker Jørgensen, a Danish microbiologist.

Single, spherical cells; occurring in empty diatom frustules; sporadic slow jerky rolling movement; vacuolated; sulfur inclusions; in sediments off Namibia; marine.

Genbank accession numbers of 16S rRNA gene sequence: FR690922–FR690925, HF954107.

"*Candidatus* Thiomargarita nelsonii" Salman et al. 2011

Thi.o.mar.ga.ri'ta. Gr. neut. n. *theion* (Latin transliteration *thium*), sulfur; L. n. *margarita* pearl; N.L. fem. n. *Thiomargarita* sulfur pearl; nel.so'ni.i. N.L. gen. n. *nelsonii* of Nelson, named in honor of Douglas C. Nelson, an American microbiologist.

Cells of highly diverse morphology and life modes; ability to divide in multiple planes, to attach, or to form gonidia; single cells or those in envelopes sporadic slow jerky rolling movement; vacuolated; sulfur inclusions; in sediments off Namibia, Chile, and Costa Rica and around cold seeps at Hydrate Ridge and Costa Rica; marine.

Genbank accession numbers of 16S rRNA gene sequence: FR690926–FR690967, FN811658–FN811659, FN811661, FN811662, HF954103, HF954105, HF954106, HF954108-110, HF954113.

Taxonomic comment: The partial 16S rRNA sequence of the original publication (AF129012) of *T. namibiensis* matches the "T. nelsonii" phylotype and not *T. namibiensis*. Obviously, the chains of cylindrical cells of "T. nelsonii" can be mistaken for the large, spherical cells in *T. namibiensis* chains.

"*Candidatus* Maribeggiatoa" Salman et al. 2011

Beggiatoa-like, large, vacuolated, nitrate-accumulating filaments from reducing marine sediments form this monophyletic lineage based on 16S rRNA gene sequences from individual filaments (Salman et al. 2011). The group contains phylotypes from the central Californian coast, Monterey Canyon (AF064543, Ahmad et al. 1999), Carmel Canyon (AY580013, Kalanetra et al. 2004), and Monterey Bay (FJ814745, FJ814753). Large individual filament diameters are found in this group: 20–76 µm for Carmel Canyon and 65–85 µm for Monterey Canyon filaments (*Fig. 6.5a–b*). With the exception of clone FJ814753, the sequences cluster tightly together and constitute the species-level candidate taxon "Maribeggiatoa vulgaris" (Salman et al. 2011). Phylotypes related to "Maribeggiatoa" were also obtained by sequencing of reverse-transcribed 16S rRNA from microbial mats in the Gulf of Mexico (partial sequences with Genbank numbers AY324499, AY324511) (Mills et al. 2004). A FISH probe for "Maribeggatoa" has been developed to distinguish "Maribeggiatoa" from "Marithioploca" (Ahmad et al. 1999).

"*Candidatus* Maribeggiatoa vulgaris" Salman et al. 2011

Ma.ri.beg.gi.a.to'a. L. gen. n. *maris* of the sea; N.L. fem. n. *Beggiatoa* a genus name; N.L. fem. n. *Maribeggiatoa* the *Beggiatoa* of the sea, the truly marine *Beggiatoa*; vul.ga'ris. L. fem. adj. *vulgaris* usual, common.

Disc-shaped cells forming multicellular filaments; constant width over the entire length of the filament, rounded terminal cells; gliding motility; vacuolated; ability to store nitrate; sulfur inclusions; marine; at seep sites and hydrothermal vents.

Genbank accession numbers of 16S rRNA gene sequence: FJ814745, AY580013, AF064543.

Taxonomic note: Two clusters of large, vacuolated, nitrate-accumulating marine *Beggiatoa*-like filaments from Guaymas Basin hydrothermal sediments consist of orange filaments with ca. 25–35 µm diameter (JN793553, JN793555, JN793556) and of very large colorless filaments of ca. 120 µm diameter (JN793554, JN793557) and form a multilineage cluster with the Candidatus taxa "Maribeggiatoa" and "Marithioploca" (McKay et al. 2012). The near-complete genome of a single orange filament has been obtained and analyzed after whole genome amplification (MacGregor et al. 2013a, b, c). Although published as "Maribeggiatoa," it became apparent that the orange Guaymas filaments share only weak bootstrap support (between 50 % and 60 %) with "Maribeggiatoa" (Salman et al. 2013). The white Guaymas filaments do not fit into currently described candidatus taxa (Salman et al. 2013). Therefore, the orange and the white Guaymas filaments are included here as separate lineages of *Beggiatoaceae* (*Fig. 6.1*).

"*Candidatus* Marithrix" Salman et al. 2011

Large, vacuolated filaments growing attached to hydrothermal vent chimneys and surrounding methane and mud seeps are exposed alternately to sulfidic and oxygenated seawater (*Fig. 6.5e*) and form this monophyletic lineage among the *Beggiatoaceae* (Kalanetra et al. 2004; Heijs et al. 2005; Kalanetra and Nelson 2010; Grünke et al. 2011, 2012). The 16S rRNA gene

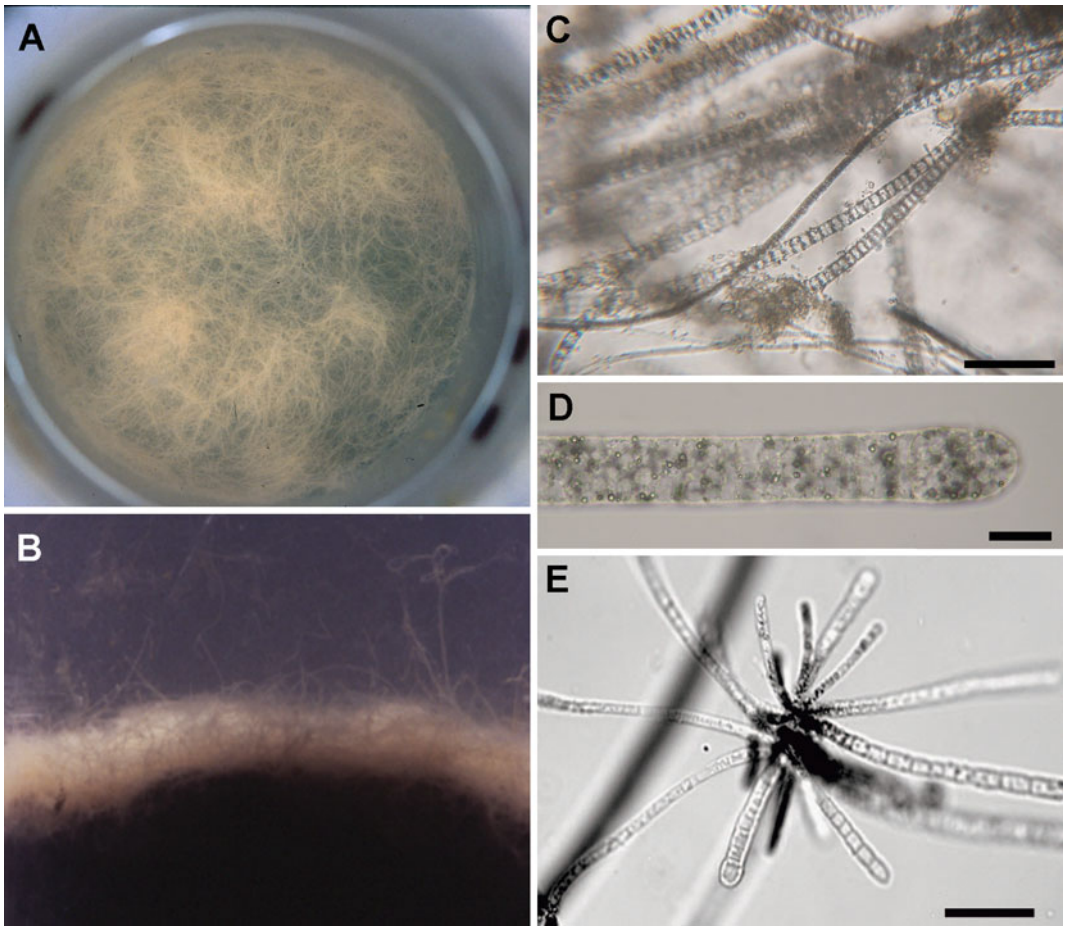

Fig. 6.5

***Marine filamentous sulfur bacteria*. (a) Mat-covered surface of a sediment core from Monterey Canyon, dominated by large, nitrate-accumulating filamentous sulfur oxidizers "*Candidatus* Maribeggiatoa." (b) Viewing the same mat from the side reveals individual filaments reaching out from the mat and into the supernatant water. (c) Microscopic image of filaments collected from a microbial mat at the Håkon Mosby mud volcano off Norway. The community consists of filaments of various diameters. Scale bar 50 μm. (d) Close-up view of a vacuolated marine filament sampled in Eckernförde Bay, Germany. The filament is in the correct size range for "Parabeggiatoa" but requires molecular identification for a definitive attribution. Scale bar 25 μm. (e) Attached filamentous sulfur bacteria ("Marithrix") sampled at White Point off Oregon forming a rosette. Scale bar 40 μm (Photos (a, b) Douglas Nelson, University of California at Davis; (c) Stefanie Meyer, Max Planck Institute for Marine Microbiology, Bremen; (d) Marc Mussmann, Max Planck Institute for Marine Microbiology, Bremen; (e) modified from Kalanetra et al. 2004)**

sequences of "Marithrix" filaments have been determined by multiple PCR amplifications with general and specifically developed group-specific primers and were validated by FISH hybridization of fresh filaments, using the rRNA equivalent of the group-specific PCR primer site as probe target (Kalanetra et al. 2004). The filament diameters of the target organism are variable: positive FISH hybridizations were obtained with filaments in the range of 10–38 μm, plus a few larger filaments (Kalanetra et al. 2004). Attached filaments from the Juan de Fuca vents ranged in diameter mostly from 9–30 μm; a minority of larger filaments reached up to 96 μm (Kalanetra and Nelson 2010). So far, filaments from two deep-sea hydrothermal areas (Juan de Fuca, Escanaba Trough) and a coastal hydrothermal vent (White Point, California) have identical 16S rRNA sequences (AY883933; AY883934; AY496953); very similar 16S rRNA transcripts were recently obtained from the Menez Gwen hydrothermal vent site (FR827864; Grünke et al. 2012) and Lucky Strike hydrothermal field (FR670384; Crépeau et al. 2011) on the Mid-Atlantic Ridge, from the Amon (FR666859, Grünke et al. 2011) and Milano (AY592917, Heijs et al. 2005) mud volcanoes in the Mediterranean Sea, and from a Storegga gas chimney off Norway (FR847874, Grünke et al. 2012). Overall, the members of this group share 16S rRNA gene sequence similarities of at least 98 %. The name of this candidate genus and species, *Candidatus* "Marithrix sessilis," reflects their distinctive surface-attached and rosette-forming growth mode that is otherwise seen in the genus *Thiothrix* (Salman et al. 2011).

"*Candidatus* Marithrix sessilis" Salman et al. 2011

Ma'ri.thrix. L. gen. n. *maris* of the sea; Gr. n. *thrix* hair; N.L. fem. n. *Marithrix* hair of the sea; ses'si.lis. L. adj. sessilis sitting, adhering to a surface.

Attached, multicellular filaments, constant width over the entire length of the filament; diameter of most filaments in the range of 10–38 µm (outliers up to 112 µm have been observed), rounded ends, sometimes forming rosettes; ability to produce gonidia; nonmotile; sulfur inclusions; vacuolated or non-vacuolated; marine; at cold seeps and hydrothermal vents.

Genbank accession numbers of 16S rRNA gene sequences: AY883933–AY883934, AY496953, FR827864.

"*Candidatus* Isobeggiatoa" Salman et al. 2011

Beggiatoa-like, vacuolated, nitrate-accumulating filamentous bacteria from a wide range of marine sediments constitute the genus-level candidate taxon "Isobeggiatoa," defined as a monophyletic lineage by 16S rRNA analysis (Salman et al. 2011). At present, this group contains representatives from Arctic fjords of Svalbard, Norway (FN561862; Jørgensen et al. 2010); Tokyo Bay, Japan (AB108786; Kojima and Fukui 2003); the Chilean coast (FJ875195; Aranda et al. 2010); and a cluster of similar sequences from Limfjorden in Denmark (AF532775) and Jadebusen in Germany (AF532769; Mussmann et al. 2003) that have been proposed as the candidate species-level taxon "Isobeggiatoa divolgata" (Salman et al. 2011). Filament diameters of geographically separated populations with distinct 16S rRNA sequences show a wide range from approximately 10–30 µm (Jørgensen et al. 2010; Aranda et al. 2010). A single filament from Eckernförde Bay in Germany (Filament PS; near 30 µm diameter) was used for whole genome amplification and subsequent pyrosequencing, yielding a partial genome of 6.769 contigs with 17x coverage and a total sequencing length of 7.6 Mb (Mussmann et al. 2007).

"*Candidatus* Isobeggiatoa divolgata" Salman et al. 2011

I.so.beg.gi.a.to'a. Gr. adj. *isos* equal, similar; N.L. fem. n. *Beggiatoa* a genus name; N.L. fem. n. *Isobeggiatoa* the bacterium similar to *Beggiatoa*; di.vol.ga'ta. L. fem. adj. *divolgata* widespread, common.

Disc-shaped cells forming multicellular filaments; constant width over the entire length of the filament, rounded terminal cells; gliding motility; vacuolated; ability to store nitrate; sulfur inclusions; brackish or marine, also arctic latitudes.

Genbank accession numbers of 16S rRNA gene sequence: AF532769, AF532775, FJ875195, AB108786, FN561862.

"*Candidatus* Parabeggiatoa" Salman et al. 2011

Morphologically similar to medium-sized, filamentous marine "Isobeggiatoa," this monophyletic group of uncultured, sulfide-oxidizing large filamentous bacteria is defined by 16S rRNA gene sequencing of single filaments (Salman et al. 2011). These bacteria occur in two distinct phylogenetic clusters: one cluster represented by filaments from brackish sediments of Limfjorden in Denmark (AF532770; AF532772-774; Mussmann et al. 2003) contains the candidatus taxon "Parabeggiatoa communis" (Salman et al 2011). The 16S rRNA sequences of this cluster are validated by FISH with a group-specific 16S rRNA probe; the FISH-stained filaments of this cluster range in diameter from approximately 33–40 µm (Mussmann et al. 2003). The second cluster consists of filaments with diameters in the range of 20–30 µm that were collected from reducing marine sediment underneath the cages of a salmon farm in southern Chile (FJ875196 to FJ875199) (Aranda et al. 2010). A single filament from Eckernförde Bay in Germany (Filament SS, ca. 30 µm diameter) was used for whole genome amplification and subsequent Sanger sequencing, yielding a low-coverage (3x) partial genome assembly of 1,091 contigs with a total sequencing length of 1.3 Mb (Mussmann et al. 2007). Recently, "Parabeggiatoa" was also found in extensive sulfide-oxidizing mats on hydrothermal sediments of Guaymas Basin in the Gulf of California, Mexico (JN793555; McKay et al. 2012).

"*Candidatus* Parabeggiatoa communis" Salman et al. 2011

Pa.ra.beg.gi.a.to'a. Gr. prep. *para* beside, like; N.L. fem. n. *Beggiatoa* a genus name; N.L. fem. n. *Parabeggiatoa* resembling the genus *Beggiatoa*; com.mu'nis. L. fem. adj. *communis* common, widespread.

Disc-shaped cells forming multicellular filaments; diameter 33–40 µm, constant width over the entire length of the filament, rounded terminal cells; gliding motility; vacuolated; ability to store nitrate; sulfur inclusions; brackish or marine.

Genbank accession numbers of 16S rRNA gene sequence: AF532770, AF532772–AF532774, FJ875196–FJ875199.

"*Candidatus* Allobeggiatoa" Hinck et al. 2011

Strains of the genus-level Candidatus group "Allobeggiatoa" (*Fig. 6.2e*) were enriched from solar salterns in Spain and hypersaline cyanobacterial mats in Spain and Mexico (Hinck et al. 2007, 2011) and represent a monophyletic lineage in 16S rRNA phylogenies (EF428583 and EU919200; Hinck et al. 2007; FR687024 to FR687036; Hinck et al. 2011). The phylogenetically clustered filaments from Spain constitute the species-level candidate group "Allobeggiatoa salina"

(Hinck et al. 2011). The 16S rRNA gene sequences have been validated by group-specific FISH probes and hybridization experiments (Hinck et al. 2011). The "Allobeggiatoa" group constitutes a sister lineage to *Beggiatoa alba* and related deeply branching *Beggiatoa*-like filamentous bacteria (Hinck et al. 2011). It differs not only by 16S rRNA phylogeny but also morphologically. The filaments of "Allobeggiatoa" are vacuolated (*Fig. 6.2e*) and have a diameter of predominantly 6–14 µm, which is distinct from the non-vacuolated, thinner (2–3 µm) filaments for *Beggiatoa alba* and its freshwater relatives (Hinck et al. 2011).

"*Candidatus* Allobeggiatoa salina" Hinck et al. 2011

Al.lo.beg.gi.a.to′a. L. gen. n. *allos* the other; *Beggiatoa* genus name; M.L. fem. n. *Allobeggiatoa*, the other *Beggiatoa*; sa.li′na. L. fem. adj. *salina* salted, saline.

Disc-shaped cells, forming filaments of 6–14 µm in diameter, constant width over the entire length of the filament, rounded terminal cells; gliding motility; intracellular storage of nitrate (up to 650 mM); each cell contains a large central vacuole; the vacuole accounts for about 80 % of cellular biovolume and is surrounded by a cytoplasmic layer; intracytoplasmic sulfur storage (up to 250 mM); facultative anaerobic and presumably performing reduction of intracellular nitrate; microaerophilic; chemolithoautotrophic sulfide oxidizer; halotolerant (tested range 3–15 % salinity); filaments do not form macroscopically visible mats, but are distributed within certain cyanobacterial mat layers that are exposed to sulfide gradients; habitat: sulfide-rich microbial mats at shallow permanently hypersaline lakes and ponds of solar saltern systems with salinities reaching up to 15 %.

Genbank accession numbers of 16S rRNA gene sequence: EF428583, EU919200, FR687024 to FR687033.

Taxonomic note: The detection of filamentous sulfur bacteria in hypersaline environments implied that the organisms were halophilic. Yet, culture studies showed identical growth at salinities ranging from 3–15 %, a characteristic that should be called halotolerant. In order to refer to the highly saline habitat where filaments were encountered, instead to an understudied physiological capability, the species name "halophila" was changed to "salina" before publication in Hinck et al. (2011). Care should be taken because the published phylogenetic tree (Hinck et al. 2011) contains the erroneous name "Allobeggiatoa halophila," which has been corrected by the erratum in Environmental Microbiology Vol. 14, Issue 12, p. 3287.

"*Candidatus* Halobeggiatoa" Grünke et al. 2012

The genus-level *Candidatus* group "Halobeggiatoa" represents a monophyletic group (95.9–100 % 16S rRNA gene sequence identity) of nitrate-accumulating marine single filaments of up to 10 µm diameter (*Fig. 6.5c*). The sequences of this phylogenetic group were obtained from filaments collected from white mats at the Håkon Mosby mud volcano offshore northern Norway (FR847864 to FR847873; Grünke et al. 2012) and from nearshore sediments in Tokyo Bay (AB106784, AB106785; Kojima and Fukui 2003).

"*Candidatus* Halobeggiatoa borealis" Grünke et al. 2012

Ha.lo.beg.gi.a.to'a. Gr. n, *hals* salt; N.L. fem. n. *Beggiatoa* a genus name; N.L. fem. n. *Halobeggiatoa* the salt *Beggiatoa*; bo.re.al.is. L. fem. adj. *borealis* northern

This species-level candidate group is based on seven identical 16S rRNA gene sequences from filaments collected at the Håkon Mosby mud volcano (HMMV) offshore northern Norway (Lichtschlag et al. 2010; Grünke et al. 2012).

Disc-shaped cells, forming filaments of 8–10 µm in diameter, constant width over the entire length of the filament; intracellular nitrate accumulation; gliding motility; filaments occur in conspicuous white mats on HMMV methane seep sediments. Genbank numbers are FR847864 to FR847870.

Taxonomic note: Given the diversity of filamentous *Beggiatoa*-like organisms in the HMMV mats, the identification of "*Candidatus* Halobeggiatoa borealis" should be regarded as preliminary and requires future validation by FISH hybridization and filament-specific physiological characterization.

"*Candidatus* Thiopilula" Salman et al. 2011

The candidate genus-level group "Thiopilula" includes large, nonfilamentous, vacuolated cells that resemble *Thiomargarita* in spherical cell morphology but occur attached to surfaces (Bailey et al. 2011), in colony-like aggregates or within diatom frustules, and form a distinct 16S rRNA lineage (Salman et al. 2011). Specimens collected from benthic marine sediments off Namibia and attached in the vicinity of seeps off Costa Rica have been sequenced (FR690968 to FR690981; FN811660 and FN811664) and are proposed as members of the species-level candidate taxon "Thiopilula aggregata" (Salman et al. 2011).

"*Candidatus* Thiopilula aggregata" Salman et al. 2011

Thi.o.pi'lu.la. Gr. neut. n. *theion* (Latin transliteration *thium*), sulfur; L. fem. n. *pilula* little ball, little globule; N.L. fem. n. *Thiopilula* little sulfur ball; ag.gre.ga'ta. L. fem. adj. aggregata joined together.

Spherical cells aggregated in variable arrangements; recorded diameters 15–65 µm; ability to attach and form gonidia; sporadic slow jerky rolling movement; vacuolated; sulfur inclusions; marine.

Genbank accession numbers of 16S rRNA gene sequence: FR690968–FR690980, FN811660, FN811664.

"*Candidatus* Thiophysa" Salman et al. 2011

The candidate genus-level group "Thiophysa" includes large, nonfilamentous, motile single spherical cells with sulfur inclusions that by 16S rRNA gene sequence (FR690982 to FR690986) form a distinct monophyletic group (Salman et al. 2011). Comparable cells have been described originally as *Thiophysa volutans* (Hinze 1903) and were later reclassified as members of the genus *Achromatium* (*A. volutans*, Van Niel 1948). However, 16S rRNA gene analysis places these bacteria clearly into the *Beggiatoaceae*, not into the *Achromatiaceae*. Therefore, the genus name *Thiophysa* has been revived and the species-level candidatus taxon "Thiophysa hinzei" proposed (Salman et al. 2011).

"*Candidatus* Thiophysa hinzei" Salman et al. 2011

Thi.o.phy'sa. Gr. neut. n. *theion* (Latin transliteration *thium*), sulfur; Gr. fem. n. *physa* bubble, breath; N.L. fem n. T*hiophysa* sulfur bubble; hin'zei. N.L. gen. n. *hinzei* of Hinze; named in remembrance of G. Hinze, a German microbiologist, who first described marine, large, spherical sulfur bacteria.

Single, spherical cells; recorded diameters from 56 to 90 µm; vacuolated; sporadic slow jerky rolling movement; sulfur inclusions; marine.

Genbank accession numbers of 16S rRNA gene sequence: FR690982–FR690986.

Phenotypic Analyses

Phenotypic Characteristics of the *Beggiatoaceae*

One of the basic, defining features of the *Beggiatoaceae* is the formation of intracellular sulfur globules by oxidation of reduced sulfur sources (e.g., *Figs. 6.2a* and *6.4b*). The physiological roles of sulfide oxidation and sulfur accumulation are complex and diverge between different physiological types of the *Beggiatoaceae*. Sulfide is a source of energy and electrons for autotrophic carbon fixation and growth (Dworkin 2012; Winogradsky 1887); it can be oxidized with oxygen or nitrate as terminal electron acceptors and can be supplemented or replaced by organic carbon compounds as energy source for heterotrophic growth; in the latter case, elemental sulfur from sulfide oxidation is stored as an alternate electron acceptor. For overview purposes, the diverse genera and candidatus groups within the *Beggiatoaceae* can be divided into several groups with shared phenotypic characteristics.

(A) The heterotrophic non-vacuolate freshwater strains with thin filament diameter are represented by several well-studied strains: the type strain of the genus *Beggiatoa*, *B. alba* B18LD (*Fig. 6.2c*, Mezzino et al. 1984); the *B. alba* strains B15LB (Strohl and Larkin 1978a, b; Strohl et al. 1982); OH-75-2a (*Fig. 6.2a*, Nelson and Castenholz 1981a, b); and the distantly related strain L1401-13 (Pringsheim 1964; Kowallik and Pringsheim 1966). (B) The autotrophic non-vacuolate marine strains with thin filament diameters are represented by the facultatively autotrophic strain MS-81-6, by the obligately autotrophic strains MS-81-1c (*Fig. 6.2b*, Nelson et al. 1982; Nelson and Jannasch 1983; Nelson et al. 1986b; Hagen and Nelson 1996, 1997), and by the marine strain 35Flor (*Fig. 6.2d*, Kamp et al. 2008; Brock et al. 2012; Schwedt et al. 2012). These strains exhibit strong chemotactic behavior and orient themselves as *Beggiatoa* "plates" in the steep oxygen/sulfide gradients which they maintain by fast sulfide oxidation under microoxic conditions (*Fig. 6.6*). (C) The small freshwater and brackish-water genus *Thioploca* is characterized by sheathed bundles of thin filaments embedded in surface sediment or decaying plant material (*Fig. 6.3g*, Høgslund et al. 2010). (D) The large, vacuolated, nitrate-accumulating autotrophic marine Candidatus groups "Maribeggiatoa," "Isobeggiatoa," "Parabeggiatoa," (*Fig. 6.5a, b, d*) and related *Beggiatoaceae* remain uncultured so far. The cells of these large, marine Beggiatoaceae filaments are hollow, i.e., composed of a thin cylinder of cytoplasm surrounding a large central vacuole. This extensive vacuolation is usually linked to high intracellular nitrate concentration (Hinze 1901; Jannasch et al. 1989; Nelson et al. 1989; Larkin and Henk 1996; McHatton et al. 1996), with the possible exception of "*Candidatus* Marithrix," in which nitrate could not yet be detected in the vacuoles (Kalanetra et al. 2004) (*Fig. 6.5e*). (E) Large size, vacuolation, and the ability to accumulate nitrate also apply to *Thiomargarita* and "*Candidatus* Marithioploca," although these organisms are set apart by their conspicuous morphology and lifestyle: the filaments of "Marithioploca" occur predominantly in bundles within sheaths embedded in surface sediment and move within their sheaths to bridge spatially separated pools of the electron donor sulfide in the sediment and the electron acceptor nitrate in the overlying seawater (Hüttel et al. 1996) (Figs. *6.3a–f* and *6.7*). (F) In contrast, *Thiomargarita*, "Thiopilula," and "Thiophysa" have very limited mobility or grow even attached to surfaces (*Fig. 6.4*); they rely primarily on their large cell size and high intracellular storage capacity to survive fluctuating redox regimes and temporary electron donor and acceptor shortages (Schulz and Jørgensen 2001).

Genus *Beggiatoa*

A physiological characterization of the genus *Beggiatoa* depends on how its taxonomic borders are drawn. If the genus is not reduced to its only recognized species, the heterotrophic

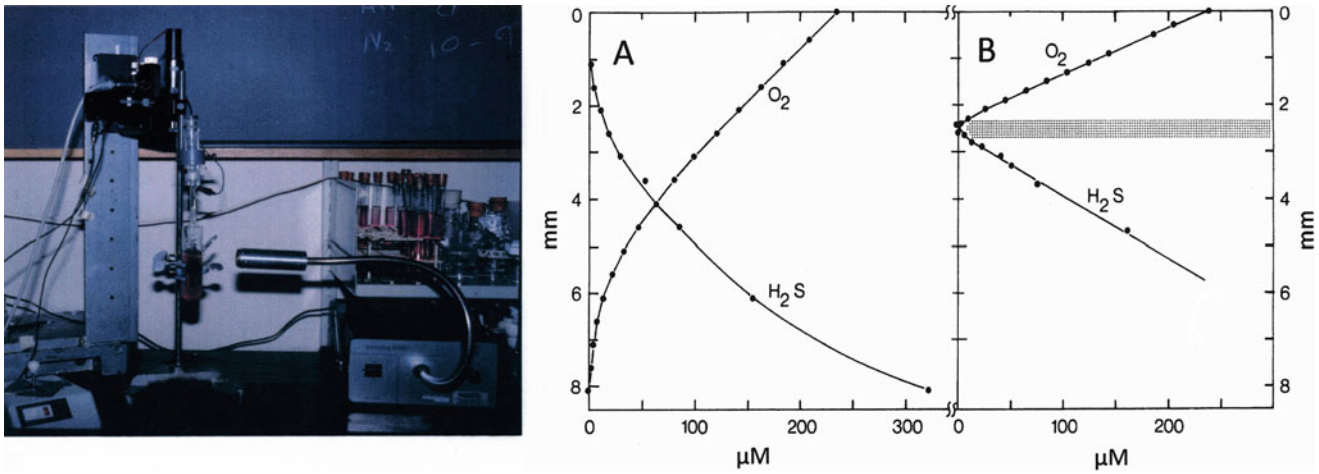

Fig. 6.6

Beggiatoa as gradient organism at the sulfide/*oxygen interface*. *Left*, photo of gradient culture of aerobic, autotrophic sulfide-oxidizing marine *Beggiatoa* spp. The oxygen profile in a gradient culture of *Beggiatoa* spp. is determined with an oxygen microelectrode mounted to a micromanipulator (Nelson et al. 1986a). The whitish *Beggiatoa* plate is visible near the surface of the gradient culture; its position is indicated by the point light source (Photo by Douglas Nelson, UC Davis). *Right*, overlap between H_2S and O_2 profiles in control medium without *Beggiatoa* spp. (a) or inoculated with a *Beggiatoa* culture after 3 days (b). Zero depth indicates the air/agar interface. The shaded area in (b) indicates the *Beggiatoa* plate (Nelson et al. 1986b)

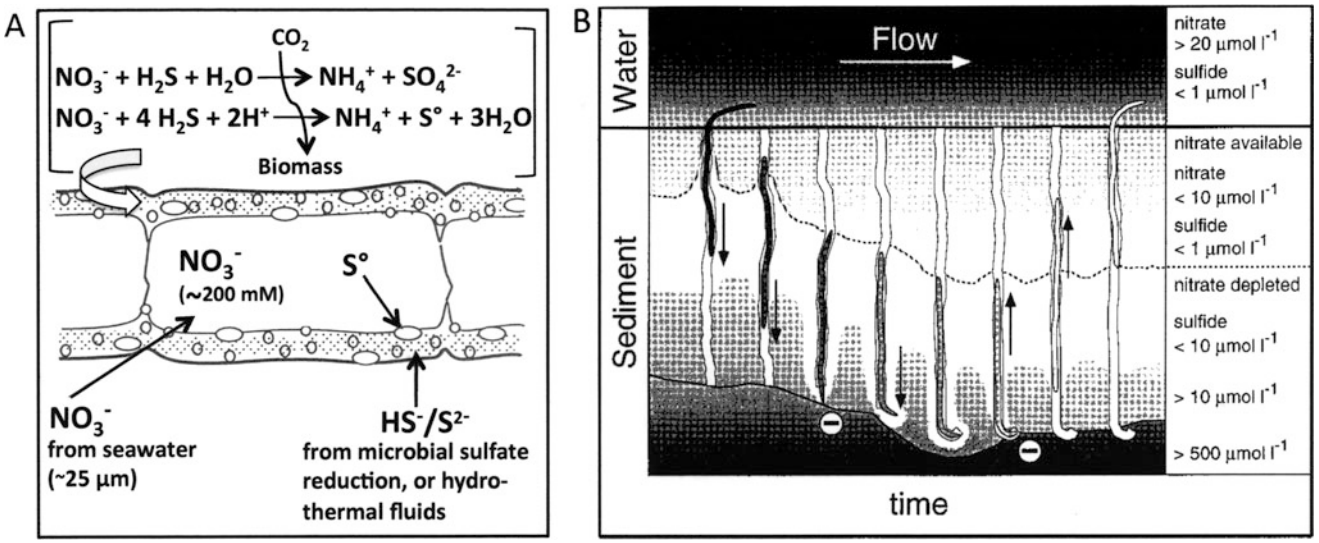

Fig. 6.7

***Physiology of nitrate-accumulating Beggiatoaceae*: *the case of "Marithioploca."* *Left*, schematic links between nitrogen, carbon, and sulfur metabolism in large, marine *Beggiatoaceae*. Nitrate is taken up from seawater, concentrated by four orders of magnitude, and stored in the central vacuole; it serves as electron acceptor for sulfide oxidation to elemental sulfur and sulfate. The resulting energy is used for autotrophic carbon fixation. The process is supposed to be localized in the cytoplasm (see *arrow*). This scenario is based on studies with "Marithioploca" (Otte et al. 1999) and "Maribeggiatoa" (McHatton et al. 1996). *Right*, diagram showing how chemotactic responses and the concentration of an internal trigger may control vertical shuttling in "Marithioploca" 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 towards sulfide and nitrate, respectively. The *minus* signs indicate phobic responses to high concentrations of oxygen or sulfide (Hüttel et al. 1996) (Diagram by Markus Hüttel, Florida State University)**

freshwater filamentous bacterium *Beggiatoa alba* B15LD and B18LD (Strohl and Larkin 1978a; Strohl et al. 1981, 1982) and its close relative OH-75-2a (Nelson and Castenholz 1981a, b), it should also include a wide variety of freshwater and marine strains with small filament diameters and a metabolic spectrum that reaches from aerobic heterotrophy coupled with auxiliary sulfur metabolism (sulfur respiration under anoxia) in freshwater strains to microaerophilic, sulfur-based autotrophy in marine strains (Nelson et al. 1982; Nelson and Jannasch 1983). *Beggiatoa* strain 35Flor is the only marine strain currently available in culture; it is capable of autotrophic growth by aerobic sulfide oxidation, and it can survive anoxic episodes by changing to anaerobic sulfur respiration (Schwedt et al. 2012).

Beggiatoa alba

Beggiatoa alba requires organic carbon substrates for aerobic, heterotrophic growth and resembles in this regard numerous freshwater strains (Faust and Wolfe 1961; Burton et al. 1966; Pringsheim 1964; Kowallik and Pringsheim 1966; Scotten and Stokes 1962; Strohl and Larkin 1978a, b; Nelson and Castenholz 1981a, b). Most *Beggiatoa* strains examined can grow with acetate as a sole source of carbon and energy. All strains of the type species *Beggiatoa alba* (B18LD, B15LD, B25RD) grow well in the presence of sulfide and additions of 0.001–0.05 % acetate (Mezzino et al. 1984), very similar to previous results on other freshwater *Beggiatoa* strains that, when grown with H_2S as energy source, required acetate additions in the range of 0.01–0.0001 % (w/v) (Kowallik and Pringsheim 1966). *Beggiatoa alba* B18LD can use acetate as an energy source and oxidize both acetate C atoms to CO_2; it can also incorporate ^{14}C-labeled acetate into a wide range of cellular and storage compounds, e.g., poly-β-hydroxyalkanoates (*Fig. 6.2c*). Also, acetate significantly increases the capability of this strain to assimilate CO_2, probably through anaplerotic reactions of the tricarboxylic acid (TCA) cycle (Strohl et al. 1981a). Recently, it was shown that *Beggiatoa alba* B18LD and its close relative OH-75-2a can grow on methanol as the sole carbon substrate, when cultured in sulfide gradient tubes (Jewell et al. 2008); the strains are therefore facultative methylotrophs. Other methylated substrates or methane did not support growth (Jewell et al. 2008).

A functional TCA cycle with a glyoxylate bypass has been demonstrated in detailed enzymological studies of *Beggiatoa* strain OH-75-2a (Nelson and Castenholz 1981b), a close relative of *Beggiatoa alba* B18DL and most likely a strain of the same species (Mezzino et al. 1984; Ahmad et al. 2006), and in the chemoheterotrophic *Beggiatoa* strain D-405 (Grabovich et al. 1993). *Beggiatoa* strain OH-75-2a can grow on acetate, ethanol, lactate, pyruvate with a small addition of yeast extract, and TCA cycle intermediates in combination with acetate.

The heterotrophic *Beggiatoa* strain OH-75-2a was quantitatively studied for autotrophic and mixotrophic growth by sulfide and thiosulfate oxidation (Nelson and Castenholz 1981a) to test whether these inorganic electron donors reduce the need for carbon oxidation, lead to increased carbon assimilation and biomass yield, and increase the ecophysiological flexibility of *Beggiatoa* in nature (Pringsheim 1967; Strohl and Schmidt 1984). Sulfide oxidation does not result in additional biomass yield for *Beggiatoa* strain OH-75-2a beyond that obtained from the oxidation of organic carbon sources; mixotrophic growth enhancement by sulfide oxidation was not found (Nelson and Castenholz 1981a). Other attempts to demonstrate mixotrophy for this strain showed experimental shortcomings and need to be revisited (Güde et al. 1981; Nelson and Jannasch 1983). Sulfur globules serve as an electron acceptor reserve that allows a rudimentary anaerobic respiration with sulfur. In *Beggiatoa* strain OH-75-2a, sulfur globules that had accumulated during aerobic thiosulfate oxidation subsequently sustained anaerobic metabolism and growth during several days of anoxia (Nelson and Castenholz 1981a). 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 helps the filaments to survive periods of anoxia in their natural interface habitat (Schmidt et al. 1987).

These results for strain OH-75-2a indicate that *Beggiatoa alba* B18LD does not use sulfur as a chemolithoautotrophic or mixotrophic source of energy. 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 obligately aerobic oxidation of reduced sulfur compounds in *Beggiatoa alba* B18LD, consistent with the presence of c-type cytochromes (Cannon et al. 1979), stops 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). During anoxic incubation, *Beggiatoa alba* B18LD reduces sulfur globules to sulfide, coupled to de novo synthesis of cell material (Schmidt et al. 1987). Thus, a major physiological role for sulfide oxidation in *Beggiatoa alba* is the formation of internal sulfur globules as an alternate electron acceptor reservoir.

Beggiatoa alba strains can use nitrate, nitrite, ammonia, and casamino acids as sole nitrogen source (Mezzino et al. 1984), and the list also includes urea, aspartate, asparagine, alanine, and thiourea that tested positive for strain B18LD (Vargas and Strohl 1985a). *Beggiatoa alba* B18LD assimilates ammonia by the glutamine synthetase–glutamate synthase pathway (Vargas and Strohl 1985a). Nitrate cannot be used as electron acceptor for growth with sulfide oxidation; it allows a limited degree of acetate oxidation, but does not sustain growth as the sole electron acceptor. The enzyme activity is associated with the soluble fraction, not with the cell membranes, and generates ammonia as the waste product. Based on its cellular localization and biochemical properties, the nitrate reductase of *Beggiatoa alba* appears to be an assimilatory nitrate reductase (Vargas and Strohl 1985b). In contrast to *Beggiatoa alba*, other freshwater

Beggiatoa strains can use nitrate as terminal electron acceptor (Sweerts et al. 1990; Kamp et al. 2006).

Beggiatoa alba tests positive for nitrogen fixation and thus contributes to total nitrogen fixation in its natural habitats. Nitrogenase activity in *Beggiatoa alba* is strongly regulated by nitrogen bioavailability: nitrate and nitrite additions to the growth medium prevent induction of nitrogenase; in vivo nitrogenase activity is inhibited by ammonia and urea (Polman and Larkin 1988). Similar nitrogenase repression was found in the heterotrophic freshwater strain OH-75-2a and several other strains isolated from a warm freshwater spring (Nelson and Castenholz 1981a, b). Tightly regulated nitrogenase activity is also shared with marine autotrophic strains MS-81-6 and MS-81-1c (Nelson et al. 1982).

Beggiatoa sp. MS-81-6 and MS-81-1c

These two marine strains, although phylogenetically distinct from *Beggiatoa alba*, remain the best-studied examples for autotrophic carbon fixation and chemolithotrophic sulfur oxidation among the *Beggiatoaceae* (*Fig. 6.2b*). 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 MS-81-6 and MS-81-1c were isolated in pure culture (Nelson et al. 1982; 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 and is almost certainly mixotrophic with regard to both carbon and energy metabolism (Hagen and Nelson 1996). Acetate additions reduce the activity of RuBPC/O to a small fraction of its activity in organic-free medium; increase the activity of 2-oxoglutarate dehydrogenase (Hagen and Nelson 1996), a key enzyme of the citric acid cycle; and open the way to respiratory oxidation of C_2 substrates. Under sulfide-induced 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* strain 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 sulfide-oxidizing bacteria (Hagen and Nelson 1996).

Autotrophic growth of *Beggiatoa* strain MS-81-6 was achieved 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 kept themselves positioned at the sulfide–oxygen interface. Their growth depends on the availability of both compounds, oxygen and sulfide, in opposed overlapping gradients (*Fig. 6.6*, Nelson and Jannasch 1983; Nelson et al. 1986b). Depending on growth stage, *Beggiatoa* strain MS-81-6 adjusts the oxidation pathways of sulfide. When sulfide is abundant, it is oxidized to the stage of elemental sulfur; when the supply of sulfide is limited and has to be used more effectively, sulfide oxidation proceeds to sulfate (Nelson et al. 1986b). 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. 1986b). In comparison to other autotrophic sulfide-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. 1986b; 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:

1. $SO_3^{2-} + AMP + acceptor_{oxidized} \rightarrow APS + acceptor_{reduced}$
2. $APS + PP_i \rightarrow SO_4^{2-} + ATP$

Both enzymes are highly active regardless of the sulfur source (H_2S 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 instead. *Beggiatoa* strain MS-81-6 completely lacks APS reductase activity. The activity of the ATP sulfurylase is two orders of magnitudes 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 systems represent a second sulfur oxidation pathway, which is found in *Beggiatoa* strains MS-81-1c, MS-81-6, and also in the heterotrophic strain OH-75-2a. Since these sulfite oxidases are localized in the cell membrane, they are most likely 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 H_2S and is at least 3 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).

DNA hybridizations were used to check the presence of dissimilatory ATP sulfurylase genes in different *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).

Strain 35Flor

The marine *Beggiatoa* strain 35Flor was isolated in 2001 from a microbial community associated with scleractinian corals suffering from black band disease off the coast of Florida. This *Beggiatoa* strain grows under chemolithoautotrophic conditions in an agar-stabilized oxygen–sulfide gradient medium gaining energy from the aerobic oxidation of sulfide (Kamp et al. 2008; Brock and Schulz-Vogt 2011), and it grows in obligate coculture with a *Pseudovibrio* strain (Brock and Schulz-Vogt 2011; Schwedt et al. 2012). Currently, strain 35Flor is the only marine, autotrophic strain within the *Beggiatoaceae* that is continuously maintained in monospecific culture. Strain 35Flor is a member of the same monophyletic lineage as strain MS-81-6 and can be considered its closest cultured relative. This strain was the first marine isolate capable of sulfur respiration with concurrent PHA dissolution under anoxic conditions (Schwedt et al. 2012). Most likely, the stored carbohydrates are oxidized and excreted, while S globules are reduced to sulfide. In this way, strain 35Flor removes excess sulfur globules that accumulate intracellularly during microoxic sulfide oxidation (Schwedt et al. 2012).

Comparative Aspects

Interestingly, the heterotrophic freshwater *Beggiatoa* strain OH-75-2a showed a similar spectrum of sulfide- and sulfur-oxidizing enzymes as the facultative autotrophs. 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, 2001; Patritskaya et al. 2001). The unusually high activities of RubisCo and of sulfur-oxidizing enzymes in strain D-402 could be connected to its high growth yield (12.2 g/mol oxidized thiosulfate). Direct comparisons of *Beggiatoa* strains 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*.

Photoresponses of *Beggiatoa* spp.

The migrations of *Beggiatoa* filaments in microbial mats and sediments are not only regulated by the combined effects of oxygen and sulfide, but are 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 that was still detectable at low ambient light levels as low as ca. 2 % of full summer sunlight intensity. Filaments were most sensitive in the blue to blue-green (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 periods of illumination and returned to the sediment surface after several hours of darkness (Nelson and Castenholz 1982). Such distinct responses to blue light invite closer examination. In the genome of an undescribed *Beggiatoa* sp., a DNA sequence encodes an adenylyl cyclase directly linked to a BLUF (blue light receptor using FAD) type light sensor domain, which led to the working hypothesis of a light-triggered cyclic AMP signaling mechanism (Stierl et al. 2011).

Genus *Thioploca*

The genus *Thioploca* is widespread in freshwater and brackish-water habitats (*Fig. 6.3g*), for example, in sediments of Lake Constance and other lakes in Germany (Lauterborn 1907; Koppe 1924; Maier and Preissner 1979), in the Neva river at St. Petersburg (Wislouch 1912), in the Rhine and in Baltic coastal lagoons (Kolkwitz 1912), in Lake Erie (Maier 1980), in Lake Ontario (Dermott and Legner 2002), in Lake Baikal in Siberia (Namsaraev et al. 1994; Zemskaya et al. 2001), and in Lake Biwa, Japan (Nishino et al. 1998; Kojima et al. 2003). Studies on field samples of freshwater and brackish-water *Thioploca* spp. (*T. ingrica*) from Japan (Kojima et al. 2007) and Denmark (Høgslund et al. 2010) have provided an initial outline of their physiology. Autoradiography experiments have shown that *T. ingrica* assimilates acetate and bicarbonate, although bicarbonate incorporation could not be detected in the specimens from Japan (Høgslund et al. 2010; Kojima et al. 2006). It is

assumed that *T. ingrica* links inorganic carbon assimilation to sulfur oxidation; however, only indirect support for sulfur oxidation is available. A correlation of elevated sulfate concentrations and *T. ingrica* biomass was shown (Kojima et al. 2007), but enzymatic or genetic studies of carbon assimilation are currently lacking. Oxygen and nitrate are required for carbon assimilation (Høgslund et al. 2010). Nitrate is accumulated intracellularly at concentrations of 2–3 mM; given environmental nitrate concentrations of ca. 10–20 µM (Lake Biwa) to ca. 100 µM (Danish fjords), *T. ingrica* concentrates nitrate by one to two orders of magnitude (Høgslund et al. 2010; Kojima et al. 2007). The large, nitrate-accumulating vacuoles that are typical of many large members of the *Beggiatoaceae* (e.g., ❯ *Fig. 6.4d*) are not found in *T. ingrica*; therefore, nitrate must be accumulated and stored in some other way, such as within smaller cytoplasmatic vacuoles, in the cytoplasm itself, or in the periplasm that can feature cytoplasmic membrane invaginations extending into the cytoplasm (Maier and Murray 1965). However, elemental sulfur, and not nitrate, is the electron acceptor of choice during prolonged anoxia. Anoxic incubation experiments in closed bottles have shown that storage globules of elemental sulfur serve as the electron acceptor during prolonged oxygen and nitrate depletion; *T. ingrica* can survive as a sulfur-reducing heterotroph for over 2–3 months (Høgslund et al. 2010). Therefore, elemental sulfur has the same role in *T. ingrica* as in *Beggiatoa alba* and other thin marine and freshwater *Beggiatoa* that rely on their intracellular sulfur reservoir under anaerobic conditions.

Genus *Thiomargarita*

Cells of the genus *Thiomargarita* show spherical, barrel-like, or bulbous shapes and either are unicellular (❯ *Fig. 6.4b*), are organized in chains surrounded by a mucous sheath (e.g., ❯ *Fig. 6.4a* and *d*), form clusters or aggregates (❯ *Fig. 6.4e*), or grow attached to surfaces (❯ *Fig. 6.4f*, Schulz et al. 1999; Kalanetra et al. 2005; Schulz 2006; Salman et al. 2011). They are among the contenders for the largest prokaryotic cells on earth; the initial discovery of *Thiomargarita* in organic-rich and sulfidic sediments on the continental shelf of Namibia reported individual cells of up to 750 µm in diameter, although sizes of 100–300 µm were more common (Schulz et al. 1999). Their nitrate-storing vacuoles fill the cell volume and restrict the cytoplasm to a thin layer sandwiched between cell membrane and vacuolar membrane; the cytoplasm presumably also contains the intracellular sulfur globules (Schulz 2006). Although some rolling motility is now reported for some morphotypes in this genus, *Thiomargarita* cannot move over long distances vertically within the surficial sediments, such as *Marithioploca*, to efficiently bridge spatially separated pools of oxidant and reductant. Instead, *Thiomargarita* relies on its extreme storage capacity for sulfur and nitrate, an essential capability in benthic habitats that experience long periods of sulfidic conditions and oxygen or nitrate depletion (Schulz et al. 1999). *Thiomargarita* is currently the only sulfur bacterium that combines four types of intracellular inclusions that ensure prolonged survival under energy limitation: nitrate-storing vacuoles and sulfur inclusions provide intracellular reserves of electron acceptor and donor, polyphosphate inclusions serve as an additional energy buffer, and glycogen (or another polyglucose) represents a carbon reserve (Schulz and Schulz 2005).

Based on analogies with previously characterized "Marithioploca" and "Maribeggiatoa" spp., *Thiomargarita* is most likely a facultative chemolithoautotrophic sulfur oxidizer. *Thiomargarita namibiensis* cells can take up and utilize acetate as a carbon source, although not as an electron donor (Schulz and de Beer 2002), and can store this organic carbon intracellularly as glycogen (Schulz and Schulz 2005). Sulfide oxidation and sulfide flux towards *Thiomargarita* cells are stimulated by the presence of oxygen; even highly oxygenated conditions (ca. 200 µM O_2 in solution) were tolerated, showing that *Thiomargarita* is not a microaerophile such as "Marithioploca" and "Maribeggiatoa" (Schulz and de Beer 2002). Oxygen additions to anoxic medium increased the sulfide flux into *Thiomargarita* cell chains by ca. 1/3 (from approximately 5–7.5 pmol sulfide per cm^2 s), indicating that while oxygen stimulated the sulfide flux, most of it was still sustained by intracellularly stored nitrate. This flexibility in usage of electron acceptor and tolerance to different electron donor and acceptor concentrations may explain the long survival of *Thiomargarita* cells in natural sediment samples in the lab.

The large cell size of *Thiomargarita*, and the spatial extent of its cytoplasm and membranes, requires a mechanism to overcome diffusional limitations to intracellular transport, signaling pathways, and coordinated gene expression across the cell volume. Assuming the absence of directional transportation via bacterial tubulins or other analogs to mitotic spindles, how does messenger RNA reach the portions of the cytoplasm that are separated by hundreds of micrometers from the location of the genome? Nucleic acid-stained condensed genomic equivalents or nucleoids are distributed throughout the cytoplasm (Schulz 2006) and suggest a polyploid genome (Angert 2012) similar to the large bacterial endosymbiont *Epulopiscium fishelsoni* (Robinow and Angert 1998; Mendell et al. 2008; Angert 2012).

"*Candidatus* Marithioploca"

Members of "*Candidatus* Marithioploca" are large, vacuolated, sulfide-oxidizing, and nitrate-reducing filamentous bacteria (❯ *Fig. 6.3 a–f*) capable of assimilating inorganic carbon as well as organic substrates for biomass production (Jørgensen and Gallardo 1999). Multiple filaments are usually surrounded by a sheath that is embedded in surficial sediment. The unique combination of high storage capacity for electron donor (sulfur) and acceptor (nitrate) and vertical migration capability between bottom seawater and sediment enables "Marithioploca" to thrive at sediment–water interfaces where electron donor and acceptor undergo major fluctuations in concentration, availability, and spatial separation (❯ *Fig. 6.7*).

All physiological studies on "Marithioploca" have been performed on freshly collected, sheathed filament bundles from the continental shelf of Chile (Maier and Gallardo 1984a, b; Fossing et al. 1995; Otte et al. 1999; Høgslund et al. 2009). The two dominant members of "Marithioploca" from the Chilean continental shelf, previously named *Thioploca chileae* and *T. araucae* and distinguished by filament diameter (12–20 µm and 30–43 µm) (Maier and Gallardo 1984b), were both able to incorporate radiolabeled bicarbonate as well as acetate and amino acids (Maier and Gallardo 1984a). In freshly collected "Marithioploca" samples, CO_2 fixation occurred at rates of 0.4–1.5 nmol carbon per minute and mg protein, similar to those of large hydrothermal vent *Beggiatoaceae* (Otte et al. 1999). Acetate was incorporated at a rate roughly equal to CO_2, with no apparent terminal oxidation, suggesting that "Marithioploca" spp. are mixotrophic with respect to carbon source (Otte et al. 1999). Mixotrophic potential was also supported by quantitative microautoradiography experiments that showed increased inorganic carbon fixation after the addition of acetate and propionate (Høgslund et al. 2009).

Oxidation of reduced sulfur compounds is linked to nitrate reduction. Incubation experiments of freshly collected "Marithioploca" with ^{15}N-labeled nitrate have shown that nitrate is taken up and subsequently reduced to ammonia at rates of 5–6.5 and 1–4 nmol nitrate uptake and reduction per minute and mg protein, respectively (Høgslund et al. 2009). "Marithioploca" filaments respond with positive chemotaxis to nitrate additions (20–30 µm) and emerge, while remaining anchored in their sediment-embedded sheaths, up to several centimeters into nitrate-amended, flowing seawater (Hüttel et al. 1996). Subsequently, the filaments can retreat (at a speed of 3–5 mm h^{-1}) into the sediment and use their stored nitrate for sulfide oxidation (Hüttel et al. 1996). Nitrate can be stored within large vacuoles at concentrations between 10 and 500 mM (Otte et al. 1999; Fossing et al. 1995; Zopfi et al. 2001); however, nitrate can be reduced to ammonia directly without prior storage and dilution of the ^{15}N-signal in the vacuole (Otte et al. 1999). Small amounts of dinitrogen are also produced (Otte et al. 1999), but these denitrification activities result from bacterial epibionts and contaminants on the "Marithioploca" sheaths (Høgslund et al. 2009). Due to nitrate ammonification, ammonia accumulates in "Marithioploca"-harboring sediment surface layers (Thamdrup and Canfield 1996; Hüttel et al. 1996) and can be microbially reassimilated; such a trend would counteract nitrogen loss through denitrification (Farias et al. 1996; Farias 1998). On the other hand, ammonia can also be reoxidized in situ by anammox bacteria (*Candidatus* Scalindua) that grow on "Marithioploca" sheaths; this microbial consortium then amplifies denitrification and enhances nitrogen efflux towards parity of ammonia and N_2 loss from the sediment (Prokopenko et al. 2006, 2013).

Oxygen can be consumed at rates comparable to nitrate, but aerobic respiration during sulfide oxidation can be sustained only at low external oxygen concentrations (ca. 30 µM, 10 % oxygen saturation) that leave the filaments within the "Marithioploca" bundles anoxic. Higher oxygen concentrations (ca. 100 µM, 30 % oxygen saturation) penetrated into the bundles and killed the filaments within 8 h of oxygen exposure (Høgslund et al. 2009). In closed-flume system experiments, dissolved oxygen concentrations of 100–150 µM in seawater triggered a retreat of seawater-exposed filaments into the sediment (Hüttel et al. 1996). Thus, "Marithioploca" can oxidize sulfur as a microaerophile, but does not survive at higher oxygen concentrations, and avoids them by retreating into the sediment.

Sulfide oxidation and ammonia production rates in "Marithioploca" samples showed a stoichiometric ratio of ca. 2, indicating that nitrate reduction to ammonia is accompanied by concomitant sulfide oxidation to elemental 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 from nitrate (requiring 8 electrons per ammonia) and elemental sulfur oxidation to sulfate (yielding 6 electrons per sulfate) is close to the predicted stoichiometric ratio of 4/3 (Otte et al. 1999). Elemental sulfur is an essential electron donor that can be accumulated and stored until needed. The high intracellular concentrations of sulfur and nitrate are correlated to the position and activity of "Marithioploca" filaments in the gradient. Deeply located "Marithioploca" 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). Given the fluctuations in sulfide supply, intracellular sulfur storage provides an essential electron donor reservoir. "Marithioploca" is capable of faster sulfide uptake than sulfide oxidation (0.5–15 compared to 2–3 nmol per minute and mg protein), indicating that sulfide can be just as limiting as nitrate and has to be taken up rapidly when available (Otte et al. 1999; Høgslund et al. 2009).

"*Candidatus* Maribeggiatoa"

Members of the genus-level candidate taxon "Maribeggiatoa" are large, vacuolated, nitrate-accumulating sulfide-oxidizing filamentous bacteria (*Fig. 6.5 a, b*) with autotrophic capabilities; they resemble "*Candidatus* Marithioploca" spp. in vacuolation and nitrate storage capacity (McHatton et al. 1996). Uncultured "Maribeggiatoa" from cold sulfide seeps in the Monterey Canyon (McHatton et al. 1996) and "Maribeggiatoa"-related filaments from the Guaymas Basin hydrothermal sediments (Nelson et al. 1989) showed high RuBisCO activity in the range of 7.5–15 and 5–6 nmol C fixed per minute and mg protein, respectively. Carbon assimilation by the Calvin cycle was consistent with the ^{13}C-isotopic signature of large "*Maribeggiatoa*"-like filaments from cold seep sediments in the Gulf of Mexico (Larkin et al. 1994).

Filaments of "*Candidatus* Maribeggiatoa" accumulate nitrate intracellularly in vacuoles and—as suggested by analogy

to "*Candidatus* Marithioploca"—use nitrate as a respiratory electron acceptor for sulfur oxidation. The "Maribeggiatoa" population at Monterey Canyon showed an intracellular nitrate concentration of ca. 160 mM (McHatton et al. 1996); the Guaymas Basin population of "Maribeggiatoa"-like mat-forming filaments accumulated intracellular nitrate in the range of 50–100 mM (McKay et al. 2012). These concentrations would not be possible with dissolved oxygen; its saturation concentration in fully aerated seawater at 1 atm pressure and 7 °C is limited to ca. 300 μM. In comparison to other Beggiatoaceae, the "Maribeggiatoa" population at Monterey Canyon showed the highest level of nitrate reductase activity. Nitrate reductase activity was predominantly found in the particulate fraction, indicating a membrane-bound location within the respiratory chain (McHatton et al. 1996). The Monterey Canyon "Maribeggiatoa" also consume oxygen at a rate considerably greater than the average rate of nitrate consumption (8–25 nmol O_2 compared ca. 1–4 nmol NO_3^- per minute and mg protein) (Kalanetra and Nelson 2010).

Nitrate transformations in mats of "Maribeggiatoa" and related *Beggiatoaceae* provide new avenues for biochemical research. The nearly complete genome sequence of a single orange filament (related to "*Candidatus* Maribeggiatoa," pending more detailed classification) from a hydrothermal microbial mat in Guaymas Basin (Gulf of California, Mexico) harbored the gene encoding an abundant soluble orange-pigmented protein in Guaymas Basin mat samples (MacGregor et al. 2013a). The predicted protein sequence grouped with octaheme cytochromes whose few characterized representatives are hydroxylamine or hydrazine oxidases. The protein was partially purified and shown by in vitro assays to have hydroxylamine oxidase, hydrazine oxidase, and nitrite reductase activities. In the context of *Beggiatoaceae* physiology, nitrite reduction was inferred as the most likely in vivo role of the octaheme protein (MacGregor et al. 2013a). The surficial sediments associated with the *Beggiatoaceae* mats in Guaymas Basin showed high denitrification activities that were inhibited by sulfide accumulation; the working hypothesis can be inferred that sulfide removal by *Beggiatoaceae* may catalyze denitrification in the Guaymas Basin sediments (Bowles et al. 2012).

Detailed studies of sulfide and sulfur oxidation pathways and activities are currently lacking for "Maribeggiatoa." However, their conspicuous content of sulfur globules, as in "Marithioploca," suggests that sulfide serves as energy source and elemental sulfur as a storage compound and electron donor when sulfide is not available. In "Maribeggiatoa"-related large, orange-colored *Beggiatoaceae* from the Guaymas Basin (30 μm 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). The near-complete genome of an orange-colored filament in this size class from Guaymas Basin showed a wide repertoire of sulfur oxidation and assimilation pathways (MacGregor et al. 2013c).

"*Candidatus* Marithrix"

The physiology of "*Candidatus* Marithrix" presents a conundrum: the large filaments contain sulfur globules and large vacuoles, but so far nitrate could not be detected in the filaments (Kalanetra et al. 2004; Kalanetra and Nelson 2010). Given that "Marithrix" grows on surfaces that are alternately exposed to mixed sulfidic vent fluids and oxygenated seawater, oxygen is the most likely electron acceptor (Kalanetra et al. 2004). Since the vacuoles are not used for nitrate storage, they could serve either as oxygen reservoirs that provide storage capacity for a few minutes of oxic respiration (Kalanetra and Nelson 2010) or as structural element contributing to filament strength. "Marithrix" has autotrophic capability (average 2.5 nmol CO_2 fixed per min and mg protein), similar to "Maribeggiatoa" and "Marithioploca" (Kalanetra and Nelson 2010).

"*Candidatus* Isobeggiatoa"

Filaments of the genus-level candidate taxon "Isobeggiatoa" are vacuolated and accumulate nitrate as well as sulfur intracellularly (Mussmann et al. 2003; Jørgensen et al. 2010). Instead of forming conspicuous microbial mats on the sediment surface, "*Candidatus* Isobeggiatoa" strains occur within surficial sediment layers where oxygen and sulfide are excluded or occur only at low concentrations. Although these sediment layers often show high sulfate reduction rates, porewater sulfide is rapidly consumed by sulfide-oxidizing bacteria or by reoxidation with metals and does not build up. In this habitat, "Isobeggiatoa" filaments do not dominate in terms of sulfide-oxidizing activity or cell number, although they constitute a larger proportion of sedimentary biomass due to large cell size (Jørgensen et al. 2010). A partial genome for an "Isobeggiatoa" filament matches the sulfide-oxidizing, nitrate-reducing physiology that can be inferred from the habitat characteristics (Mussmann et al. 2007). The partial genome contains a sulfide quinone oxidoreductase and flavocytochrome c-sulfide dehydrogenase for sulfide oxidation, the reverse dissimilatory sulfate reductase pathway for sulfur oxidation, and a partial sox pathway for thiosulfate oxidation (Mussmann et al. 2007). Sulfur respiration is also supported by the presence of genes for the respiration of dimethyl sulfoxide and the reduction of thiosulfate (Mussmann et al. 2007). The genome has a partial nitrate reduction pathway, but the preferred end product of nitrate reduction (N_2 or NH_3) cannot be inferred. Two cytochrome c oxidases that most likely differ by oxygen affinity indicate the capability for aerobic respiration (Mussmann et al. 2007). The "Isobeggiatoa" genome combines a nearly complete tricarbonic acid cycle with some key genes of the Calvin cycle, including form I RubisCO. "*Candidatus* Isobeggiatoa" has genes for glycolate oxidation, for the synthesis of the storage compound poly-beta-hydroxybutyric acid, for glycogen synthesis, and for ATP synthesis through substrate-level phosphorylation by fermenting

pyruvate to lactate (Mussmann et al. 2007). The genome indicates a major role for phosphate uptake and storage; it encodes a phytase for accessing inorganic phosphates, selective porins and ABC phosphate transporters for phosphate uptake, and a polyphosphate kinase for intracellular polyphosphate synthesis (Mussmann et al. 2007). Non-ribosomal peptide synthetases and polyketide synthetases indicate the potential for secondary metabolite synthesis. Numerous glycoproteins in "*Candidatus* Isobeggiatoa" are most likely involved in cell adhesion and aggregation. These two gene categories have often cyanobacterial affinities, suggesting horizontal gene transfer between *Cyanobacteria* and *Beggiatoaceae* in shared sedimentary and microbial mat habitats during long periods of coexistence in the earth's past (Mussmann et al. 2007).

"*Candidatus* Parabeggiatoa"

Members of "*Candidatus* Parabeggiatoa" were originally found in surficial sediments of a brackish fjord, Limfjorden, in Denmark, and formed a monophyletic cluster of large (33–40 µm filament diameter), vacuolated, presumably nitrate-accumulating filaments (Mussmann et al. 2003). "Parabeggiatoa" and "Isobeggiatoa" were identified during a reexamination of previously discovered *Beggiatoa*-like filaments within surficial sediments of Limfjorden (Jørgensen 1977). These filaments did not occur in the narrow zone of overlapping oxygen and sulfide gradients at the sediment surface, but in the surficial sediment interval where oxygen was no longer available but sulfide did not visibly accumulate. In retrospect, this was the first indication of the nitrate-reducing and potentially sulfur-reducing mode of metabolism that sustains these bacteria in their anoxic habitat. The filaments of "Parabeggiatoa" were larger than those of their sister group "Isobeggiatoa" (33–40 µm vs. 9–17 µm filament diameter, respectively) and occurred preferentially deeper in the sediment in close proximity to the sulfidic zone (Mussmann et al. 2003), possibly due to a greater reservoir of intracellular electron acceptors, nitrate, and elemental sulfur. At present, nitrate and sulfur content of the Limfjorden "Parabeggiatoa" are incompletely reported; the smaller "Isobeggiatoa" filaments from Limfjorden contain 156 ± 71 mM nitrate, but the nitrate content of the larger "Parabeggiatoa" filaments is unspecified. The elemental internal sulfur concentration of all measured Limfjorden filaments was 369 ± 176 mM, indicating large sulfur storage capacity in a similar range as nitrate concentrations (Mussmann et al. 2003). A single filament from Eckernförde Bay in Germany (Filament SS) was used for whole genome amplification and subsequent Sanger sequencing, yielding a low-coverage (3x) partial genome assembly of 1,091 contigs with a total sequencing length of 1.3 Mb (Mussmann et al. 2007). The"Parabeggiatoa" partial genome is more incomplete and fragmented than the "Isobeggiatoa" partial genome that was reported in the same study. Both partial genomes contain genes for the reverse dissimilatory sulfate reductase pathway, for putative nitrate reductases, and for genes that channel acetate into general metabolism (acetate/cation symporters, acetate kinase, and acetyl-CoA (coenzyme A) synthase) (Mussmann et al. 2007).

"*Candidatus* Allobeggiatoa"

Thin, sulfur- and nitrate-accumulating vacuolated filaments with diameters of 6–14 µm from hypersaline cyanobacterial mats (*Fig. 6.2e*) constitute the candidate genus "*Candidatus* Allobeggiatoa." So far, "Allobeggiatoa" has been observed and identified by 16S rRNA sequencing and FISH in hypersaline cyanobacterial mats in saline lagoons and salterns in Spain and Mexico (Hinck et al. 2007, 2011). Given the preference of this group for hypersaline conditions, previous observations of *Beggiatoa*-like filaments in the hypersaline cyanobacterial mat of Guerrero Negro, Mexico, most likely include populations of "Allobeggiatoa" (Garcia-Pichel et al. 1994; Jørgensen and DesMarais 1986). Within hypersaline cyanobacterial mats, "Allobeggiatoa" occur predominantly at the upper sulfide horizon where oxygen and sulfide meet during daytime photosynthetic activity; they remain almost stationary at night and do not follow the retreating oxygen gradients towards the mat surface (Hinck et al. 2007). Intracellular sulfur and nitrate concentrations are highly variable. In sulfide-gradient enrichment culture, where they could be sustained for several weeks, "Allobeggiatoa" grow at the sulfide–oxygen interface where they most likely respire with oxygen; under these conditions they accumulate nitrate to high concentrations (430–650 mM) and deplete their intracellular sulfur store (6–25 mM). In their cyanobacterial mat habitat, intracellular nitrate concentrations are depleted (4 mM), and elemental sulfur accumulates (250 mM); thus, "Allobeggiatoa" appears to be nitrate-limited in its natural habitat (Hinck et al. 2011).

"*Candidatus* Halobeggiatoa"

The preliminary phenotypic characterization of this filamentous group is based on a habitat study of "Halobeggiatoa" from white mat patches at the Håkon Mosby mud volcano at 1,260 m depth in the Barents Sea (*Fig. 6.5c*, Lichtschlag et al. 2010; Grünke et al. 2012). These filamentous sulfur oxidizers grow in an intermediate zone of the mud volcano where the sulfide supply is high enough to support growth, but not to exceed the available oxygen and nitrate supply. Sulfide is sequentially oxidized with elemental sulfur as intermediate. The filaments have a diameter of approx. 8–10 µm and accumulate nitrate intracellularly (average 110 mM, range 73–149 mM) in the same range as intracellular S° (average 120 mM, range 45–289 mM) (Lichtschlag et al. 2010). The cells of this population disintegrate at temperatures higher than 8 °C and therefore represent genuine psychrophiles that are adapted to the permanently cold (−0.7 °C) in situ temperature in their habitat (Grünke et al. 2012).

"*Candidatus Thiopilula*"

The candidate genus "*Thiopilula*" was described from free-living specimens in Namibian sediments and from specimens attached to solid substrates in sediments of the Costa Rica margin (Salman et al. 2011). These large, spherical cells (25–67 µm diameter) occur mostly in aggregates within a thick mucus envelope; smaller individual cells (11–24 µm diameter) of the same phylogenetic lineage reside in diatom frustules (Salman et al. 2011). Similar-sized cells in mucus-ensheathed clusters were also documented earlier from Namibian sediment samples (Schulz 2006). The cells contain sulfur inclusions. Most cells are vacuolated and could store nitrate, but intracellular nitrate concentrations have not been tested yet. The attached cells are most likely dividing by budding from mother cells attached to solid substrates (Bailey et al. 2011). Cells within aggregates and diatom frustules show binary division stages and occasional motility by jerking, rolling movement (Salman et al. 2011).

"*Candidatus* Thiophysa"

The candidate genus "*Candidatus* Thiophysa" is physiologically almost entirely uncharacterized. These large, single, spherical cells (56–90 µm diameter) from Namibian sediments contain sulfur inclusions and also vacuoles that could store nitrate, but intracellular nitrate concentrations remain to be tested (Salman et al. 2011). They are motile by slow, rolling, and jerking motions (Salman et al. 2011). The genus was originally described from sandy sediments of shallow marine sulfur springs in the Gulf of Naples, strongly smelling of hydrogen sulfide (Hinze 1903). These specimens lost their sulfur inclusions during 1 or 2 days of incubation in oxygenated seawater, consistent with a sulfur-oxidizing metabolism (Hinze 1903). The Namibian and Neapolitan specimens resemble each other in morphology; the original, beautifully detailed microscopic drawings (Hinze 1903) are close equivalents of the modern microphotographs (Salman et al. 2011). Yet, the cell diameter of the Neapolitan "Thiophysa" is given as 7–18 µm (Hinze 1903), approx. 1/5 to 1/8 of the Namibian specimens. This difference suggests unexplored morphotype and species diversity either within the candidate genus "*Thiophysa*" or other taxa of the family *Beggiatoaceae*.

Cell Structure

Vacuolation

The cells of many large members of the *Beggiatoaceae* appear hollow in microscopic examination; they are composed of a thin cylinder of cytoplasm surrounding a large central vacuole, or several large vacuoles, a characteristic that has been initially observed and described more than a century ago (Hinze 1901, ❯ *Figs. 6.4d* and ❯ *6.8a*, *b*). This extensive vacuolation is characteristic for most large *Beggiatoaceae* (Jannasch et al. 1989; Nelson et al. 1989) above a size threshold of approx. 10 µm (Larkin and Henk 1996). Since vacuolation commonly coincides with high intracellular nitrate concentration (McHatton et al. 1996), the vacuoles are presumed to be the locations where nitrate is accumulated for respiration, either by denitrification or by dissimilatory reduction to ammonia. However, important caveats apply: the small, freshwater species *Thioploca ingrica* lacks the extensive vacuolation of large, marine *Beggiatoaceae* (Maier and Murray 1965), but is still capable of nitrate accumulation (Høgslund et al. 2009). Analyses of "Candidatus Marithrix" demonstrate that large vacuoles cannot be equated with nitrate accumulation (Kalanetra et al. 2004).

The large intracellular vacuoles have been studied in greater physiological detail in "Allobeggiatoa," using a combination of vacuolar pH measurements (Beutler et al. 2009), immunostaining, and selective inhibition of membrane proteins (Beutler et al. 2012). Nitrate addition increased the vacuolar proton motive force and acidified the vacuole; the resulting proton gradient from vacuole interior to cytoplasm can be used for ATP and pyrophosphate generation in the cytoplasm. Nitrate addition also led to the production of nitric oxide (NO) from its precursor nitrite in the vacuole (Beutler et al. 2012); subsequently, NO could be reduced to the denitrification intermediate nitrous oxide (N_2O).

Cell Envelope

Ultrastructural analyses of cell envelope and cell inclusions have focused on freshwater *Beggiatoa alba* (Strohl et al. 1982; Strohl and Larkin 1978b), on *Thioploca ingrica* (Maier and Murray 1965), and on marine filamentous *Beggiatoaceae* (de Albuquerque et al. 2010; Larkin and Henk 1996). Thin sectioning and freeze-etching techniques have shown that filaments of the freshwater species *Beggiatoa alba* (strain B15LD) have a cell envelope consisting of five distinct layers that is continuous over the entire filament (❯ *Fig. 6.8c*); the individual cells within each filament are separated only by their cytoplasmic membranes plus a septum that is contiguous with the inner layer of the envelope (Strohl et al. 1982). In other freshwater *Beggiatoa* strains, similar multilayer cell envelops and single-layer cell septa have been observed, in lower resolution due to technical limitations (Morita and Stave 1963; Drawert and Metzner-Küstner 1958). Recently, marine *Beggiatoa* filaments (non-vacuolated, average diameter 4.4 µm, related to the marine autotrophic strain MS-81-6) were shown to have a five-layer envelope and a single-layer cell septum (presumably murein) that is contiguous with the inner layer of the envelope (de Albuquerque et al. 2010). Large, vacuolated marine *Beggiatoaceae* (size not explicitly specified, but most likely 10–30 µm filament diameter) showed a similarly complex cell envelope composed of four distinct layers (de Albuquerque et al. 2010) (❯ *Fig. 6.8d*, *e*).

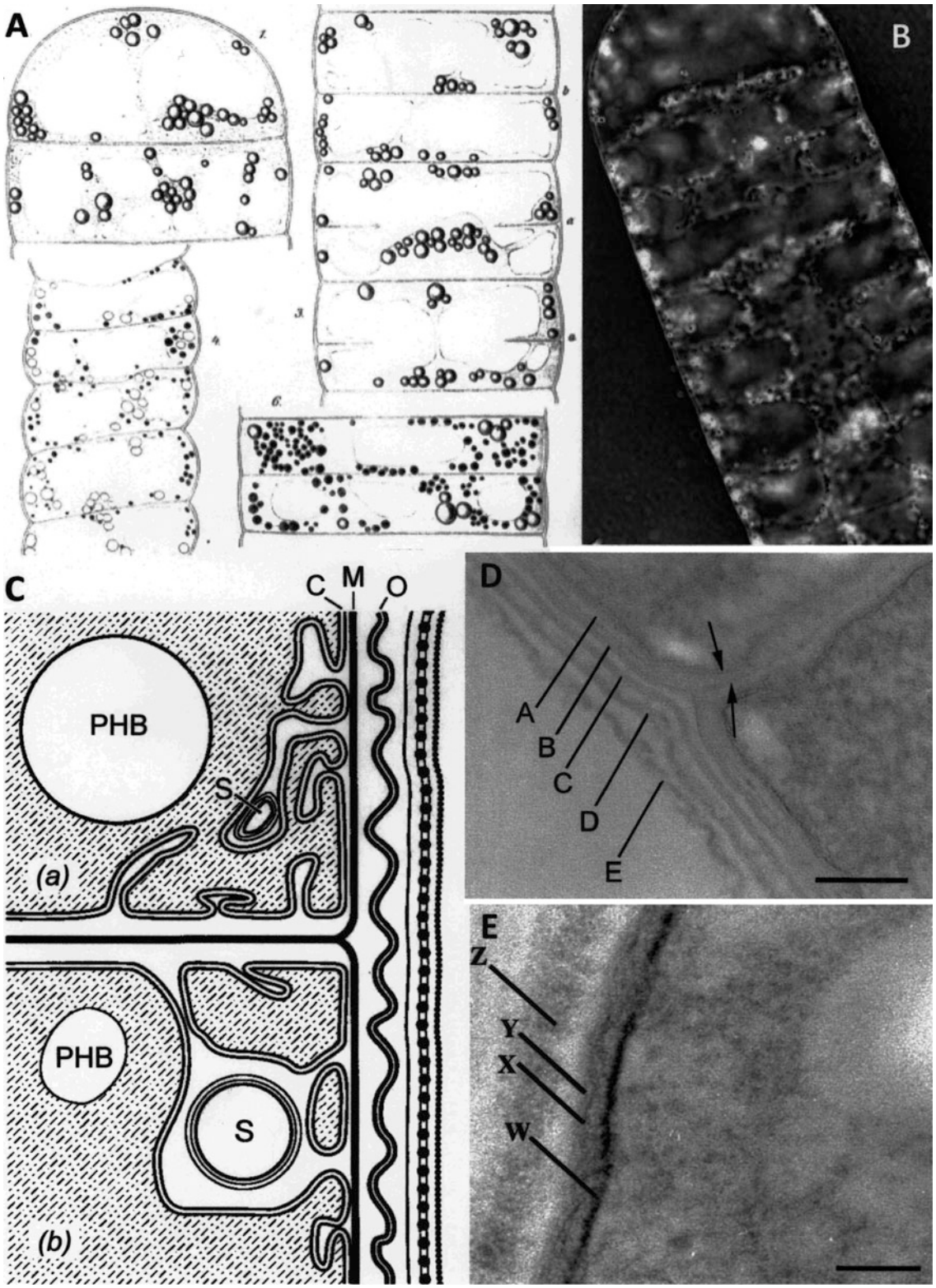

Fig. 6.8

***Cell structure of Beggiatoaceae*.** (**a**) Drawings from the original publication on the structure of large *Beggiatoaceae* filaments (Hinze 1901) show the extensive vacuoles that take up most of the cell volume, the sulfur globules embedded into the cytoplasm (drawn as three-dimensionally shaded globules), empty membranes after dissolution of sulfur globules, and small carbohydrate inclusions (in *black*), all based on microscopic observation of large *Beggiatoaceae* filaments (ca. 45 μm diameter) collected in Kiel Harbor. (**b**) Microphotograph of a large vacuolated *Beggiatoaceae* filament collected from the Chilean continental shelf shows the close

Surrounding the cell envelope and embedding the thin marine filaments, a sheath-like outer layer of fibrillar or striated material was observed (de Albuquerque et al. 2010). Large marine filaments show small round pores, ca. 15 nm in diameter, arranged in rows on the outer surface of a filament; a possible role in mucus secretion was suggested (Larkin and Henk 1996). Linearly arranged longitudinal fibrils have been observed on the cell envelope surface layer of *Beggiatoa alba*; most likely, they expel mucus forming a trail of mucilage around the filaments, inside which they can glide (Strohl et al. 1982). In *Beggiatoa alba*, the mucilage trail is composed of neutral polysaccharides (mannose and glucose) (Larkin and Strohl 1983). Earlier electron microscopic observations of conspicuously striated sheath material in freshwater *Beggiatoa* spp. (Drawert and Metzner-Küstner 1958) could correspond to such an outer cell envelope layer of longitudinal fibrils, or to the mucilaginous coating itself. Mucilaginous coats surrounding individual *Beggiatoa* are significant for filament movement (Møller et al. 1985); they could be a direct homolog for the larger sheaths surrounding *Thioploca* and "Marithioploca" filament bundles. Consistent with such a derivation, these sheaths appear to have a striated texture that runs parallel to the filaments; epibiotic filamentous bacteria on and within the sheath matrix tend to be aligned parallel to the sheath striation and the "Marithioploca" filaments (Fukui et al. 1999; Muyzer and Ramsing 1995) (*Fig. 6.3f*).

A cell envelope similar to *Beggiatoa* spp. has been observed in an electron microscopic ultrastructure survey of *Thioploca ingrica* filaments (Maier and Murray 1965). The envelope surrounds the entire filament, including the filament tips, and consists of multiple distinct layers. Adjacent to the cytoplasmic membrane, an electron-dense inner layer is contiguous with the cell septa and then follows a complex, multilayered "quadruple profile" and on the outside two distinct outer envelope layers (Maier and Murray 1965). A similar cell envelope is found in the Chilean "Marithioploca" filaments (Maier et al. 1990). The inner layer, which is continuous with cell septa, is followed by interstitial material and the adjacent undulating "triple layer"; after a gap, two outer layers are completing the cell envelope. On the inside of the cell envelope, periplasmic spaces appear in some locations, followed by the cytoplasmic membrane. Incomplete cell septa branch off from the cell envelope inner layer and extend into the cytoplasm and the ventral vacuole. However, they do not close off a cell, but they remain surrounded by the cytoplasmic membrane and the vacuolar membrane (Maier et al. 1990). The incomplete septa seen in various lengths suggest a mode of cell division where growing septa bisect a cell and its central vacuole. Interestingly, the early study by Hinze (1901) records a similar mechanism of cell division in microscopic drawings of large *Beggiatoaceae*.

Cell Inclusions

Four types of inclusions have been reported for members of the *Beggiatoaceae*: polyhydroxyalkanoates (PHA) (Pringsheim 1964; Pringsheim and Wiessner 1963; Strohl and Larkin 1978a; Strohl et al. 1982; Schwedt et al. 2012), glycogen or a similar polyglucose (Schulz and Schulz 2005), polyphosphate (Maier and Murray 1965; Schulz and Schulz 2005; Brock et al. 2012), and sulfur (Strohl et al. 1981b, 1982; Winogradsky 1887).

PHA Inclusions

Production of PHA appears to be a universal feature of heterotrophic freshwater strains (*Fig. 6.2c*). Interestingly, in heterotrophic *Beggiatoa* spp. the deposition of the alkanoate polyhydroxybutyrate seems to correlate primarily with high aeration (Pringsheim 1964), and it can account for up to 50 % of total dry weight under aerated conditions in the absence of sulfide (Güde et al. 1981). Numerous cell inclusions that resemble PHA were also found by TEM in the freshwater and brackish-water species *Thioploca ingrica* (Kojima et al. 2003). In the marine *Beggiatoaceae* strain 35Flor, PHA is used up under prolonged anoxia, most likely by oxidation using intracellular sulfur as electron acceptor (Schwedt et al. 2012).

Sulfur Inclusions

The sulfur inclusions of *Beggiatoa* are periplasmic in location, being enclosed in invaginations of the cell membrane. The sulfur globules are surrounded by their own electron-dense monolayered envelope, followed by the cytoplasmic membrane (de Albuquerque 2010). In large, vacuolated filaments, the sulfur inclusions are located in the narrow cytoplasmic space between

Fig. 6.8 (continued) correspondence between the drawings by Hinze and modern observations (Photo by Jan Küver, Institute for Materials Testing, Bremen). The positive photo slide was scanned as a negative slide for improved contrast. (c) Ultrastructure drawing of *Beggiatoa alba* strain B15LD (ATCC#33554). Symbols: C, cell membrane; M, presumed murein layer; O, presumed outer membrane layer; S, globule of S°; PHB, poly-β-hydroxybutyrate granule. (1) Note large PHB inclusion and rudimentary S° globule typical of cells grown in acetate-supplemented mineral medium. (2) Note small PHB inclusion and large S° globule typical of cell grown in the presence of sulfide or thiosulfate and a low concentration of acetate (Figure adapted from Strohl et al. 1982). (d) Transmission electron micrograph of cell surface envelope with five layers from a small non-vacuolated filament of marine *Beggiatoaceae*; the two small arrows indicate the septum that separates two cells within the same filament. (e) Transmission electron micrograph of cell envelope with four layers from a large marine vacuolated filament. Both filaments were collected from coastal lagoons in Brazil (Figure adapted from de Albuquerque et al. 2010)

the cell membrane and the large central vacuole (Jannasch et al. 1989; Larkin and Henk 1996; de Albuquerque et al. 2010). Very similar observations were made for the Chilean "Marithioploca" species; a thin cytoplasmic layer containing membrane-enclosed sulfur globules surrounds the central vacuole (Maier et al. 1990). The vacuole is surrounded by its own vacuolar membrane in addition to the cytoplasmic membrane.

In *Beggiatoa alba* strain B15LD, the sulfur globules are enclosed within a multilayered sulfur inclusion envelope of 12–14 nm thickness (Strohl et al. 1982), while in other strains the S° globule envelope appears to be composed of a single protein layer 4–5 nm thick (Strohl et al. 1981b). The extraction of S° globules with solvents such as pyridine and their refractile appearance when intact cells are viewed with phase contrast microscopy have proven very useful in confirming their presence (Skerman et al. 1957). *Beggiatoa* cells grown in the absence of reduced sulfur compounds apparently contained small, "rudimentary" S° inclusion envelopes (Strohl et al. 1982). Dehydration solvents (e.g., ethanol) that are necessary for preparation of electron microscopy dissolve the S° and complicate the determination whether the rudimentary inclusions completely lack elemental sulfur. The sulfur globules consist of fine-grained, microcrystalline elemental sulfur in the common, stable S_8 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.

Polyphosphate Inclusions

Cells of *Thiomargarita namibiensis* accumulate phosphate intracellularly and store it as polyphosphate granules (Schulz and Schulz 2005). Polyphosphate accumulation and phosphate release by *Thiomargarita* lead to the precipitation and accumulation of phosphate-rich minerals (Schulz and Schulz 2005). These observations link microbially catalyzed precipitation of authigenic phosphate minerals in marine sediments to the in situ activities of sulfur-oxidizing bacteria and sulfate-reducing bacteria (Arning et al. 2009; Williams and Reimers 1983) and realize earlier suggestions made after the discovery of the Chilean Marithioploca mats (Gallardo 1977b). Although it is very likely that these granules provide an energy buffer for *Thiomargarita* cells, the environmental or biogeochemical triggers for polyphosphate accumulation and degradation in *Thiomargarita* remain to be identified.

In addition to *Thiomargarita*, polyphosphate inclusions have been documented for different filamentous *Beggiatoaceae* (Maier and Murray 1965; de Albuquerque et al. 2010; Brock and Schulz-Vogt 2011); they appear to be absent from "Marithioploca," at least at the time of sampling (Holmkvist et al. 2010). Experiments with *Beggiatoa* strain 35Flor—a strain that accumulates phosphate intracellularly as large inclusions surrounded by lipids, most likely a membrane (Brock et al. 2012)—have shown that phosphate is released in response to increasing sulfide concentrations; acetate additions have a similar effect on *Thiomargarita* but most likely act by stimulation of sulfate reduction and concomitant sulfide production in the sediment (Brock and Schulz-Vogt 2011).

Isolation, Enrichment, and Maintenance Procedures

Enrichments from Natural Environments

Natural enrichments of *Beggiatoaceae* can be transplanted into the laboratory for observation and community succession studies; a highly reducing *Beggiatoa*-rich sediment can be maintained in an aquarium for continued observation. For example, the development of a *Beggiatoa* mat in the laboratory unfolded over 10 days and revealed a succession of different *Beggiatoa* size classes (Bernard and Fenchel 1995).

Similar aquarium setups can be used for selective enrichments (Nelson 1992). The bottom of a shallow pan or aquarium (approximately 30 × 30 × 12 cm) is covered with a few centimeters of sand; seaweed or shredded paper is added as a source of complex organic polymers, plus approx. 20 g of $CaSO_4$ and a few grams of K_2HPO_4, followed by several centimeters of sulfide-rich marine mud and sufficient seawater to overlay the entire enrichment by about 1–2 cm. Subsequent dark incubation 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 airstone. 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 (10 h:14 h) provided viable tufts of *Beggiatoa* spp. for almost 1 year (Nelson and Castenholz 1982). Sewage treatment plants are also an excellent source of enrichment material (Burton and Lee 1978; Williams and Unz 1985).

Enrichment in extracted hay medium (Cataldi 1940) provides a useful strategy for enriching *Beggiatoaceae*, and several modifications of this method have been employed successfully for enriching freshwater *Beggiatoa* strains from decaying plant material and aquatic sediment (Faust and Wolfe 1961; Joshi and Hollis 1976; Saravanakumar et al. 2012). These materials provide good inocula even when conspicuous mats are absent; as filaments break at necridia or "sacrifical cells," trichomes as short as 3–10 μm are produced and widely dispersed (Pringsheim 1964; Strohl and Larkin 1978b; Kamp et al. 2008). Enriched tufts of *Beggiatoa* filaments then provide start material

for single filament isolation by excising individual filaments after transfer of tufts on agar plates (Faust and Wolfe 1961).

Liquid Media

Liquid media can be used for enrichment, MPN enumeration, and bulk cultivation of *Beggiatoa*. 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* B18LD, and related strains, are generally grown in liquid 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).

In an extensive study, Strohl and Larkin (1978a) have tested several liquid media formulations for isolation and MPN enumeration of heterotrophic *Beggiatoa* filaments from organic-rich freshwater ditches and lakes. A soil extract amended with 0.05 % (w/v) acetate, 15–35 U catalase per mL, and 1 % (w/v) hay extract yielded the best results. Following a recently published version of this protocol (Saravanakumar et al. 2012), hay is extracted by boiling in tap water for about 30 min and the water is decanted. Repeated boiling and decanting is carried out at least five times with cold tap water rinses between each boiling/decanting step. The extracted hay is left in water overnight and then decanted and dried at room temperature for 2 days. Approximately 1 g of dried hay is added to 100 mL of artificial seawater in a 250-mL Erlenmeyer flask and autoclaved. Filter-sterilized catalase is added to the medium at a final concentration of 35 U/mL. The medium is subsequently inoculated with 1–2 g of sediment sample. After 1–2 weeks incubation at 28 °C in the dark, the enrichments are checked for the presence of whitish threadlike mats and tufts, and these are examined microscopically for the presence of typical *Beggiatoa* filaments. The tufts from enrichment cultures are washed twice with sterile 0.01 % sodium azide solution (Strohl and Larkin 1978a) prepared in artificial seawater, followed by two washes and a 5-min soak in filtered artificial seawater containing catalase (35 U/mL). Washed filaments are then used for pure culture isolation procedures using heterotrophic media (Burton and Lee 1978; Strohl and Larkin 1978a) or sulfide gradient cultures (Nelson and Jannasch 1983).

Isolations on Agar Plates

Agar plate enrichments have commonly led to the isolation of heterotrophic *Beggiatoa* strains, but can be adjusted for autotrophic enrichments by minimizing or removing the organic carbon source in the agar medium. Tufts of *Beggiatoa* filaments are collected from the environment or an enrichment, washed in a sterile washing solution, and placed on an agar plate that contains dilute organic substrates, such as small amounts (1–0.25 ‰ w/v) of peptone or yeast extract or 0.5 mM acetate. 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); individual filaments can also be pulled away from the inoculum with a suitable micromanipulation needle or finely pointed watchmaker's forceps (Nelson 1992). The surface of the agar plates should be dry and free of condensation water droplets.

To enrich and isolate heterotrophic freshwater *Beggiatoa*, the representative DTA medium (Nelson 1992) for agar plates is prepared as follows. The pH is adjusted to 7.0 prior to autoclaving.

ND stock solution	50 mL
$(NH_4)_2SO_4$	0.13 g
Sodium acetate	0.68 g (may be reduced)
K_2HPO_4	0.027 g
$Na_2S_2O_3 \times 5H_2O$	0.50 g
$CaCl_2$	0.10 g
Distilled water	950 mL
Agar	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/L) 20 mL
$CaSO_4 \times 2H_2O$	1.2 g
$MgSO_4 \times 7H_2O$	2.0 g
NaCl	0.16 g
Na_2HPO_4	1.4 g
KH_2PO_4	0.72 g
Micronutrient solution	
Distilled water	1,000 mL
H_2SO_4 (concentrated)	0.5 mL
$MnSO_4 \times H_2O$	2.28 g
$ZnSO_4 \times 7H_2O$	0.50 g
H_3BO_3	0.50 g
$CuSO_4 \times 5H_2O$	0.025 g
$Na_2MoO_4 \times 2H_2O$	0.025 g
$CoCl_2 \times 6H_2O$	0.045 g

By using defined mineral media and reducing the organic carbon content of the agar medium (Nelson and Castenholz 1981b), heterotrophic contaminants are selected against, and the enrichment and isolation of autotrophic *Beggiatoa* is favored. Agar plates made with filtered seawater, trace elements, and 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. 1982). The marine basal medium (J3) without carbon substrate amendments can be

used for agar plates selecting for autotrophic *Beggiatoa* spp. (Nelson 1992) and is prepared starting with three solutions that are separately autoclaved in Erlenmeyer flasks.

Solution 1:
Aged natural seawater (salinity 3.2–3.5 %), 500 mL
Prefiltered (Whatman #1 or Gelman GF/F) and filtered (0.45 µm)

Solution 2 (in larger flask):	
Distilled water	200 mL
Agar	9.0 g
Solution 3:	
NH_4NO_3	0.06 g
Trace element solution SL8	0.75 mL
Mineral stock	50 mL

The aged natural seawater can be replaced by artificial seawater, containing per liter NaCl, 27.5 g; $MgCl_2 \times 6H_2O$; $MgSO_4 \times 7H_2O$, 4.1 g; $CaCl_2 \times 2H_2O$, 0.66 g; and KCl, 1.02 g (Kamp et al. 2008).

The trace element solution SL8 (Pfennig and Biebl 1981) contains per liter Na_2EDTA, 5.2 g; $FeCl_2 \times 4H_2O$, 1.5 g; $ZnCl_2$, 0.070 g; $MnCl_2 \times 4H_2O$, 0.100 g; H_3BO_3, 0.062 g; $CoCl_2 \times 6H_2O$, 0.19 g; $CuCl_2 \times 2H_20$, 0.017 g; $NiCl_2 \times 6H_2O$, 0.024 g; and $Na_2MoO_4 \times 2H_2O$, 0.036 g.

The mineral stock contains per liter K_2HPO_4, 0.52 g; Na_2MoO_4, 0.05 g; $FeCl_3 \times 6H_2O$, 0.29 g; $Na_2S_2O_5$ (sodium pyrosulfite), 0.75 g; and phenol red, 10 mL of a sterile 0.5 % solution (Gibco) (Nelson 1992).

After cooling to 50 °C, the autoclaved solutions are aseptically combined in the solution 2 flask (volume > 750 mL) and supplemented with 0.2 mL of Va vitamin solution, which contains (in mg per liter) B_{12}, 1; thiamine, 200; biotin, 1; folic acid, 1; para-aminobenzoic acid, 10; nicotinic acid, 100; inositol, 1; and calcium pantothenate, 100.

J3 basal medium is amended to produce an isolation medium (J-TS) by adding the following three sterile stocks, with final concentrations in parentheses (Nelson 1992): (1) 7.5 mL of 200 mM $Na_2S_2O_3$ (2 mM) and (2) 3.75 mL of freshly neutralized 200 mM Na_2S (1 mM). The Na_2S stock is autoclaved as a basic solution and then neutralized with an equimolar quantity of sterile HCl just prior to use. The Na_2S stock solution is kept for approx. a month during oxic storage, unless it is stored under N_2 gas. (3) 15 mL of 1 M $NaHCO_3$ (20 mM). To make this stock, autoclave 8.4 g of $NaHCO_3$ (dry), and add 100 mL sterile water when cool. The medium is buffered by the bicarbonate in conjunction with the atmospheric CO_2.

Immediately after solidification, plates are incubated in a bell jar for 24 h or more under anoxic conditions (99.5 % N_2, 0.5 % CO_2), with desiccant present to absorb water evaporating from the surface of the plates. After inoculation with a tuft of *Beggiatoa* spp., plates are placed in a microoxic atmosphere (0.5 % CO_2; 0.2 % O_2; balance N_2). The medium and the bacteria tolerate temporary air exposure during inoculation or single-filament isolation (Nelson 1992).

Methods for isolating marine *Beggiatoa* strains on agar under microoxic conditions should in principle work for freshwater *Beggiatoa* as well. While freshwater strains were typically isolated on a variety of media equilibrated with full air (Nelson and Castenholz 1981b; Strohl and Larkin 1978a; Williams and Unz 1985), microoxic conditions may be required on occasion: the cultivation of a freshwater *Beggiatoa* strain in liquid mineral medium on thiosulfate and HCO_3^- required dissolved oxygen concentrations in the range of 3–16 µM (0.1–0.5 mg O_2/L) (Patritskaya et al. 2001).

Isolations and Cultivation in Gradient Media

Motile *Beggiatoa* spp. display strong chemotactic movement and adjust their position in gradients of oxygen and sulfide; they form platelike aggregates near the microoxic surface of semisolid agar tubes or—in liquid culture—reticulate networks of filaments surrounding an FeS pellet as sulfide source (Faust and Wolfe 1961). These observations were extended into a cultivation approach based on gradient media, where two layers of agar (a sulfide-rich agar plug at the bottom overlaid with sulfide-free soft agar) containing opposed sulfide and oxygen gradients provide a suitable gradient habitat for maintaining and propagating marine, autotrophic, non-vacuolate *Beggiatoa* spp. (*Fig. 6.6*) (Nelson and Jannasch 1983).

Marine gradient medium JG8 (Nelson 1992) is constructed as follows: first a 4 mL quantity of J3 agar (pH 8.4; the $NaHCO_3$ concentration is lowered to 2.0 mM; thiosulfate may be omitted) supplemented with freshly neutralized Na_2S is solidified in the bottom of a screw-capped tube (Hungate tube, 16 × 150 mm or similar). The bottom agar plug contained initially a sulfide concentration of 8 mM (Nelson and Jannasch 1983), but 3–4 mM sulfide is sufficient (Kamp et al. 2008; Jewell et al. 2008); agar strength can be reduced from 1.5 % to 0.75 % (Kamp et al. 2008). This bottom agar is then overlaid with 8.0 mL of semisolid J3 agar (0.25 % agar; $NaHCO_3$ concentration lowered to 2.0 mM; no sulfide or thiosulfate, but may contain nitrate). 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. 1986a, b). The overlying air headspace reservoir in the tube constitutes an oxygen reservoir. Tubes are loosely capped to permit exchange of headspace gasses with the atmosphere.

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 fluxes; however, molecular diffusion and nonbiological reactions between sulfide and oxygen gradually alter the gradient (Nelson et al. 1986a, b). The sulfide–oxygen interface near the top of the agar column spreads out in the absence of a *Beggiatoa* inoculum and contracts after inoculation. For example, sulfide and oxygen overlapped for 6–7 mm in uninoculated medium during slow, nonbiological sulfide

oxidation (Nelson et al. 1986b), in marked contrast to an overlap of 0.2 mm or less in active *Beggiatoa* cultures where sulfide oxidation proceeded quickly (Nelson 1992). 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 variable thickness. Gliding motility and negative chemotactic responses allow these bacteria to track this interface as it slowly descends due to the gradual depletion of the sulfide reservoir.

The gradient approach is not limited to marine *Beggiatoa*. 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 was supplemented with ammonium nitrate (J2 Medium) (Nelson and Jannasch 1983). For gradient culture of freshwater *Beggiatoaceae* (Kamp et al. 2006), the basal mineral medium is adjusted accordingly (per liter: EDTA, 0.010 g; $CaSO_4 \times 2\ H_2O$, 0.120 g; $MgSO_4 \times 7\ H_2O$, 0.200 g; NaCl, 0.016 g; Na_2HPO_4, 0.140 g; NaH_2PO_4, 0.138 g; $CaCl_2 \times 2\ H_2O$, 0.264 g; $FeCl_3$ solution [0.290 g/L]; 1 mL micronutrient solution). The micronutrient solution contains per liter 0.5 mL H_2SO_4 (>98 %); $MnSO_4 \times H_2O$, 2.28 g; $ZnSO_4 \times 7\ H_2O$, 0.5 g; H_3BO_3, 0.5 g; $CuSO_4 \times 5\ H_2O$, 0.025 g; $Na_2MoO_4 \times 2\ H_2O$, 0.025 g; and $CoCl_2 \times 6\ H_2O$, 0.045 g (Kamp et al. 2006); the vitamin solution remains the same.

Coculture and Obligate Associations

Some *Beggiatoaceae* could only be cultured in association with nonfilamentous bacteria (Kamp et al. 2006). One of these cocultures, an obligate association of the marine *Beggiatoa* strain 35Flor with a single, specific *Pseudovibrio* strain has been studied in more detail (Kamp et al. 2008; Brock and Schulz-Vogt 2011; Schwedt et al. 2012). Strain 35Flor grows only in the presence of an accompanying *Pseudovibrio* sp. strain that can be cultured without the *Beggiatoa* sp., but not vice versa. Since members of the genus *Pseudovibrio* are commonly isolated from marine invertebrates, the 35Flor-associated strain may have originated from the coral from which *Beggiatoa* sp. 35Flor had been isolated (Bondarev et al. 2013). Genome sequencing revealed that the *Pseudovibrio* symbiont has the genomic potential to attach to host cells, to produce secondary metabolites, and to provide the host organism with enzymatic cofactors (Bondarev et al. 2013).

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 freshwater *Thioploca*. Filaments may be maintained in jars overlaid with 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 H_2S development, *Thioploca* bundles are added, and incubation continues for many weeks with intermittent inspection.

Attempts to enrich marine "Marithioploca" spp. have met 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-Vogt, pers. comm.). Physiological studies with harvested "Marithioploca" filaments required careful handling of the filaments, and avoidance of oxygen and air exposure, in order to prevent significant losses in enzymatic activities (Otte et al. 1999). Future cultivation approaches have to take into account the sensitivity of "Marithioploca" to high sulfide concentrations and to oxygen exposure and should 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. can be maintained in sulfide–oxygen gradient media. The smooth oxygen and sulfide gradients coupled with the chemotactic motility of the bacteria, which directs them to the proper microenvironment, make this approach especially attractive for obligately microaerophilic strains (Nelson et al. 1986b). A low concentration of acetate must be provided for the strains that do not show autotrophic capacity. For the typical medium geometry employed (Nelson 1992), transfers to new tubes of gradient medium should be made every 2–3 weeks. Gradient medium should be stored no more than 4–6 weeks; transfers into aged gradient media will not survive as long as those into freshly prepared media (Nelson 1992). 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 microoxic regimes in bell jars is best reserved for initial isolation and any required repurification steps. Cryopreservation of strains has proved problematic (Nelson and Schulz, pers. comm.).

Ecology

The *Beggiatoaceae* are gradient bacteria that occupy an ecological niche at the interface where fluxes of sulfide as electron donor and oxygen or nitrate as electron acceptor meet; different

genera have adapted to this niche in characteristic ways and prefer different types of interface habitats. These ecophysiological strategies of efficient sulfur oxidation are directly reflected in the structure of the mats and in the arrangement and the movements of the filaments and cells within the oxic/anoxic gradient. Thus, physicochemical habitat characteristics are directly linked to occurrence patterns, morphology, and physiology of *Beggiatoaceae* in nature.

Their ecophysiological flexibility allows the *Beggiatoaceae* to colonize a wide spectrum of freshwater and marine environments; *Beggiatoaceae* can be found in a wide range of habitats, including organic-rich, coastal marine sediments (Jørgensen 1977; Klas 1937; Mussmann et al. 2003; Rosenberg and Diaz 1993); benthic microbial mats (Teske and Stahl 2002) salt marshes (Nelson et al. 1982); eutrophic, oxygen-depleted bays (Graco et al. 2001; Vallius 2006); marine oxygen-minimum zones (Schmaljohann et al. 2001); oxygen-depleted marine basins (Williams and Reimers 1983); geothermally active submarine caves (Mattison et al. 1998); hydrothermal vents (Jannasch et al. 1989; Nelson et al. 1989); cold sulfide seeps (Sassen et al. 1994); and hydrocarbon seeps (Larkin et al. 1994). Freshwater habitats include sulfur springs (Uphof 1927; Caldwell et al. 1975; Nelson and Castenholz 1981b; Fukui et al. 1999); freshwater ditches, puddles, wetlands, and lake sediments (Koppe 1924; Pringsheim 1964; Scotten and Stokes 1962; Strohl and Larkin 1978a); terrestrial salt springs (Kolkwitz 1918); and sulfidic cave streams (Macalady et al. 2006, 2008). The cave stream study is of special interest for the ecology of the *Beggiatoaceae* since it outlines the environmental preferences of *Beggiatoa*-like filaments against single-celled sulfur-oxidizing epsilonproteobacteria and filamentous *Thiothrix* spp. that compete for different microhabitats within the same cave ecosystem. The Epsilonproteobacteria dominated extremely oxygen-depleted stagnant water with very little turbulent flow and oxygen in-mixing; the *Thiothrix*-like filaments preferred locations characterized by strong turbulent mixing, higher oxygen availability, and reduced sulfide concentrations, whereas the *Beggiatoa*-like filaments inhabited intermediate habitats over a wide range of oxygen and sulfide concentrations, as long as a sedimentary substrate allowing for mat formation was available (Macalady et al. 2008).

The Oxygen–Sulfide Interface at the Sediment Surface

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. High sulfate reduction rates in surficial sediments maintain high fluxes of sulfide in the range of 10–100 mmol m^{-2} day^{-1} (see literature compilation in Schwedt et al. 2012); sulfide is then oxidized within *Beggiatoa* mats at the sediment–water interface. Oxygen from the overlying water is also consumed within *Beggiatoa* mats and does not penetrate the underlying sulfidic sediment (Jørgensen and Revsbech 1983; Møller et al. 1985; Nelson et al. 1986a; Fenchel and Bernard 1995). The microoxic growth zone of *Beggiatoa* is characterized by oxygen concentrations in the range of 1–2.5 μM (Nelson et al. 1986a). This microoxic niche of *Beggiatoa* is created by the highly dynamic sulfide-oxidizing metabolism of the *Beggiatoa* filaments themselves, but once established, it is remarkably stable (◉ *Fig. 6.6*). Laboratory gradient cultures remain active for several weeks and died only when the sulfide in the bottom agar was exhausted (Nelson et al. 1986a). 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_2O_2 exposure inhibited fumarate hydratase, an essential TCA cycle enzyme, in the heterotrophically growing *Beggiatoa* freshwater strain D-405 (Grabovich et al. 1993).

The Anoxic, Non-sulfidic Surficial Sediment

In coastal, organic-rich marine sediments, *Beggiatoaceae* are often found in the intermediate sediment layer where porewater oxygen and nitrate are already depleted, but sulfide does not yet accumulate—a conspicuous departure from growth in overlapping oxygen–sulfide gradients at the sediment–water interface (Jørgensen 1977; Mussmann et al. 2003; Preisler et al. 2007; Jørgensen et al. 2010). These sediment populations do not form visually conspicuous mats at the sediment surface; their abundance becomes clear only after microscopic counts of the sediment-embedded filaments. Especially "Isobeggiatoa" and "Parabeggiatoa" occur in this habitat; their intracellular storage capacity for nitrate and sulfur is crucial in bridging the gap between the sedimentary porewater pools of electron acceptor and donor (Mussmann et al. 2003; Preisler et al. 2007). Even when they are abundant, these *Beggiatoa*-like filaments contribute only to a minor extent to overall anaerobic sulfide oxidation; precipitation with Fe^{2+} and oxidation with Fe^{3+} dominated sedimentary sulfide oxidation (Preisler et al. 2007). Filaments position themselves in the anoxic, sulfide-free zone by responding chemotactically to porewater concentrations of oxygen and sulfide; filaments in the favored zone reversed course frequently and were gliding shorter distances in randomized directions between reversals, whereas filaments in oxic or sulfidic sediments took more time between reversals and therefore glided longer distances (Dunker et al. 2010).

Hypersaline Cyanobacterial Mats

Beggiatoaceae, including members of the halotolerant Candidatus genus "*Allobeggiatoa*," occur in hypersaline benthic cyanobacterial mats (Hinck et al. 2007, 2011; Dillon et al. 2009). Cyanobacterial mats form a diurnally shifting gradient habitat (Jørgensen 1982). At daytime, the upper layers of cyanobacterial mats are photosynthetically active and become supersaturated with oxygen. At night, oxygen production ceases, and sulfide produced by sulfate reduction moves up towards the mat surface (Jørgensen et al. 1979; Jørgensen and Revsbech 1983). If this interface remains outside of the photic zone during the day, photosynthetic sulfide-oxidizing bacteria are excluded, and colorless sulfur bacteria grow along the oxic/anoxic interface (Jørgensen and DesMarais 1986). The response of motile filaments in the mat is modulated by their physiology. Migrating *Beggiatoa*-like filaments closely follow the diel up-and-down movement of the oxygen–sulfide interface, whereas a nonmigratory population in the same mat remains stationary at ca. 1 mm depth (Garcia-Pichel et al. 1994). Interestingly, nitrate-storing *Beggiatoaceae* do not migrate towards the mat surface to escape nighttime anoxia, but remain in the mat and stay several millimeters below the oxic surface layer (Hinck et al. 2007).

Close associations with cyanobacteria in microbial mat habitats may have left a genomic imprint in some *Beggiatoaceae* that is especially visible in genetic elements involved in cell differentiation (MacGregor et al. 2013b). The draft genome sequence of a single orange "Maribeggiatoa"-related filament from hydrothermal mats shows evidence of extensive genetic exchange with cyanobacteria, in particular for sensory and signal transduction genes. A putative homing endonuclease gene and Group I intron within the 23S rRNA gene; several Group II catalytic introns; GyrB and DnaE inteins, also encoding homing endonucleases; and multiple copies of sequences similar to the fdxN excision elements XisH and XisI (required for heterocyst differentiation in some cyanobacteria) all have close non-*Beggiatoaceae* matches with cyanobacterial sequences (MacGregor et al. 2013b). Sequences similar to the uncharacterized ORF and Xis elements are found in other *Beggiatoaceae* genomes, a variety of cyanobacteria, and a few phylogenetically dispersed pleiomorphic or filamentous bacteria (MacGregor et al. 2013b). Thus, gene transfer and evolutionary linkages between *Beggiatoaceae* and other filamentous bacteria (Reichenbach and Dworkin 1981) might be more significant than expected.

Hydrothermal Vents

Large filamentous sulfide oxidizers ("Maribeggiatoa" spp. and "Marithrix" spp.) occupy a distinct ecological niche characterized by fluctuating sulfide and oxidant levels. Conspicuous examples of this habitat have been studied in the hydrothermal sediments of Guaymas Basin in the Gulf of California (Jannasch et al. 1989; Nelson et al. 1989). Here, massive "Maribeggiatoa"-like mats of several cm thickness growing on hydrothermally active sediments are exposed to irregularly fluctuating pulses of oxygenated seawater and sulfidic sediment fluids (Gundersen et al. 1992) (*Fig. 6.9a*, *b*). The gradients of sulfide, DIC, and (most likely) low molecular weight organic compounds become steeper towards the center of a hydrothermal hot spot and appear to select for different morphotypes and genotypes in the center and in the periphery of the hydrothermally active sediment region (McKay et al. 2012). Orange filaments with a diameter of ca. 25–40 µm (*Fig. 6.9c*) dominate the center of the mat (*Fig. 6.6c*), whereas larger white filaments (>120 µm diameter, *Fig. 6.9c–e*) form the periphery of the mat (McKay et al. 2012). High temperature is unlikely to select for these different populations; in situ temperatures at the sediment–water interface across the multicolored mats remained cool (near 10 °C when measured with the Alvin temperature probe) and suitable for psychrophilic bacteria (McKay et al. 2012).

Filamentous mats do not only grow on hydrothermal sediments, but essentially on all solid substrates with a suitable regime where oxygenated seawater and reduced hydrothermal fluids mix, including the exterior of gradually seeping chimneys (*Fig. 6.9b*) and gaps and cracks in rocky debris that channel the flow of reduced fluids (*Fig. 6.9f*). Convective mixing of reduced vent fluids and oxygenated seawater also characterizes the habitat of "Marithrix" filaments; substrate-attached growth as rosettes allows "Marithrix" filaments to persist on exposed surfaces, such as hydrothermal chimneys (Kalanetra et al. 2004; Heijs et al. 2005; Kalanetra and Nelson 2010; Grünke et al. 2012). Most likely, these filaments have a higher oxygen tolerance than those in the Guaymas Basin mats.

Hydrocarbon Seeps

At hydrocarbon seeps, sediment areas characterized by active seepage of methane- and sulfide-rich fluids host conspicuous mats of *Beggiatoa*-like filaments (*Fig. 6.10*). Currently, most observations of this habitat type come from the continental slope of the northern Gulf of Mexico, an area exceptionally rich in hydrocarbon seeps (Larkin et al. 1994; Nikolaus et al. 2003). A cross section from the center to the margin of a mat-covered sediment area in the Gulf of Mexico (MC118) showed that the mat area coincided with high sulfate reduction and anaerobic methane oxidation rates in the surficial sediments; the microbial community underneath the mat was dominated by deltaproteobacterial sulfate-reducing bacteria and by methane-oxidizing archaea (Lloyd et al. 2010). The rates declined in bare sediments adjacent to the mat, and the microbial communities in the surficial sediments diversified considerably (Lloyd et al. 2010). The sulfidic seep sediments underneath the mats select for a specialized sulfur- and methane-cycling microbial community of reduced diversity (Lloyd et al. 2010).

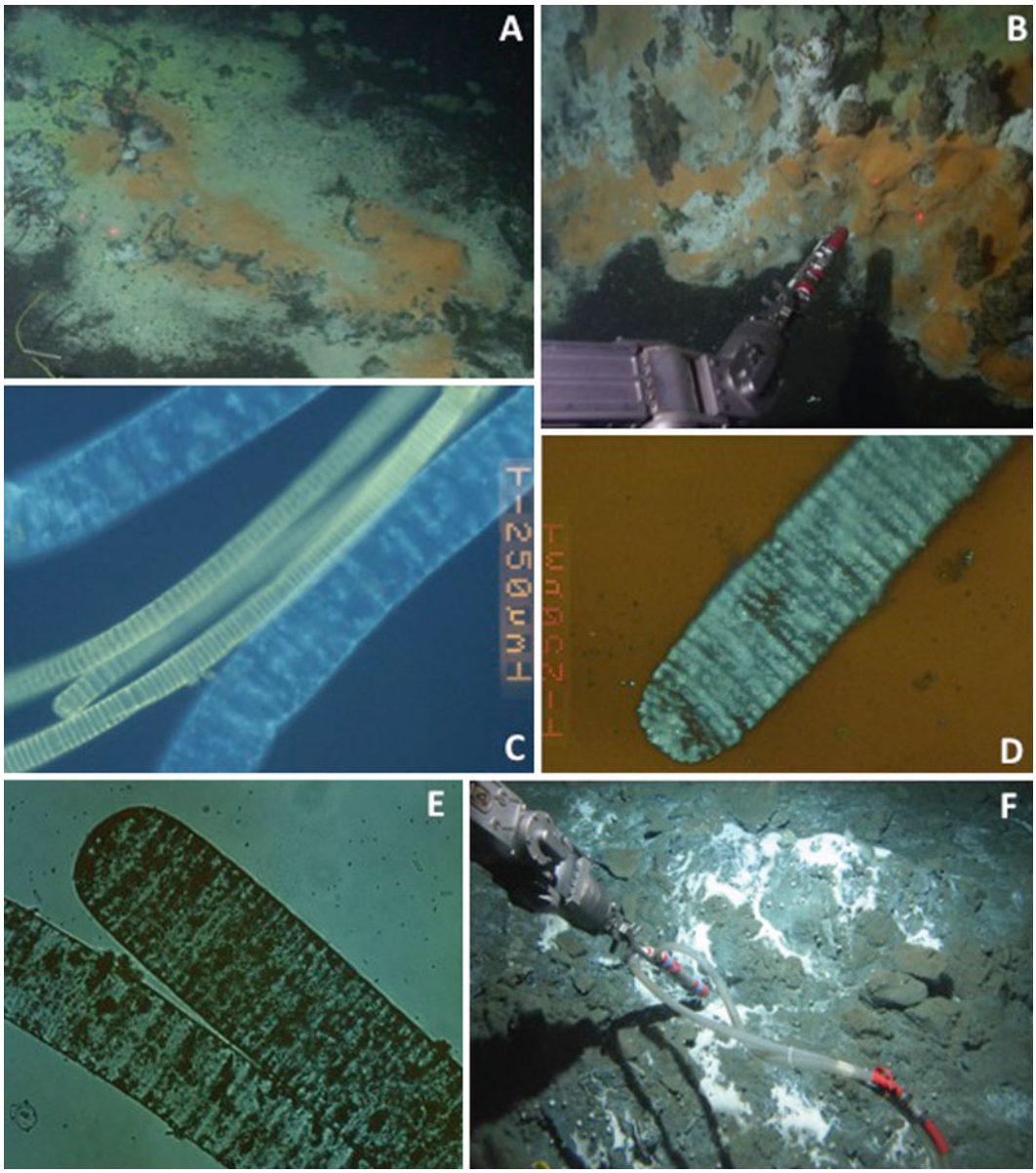

Fig. 6.9

***Hydrothermal vent Beggiatoaceae*. (a) Typical structure of Guaymas Basin mat of filamentous sulfur oxidizers: orange filaments at the center and white filaments at the periphery, surrounded by bare sediment (Alvin Dive 4569, N 27°00.47, W 111°24.431, 2,009 m depth). For this mat (M14), in situ temperature gradients, porewater geochemistry, filament types, and their 16S rRNA gene sequences are described in context (McKay et al. 2012). (b) Mats on chimney structure "Cathedral Hill" in Guaymas Basin (Alvin Dive 4573, near N 27°00.696, W 111°24.265, 2,013 m depth). The Alvin sampling device ("slurp gun") is visible in the foreground. (c) Epifluorescence microphotograph of the two dominant types of Guaymas Basin *Beggiatoaceae*, large white (ca. 120 μm diameter), and smaller orange (ca. 40 μm diameter) filaments under UV excitation light. Vertical scale bar, 250 μm. (d) Dark field microphotograph of large *Beggiatoaceae* filament, showing the sulfur globules and the salami-like arrangement of individual cells in the filament. Vertical scale bar, 250 μm. (e) Transmission light microphotograph of large *Beggiatoaceae* filament, same scale as photos (c) and (d). (f) Sampling of *Beggiatoaceae* mats at Costa Rica Jaco Scarp (Alvin Dive 4509, N 09°07.030, W 84°50.550, 1,866 m depth) (Photos (a, b) Woods Hole Oceanographic Institution; (c–e) Andreas Teske, University of North Carolina at Chapel Hill; (f) Jake Bailey, University of Minnesota)**

The large filamentous sulfur oxidizers from Gulf of Mexico hydrocarbon seeps (with filament diameters up to 200 μm; Larkin and Henk 1989, 1996) are taxonomically unidentified but resemble large "Maribeggiatoa" filaments (e.g., *Fig. 6.10e*). In several investigations of cold hydrocarbon seeps in the Gulf of Mexico, colorful *Beggiatoaceae* mats showed a Guaymas-like spatial distribution of orange mats with white peripheries (Wirsen et al. 1992; Sassen et al. 1994; Larkin and Henk 1996;

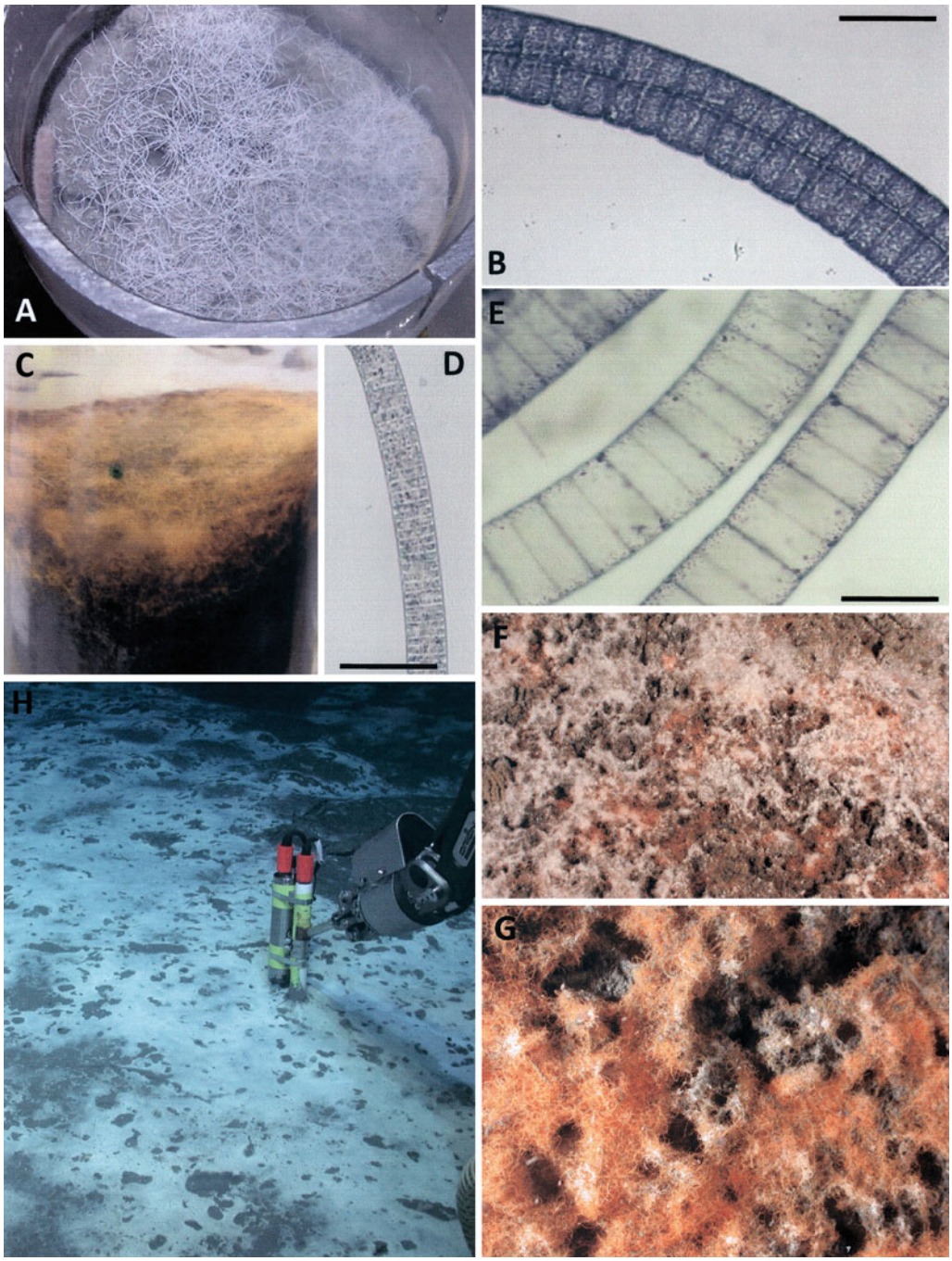

Fig. 6.10

***Cold seep Beggiatoaceae*.** (**a**) Sediment core, 6 cm diameter, with mat of white filaments collected on Alvin dive 4645 near Garden Banks 425 mud volcano in the Gulf of Mexico (N 27°33.140, W 92°32.437; 597 m depth). (**b**) Phase contrast microphotograph of single white filament from this mat; filament diameters range from 78 to 94 μm. Note central ''pipeline''-like structure running through the filament. Scale bar 100 μm. (**c**) Orange mat in sediment core, 6 cm diameter, collected on Alvin dive 4653 near Green Canyon 233 brine lake (N 27°43.429′, W. 91°16.777, 633 m depth). Scale bar 100 μm. (**d**) Transmission microphotograph of individual filament from this mat, average diameter 38 μm. Scale bar 100 μm. (**e**) Transmission microphotograph of large, vacuolated, white filaments (collected on Alvin dive 4652 in Green Canyon 426 Mud volcano area (N 27°42.098, W 90°38.887; 837 m depth), average diameter 119 μm. Scale bar 100 μm. (**f**, **g**) In situ close-up photograph of predominantly orange filamentous mats at Green Canyon 185, a hydrate-rich seep site in the Gulf of Mexico (N 27°44.930, W 91°30.450; 540 m depth). Note the complex mat architecture containing spherical sulfur bacteria (most likely *Thiomargarita* spp.) and white and orange filaments growing in tufts. (**h**) ROV sampling of white filamentous mats (''*Candidatus* Halobeggiatoa'') at Håkon Mosby mud volcano, Barents Sea (Niemann et al. 2006; Grünke et al. 2012) (Photos (**a**–**e**) Andreas Teske, University of North Carolina; (**f**, **g**) Ian McDonald, Florida State University; (**h**) MARUM, Bremen University)

Nikolaus et al. 2003). Hydrocarbon analysis of sediments under *Beggiatoaceae* mats revealed that, in comparison to white filaments, adjacent orange filaments reside over sediments with elevated concentrations of unresolved petroleum hydrocarbons, and 1–3 orders of magnitude higher methane and ethane concentrations (Sassen et al. 1994). A recent survey in the Gulf of Mexico identified colorless, vacuolated, large filaments with diameters near 90–120 µm and smaller, vacuolated, orange-colored filaments near 35 µm (Fig. 6.10a–e); these coloration and size classes resembled those of the Guaymas Basin *Beggiatoaceae* (Teske, unpublished results). Rates of hydrocarbon seepage could control the composition of Gulf of Mexico mats similarly as hydrothermal seepage in Guaymas Basin.

The physiological capacity of hydrocarbon seep-associated *Beggiatoaceae* is an open research field. White filaments from Gulf of Mexico seeps assimilate CO_2 autotrophically, whereas orange filaments show strongly reduced capacity for CO_2 uptake and appear to be heterotrophs (Wirsen et al. 1992; Nikolaus et al. 2003). When methane-derived DIC or low molecular weight organic compounds reach the sediment surface, *Beggiatoaceae* mats can take up ^{13}C-depleted CO_2 derived from methane oxidation and acquire the isotopically light signature ($\delta^{13}C$ in the range of −50 to −60‰) of partially methane-derived biomass (Paull et al. 1992; Orphan et al. 2002). In most cases, mixed *Beggiatoaceae* mat samples collected at cold seeps in the Gulf of Mexico have $\delta^{13}C$ isotopic signatures near −26 to −29‰, indicating that carbon sources of planktonic, photosynthetic origin are assimilated (Zhang et al. 2005).

In contrast to the commonly studied filamentous mats at hydrocarbon seeps, *Beggiatoaceae* occurring at a methane seep off Costa Rica have an attached habitus (Fig. 6.4f). Nonfilamentous sulfur bacteria (certain *Thiomargarita* sp. and *Thiopilula* sp.) are attached to solid surfaces like rocks, shells, or the byssus of mussels, where they are exposed to turbulent mixing of alternating sulfidic and oxygenated water (Bailey et al. 2011).

Mud Volcanoes

When fluidized mud flows from the subsurface reach the seafloor, they form extensive mud volcanoes characterized by high gas flow and an unstable sediment–water interface. At the center of mud volcanoes, the highly dynamic sediment–water interface does not allow the formation of sulfide-oxidizing microbial mats; more quiescent regions towards the periphery of the mud volcano provide the stable sediment–water interface that is required for growth of filamentous sulfide-oxidizing bacteria (Niemann et al. 2006; de Beer et al. 2006; Grünke et al. 2011; Girnth et al. 2011) (Fig. 6.10h).

Mud volcanoes with periodic flows of subsurface-derived brines (for example, the Amon mud volcano on the Nile Deep Sea fan in the Eastern Mediterranean; Girnth et al. 2011) present a special habitat. The brine flows cover the sediment surface at irregular intervals with dense, highly sulfidic brine; under these conditions, stationary *Thiomargarita* spp. have an advantage against "Maribeggiatoa" and "Marithioploca." The latter two would spend energy by chemotactic responses to the shallow brine flow, whereas the stationary *Thiomargarita* cells avoid this energy expenditure and await the end of the sulfidic brine flow episode (Girnth et al. 2011). This strategy resembles the stationary survival mode of *Thiomargarita namibiensis*, which relies on environmental perturbations for sulfide and nitrate exposure and uptake (Schulz 2006). Attached *Beggiatoaceae* ("Marithrix" spp.) have a similar ecological advantage under the highly fluctuating regimes of oxidized and reduced bottom waters and passing brine flows (Heijs et al. 2005; Grünke et al. 2011, 2012).

Nearshore Upwelling Areas: The Chilean Continental Shelf

Nearshore upwelling areas are characterized by oxygen-depleted or anoxic bottom water overlying organic-rich sediments; sulfate reduction in the sediments produces sulfide that is oxidized by microaerophilic and nitrate-reducing mats of large filamentous sulfide oxidizers (Fig. 6.3a–c). By geographical extent, these mats are probably the most widespread sulfide-oxidizing mat ecosystem on earth. Currently, the ecologically and oceanographically best documented mat systems are the complex sulfide-oxidizing bacterial mats on the Pacific continental shelf of Chile and Peru dominated by the large nitrate-accumulating filaments of "*Candidatus* Marithioploca" (Gallardo 1963, 1977a, b; Fossing et al. 1995; Gallardo and Espinoza 2007) (Fig. 6.3a–f).

The predominantly vertically oriented "Marithioploca" filaments can bridge and exploit the vertically separated pools of sediment sulfide and seawater nitrate (Hüttel et al. 1996; Schulz et al. 1996) and thus improve on the "holding your breath" strategy of nitrate accumulation and respiration of large, marine "Maribeggiatoa" spp. Large "Marithioploca" spp. from the Chilean continental shelf have turnover times of 8–10 days for their intracellular nitrate and sulfur reserves (Otte et al. 1999). For long-term survival, "Marithioploca" filaments require just the right balance of nitrate availability in oxygen-depleted bottom water and sulfide availability in the sediment. The annual fluctuations in mat abundance during a seasonal upwelling cycle (Schulz et al. 2000) can be exacerbated by prolonged summer anoxia, when the mats cannot cope with increased sulfate reduction and sulfide inundation; the result is mat die-off (Gallardo 1992) and high porewater sulfide concentrations in previously sulfide-free surficial sediments (Holmkvist et al. 2010). On the other hand, increased oxygen exposure and decreased water column productivity and organic matter input during El Niño years adversely affect the "Marithioploca" mats. During such events, bottom water oxygen concentrations increase from near detection limit (<2 µM) to ca. 20–40 µm; at the same time, "Marithioploca" biomass decreases considerably, from up to 160 g m^{-2} (Schulz et al. 2000) to <1 to 5 g m^{-2} (Schubert et al. 2000; Neira et al. 2001).

The spatial structure of the Chilean "Marithioploca" mats is conducive to the microbial lifestyle of bridging sulfide and nitrate pools. The densest mat matrix of randomly oriented filaments and bundles is found in the uppermost centimeter layer, while predominantly vertically oriented, less densely packed "Marithioploca" bundles reach down to a depth of generally 4–8 cm; they peter out at approx. 10–15 cm (Schulz et al. 1996) and are only rarely found in deeper sediment layers. The surface layer of the mat is generally well supplied with nitrate; it can penetrate several centimeters into the hydraulically conductive, porous, and soft "Marithioploca" mat sediments (Hüttel et al. 1996). The upper 1–5 cm of the sediment also shows the highest sulfate reduction rates, up to 1,500 nmol cm^{-3} d^{-1}, which are extremely high rates for marine sediments. Nevertheless, efficient in situ reoxidation of sulfide keeps the sulfide concentrations in the "Marithioploca" mat sediments low, mostly in the range of 5–50 µm, while sulfate concentrations were never depleted below bottom water concentrations (Ferdelman et al. 1997; Thamdrup and Canfield 1996). "Marithioploca" mats contribute significantly to in situ anaerobic oxidation of sulfide produced by sulfate reduction; their share can range from 6 to 91 %, but most measurements indicate a contribution between 20 % and 30 % (Fossing et al. 1995; Thamdrup and Canfield 1996; Ferdelman et al. 1997; Otte et al. 1999). The sulfide-oxidizing activity of "Marithioploca" is most significant in the upper 4 or 5 cm of sediment and quickly declines towards deeper sediment layers (Zopfi et al. 2008).

Sulfur recycling within the "Marithioploca" mats most likely benefits from the close spatial association between sulfate-reducing and sulfide-oxidizing bacteria in "Marithioploca" mats. Mat biomass and cultivable MPN numbers of sulfate-reducing bacteria both peak in the surface layer of the mat (Teske et al. 2009). Filamentous sulfate reducers of the genus *Desulfonema* grow on and within the "Marithioploca" sheaths and thus contribute to a cycle of sulfate reduction and reoxidation within a single "Marithioploca" bundle (Fukui et al. 1999; Teske et al. 2009).

Nearshore Upwelling Areas: The Benguela Upwelling System

The survival strategy of "Marithioploca" spp. contrasts with the ecophysiology of its relative *Thiomargarita namibiensis*. This immobile, giant sulfide oxidizer, the largest known prokaryote by volume, relies on its enormous storage capacity for sulfur and nitrate, to carry it through irregular natural fluctuations of sulfide and nitrate availability in its sedimentary habitat in the Benguela upwelling region offshore Namibia (Schulz et al. 1999). The high input of diatom-dominated phytoplankton debris fuels extremely high sulfate reduction rates that deplete sulfate within a few centimeters of the sediment surface and generate extremely sulfidic conditions (up to 20 mM) in the extremely soft diatomaceous ooze bottom sediments (*Fig. 6.4a*) (Brüchert et al. 2003). In this habitat, *Thiomargarita* cells reach a biomass density of up to 170 g m^{-2} sediment, similar to the "Marithioploca" mats offshore Chile (Brüchert et al. 2003). Filamentous sulfur bacteria, free-living or bundled, are scarce in the sulfidic Namibian liquid sediments; although previous reports suggest the possibility, only a few specimens were found in recent surveys (Gallardo et al. 1998; Salman et al. 2013). In contrast to the oxygen-sensitive Chilean "Marithioploca" 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 (Schulz and de Beer 2002). This respiratory flexibility in combination with its large intracellular storage capacity helps *Thiomargarita namibiensis* to tolerate fluctuations of sulfide, nitrate, and oxygen during irregular resuspension episodes due to massive sulfide and methane outgassing events in its natural habitat (Emeis et al. 2004; Weeks et al. 2002, 2004).

Ecosystem Roles of *Beggiatoaceae*

The *Beggiatoaceae* serve as indicator organisms of beginning or advanced oxygen depletion and sulfidic bottom conditions in aquatic habitats; these conditions go generally together with increased oxygen demand due to seasonal biomass degradation (Bernard and Fenchel 1995). Point sources of anthropogenic pollution (Elliott et al. 2006) and fish farm eutrophication (Bissett et al. 2007; Gallardo and Espinoza 2007; Aranda et al. 2010) favor the development of *Beggiatoaceae* mats; sea grass beds (*Zostera marina*) are replaced by sulfidic bottom mud with bacterial mats (Elliott et al. 2006).

As a result of their growth pattern at the sediment–water interface, *Beggiatoaceae* mats play a significant role for the benthic–pelagic exchange of sulfur in the marine environment. They act as a sulfide trap that prevents 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).

A significant ecosystem service of *Beggiatoaceae* mats, catalyzing phosphorous retention in benthic sediments, was recently proposed in a geochemical modelling study (Yekta and Rahm 2011). Sulfide oxidation by *Beggiatoaceae* contributes to shifting the redox balance of iron in surficial marine sediments from Fe-II to Fe-III and changes the balance of the resulting iron solid phases in surficial sediments from iron sulfide and pyrite to ferric oxyhydroxides; the latter absorb and immobilize phosphate in surficial sediments. This mechanism could provide a strategy for phosphorus retention in hypoxic marine sediments affected by eutrophication, a widespread problem in the Baltic Sea (Yekta and Rahm 2011). It remains a matter of debate whether the sulfur-oxidizing activities and biomass of *Beggiatoaceae* in situ are sufficient to turn around the redox state of the sedimentary iron pool (Preisler et al. 2007).

Local "hot spots" of decaying biomass that sustains mat-forming *Beggiatoaceae* can range from whale carcasses (Smith et al. 1989; Deming et al. 1997) to coral heads, where

environmental stressors can induce the formation of mucus, which is then colonized by sulfate reducers and filamentous sulfur bacteria (Mitchell and Chet 1975). In black band coral disease, cyanobacterial mats and diverse heterotrophic bacteria are overgrowing and degrading coral tissue; colorless sulfur bacteria are a significant component of these mats growing on necrotic coral tissue (Carlton and Richardson 1995). Functional gene studies using the widely distributed sulfur oxidation key gene soxB show that uncultured *Alphaproteobacteria* are the dominant component of these mats; the *Beggiatoaceae* are morphologically conspicuous but appear as a minority in functional gene surveys (Bourne et al. 2013) or remain undetected in standard 16S clone library surveys (Sekar et al. 2006).

Many associations of *Beggiatoaceae* with other organisms benefit the partner organisms. The addition of cultured *Beggiatoa* filaments and tufts to different soils with rice plant seedlings reduced hydrogen sulfide levels in the flooded soil and increased oxygen production by the rice seedlings (Pitts et al. 1972; Joshi and Hollis 1977). Sulfide removal and detoxification has also been invoked to explain the conspicuous association of filamentous marine *Beggiatoaceae* with protists and nematodes in highly reducing marine sediments (Bernhard et al. 2003). Specific nematodes also inhabit *Beggiatoaceae* mats and the underlying sediment at the Håkon Mosby mud volcano and shape a meiofaunal community that is taxonomically distinct from and less diverse than its counterpart outside of the mat area (Van Gaever et al. 2010). On the Pacific continental shelf offshore Chile, benthic invertebrates (polychaetes, crustaceans, mollusks, anthozoa) occurred in greater abundance and diversity at sampling locations with well-developed "Marithioploca" mats than at sites where mats were sparse or absent (Carrasco et al. 1999). The Chilean "Marithioploca" mats also provide nursery habitat for marine invertebrates, such as squat lobster larvae (Roa et al. 1995; Gallardo et al. 1994). These effects are not only attributable to food source availability but also to sulfide sequestration.

Due to nitrate reduction and ammonification by "*Candidatus* Marithioploca," ammonia accumulates at high rates in "Marithioploca"-harboring sediment surface layers (Thamdrup and Canfield 1996). The "Marithioploca" mats turn the sediments from a denitrifying nitrogen sink into an ammonia-producing nitrogen source; ammonia would constitute a readily utilized and recycled nitrogen source for the water column (Farias et al. 1996; Farias 1998). Similar findings have been reported for mats of large, vacuolated *Beggiatoa*-like filaments in Tokyo Bay (Sayama 2001) and in Aarhus Bay marine sediments (Sayama et al. 2005). Here, *Beggiatoaceae* separate the sulfide and nitrate porewater pools by pushing the nitrate-reducing sulfide oxidation horizon down into the sediment; in parallel, the predominant nitrate and nitrite reduction pathway in the *Beggiatoa*-inoculated sediment shifts from denitrification to N_2 towards dissimilatory nitrate reduction to ammonia (Sayama et al. 2005). The product of anaerobic sulfide oxidation, sulfur, is then transported to the sediment surface and oxidized aerobically to sulfate. This spatial separation of anaerobic and aerobic sulfur oxidation pathways, and parsimonious use of nitrate only for the initial oxidation step (of sulfide to sulfur), counteracts nitrate depletion during complete oxidation of sulfide to sulfate.

In a fascinating twist, microbial associations of "Marithioploca" filaments and sheath-associated anaerobic ammonia-oxidizing bacteria ("*Candidatus* Scalindua") can recycle ammonia by anaerobic oxidation to dinitrogen via conproportionation with nitrite (Prokopenko et al. 2006, 2013). Freshly generated bioavailable reduced nitrogen in the sediments would be lost in a coupled nitrate ammonification/anammox process that, from the outside, looks like straightforward denitrification. In a detailed case study of the Soledad basin offshore Baja California, the "Marithioploca"-catalyzed nitrogen loss from the sediment was very similar to the measured efflux of ammonia; anammox rate measurements and geochemical modelling showed that the Marithioploca/Scalindua consortium contributed ca. 20–57 % of the total N_2 production. In this way, "Marithioploca" catalyzes nitrogen loss in organic-rich, highly reducing sediments where otherwise limited diffusion of the oxidants nitrate and nitrate into the sediment limits denitrification (Prokopenko et al. 2011, 2013). Thus, benthic mats of nitrate-accumulating, sulfur-oxidizing *Beggiatoaceae* represent simultaneously a source and a sink of bioavailable nitrogen, and the relative contributions of these processes will depend to a large extent on the redox state and organic carbon load of benthic sediments.

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7 The Family *Cardiobacteriaceae*

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Abstract

Cardiobacteriaceae, within the order, *Cardiobacteriales*, in the class, *Gammaproteobacteria*, was described as a novel family, based upon the 16S rRNA sequence-based relationships of *Cardiobacterium hominis*, *Dichelobacter nodosus*, and *Suttonella indologenes*, representing distinct lineages within a common phylogenetic cluster. The two species of *Cardiobacterium*, one species of *Dichelobacter*, and two species of *Suttonella* are Gram-negative, rod-shaped, fastidious, facultatively anaerobic, strictly anaerobic or microaerophilic chemoorganotrophs, manifesting pathogenicity in humans and animals. Strains of the species are isolated from human and animal samples. *C. hominis*, *C. valvarum*, and *S. indologenes* are typically recovered from the blood cultures of patients with endocarditis. *D. nodosus* is a causative agent for foot-rot disease in cloven-hoofed animals, and *S. ornithocola* is implicated in pulmonary necrosis in birds of the tit family. Rarely are bacteria of the *Cardiobacteriaceae* detected in nonclinical samples.

Taxonomy, Historical and Current

Short Description of the Family

Cardiobacteriaceae Dewhirst, Paster, La Fontaine and Rood 1990, 431[VP]; Car.di.o.bac.te.ri.a'ce.ae; N.L. neut. n. *Cardiobacterium*, type genus of the family; *-aceae* suffix denoting a family; M.L. fem. pl. n. *Cardiobacteriaceae*, the family of bacteria of the heart (Dewhirst and Paster 2005a).

Cardiobacteriaceae is Family I, and the only family, of the *Cardiobacteriales* (Garrity et al. 2005b), Order IV within the class *Gammaproteobacteria* (Garrity et al. 2005a). *Cardiobacteriaceae* was described by Dewhirst et al. (1990), as a monophyletic novel taxon, based upon the phylogenetic analyses inferred from the 16S rRNA sequences of bacteria classified as *Cardiobacterium hominis*, *Bacteroides nodosus*, and *Kingella indologenes*. Comparative sequence analyses confirmed that *B. nodosus* and *K. indologenes* are not related phylogenetically to the genera in which they had been classified, but are related to each other and to *C. hominis*, as distinct lineages within a common family.

The family *Cardiobacteriaceae* (Dewhirst et al. 1990) was described as a familial-level taxon, encompassing three genera, including the type genus, *Cardiobacterium* (Slotnick and Dougherty 1964), *Dichelobacter* (Dewhirst et al. 1990), and *Suttonella* (Dewhirst et al. 1990), with a single species for each genus; since 1990, only two new species, *C. valvarum* (Han et al. 2004) and *S. ornithocola* (Foster et al. 2005), have been described and validly published (*Table 7.1*). All species of the genera within *Cardiobacteriaceae* are Gram-negative, rod-shaped, fastidious, facultatively anaerobic or strictly anaerobic chemoorganotrophs, associated with particular pathogenic conditions of humans or animals.

Cardiobacterium Slotnick and Dougherty 1964, 271[AL] (type genus of the family *Cardiobacteriaceae*); Car.di.o.bac.té.ri.um; Gr. n. *cardia*, heart; Gr. n. *bakterion*, small rod; M.L. neut. n. *Cardiobacterium*, bacterium of the heart.

Cardiobacterium is the type genus within the family *Cardiobacteriaceae*. The genus was created from Gram-negative, pleomorphic bacilli, isolated from the blood of patients with endocarditis (Tucker et al. 1962). Nearly 30 years later, the genus was placed within the newly created family, *Cardiobacteriaceae*, based upon the elucidation of the close phylogenetic relationships of *C. hominis* with two other species, *Bacteroides nodosus* and *Kingella indologenes*, that were reclassified in new and distinct genera, *Dichelobacter nodosus* and *Suttonella indologenes*, respectively (Dewhirst et al. 1990). These three genera were observed to branch deeply within the γ-division of the *Proteobacteria*, distinct from any established taxon. Before nucleic acid sequence-based analyses helped define the phylogenetic and systematic position of *Cardiobacterium*, the genus had been established by the classification of the Gram-negative bacilli, "Group II D bacteria," as *Cardiobacterium hominis* (Slotnick and Dougherty 1964). However, the taxonomic placement of the genus was uncertain, exhibiting significant

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Table 7.1
The genus and species members of the family *Cardiobacteriaceae*

Genus	Species	Type strain	16S rRNA sequence	Gram-reaction	Metabolism	Size of genome (bp)	DNA G+C (mol%)	Reference
Cardiobacter	*hominis*	ATCC 15826	M35014	Gram-negative	Facultatively anaerobic	2,578,897	59–60 59.3*	Slotnick and Dougherty 1964
Cardiobacter	*valvarum*	CCUG 48245	AF506987	Gram-negative	Facultatively anaerobic	2,553,535	58.2*	Han et al. 2004
Dichelobacter	*nodosus*	ATCC 25549	JN175347	Gram-negative	Anaerobic	1,389,350	45 44.4*	Dewhirst et al. 1990
Suttonella	*indologenes*	ATCC 25869	M35015	Gram-negative	Microaerophilic	nd	49	Dewhirst et al. 1990
Suttonella	*ornithocola*	CCUG 49457	AJ717394	Gram-negative	Microaerophilic	nd	nd	Foster et al. 2005

Taxonomic and type strain information obtained from list of prokaryotic names with standing in nomenclature (http://www.bacterio.net/)
Genomic information obtained from the National Center for Biotechnology Information (*NCBI*) (http://www.ncbi.nlm.nih.gov/genome/browse/)
Genomic DNA G+C contents determined by whole genome sequencing are indicated by asterisk (*)
nd = not determined

differences from the genera that were presumed to be related, such as *Pasteurella*, *Moraxella*, *Bacteroides*, and other genera. This uncertainty was reflected 10 years after the publication of the genus, in the placement of *Cardiobacterium* and *C. hominis* in "Genera of Uncertain Affiliation," within the facultatively anaerobic Gram-negative rods (Lapage 1974), in *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbons 1974) and, again, 10 years later (Weaver 1984), in *Bergey's Manual of Systematic Bacteriology* (Krieg and Holt 1984). *Cardiobacterium* includes the species, *C. hominis* (Slotnick and Dougherty 1964) and *C. valvarum* (Han et al. 2004) (*Table 7.1*). *Cardiobacterium* are typically associated with cases of endocarditis, isolated from blood cultures and tissue samples of the endocardium, heart valves, heart septa, and other structures connected to the heart or intracardiac prosthetic devices. *Cardiobacterium* are rarely detected as causative agents of infection outside the cardiovascular system, although they have been recognized to colonize the gastrointestinal tract of healthy individuals (Slotnick et al. 1964) and isolations have been reported from peritoneal fluid (Bhan et al. 2006) and from dacryocystitis with lachrymal abscess (Manderwad et al. 2014). These organisms can be found as part of the flora of the upper respiratory tract in a majority of humans (Slotnick and Dougherty 1964). Detection in the upper respiratory tracts of bottlenose dolphins has been reported (Johnson et al. 2009).

Cardiobacterium hominis Slotnick and Dougherty 1964, 271^{AL} (type species of the genus *Cardiobacterium*); hó.mi.nis; L. gen. n. *hominis*, of man.

C. hominis is the type species within the genus *Cardiobacterium*. The type strain of the species is strain 6573, deposited by I. J. Slotnick with the American Type Culture Collection (ATCC) and with the National Collection of Type Cultures (NCTC); strains from other collections are derived from strains ATCC 15826^{T} or NCTC 10426^{T}.

C. hominis was isolated from the blood of endocarditis patients, initially described as "*Pasteurella*-like" and designated by the US Centers for Disease Control and Prevention as "Group II D bacteria" (Tucker et al. 1962). In addition to infections of the endocardium and cardiac valves, *C. hominis* has been implicated as the causative agent in cases of bacteremia, sepsis, septic arthritis, peritonitis, pericarditis, meningitis, osteomyelitis, periodontal infections, ocular infections, and abscesses (Kuzucu et al. 2005; Bhan et al. 2006; Malani et al. 2006; Colombo et al. 2012; Manderwad et al. 2014). *C. hominis* may be detected as a resident of the human oral cavity and respiratory tracts (Wormser and Bottone 1983).

Cardiobacterium valvarum Han et al. 2004, emend. Han and Falsen 2005, 1593^{VP}; val.vaŕ.um; L. fem. n. *valva*, a folding door, referring to the heart valve; L. gen. n. *valvarum*, of heart valve.

C. valvarum is the second species described in the genus *Cardiobacterium*. The type strain of the species is strain MDA-3079, deposited by X. Y. Han with the ATCC, the Culture Collection University of Gothenburg (CCUG), and the NCTC; strains from other collections are derived from strains ATCC BAA-694^{T}, CCUG 48245^{T}, or NCTC 13294^{T}.

C. valvarum was isolated from the blood of a patient with endocarditis and cerebral aneurysm; the origin of the infectious bacterium was probably the oral cavity (Han et al. 2004). Although both *C. hominis* and *C. valvarum* are implicated as etiological agents of endocarditis, *C. valvarum* has a much lower incidence than *C. hominis*. *C. valvarum*, like *C. hominis*, is known to be associated with the human oral cavity and respiratory tract (Han and Falsen 2005).

Dichelobacter Dewhirst, Paster, La Fontaine and Rood 1990, 430^{VP}; Di.ché.lo.bac.ter; Gr. adj. *Dichelos*, cloven hoofed; N. L. masc. n. *bacter*, rod; N. L. masc. n. *Dichelobacter*, cloven-hoofed rod, referring to the rod-shaped bacterium responsible for

foot-rot disease in sheep, cattle, and other animals of the order *Artiodactyla*. The genus *Dichelobacter* was created to accommodate the incorrectly classified *Bacteroides nodosus* and categorized with the phylogenetically related bacteria, *Cardiobacterium hominis* and *Suttonella* (*Kingella*) *indologenes*, within the family, *Cardiobacteriaceae* (Dewhirst et al. 1990). *Dichelobacter* includes one species, *D. nodosus* (Dewhirst et al. 1990) (Table 7.1).

Dichelobacter nodosus (*Fusiformis nodosus* Beveridge 1941), Dewhirst, Paster, La Fontaine and Rood 1990, 426^{VP} (type species of the genus); no.dó.sus; L. masc. adj. *nodosus*, full of knots, referring to the shape of the cells.

D. nodosus is the type species within the genus *Dichelobacter*. The type strain of the species is "Organism K" (Beveridge 1938), obtained by L. D. S. Smith (strain 11342), who sent it to L. V. Holdeman (VPI 2340), who deposited the strain with the ATCC; strains from other collections are derived from strain ATCC 25549^{T}.

D. nodosus was isolated from the hoof tissue of sheep, initially described and referred to as "Organism K" (Beveridge 1938). Subsequent taxonomic study included *D. nodosus* being classified as *Fusiformis nodosus* (Beveridge 1941), *Ristella nodosus* (Prévot 1948), and *Bacteroides nodosus* (Mráz 1963). Comparative 16S rRNA sequence analyses demonstrated that *B. nodosus* was taxonomically misplaced in the genus *Bacteroides*, within the Firmicutes, and belonged phylogenetically within the γ-division of the Proteobacteria (La Fontaine and Rood 1990) and related to *Cardiobacterium hominis* and *Suttonella* (*Kingella*) *indologenes* (Dewhirst et al. 1990). *B. nodosus*, as a strict anaerobe, was differentiated from *C. hominis* and *S. indologenes*, as facultative anaerobes, and was designated within a new and distinct genus, *Dichelobacter nodosus* (Dewhirst et al. 1990).

Studies have detected benign strains of *D. nodosus* in asymptomatic animals and virulent strains in clinically affected animals (Stäuble et al. 2014). While virulence factors have been identified, the issue of how strains of *D. nodosus* are induced to virulence is not clear. Furthermore, although implicated as the primary pathogen of foot-rot, inflicting the epidermal tissues of the hooves of cloven-hoofed animals and recovered from foot-rot lesions, recent reports point out that *D. nodosus*, alone, has been unable to fulfill Koch's postulates as the etiological agent of disease (Bennett et al. 2009); *Fusobacterium necrophorum* is reported to be required, as a second pathogen, to manifest the symptoms of foot-rot, through a synergistic interaction between the two bacteria (Roberts and Egerton 1969; Bennett et al. 2009). Foot-rot and the association of *D. nodosus* with the disease appear to be multifactorial, depending, not only upon infection by other bacterial species, but also upon host animal genetics, immunity, and nutrition, as well as management practices and environmental conditions (Graham and Egerton 1968; Lechtenberg et al. 1998; Bennett et al. 2009).

Suttonella Dewhirst, Paster, La Fontaine and Rood 1990 emend. Foster, Malnick, Lawson, Kirkwood, MacGregor and Collins 2005, 429^{VP}; Sut´.ton.el´la; L. dim. ending, *ella*; N.L. fem. n. *Suttonella*, named after R. G. A. Sutton.

The genus *Suttonella* was established as a new genus for the reclassification of the incorrectly classified *Kingella indologenes* with phylogenetically related species, *Cardiobacterium hominis* and *Dichelobacter* (*Bacteroides*) *nodosus*, within the created family, *Cardiobacteriaceae* (Dewhirst et al. 1990). *Suttonella* includes two species, *S. indologenes* (Dewhirst et al. 1990) and *S. ornithocola* (Foster et al. 2005) (Table 7.1).

Suttonella indologenes (*Kingella indologenes* Snell and Lapage 1976), Dewhirst, Paster, La Fontaine, and Rood 1990, 430^{VP} (type species of the genus); in.dol.ó.ge.nes; M.L. n. *indolum*, indole; M.L. suff. *-genes*, producing; M.L. adj. *indologenes*, indole-producing.

S. indologenes is the type species within the genus *Suttonella* (Dewhirst et al. 1990). The type strain of the species was deposited with the ATCC and the NCTC (van Bijsterveld 1970); strains from other collections are derived from strains ATCC 25869^{T} or NCTC 10717^{T}.

The first isolates of *S. indologenes* were considered to be new species of *Moraxella* (van Bijsterveld 1970; Sutton et al. 1972). Subsequently, the strains were recognized to be distinct from *Moraxella* species and were described and validly published as *Kingella indologenes* (Snell and Lapage 1976). However, comparative 16S rRNA sequence analyses determined that *K. indologenes* is not related phylogenetically to other *Kingella* species, but is related to *Dichelobacter* (*Bacteroides*) *nodosus* and *Cardiobacterium hominis*, branching deeply within the γ-division of the *Proteobacteria* (Dewhirst et al. 1990). These three species were combined, as members of distinct genera, within the family, *Cardiobacteriaceae* (Dewhirst et al. 1990).

S. indologenes has been isolated from the blood cultures of patients with endocarditis (Jenny et al. 1987; Yang et al. 2011; Özcan et al. 2011). *S. indologenes* endocarditis was associated with splenic infarction and lethal intracranial hemorrhage in a patient with aortic valve replacement (Özcan et al. 2011). *S. indologenes* has been implicated also with human eye infections (van Bijsterveld 1970; Sutton et al. 1972; Barberis et al. 2007). The incidence of endocarditis from *S. indologenes* infection is much lower than that instigated by *C. hominis*. The only instance of *S. indologenes* from a nonclinical source is from a report of the isolation of strains from samples of seawater from the South China Sea (Lai et al. 2004).

Suttonella ornithocola Foster et al. 2005; 2271^{VP}; oŕ.ni.tho.có.la; Gr. n. *ornis*, bird; L. suff. *-cola*, dweller; N.L. n. *ornithocola*, bird dweller. *S. ornithocola* is the second species within the genus *Suttonella*. The type strain of the species is B6/99/2^{T} (Foster et al. 2005), which was deposited with the CCUG and with the NCTC; strains from other collections are derived from strains CCUG 49457^{T} or NCTC 13337^{T}.

Strains were isolated from the lungs of birds, of the tit family (*Paridae* and *Aegithalidae* species), that were victims of unusual mass mortalities of unexplained origin in Britain. Examination of lung tissue of the deceased birds observed pulmonary necrosis associated with bacterial isolates that were characterized and classified as *S. ornithocola* (Foster et al. 2005; Lawson et al. 2011). Since 2005, only two publications have appeared on *S. ornithocola* and disease in birds.

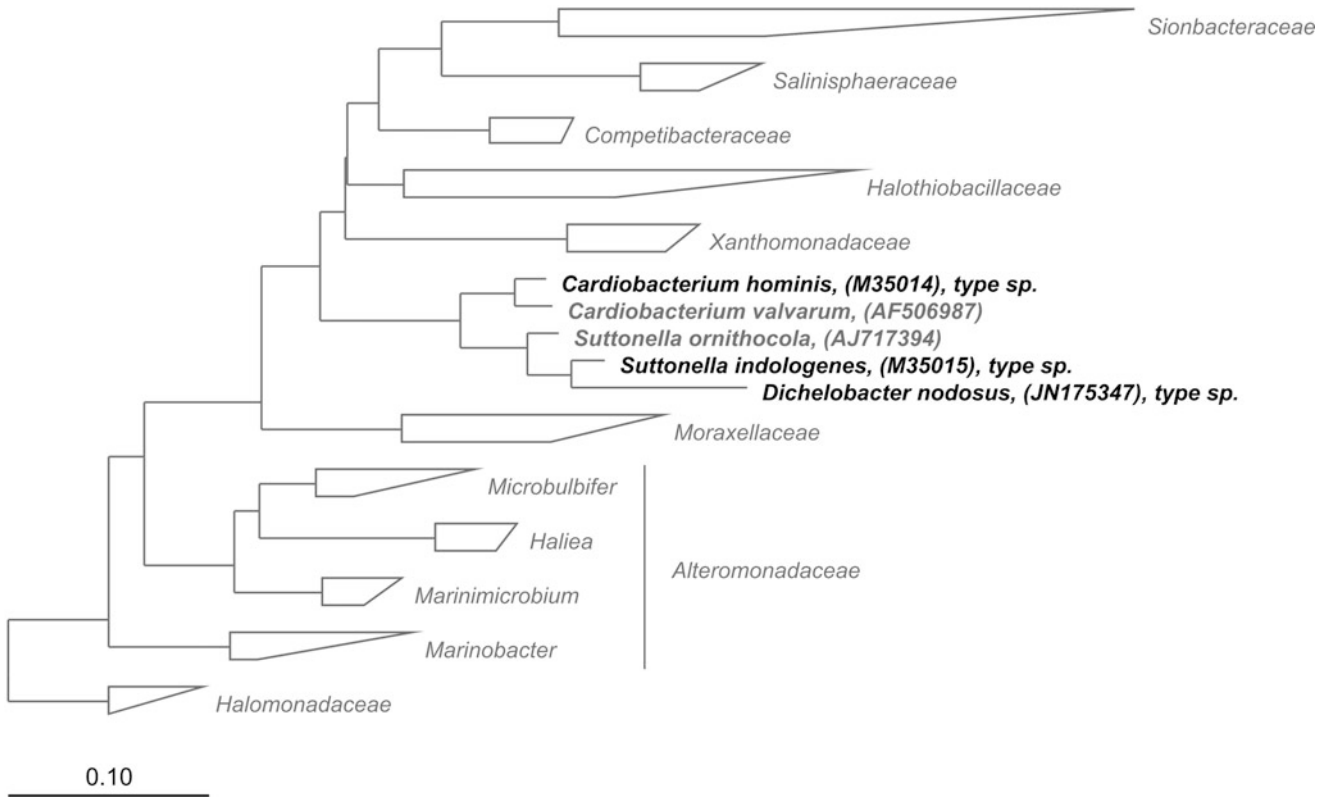

Fig. 7.1
The intra- and inter-generic phylogenetic relationships of the Cardiobaceriaceae, derived from comparative 16S rRNA gene sequence analysis (Yarza et al. 2013)

Phylogenetic Structure of the Family and Its Genera

The family, *Cardiobacteriaceae*, within the order, *Cardiobacteriales*, within the class, *Gammaproteobacteria*, encompasses three genera, including five species. The basis for the establishment of *Cardiobacteriaceae* was the recognition, by comparative 16S rRNA sequence analyses, that some species of established genera did not belong within their respective genera; *Bacteroides nodosus* and *Kingella indologenes* were not phylogenetically related to the genera in which they had been classified, but were observed to be related to each other and to *Cardiobacterium hominis*, as distinct lineages within a common family (Dewhirst et al. 1990). The three genera produced a monophyletic lineage that branched deeply within the γ-division of the *Proteobacteria*. The estimated phylogenetic intra- and intergeneric relationships of the five species of the three genera of *Cardiobacteriaceae* and the phylogenetic position of *Cardiobacteriaceae* within the spectra of related taxa are shown in *Fig. 7.1*. From the 16S rRNA and rRNA gene sequence data maintained in the Living Tree Project (Yarza et al. 2013), the *Cardiobacteriaceae* are seen to be only distantly related to *Xanthomonadaceae*, *Halothiobacillaceae*, and *Moraxellaceae*. The 16S rRNA sequence similarities between the species of the genera and between the genera are shown in *Table 7.2*. The sequence similarity between the type strains of the two species of *Cardiobacterium*, *C. hominis* and *C. valvarum*, is 96.5 % (3.5 % sequence difference). The sequence similarity between the type strains of the two species of *Suttonella*, *S. indologenes* and *S. ornithocola*, is 95.1 % (4.9 % sequence difference). These overall levels of 16S rRNA sequence dissimilarity (3.5–4.9 %) are within the range observed between

Table 7.2
Similarity matrix of the 16S rRNA sequences of the species of the genera in the *Cardiobacteriaceae*

	1	2	3	4	5
1. *C. hominis*	100				
2. *C. valvarum*	96.5	100			
3. *D. nodosus*	90.9	90.9	100		
4. *S. indologenes*	91.6	91.5	93.1	100	
5. *S. ornithocola*	92.7	92.7	91.8	95.1	100

1. *Cardiobacterium hominis* ATCC 15826^T (M35014); 2. *Cardiobacterium valvarum* MDA 3079^T = CCUG 48245^T (AF506987); 3. *Dichelobacter nodosus* ATCC 25549^T = CIP 107042^T (JN175347); 4. *Suttonella indologenes* ATCC 25869^T (M35015); 5. *Suttonella ornithocola* B6/99/2^T = CCUG 49457^T (AJ717394)

species of a genus. The 16S rRNA sequence similarities between the three genera of *Cardiobacteriaceae* range from 90.9 % (between *Cardiobacterium* and *Dichelobacter*) to 93.1 % (between *Dichelobacter* and *Suttonella*).

Molecular Analyses

The initial descriptions of the species *Cardiobacterium hominis*, *Dichelobacter nodosus*, and *Suttonella indologenes* relied upon morphological and phenotypic characterizations, resulting in their classifications within genera that were tentative at the time and would later be shown to be incorrect. Molecular analyses elucidated the phylogenetic and taxonomic placements of the three genera.

Genomic DNA G+C Content

The G+C content of the genomic DNA of *Cardiobacterium hominis* was reported initially as 61.7 mol% (Hill et al. 2010), although slightly lower G+C contents of 59–60 mol% were reported later (Weaver 1984). The first taxonomic reviews of *C. hominis* noted these values to be significantly higher than the G+C contents of bacteria that were phenotypically similar and presumed to be related, i.e., *Bacteroides*, *Moraxella*, *Haemophilus*, *Actinobacillus*, *Kingella*, *Pasteurella*, etc., all of which exhibit G+C contents below 50 mol% (Lapage 1974; Weaver 1984). The genomic DNA G+C content also distinguishes *C. hominis* from the species of the other two genera within the *Cardiobacteriaceae* (Dewhirst et al. 1990) (*Table 7.1*). The G+C content of genomic DNA of *Dichelobacter nodosus* is 45 mol% (Dewhirst et al. 1990); the genomic DNA G+C content of *Suttonella indologenes* is 49 mol% (Dewhirst et al. 1990). There are no published reports of the genomic DNA G+C contents of *C. valvarum* or *S. ornithocola* having been determined. Subsequent analyses by whole genome sequence determination of *C. hominis* (ATCC 15826) ascertained the G+C content as 59.3 mol% (http://www.ncbi.nlm.nih.gov/nuccore/ACKY00000000.1/). The whole genome sequence analysis of *C. valvarum* (F0432) determined the G+C content as 58.2 mol% (http://www.ncbi.nlm.nih.gov/nuccore/AGCM00000000.1/). Whole genome sequence analysis of *D. nodosus* (VCS1703A) (Myers et al. 2007) determined the G+C content as 44.4 mol% (http://www.ncbi.nlm.nih.gov/nuccore/NC_009446.1) (*Table 7.1*).

Genomic Analyses

The genome sequences of two strains, representing both species of *Cardiobacterium*, have been determined. *Cardiobacterium hominis* ATCC 15826^T (the type strain of the species) and *C. valvarum* FO432 were sequenced as part of the Human Microbiome Project (HMP) (http://www.hmpdacc.org/). The genome sequence of *C. hominis* (NZ_ACKY01000000) is derived from 26 scaffolds (NZ_GG694025-NZ_GG694050) of overlapping sequence contigs. The total sequence length is 2,578,897 bp (59.3 mol% G+C content) with 2,632 putative genes detected. The sequence assembly comprises more than 90 % of the genome. The genome sequence of *C. valvarum* (AGC00000000) is derived from 98 sequence scaffolds (JH417875-JH417972) with a total sequence length of 2,553,535 bp (58.2 mol% G+C content) and 3,076 putative genes detected. The genome sequences represent reference genomes for the HMP.

The genome sequence of *Dichelobacter nodosus* VCS1703A was determined (Myers et al. 2007), representing the smallest genome of an obligately anaerobic bacterium, with approximately 20 % of the sequence derived from horizontal genetic transfer. The total sequence (NC_009446) is 1,389,350 bp (44.4 mol% G+C content) with 1,351 putative genes detected.

Phenotypic Analyses

Cardiobacteriaceae (Dewhirst et al. 1990). The species of the genera of the family are chemoorganotrophic, facultatively anaerobic strictly anaerobic or microaerophilic, oxidase-positive or oxidase-negative, and catalase-negative. Species of the genera exhibit fermentative metabolisms or no carbohydrate fermentation. When fermentation of sugars occurs, acid but not gas is produced. Species of the genera are urease-negative, negative for nitrate reduction and positive for production of H_2S. Cells stain Gram-negative are nonspore-forming and rod-shaped with rounded ends, ranging in size from 1.0 to 6.0 μm long and 0.5–1.7 μm in diameter. A comprehensive overview of the phenotypic features of all members of the *Cardiobacteriaceae* is found in *Table 7.3*.

Cardiobacterium (Paster and Dewhirst 2005). The species of the genus are chemoorganotrophic, facultatively anaerobic, oxidase-positive, and catalase-negative. The species exhibit strictly fermentative metabolism; acid, but not gas, is produced from the fermentation of some sugars. The species are positive for indole production, negative for nitrate reduction, urease-negative, and ornithine decarboxylase-negative. Cells are Gram-negative, nonmotile, rod-shaped with rounded ends, 1.0–4.0 μm long, and 0.5–1.0 μm in diameter; filaments sometimes occur. A comprehensive overview of the phenotypic features of the species of *Cardiobacterium* is found in *Table 7.3*.

Cardiobacterium hominis (Slotnick and Dougherty 1964; Paster and Dewhirst 2005). Cells of strains of *C. hominis* are Gram-negative, nonmotile, pleomorphic rod-shaped with rounded ends, 1.0–4.0 μm long, and 0.5–0.7 μm in diameter. A spectrum of cell morphological types has been observed, including pairs, chains, rosette clusters, as well as occasional filamentous forms. Colonies on blood agar are 1–2 mm in diameter after 72 h, appearing circular, convex, smooth, entire, glistening, opaque, and butyrous. Strains are typically cultivated on blood agar media with 5 % human, sheep, or horse blood. Strains are oxidase-positive, catalase-negative, positive for indole production, negative for nitrate reduction,

Table 7.3

Table of phenotypic features characterizing the species of the different genera of the *Cardiobacteriaceae*. + positive, − negative, *w* weak, *nd* not determined, and *D* differs among strains

Characteristic	*Cardiobacterium hominis*	*Cardiobacterium valvarum*	*Dichelobacter nodosus*	*Suttonella indologenes*	*Suttonella ornithocola*
Rods	+	+	+	+	+
Cell width (μM)	0.5−0.7	1.0	1.0−1.7	1.0	nd
Cell length (μM)	1−4	1−3	3−6	2−3	2−3
Gram-negative	+	+	+	+	+
Motility	−	−	−[a]	−	−
Aerobic	+	+	−	+	+
Oxidase	+	+	−	+	+
Catalase	−	−	−	−	+
Growth in 6 % NaCl	nd	nd	nd	−	+
Hemolysis	nd	nd	nd	−	+
Hydrolysis of tween 20	−	nd	nd	+	D
Hydrolysis of tween 80	−	nd	+	−	D
Alkaline phosphatase activity	−	nd	W	+	+
Arginine dihydrolase	−	−	−	−	−
DNase activity	−	nd	−	−	−
H_2S production	+	+	+	+	−
H_2S production (TSI)	nd	nd	nd	−	−
Indole production	+	+	−	+	−
Lysine decarboxylases	−	nd	−	−	−
Nitrate reduction	−	−	−	−	−
Nitrite reduction	D	nd	nd	+	−
Ornithine decarboxylases	−	nd	+	−	−
Urease production	−	−	−	−	−
Acid was produced from:					
Adonitol	−	nd	−	−	nd
Arabinose	−	nd	−	−	nd
Arabitol	−	nd	−	nd	nd
Cellobiose	−	nd	−	−	nd
Dextrin	nd	nd	−	+	+
Dulcitol	−	nd	−	−	nd
Erythritol	−	nd	−	−	nd
Ethanol	−	nd	−	−	nd
Fructose	+	D	−	+	+
Galactose	−	−	−	−	−
Glucose	+	+	−	+	+
Glycerol	−	nd	−	−	nd
Inositol	−	nd	−	−	−
Inulin	−	nd	−	nd	nd
Lactose	−	−	−	−	−
Levulose	+	nd	−	nd	nd
Maltose	+	−	−	+[b]	−
Mannitol	+	−	−	−	−
Mannose	+	+	−	+	+
Melezitose	−	nd	−	nd	nd

Table 7.3 (continued)

Characteristic	*Cardiobacterium hominis*	*Cardiobacterium valvarum*	*Dichelobacter nodosus*	*Suttonella indologenes*	*Suttonella ornithocola*
Melibiose	–	nd	–	–	nd
Raffinose	–	–	–	–	–
Rhamnose	–	nd	–	–	nd
Salicin	–	nd	–	–	–
Sorbitol	+	+	–	–	–
Sucrose	+	–	–	+	+
Trehalose	–	nd	–	–	+
Xylose	–	–	–	–	–
Ammonia produced from:					
Arginine, asparagine, serine, threonine	nd	nd	+	nd	nd
Phenylalanine, cystine, citrulline, ornithine	nd	nd	–	nd	nd

[a]No flagella observed but cells exhibit possible twitching motility
[b]The maltose reaction is weak and delayed (positive at 28 days) but is negative as determined by rapid (4–24 h) protocols (Dewhirst et al. 1990)

urease-negative, and ornithine decarboxylase-negative. Carbohydrates fermented by strains of *C. hominis* include glucose, levulose, maltose, mannitol, mannose, sorbitol, and sucrose. Glucose is fermented predominantly to lactic acid, with lesser amounts of pyruvate, formate, and propionate and a trace of ethanol produced; acetate is not produced and no CO_2 or H_2 is generated. Substrates not utilized by *C. hominis* include adonitol, arabinose, cellobiose, dulcitol, erythritol, galactose, glycerol, inositol, inulin, lactose, melezitose, melibiose, rhamnose, salicin, trehalose, and xylose. A comprehensive overview of the phenotypic features of *C. hominis* is found in *Table 7.3*.

Cardiobacterium valvarum (Han et al. 2004). Cells of strains of *C. valvarum* are Gram-negative, nonmotile, pleomorphic bacillus measuring 1.0–3.0 μm in length and approximately 1.0 μm in diameter, with varying morphologies, depending upon the cultivation conditions. Colonies on blood agar may reach 1 mm in diameter after 72 h, appearing round, elevated, opaque, smooth, and glistening. Strains are typically cultivated on blood agar media with 5 % human, sheep, or horse blood. *C. valvarum* is more fastidious than *C. hominis*, growing slower and producing smaller colonies on agar media. Carbohydrate utilization by strains of *C. valvarum* differentiating it from *C. hominis* include maltose, mannitol, and sucrose. A comprehensive overview of the phenotypic features of *C. valvarum* is found in *Table 7.3*.

Dichelobacter. Only one species has been described for the genus; the phenotypic description of the genus is the same as that of the species, *D. nodosus*.

Dichelobacter nodosus (Dewhirst et al. 1990; Rood et al. 2005). Strains of the species are obligately anaerobic but are not readily killed by exposure to oxygen; the addition of 10 % CO_2 enhances growth. Strains do not ferment carbohydrates; no acid or gas is produced from sugars. Strains are oxidase-negative, catalase-negative, coagulase-negative, indole-negative, urease-negative, negative for nitrate reduction, positive for production of H_2S, and positive for production of ornithine decarboxylase. Ammonia is produced from arginine, asparagine, serine, and threonine. Cells are Gram-negative, non-spore-forming, straight, or slightly curved rods, 3.0–6.0 μm long and 1.0–1.7 μm in diameter, with rounded ends, often with terminal swellings. The cells exhibit large numbers of polar fimbriae or pili that may be correlated with virulence and changes in colony morphologies (Weaver 1984). A comprehensive overview of the phenotypic features of *D. nodosus* is found in *Table 7.3*.

Suttonella (Dewhirst and Paster 2005b). The species are chemoorganotrophic, microaerophilic with enhanced growth in the presence of CO_2, acid, but not gas, is produced from the metabolism of some sugars. The species are oxidase-positive, catalase-positive or catalase-negative, positive or negative for indole production, negative for nitrate reduction, urease-negative, ornithine decarboxylase-negative, alkaline phosphatase-positive, and DNase-negative. Cells are Gram-negative, nonmotile, non-spore-forming, rod-shaped, 2.0–3.0 μm long, and 0.5–1.0 μm in diameter, with rounded ends; filaments sometimes occur. A comprehensive overview of the phenotypic features of the species of *Suttonella* is found in *Table 7.3*.

Suttonella indologenes (Dewhirst et al. 1990; Dewhirst and Paster 2005b). Cells of strains of *S. indologenes* are Gram-negative and nonmotile with no detectable flagella, although they may exhibit fimbriae and pili with twitching motility, pleomorphic rod-shaped with rounded ends, 2.0–3.0 μm long, and approximately 1.0 μm in diameter. Cell morphologies may exhibit pairs, chains, or rosette clusters. Colonies on blood agar grown under microaerophilic conditions are 0.1–0.5 mm in diameter after 24 h. Strains of *S. indologenes* are oxidase-positive, catalase-negative, positive for indole production, negative for

nitrate reduction, urease-negative, ornithine decarboxylase-negative, alkaline phosphatase-positive, and DNase-negative. Carbohydrates utilized by strains of *S. indologenes* include dextrin, fructose, glucose, maltose, mannose, and sucrose, with the production of acid. Substrates not utilized include adonitol, arabinose, cellobiose, dulcitol, ethanol, erythritol, galactose, glycerol, inositol, lactose, mannitol, melibiose, raffinose, rhamnose, salicin, sorbitol, starch, trehalose, and xylose. A comprehensive overview of the phenotypic features of *S. indologenes* is found in ❯ *Table 7.3*.

Suttonella ornithocola (Foster et al. 2005). Cells of strains of *S. ornithocola* are Gram-negative, nonmotile, pleomorphic rod-shaped with rounded ends, 2.0–3.0 µm long, and approximately 1.0 µm in diameter. Cell morphologies may exhibit pairs, chains, or rosette clusters. Colonies on blood agar grown under microaerophilic conditions are 0.1–0.5 mm in diameter after 24 h. Strains are oxidase-positive, catalase-positive, negative for indole production, negative for H_2S production, negative for nitrate reduction, urease-negative, ornithine decarboxylase-negative, alkaline phosphatase-positive, and DNase-negative. Carbohydrate utilization by strains of *S. ornithocola* differentiating it from S. indologenes include maltose and trehalose. A comprehensive overview of the phenotypic features of *S. ornithocola* is found in ❯ *Table 7.3*.

Isolation, Enrichment, and Maintenance Procedures

Strains of *Cardiobacterium hominis* and *C. valvarum* have been isolated from human blood samples and nose and throat samples, using media supplemented with 5 % human, horse, rabbit, or sheep blood, as well as on trypticase soy agar medium without blood enrichment. Chocolate agar, PPLO agar, Cystine-Heart agar, Casman's agar, and Brain-Heart Infusion agar with and without sodium polyanetholsulfonate (SPS) and *p*-aminobenzoic acid, casein soy broth with SPS, thioglycolate broth with SPS, and glucose broth also support growth (Slotnick and Dougherty 1964; Midgley et al. 1970). Colonies of *C. hominis* on blood agar are 1–2 mm in diameter after 72 h (Slotnick and Dougherty 1964), whereas *C. valvarum* will produce pinpoint colonies, approximately 0.2 mm in diameter at 48 h and 0.5 mm at 72 h; extended incubation does not result in colonies larger than 1.0 mm. Incubations are typically at 37 °C for 48–72 h. Strains of *Cardiobacterium* species may be preserved by lyophilization or by freezing.

Strains of *Dichelobacter nodosus* have been isolated under anaerobic conditions with media containing trypticase and also 0.02–0.05 M arginine, as well as 10 % horse serum, for maximum growth. Growth can be enhanced by cultivation in 10 % CO_2. Growth is best at pH 6.4–7.6 and at 37–45 °C and poor growth at 30 °C. After 2–6 days, colonies on agar media are 0.5–2.0 mm, appearing smooth, convex, translucent, or semi-opaque (Skerman 1989). Colonies may etch or pit the surface of agar media, producing a sunken appearance. Strains may be preserved by lyophilization or by freezing.

Strains of *Suttonella indologenes* and *S. ornithocola* are typically isolated in blood cultures or on blood agar medium supplemented with 5 % horse or sheep blood or chocolate agar medium, at 37 °C and under microaerophilic conditions. Aerobic growth is enhanced by 5 % CO_2 and high humidity atmosphere. After 48 h, colonies will appear 2–3 mm. Strains may be preserved by lyophilization or by freezing.

Pathogenicity, Clinical Relevance

Cardiobacterium is one of five genera comprising the "HACEK" bacteria (the acronym for "*Haemophilus*, *Actinobacillus*, *Cardiobacterium*, *Eikenella*, *Kingella*"). These Gram-negative bacteria constitute members of human flora that exhibit a capacity to cause infections of endocardium, often in children (Das et al. 1997; Feder et al. 2003). Endocarditis represents a life-threatening disease with substantial morbidity, as well as mortality. *C. hominis* has been reported to cause endocarditis but is a relatively uncommon etiological agent of this infection (Walkty 2005; Malani et al. 2006; Pousios et al. 2012; Chambers et al. 2013). However, because of their fastidious and slow growth, *C. hominis* may be a cause of culture-negative and undefined endocarditis (Baron et al. 2005). In addition to cardiac valve infections, *C. hominis* is the cause of other infections, including bacteremia, sepsis, septic arthritis, peritonitis, pericarditis, meningitis, osteomyelitis, periodontal infections, ocular infections, and abscesses, including brain abscess.

In assessing the risks of potential infection, several factors are thought to predispose patients to *C. hominis* endocarditis. Since the organism is a constituent of the normal flora of the oral cavity and upper respiratory tract (Slotnick and Dougherty 1964; Slotnick et al. 1964), oral, dental, esophageal, and respiratory tract procedures may be potential causes of bacteremia and have been cited as risk factors associated with endocarditis (Taubert and Dajani 2001). *C. hominis* is known to colonize the gastrointestinal tract of healthy individuals (Slotnick et al. 1964). Gastrointestinal endoscopy and colonoscopy have been reported as risk factors (Pritchard et al. 1991; Malani et al. 2006). Different structural cardiac abnormalities predispose to infection with *C. hominis*. Previous valve replacement, history of rheumatic heart disease, past endocarditis, ventricular septal defect, bicuspid aortic valve, congenital aortic valve disease, mitral valve prolapse with murmur, and dilated cardiomyopathy have all been described as potential to predisposition for cardiac lesions in cases of *C. hominis* endocarditis (Walkty 2005). Infection has been reported in men and women and in patients of ages 18 days to 82 years (Apisarnthanarak et al. 2002; Vidal-Lampurdanes et al. 2012). In cases of *C. hominis* endocarditis, the aortic and mitral valves are most often infected, leading to complications, including congestive heart failure, central nervous system embolism, arrhythmia, and mycotic aneurysm (Malani et al. 2006). *C. hominis* can cause pericarditis and pericardial effusion without affecting the cardiac valves (Kuzucu et al. 2005). *C. hominis* is rarely implicated in infections outside the vascular system, although sepsis without endocarditis has

been reported (Vidal-Lampurdanes et al. 2012). A patient on peritoneal dialysis with evidence of *C. hominis* infection as the cause of peritonitis but with no apparent endocarditis has been presented (Bhan et al. 2006). The first documented finding of *C. hominis* infection outside the cardiovascular system was in a patient with bacterial meningitis, although septic embolism from an underlying endocarditis was probably the source of the infection (Francioli et al. 1983). *C. hominis* has been isolated and identified from an abdominal abscess and blood of a patient with diabetes mellitus and adenocarcinoma (Rechtman and Nadler 1991). *C. hominis* valve endocarditis has also been shown to be presented as septic arthritis (Apisarnthanarak et al. 2002). *C. hominis* bacteremia has been described with unique features: pacemaker lead infection and osteomyelitis without valvular affection (Nurnberger et al. 1998). Recently, isolations have been reported from dacryocystitis with lacrimal abscess (Manderwad et al. 2014).

Initial isolates of *C. hominis* were ampicillin sensitive, although β-lactamase-producing strains of *C. hominis* have been identified. Since antimicrobial susceptibility testing may be difficult to perform on HACEK bacteria, the American Heart Association recommends that all microorganisms from the group be considered to be ampicillin resistant, with third-generation cephalosporins (ceftriaxone) the treatment of choice. Other options include ampicillin-sulbactam and fluoroquinolones (ciprofloxacin, levofloxacin, gatifloxacin, or moxifloxacin) (Baddour et al. 2005). The prognosis of patients diagnosed with *C. hominis* native and prosthetic valve endocarditis is favorable (Malani et al. 2006).

The first isolation of *C. valvarum* was from the blood sample of an insidious endocarditis patient with a sudden rupture of a cerebral aneurysm (Han et al. 2004). Several more cases of infective endocarditis caused by *C. valvarum* have been described worldwide (Han et al. 2004; Hoover et al. 2005; Bothelo et al. 2006; Geißdörfer et al. 2007; Gonzales et al. 2007; Hoffman et al. 2010; Vaněrkova et al. 2010; Chen et al. 2011). Most reported *C. valvarum* infective endocarditis cases had histories of recent dental procedures.

Among *Cardiobacteriaceae* genera other than *Cardiobacter* that have demonstrated pathogenicity, *Suttonella indologenes* has been isolated from blood samples of patients with endocarditis (Jenny et al. 1987; Yang et al. 2011; Özcan et al. 2011). *S. indologenes* endocarditis also has been associated with splenic infarction and lethal intracranial hemorrhage in a patient with aortic valve replacement (Özcan et al. 2011). The strain was sensitive to ampicillin, cephalosporins, and ciprofloxacin but resistant to imipenem and meropenem. The patient succumbed to respiratory arrest.

S. indologenes has been associated also with human eye infections (van Bijsterveld 1970; Sutton et al. 1972; Barberis et al. 2007). The type strain of the species (ATCC 25869^T = NCTC 10717^T) was isolated as the causative agent of angular conjunctivitis (Snell and Lapage 1976). The activities of 12 antimicrobial agents were tested on a strain of *S. indologens* associated with corneal abscess. The Minimum Inhibitory Concentrations (MICs) for antibiotics were determined as follows: penicillin, 0.002 μg/ml; ampicillin, 0.032 μg/ml; ampicillin-sulbactam, 0.032 μg/ml; cephalothin, 0.25 μg/ml; ceftriaxone, 0.004 μg/ml; ceftazidime, 0.008 μg/ml; amikacin, 0.75 μg/ml; gentamicin, 0.25 μg/ml; clarithromycin, 0.38 μg/ml; trimethoprim-sulfamethoxazole, 0.004 μg/ml; ciprofloxacin, 0.008 μg/ml; and levofloxacin, 0.012 μg/ml. The strain did not produce β-lactamase when tested by the nitrocefin test (Barberis et al. 2007). All strains of *S. indologenes* have been found to be sensitive to streptomycin, oxytetracycline, chloramphenicol, erythromycin, and many other commonly used antibiotics (van Bijsterveld 1970).

S. ornithocola is a causative agent of pulmonary disease in birds of the tit family (Foster et al. 2005; Kirkwood et al. 2005; Lawson et al. 2011). Incidents of tit mortality associated with this bacterial species have been reported only in Britain.

Dichelobacter nodosus (formerly "*Bacteroides*" *nodosus*) is the major agent of foot-rot, a contagious debilitating disease affecting the hooves of sheep and other cloven-hoofed animals, such as goats and cattle, with major economic significance to the meat and wool industries (Skerman 1989). *D. nodosus* has several described virulence factors, such as fimbriae, proteases, and outer membrane proteins. The fimbriae of *D. nodosus*, required for binding to epithelial cells, are involved also in the uptake of extracellular DNA and are part of an extracellular protease secretion system (Myers et al. 2007). *D. nodosus* extracellular protease secretion is particularly important, as the bacterium is not able to synthesize amino acids (Myers et al. 2007). Rather, *D. nodosus* derives its amino acids by importing them from digested extracellular protein. Outer membrane proteins of *D. nodosus* are not known to play a direct role in attacking the host; rather, they are thought to interfere with the host's immune response (Myers et al. 2007).

Foot-rot disease in Australia is categorized as benign or virulent; in cases of benign foot-rot, the strains of *D. nodosus* do not exhibit known virulence factors and only the interdigital skin is inflamed, whereas virulent *D. nodosus*, exhibiting virulence factors, produces under-running, or separation of the hard horn from the foot of the animal (Egerton and Parsonson 1969). In Europe, *D. nodosus* is not typically categorized into benign and virulent strains, and affected animals are typically classified by their clinical signs, making the occurrence of lameness and the severity of damage to the foot the key factors used in clinical diagnosis (Moore et al. 2005). In Spain, *D. nodosus* has been isolated from pigs, where the associated condition is known as "bush-foot" (Piriz et al. 1996).

Treatment of sheep with foot-rot infections includes topical medications, vaccination, and parenteral antibiotic therapy (Abbott and Lewis 2005). The application of antibiotics and other antibacterial solutions to sheep is inefficient and usually reserved for occasions when sheep are handled individually. The use of an antimicrobial footbath facilitates treating large numbers of sheep with frequent repetition of treatments (Stewart 1989). The most commonly used and most effective footbath solutions are zinc- and copper-based solutions (Beveridge 1941; Reed and Alley 1996).

The serological diversity among isolates of *D. nodosus* has constrained vaccine development because heterologous protection is limited; multi-serogroup infections within a flock of sheep are common and the serogroups in outbreaks are usually not determined (Egerton and Morgan 1972). The only commercial vaccine available in the European Union, USA, Malaysia, Australia, and New Zealand is multivalent (Footvax, Schering-Plough Animal Health Ltd.), containing ten serotypes (Abbott and Lewis 2005).

A number of broad-spectrum antibiotics have been shown to be effective against foot-rot, following intramuscular injection. Both monotherapy (erythromycin, oxytetracycline, enrofloxacin) and combined therapies (penicillin + streptomycin + oxytetracycline and lincomycin + spectinomycin) have been applied (Venning et al. 1990; Webb Ware et al. 1994; Jordan et al. 1996; Rendell and Callinan 1997; Píriz et al. 2001; Saglyan et al. 2008; Kaler et al. 2012). Antibiotic treatment has been shown to be effective against severe under-running lesions, with recovery rates greater than 85 % (Egerton et al. 1968; Venning et al. 1990; Grogono-Thomas et al. 1994; Jordan et al. 1996).

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8 The Family *Celerinatantimonadaceae*

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Abstract
The *Celerinatantimonadaceae* are members of the *Alteromonadales* in the *Gammaproteobacteria* Cramer et al. (Int J Syst Evol Microbiol 61:1053-1060, 2011). As such they are Gram negative, do not form endospores, and are motile by means of a single flagellum. *Celerinatantimonadaceae* is a monogeneric family containing at present a single species, *Celerinatantimonas diazotrophica*. Phylogenetic neighbors include the families *Alteromonadaceae* and *Colwelliaceae*.

Taxonomy, Historical, and Current

The *Celerinatantimonadaceae* of the *Alteromonadales* are Gram negative, do not form endospores, and are motile by means of a single flagellum. The family was established mainly on the basis of phylogenetic analyses and includes the genus *Celerinatantimonas* (Cramer et al. 2011). Neighboring families include the *Alteromonadaceae* and *Colwelliaceae*, and differentiating characteristics are given in *Table 8.1*.

Celerinatantimonadaceae Cramer, Haghshenas, Bagwell, Matsui, and Lovell 2011

Cel.er.i.na.tant.i.mo.nad.a´ ce.ae. N.G. fem. n. *Celerinatantimonas* type genus of the family, *-aceae* ending to denote a family; G.L. fem. pl. n. *Celerinatantimonadaceae* the *Celerinatantimonas* family.

Type genus: *Celerinatantimonas* Cramer, Haghshenas, Bagwell, Matsui, and Lovell 2011.

Molecular Analyses

Phylogenetic Structure of the Family

The phylogenetic analysis (*Fig. 8.1*) indicates that *Celerinatantimonadaceae* is a distinct clade within the *Alteromonadales* and has no close sister clades. The family was created by Cramer et al. (2011) on the basis of phylogenetic position and contains a single genus, *Celerinatantimonas*, which contains a single species, *diazotrophica*. 16S rRNA gene sequences from all strains reported to date were 99–100 % similar to that of the type strain S-G2-2^T.

Genome Analyses

No *Celerinatantimonas* genomes have been sequenced to date. GC content of DNA ranged from 41.5 to 44.4 mol%, determined using the HPLC method of Mesbah et al. (1989). DNA-DNA hybridization studies have been performed on several strains of *C. diazotrophica*, and all hybrid genome pairings yielded a $\Delta T_m \leq 1.2$ °C.

Phenotypic Analyses

Gram-negative, facultatively anaerobic, motile, polarly monotrichous thick rods. Colonies grown on Bacto Marine Agar were tiny, circular, convex, and colorless. Catalase positive, oxidase negative, and required sea water salts or NaCl and mesophilic conditions. Nitrate was not reduced. The G + C content is 41.5–44.4 mol% (*Table 8.1*). A wide variety

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Table 8.1
Diagnostic properties in which the family *Celerinatantimonadaceae* differs from neighboring families in the *Gammaproteobacteria*

Characteristic	*Celerinatanti-monadaceae*	*Alteromonadaceae*		*Colwelliaceae*		*Pseudoalteromonadaceae*		*Ferrimon-adaceae*	*Idiomarin-aceae*	*Moritell-aceae*	*Psychromon-adaceae*	*Shewanel-aceae*
		Alteromonas	*Glaciecola*	*Colwellia*	*Thalass-omonas*	*Pseudoaltero-monas*	*Algicola*					
Growth parameters												
Requires NaCl	+	+	+	+	+	+	+	+	+	+	–	–
Halotolerance (% NaCl)	8	6	8	20	3	15	6	7.5	15	4	4	10
Growth at (°C)												
4	–	–	+	+	–	+	–	–	+	+	+	+
37	+	+	–	–	+	+	–	+	–	–	–	–
42	+	–	–	–	–	–	–	+	–	–	–	–
Nitrate reduction	–	–	–	+	–	V	–	+	–	+	V	+
Oxidase activity	–	+	+	+	+	V	+	+	+	+	+	+
Catalase activity	+	+	+	+	+	V	+	+	+	+	+	+
DNA G + C content (%mol)	41.5–44.4	44–48	40–46	35–46	48.4	37–50	44–46	54	48.9–50.4	44–45	42.8	38–54

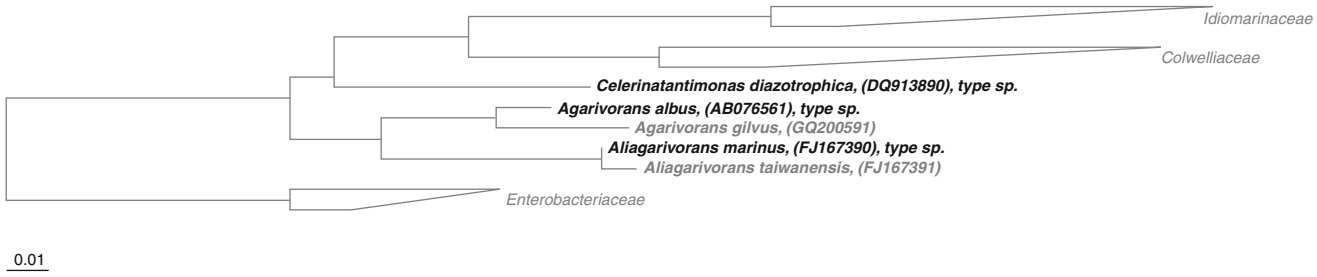

Fig. 8.1
Neighbor-joining phylogenetic analysis showing relationships

of carbohydrates and carboxylic acids were utilized and acid was produced from glucose. Few amino acids or betaines were used. Key chemotaxonomic characters are shown in *Table 8.1*.

The type species is *Celerinatantimonas diazotrophica* Cramer 2011. The type strain is S-G2-2^T (= ATCC BAA-1368^T = DSM 19577^T).

According to API 20E test strips, the type strain *C. diazotrophica* ATCC BAA-1368^T expressed β-galactosidase activity, utilized citrate, and fermented D-glucose with acid products. According to API 20NE test strips, β-glucosidase was expressed and *N*-acetyl-glucosamine, L-arabinose, gluconate, malate, maltose, D-mannitol, and D-mannose were also utilized. According to the BIOLOG GN2 substrate panel, the following substrates were also used: acetic acid, *cis*-aconitic acid, D-arabitol, bromosuccinic acid, D-cellobiose, dextrin, D-fructose, D-galactose, D-galacturonic acid, gentiobiose, D-gluconic acid, D-glucuronic acid, glucuronamide, L-glutamic acid, glycerol, glycogen, *m*-inositol, α-ketoglutaric acid, D,L-lactic acid, D-lactose, lactulose, D-melibiose, β-methyl-D-glucoside, L-proline, D-psicose, D-rhamnose, succinic acid, sucrose, and D-trehalose. According to API Zyme test strips, all strains produced acid and alkaline phosphatases, esterase, esterase lipase, leucine arylamidase, α-chymotrypsin, naphthol-AS-B1-phosphohydrolase, α- and β-galactosidase, and β-glucosidase. In addition, strain S-G2-2^T produced *N*-acetyl-β-glucosaminidase (chitinase).

Temperature limits for growth of *C. diazotrophica* are 17–49 °C, with optimal growth at 31 °C. NaCl concentrations for growth are 2.5–8.0 %, with optimal growth at 7.0–7.5 %. The pH range for growth is 3.5–8.0, with optimal growth at pH 6.0. It does not produce amylase, gelatinase, casein protease, or lipase. Catalase positive, oxidase negative. Polar lipids included diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine, aminophospholipid, phospholipids, and unidentified aminolipids. Major menaquinone and ubiquinones were MK8 (100 %) and Q8 (93 %), respectively. Predominant fatty acids present were $C_{12:0}$ aldehyde and/or unknown fatty acid 10.9525 (MIDI designation) and/or $C_{16:1}$iso I/$C_{14:0}$3OH, $C_{16:1}$ω7c/$C_{16:1}$ω6c, $C_{16:0}$, $C_{17:0}$cyclo, and $C_{18:1}$ω7c and/or C18:1ω6c. The near full-length 16S rRNA gene sequences of strains were very similar (99–100 % similarity), and strains were identified as members of the same species by DNA-DNA relatedness. 16S rRNA analysis of strains formed a monophyletic lineage within the order *Alteromonadales*. All strains fixed atmospheric N_2. Partial *nifH* gene sequences also formed a monophyletic lineage within the *Gammaproteobacteria* and were dissimilar to any previously described diazotroph (Cramer et al. 2011). Differences between *C. diazotrophica* and genera belonging to the *Alteromonadales* include the inability to produce cytochrome oxidase and growth at 42 °C.

All known representatives are capable of nitrogen fixation. The only genus is *Celerinatantimonas*, which was described in 2011.

Isolation, Enrichment, and Maintenance Procedures

Isolation and Enrichment

Celerinatantimonas diazotrophica was isolated from the roots of the salt marsh plants *Juncus roemerianus* (black needle rush) and *Spartina alterniflora* (smooth cordgrass) by stab inoculating whole roots into combined nitrogen free semi-solid tube media that employed glucose as carbon and energy source and were adjusted to pH 7.0 or 7.5 (Bagwell et al. 1998). Enrichments were incubated at 30 °C for 48 h. Pure cultures were isolated by streaking outgrowth from the roots on plates of the combined nitrogen free media.

Maintenance

Cultures can be refrigerated for short-term storage. Medium-term storage requires supplementation of broth cultures with 5 % dimethylsulfoxide and 5 % glycerol, followed by freezing at −80 °C. Long-term storage is by lyophilization or in liquid nitrogen. Strains can be routinely cultivated on Bacto Marine Broth (BD Biosciences) supplemented with 15 g l^{-1} Bacto Agar or on Luria-Bertani Broth supplemented with 28 g NaCl l^{-1} (to meet the salinity requirement of these organisms) and 15 g l^{-1} Bacto Agar (Cramer et al. 2011).

Ecology

All strains were isolated from roots of the salt marsh grasses *Juncus roemerianus* or short form *Spartina alterniflora* collected from Goat Island in the North Inlet salt marsh near Georgetown, SC, USA (79° 12′W, 33° 20′N) (see Dame and Kenny 1986; Morris and Haskin 1990 for site descriptions). Isolation of these strains employed combined nitrogen-free media and the strains are all diazotrophic. The importance of nitrogen fixation in the nitrogen-limited salt marsh ecosystem is well understood (Hanson 1983; Patriquin and McClung 1978; Whiting and Morris 1986). The ability of *C. diazotrophica* strains to use a wide range of carbohydrates and carboxylic acids is consistent with the rhizoplane environment from which they were isolated as these compounds are abundant in plant root exudates (e.g., Boyle and Patriquin 1981; Hale et al. 1978). The only amino acid used by all strains of *C. diazotrophica* was proline, a common osmolyte which may be of particular importance in an environment subject to periodically high salinity.

Diazotrophs are remarkably diverse in salt marsh ecosystems (Gamble et al. 2010; Lovell and Davis 2012; Lovell et al. 2000, 2008), and the functional role of some organisms has been established from recovery of mRNA expressed from *nifH*, the gene that encodes nitrogenase reductase (Brown et al. 2003). No expressed *nifH* sequence was highly similar to that from *C. diazotrophica*, but a comprehensive survey has not been performed. Sequences of *nifH* from the *C. diazotrophica* strains were allied with those from authentic *Gammaproteobacteria*, but formed a monophyletic grouping well separated from sequences from formally described diazotrophs (Cramer et al. 2011).

Pathogenicity, Clinical Relevance

C. diazotrophica has no known pathogenic features or clinical relevance.

Application

As a salt tolerant diazotroph, *C. diazotrophica* may have value as a biofertilizer in saline soils, but no application has appeared to date.

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9 The Family *Chromatiaceae*

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Abstract
The *Chromatiaceae* is a family of the *Chromatiales* within the Gammaproteobacteria and closely related to the *Ectothiorhodospiraceae*. Representatives of both families are referred to as phototrophic purple sulfur bacteria and typically grow under anoxic conditions in the light using sulfide as photosynthetic electron donor, which is oxidized to sulfate via intermediate accumulation of globules of elemental sulfur. In *Chromatiaceae* species, the sulfur globules appear inside the cells; in *Ectothiorhodospiraceae*, they are formed outside the cells and appear in the medium. Characteristic properties of these bacteria are the synthesis of photosynthetic pigments, bacteriochlorophyll *a* or *b*, and various types of carotenoids and the formation of a photosynthetic apparatus with reaction center and antenna complexes localized within internal membrane systems. Phototrophic growth, photosynthetic pigment synthesis, and formation of the photosynthetic apparatus and internal membranes are strictly regulated by oxygen and light and become derepressed at low oxygen tensions. Typically, *Chromatiaceae* are enabled to the photolithoautotrophic mode of growth. A number of species also can grow photoheterotrophically using a limited number of simple organic molecules. Some species also can grow under chemotrophic conditions in the dark, either autotrophically or heterotrophically using oxygen as terminal electron acceptor in respiratory processes.

Taxonomy, Historical and Current

The *Chromatiaceae* represent a family of the *Chromatiales* order in the Gammaproteobacteria. *Chromatiaceae* species are anaerobic bacteria performing an anoxygenic mode of photosynthesis, use a special membrane-bound photosynthetic apparatus, and synthesize specific bacteriochlorophyll molecules and carotenoids as photosynthetic pigments. For their photosynthetic mode of growth, they are dependent on the availability of light. Some species are able to perform a dark respiratory metabolism in addition to the performance of photosynthesis in the light. Sulfide is an important photosynthetic electron donor and enables photolithoautotrophic growth.

Chemotaxonomic data and phylogenetic considerations have led to a redefinition of the *Chromatiaceae*, and this family

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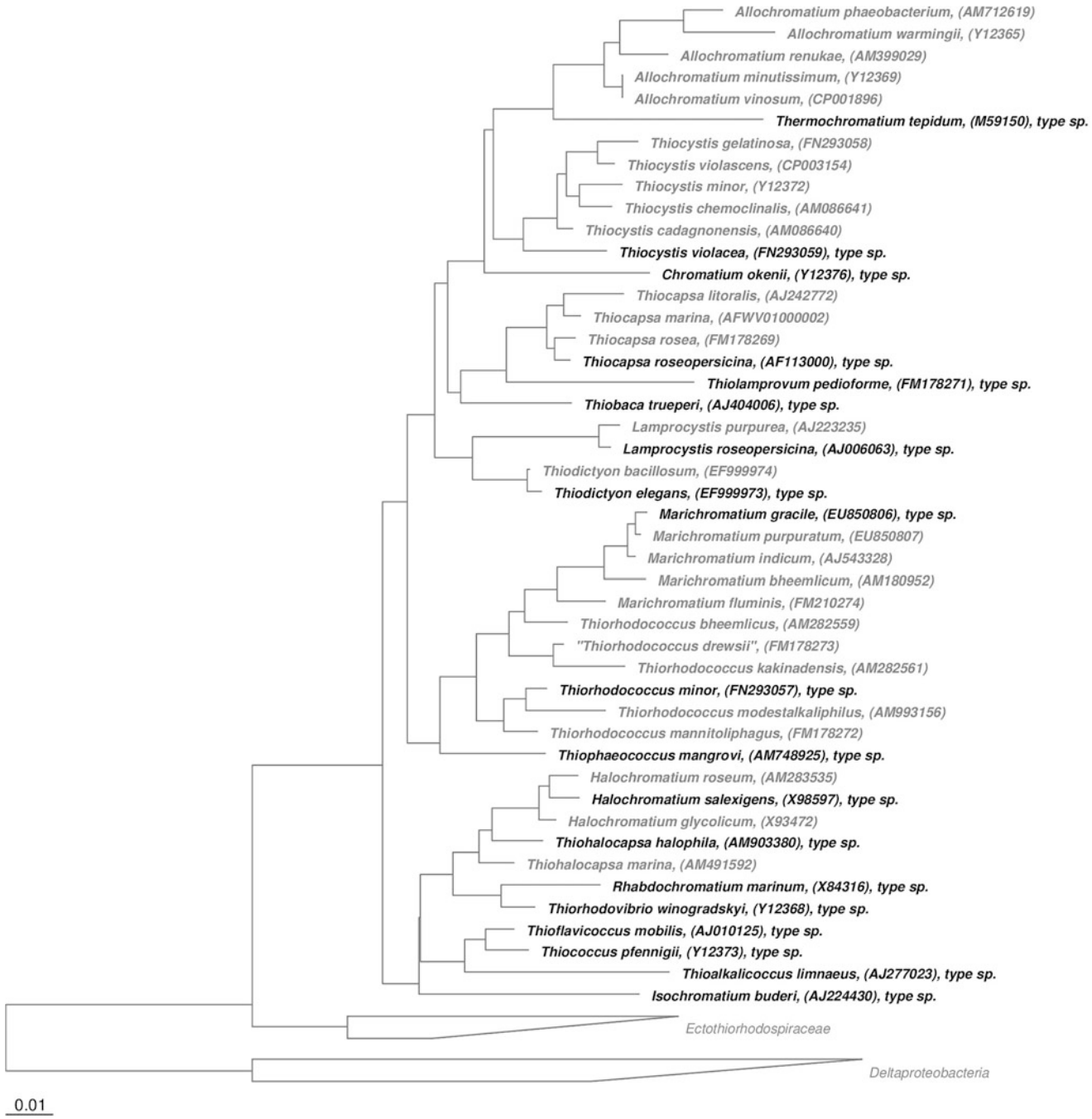

Fig. 9.1

Phylogenetic tree of the *Chromatiaceae* family. The construction is based on 16S rRNA and created using the PHYML algorithm (Guindon and Gascuel, 2005). The sequence dataset and alignment were used according to the All-Species Living Tree Project (LTP) database (Yarza et al., 2010; http://www.arb-silva.de/projects/living-tree) and Straininfo project (Dawyndt et al., 2006; http://www.straininfo.net). Representative sequences from closely related taxa were used as outgroups. The scale bar indicates estimated sequence divergence

includes those phototrophic purple sulfur bacteria that deposit elemental sulfur inside the cells (Imhoff 1984a). This definition agrees with Molisch's (1907) definition of the "*Thiorhodaceae*." It is in contrast to that of Bavendamm (1924) who included all phototrophic purple bacteria using sulfide as photosynthetic electron donor and accumulating globules of elemental sulfur, either inside or outside the cells (Pfennig and Trüper 1971, 1974). The separation from *Ectothiorhodospiraceae*, anoxygenic

phototrophic purple sulfur bacteria, which accumulate the elemental sulfur outside the cells, is in agreement with the clear phylogenetic separation of the two families (Fowler et al. 1984; Imhoff 1984a; Imhoff and Süling 1996; Guyoneaud et al. 1998; Imhoff et al. 1998).

Exclusion from the Chromatiaceae: Despite the mentioning of several online portals of the genera *Rheinheimera* and *Arsukibacterium*, these two form a separate phylogenetic lineage together with *Alishewanella*, which is clearly outside the frame of the family *Chromatiaceae*. There has been no formal inclusion into the family *Chromatiaceae* and both phenotypic properties and phylogenetic data do preclude the inclusion into the *Chromatiaceae*. The same holds for the genus *Nitrosococcus*.

Differentiation of *Chromatiaceae* and *Ectothiorhodospiraceae*

The two families of the *Chromatiales* order, the *Chromatiaceae* and *Ectothiorhodospiraceae*, include the phototrophic bacteria traditionally known as purple sulfur bacteria. The most easily recognized distinguishing property between *Chromatiaceae* and *Ectothiorhodospiraceae* is the deposition of elemental sulfur during growth on sulfide. Elemental sulfur globules accumulate as an intermediate oxidation product and appear microscopically visible either inside (*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 species of the *Ectothiorhodospiraceae*, *Thiorhodospira sibirica*, sulfur globules remain attached to the cells and, according to microscopic observations, appear in the cell periphery or the periplasmic space of the cells (Bryantseva et al. 1999).

Species of both families can be differentiated by the form of the internal membrane system, vesicles, or tubules in *Chromatiaceae* and stacks in *Ectothiorhodospiraceae*. The preference for alkaline pH and marine to extremely high salt concentrations is a typical property for *Ectothiorhodospiraceae* species, while almost neutral pH and freshwater, brackish water or marine, in one cluster also hypersaline conditions are typical for most *Chromatiaceae*. Alkaline salt lakes typically are inhabited by *Ectothiorhodospiraceae* species, while marine salterns and hypersaline coastal lagoons give rise to the development of halophilic *Chromatiaceae*. The salt response is a property of taxonomic relevance of *Chromatiaceae* species (see Imhoff 2001).

Marked differences between representatives of the two families are seen in their phylogenetic properties (sequence similarities of *16S rRNA* and *pufLM* genes) and depicted in a number of characteristic signatures and in their overall sequence distance.

A clear distinction of the two families is also possible 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 *Ecto-thio-rhodo-spira* (Imhoff et al. 1982). While C-16 fatty acids (C-16:1 and C-16:0) together with C-18:1 are the major components in *Chromatiaceae*, C-18 fatty acids (in particular C-18:1) are clearly dominant in *Ectothiorhodospiraceae* and C-16:1 is only a minor component in this latter group (Imhoff and Bias-Imhoff 1995). In addition, the lipopolysaccharides show significant differences between members of the two families (Weckesser et al. 1979, 1995). The lipid A of *Chromatiaceae* (*Allochromatium vinosum*, *Thermochromatium tepidum*, *Thiocystis violacea*, *Thiocapsa roseopersicina*, *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 *Ecto-thio-rho-do-spi-ra* (*Ect. vacuolata*, *Ect. shaposhnikovii*, *Ect. haloalkaliphila*, *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 (Weckesser et al. 1995; Zahr et al. 1992). These distinctive properties of the lipid A are characteristic features of the two families.

Genera and Species of *Chromatiaceae*

According to their phylogenetic relationship, the taxonomic relevance of a number of phenotypic properties was reevaluated and several *Chromatiaceae* species have been reassigned to other and in part new genera (Guyoneaud et al. 1998; Imhoff et al. 1998). In the traditional classification, easily recognizable properties such as cell morphology, motility, presence of gas vesicles, and formation of cell aggregates had been of major relevance (Pfennig and Trüper 1971, 1974, 1992). Because this classification was in clear contrast to the phylogenetic relationships of *Chromatiaceae* species, a reclassification was proposed on the basis of similarities of 16S rRNA gene with support of selected phenotypic properties (Guyoneaud et al. 1998; Imhoff et al. 1998). On this basis, during the past 10 years, one new genus and a number of new species of the *Chromatiaceae* have been described. These include the new genus and species *Thiophaeococcus mangrovi* (Kumar et al. 2008a) and the new species *Allochromatium phaeobacterium* (Srinivas et al. 2009), *Allochromatium renukae* (Kumar et al. 2008b), *Halochromatium roseum* (Kumar et al. 2007a), *Marichromatium fluminis* (Sucharita et al. 2010a), *Marichromatium bheemlicum* (Kumar et al. 2007a), *Marichromatium indicum* (Arunasri et al. 2005), *Thiobaca trueperi* (Rees et al. 2002), *Thiocapsa marina* (Caumette et al. 2004), *Thiocystis cadagnonensis* (Peduzzi et al. 2011), *Thiocystis chemoclinalis* (Peduzzi et al. 2011), *Thiohalocapsa marina* (Kumar et al. 2009), *Thiorhodococcus bheemlicus* (Kumar et al. 2007b), *Thiorhodococcus kakinadensis* (Kumar et al. 2007b), *Thiorhodococcus mannitoliphagus* (Rabold et al. 2006), and *Thiorhodococcus modestalkaliphilus* (Sucharita et al. 2010b). A number of well-characterized new bacteria appear as invalidly described species according to the Code of Nomenclature of bacteria (Lapage et al. 1992). These include the

species *Marichromatium chrysaorae* and *Marichromatium litoris* (Shivali et al. 2011), *Thiocapsa bogorovii* (Tourova et al. 2009), *Thiocapsa imhoffii* (Asao et al. 2007), and *Thiorhodococcus drewsii* (Zaar et al. 2003).

The genera and species of the *Chromatiaceae* and some selected characteristic properties are shown in Table 9.1.

Phylogeny and Molecular Analyses

Phylogenetic analyses, phenotypic characteristics, and chemotaxonomic information form the basis for the taxonomy of the *Chromatiaeceae* (Caumette et al. 1997; Guyoneaud et al. 1998; Imhoff et al. 1998). Positive correlation between similarities of 16S rRNA gene 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 informations for classification (Imhoff et al. 1998).

Major phylogenetic branches of the *Chromatiaceae* are as follows:

1. One branch is formed by *Allochromatium* and *Thermochromatium*, *Thiocystis* species together with *Chromatium okenii*. Cells are rod-shaped or spheres, motile by polar flagella, do not contain gas vesicles, and are primarily freshwater species without requirement for salt, though some may tolerate low salt concentrations and thrive in brackish and marine habitats.

   Most of these bacteria do not have a specific requirement for salt and according to the definitions given by Imhoff (2001) 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 bacteria, they may compete with the marine species of purple sulfur bacteria, in particular at low salt concentrations.
2. In a second branch related to this, the *Thiocapsa* species, *Thiolamprovum* and *Thiobaca* are found and associated to these are *Lamprocystis* and *Thiodictyon* species.

   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. In addition, *Thiodictyon* species are associated to this group. Also bacteria of this group are primarily freshwater bacteria. Some species may tolerate low salt concentrations and their salt tolerance may be sufficient to enable development and competition in coastal habitats. *Thiocapsa litoralis* (Puchkova et al. 2000) even can grow better at minor salt concentrations (1 % NaCl) than in its complete absence.
3. A third major branch is formed by the marine rod-shaped *Marichromatium* and spherical *Thiorhodococcus* species. Species of both genera are typical marine bacteria motile by flagella. Associated to these two genera is *Thiophaeococcus mangrovi*.
4. Another major branch which is more distant to the others includes the halophilic *Halochromatium* and *Thiohalocapsa* species, the marine *Thiorhodovibrio* and *Rhabdochromatium*, the marine *Isochromatium*, as well as the bacteriochlorophyll b-containing genera *Thiococcus*, *Thioflavicoccus*, and *Thioalkalicoccus* in distinct lines. *Thiococcus pfennigii*, *Thioalkalicoccus sibiricus*, and *Thioflavicoccus mobilis*, in contrast to all other *Chromatiaceae*, have tubular internal membranes (Bryantseva et al. 2000; Imhoff and Pfennig 2001).

The 16S rRNA gene sequences from *Thiospirillum jenense*, *Lamprobacter modestohalophilus*, and *Thiopedia rosea* are not yet available.

Phenotypic Properties

The phenotypic appearance of *Chromatiaceae* species is characterized by the presence of photosynthetic pigments, which colorize cultures and colonies and in nature are responsible for the formation of colored blooms of these bacteria, when appearing in masses. Pigments of *Chromatiaceae* are bacteriochlorophyll *a* or bacteriochlorophyll *b* (in a distinct phylogenetic line with three genera) and various types of carotenoids. These pigments are localized in photosynthetic membranes originating from the cytoplasmic membrane and forming either vesicles in most of the *Chromatiaceae* or tubules in the species with bacteriochlorophyll b. These internal membranes can be seen with the electron microscope.

Chromatiaceae are variable in cell size and shape (sphere, rod, vibrio, spirillum), in motility (motile by polar flagella or nonmotile), and the presence of gas vesicles, and some species may form cell aggregates.

Some properties of the *Chromatiaceae* species are shown in Table 9.1. More detailed information, including the utilization of substrates, relations 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, are found in the species descriptions and in chapters on *Chromatiaceae* of *Bergey's Manual of Systematic Bacteriology* (Imhoff 2005; Pfennig 1989a).

The physiology of *Chromatiaceae* is clearly dominated by the photosynthetic way of life and anoxygenic photosynthesis under anaerobic conditions. Many can grow photolithoautotrophically with reduced sulfur compounds or hydrogen as electron sources and photoassimilate a small number of simple organic molecules. Some species are quite restricted in the use of organic compounds, while others grow very well as photoheterotrophic bacteria.

Table 9.1
Selected characteristics of genera and species of the *Chromatiaceae*

Genera	Species	Motile	Gas vesicles	Cell form and size [um]	Optimum salinity	Topt [°C]	Vitamins required	G+C content [mol%]	Chemolithotrophy	Carotenoid group
Chromatium		m	No	Rod	bchl a, vesicular internal membranes					
Chr.	*Okenii*			4.5–6.0	None	20–35	B_{12}	48.0–50.0	No	Ok
Chr.	*Weissei*			3.5–4.0	None	20–35	B_{12}	48.0–50.0	No	Ok
Thiocystis		m	No	sphere/rod	bchl a, vesicular internal membranes					
Tcs.	*Gelatinosa*			3.0	None	30	None	61.3	Yes	Ok
Tcs.	*Minor*			2.0	None	30	None	62.2	Yes	Ok
Tcs.	*Violacea*			2.5–3.5	o	25–35	None	62.8–67.9	Yes	ra
Tcs.	*Violascens*			2.0	o	30–35	None	61.8–64.3	Yes	ra
Tcs.	*Chemoclinalis*			2.3–2.6	None	20		68.1	Yes	Ok
Tcs.	*Cadagnonensis*			2.3–4.7	None	20		69.5	Yes	Ok
Allochromatium		m	No	Rod	bchl a, vesicular internal membranes					
Alc.	*Vinosum*			2.0	o	30–35	None	61.3–66.3	Yes	sp
Alc.	*Minutissimum*			1.0–1.2	None	30–35	None	63.7	Yes	sp
Alc.	*Warmingii*			3.5–4.0	None	25–30	B_{12}	55.1–60.2	No	ra
Alc.	*Phaeobacterium*			1.0–1.5	None	30	None	59.8	No	ra
Alc.	*Renukae*			2.0–2.5	None	28–32	None	62.3	No	ly
Thermochromatium		m	No	Rod	bchl a, vesicular internal membranes					
Tch.	*Tepidum*			1–2	None	48–50	None	61.5	No	sp
Thiophaeococcus		m	No	Sphere	bchl a, vesicular internal membranes					
Tph.	*Mangrovi*			2.0–2.5	1 %	20–35	YE	68.5	No	ly
Thiocapsa		No		Sphere	bchl a, vesicular internal membranes					
Tca.	*Roseopersicina*		No	1.2–3.0	o	20–35	None	63.3–66.3	Yes	sp
Tca.	*Rosea*		GV	2.0–3.0	None	20–35	B_{12}	64.3	Yes	sp
Tca.	*Pendens*		GV	1.5–2.0	None	20–35	B_{12}	65.3	No	sp
Tca.	*Litoralis*		no	1.5–2.5	1 %	30	B_{12}	64	Yes	sp
Tca.	*Marina*		no	1.5–3.0	1–2 %	30–35	None	62.7–63.2	Yes	Ok
Tca.	*Imhoffii*			1.7–2.0	None	32	None	nd	No	sp
Tca.	*Bogorovi*			1.0–1.5	1–2 %	nd	B_{12}	66.7	Yes	sp
Thiolamprovum		no	GV	Sphere	bchl a, vesicular internal membranes					
Tlp.	*Pedioforme*			2.0	None	37	None	65.5	Yes	sp
Thiobaca		m	No	Rod	bchl a, vesicular internal membranes					
Tba.	*Trueperi*			1.6	None	25–30	o	62.9–63.9	O	ly

Table 9.1 (continued)

Genera	Species	Motile	Gas vesicles	Cell form and size [um]	Optimum salinity	Topt [°C]	Vitamins required	G+C content [mol%]	Chemolithotrophy	Carotenoid group
Lamprocystis				Sphere	bchl a, vesicular internal membranes					
Lpc.	*Roseopersicina*	m	GV	3.0–3.5	None	20–30	None	63.8	No	ra (la, lo)
Lpc.	*Purpurea*	No	GV	1.9–2.3	None	23–25	o	63.5	Yes	Ok
Thiodictyon		No	GV	Rod	bchl a, vesicular internal membranes					
Tdc.	*Elegans*			1.5–2.0	None	20–25	None	65.3	No	ra
Tdc.	*Bacillosum*			1.5–2.0	None	20–30	None	66.3	No	ra
Thiorhodococcus		m	No	Sphere	bchl a, vesicular internal membranes					
Trc.	*Minor*			1.0–2.0	2 %	30–35	None	66.9	Yes	sp
Trc.	*Bheemlicus*			4.0–6.0	1.0–3.0 %	25–30	None	65.5	No	(sp)
Trc.	*Drewsii*			2.0–3.5	2.4–2.6 %	30–35	None	64.5	nd	sp
Trc.	*Kakinadensis*			3.0–5.0	1.0–2.0 %	25–30	n,b,p	57.5	No	(sp)
Trc.	*Mannitoliphagus*			1.5–2.5	0.5–2.0 %	25–30	B_{12}	61.8	No	sp
Trc.	*Modestoalkaliphilus*			1.5–2.5	1.5 %	25–30	none	57.8	No	ly
Marichromatium		m	No	Rod	bchl a, vesicular internal membranes					
Mch.	*Gracile*			1.0–1.3	2–3 %	30–35	None	68.9–70.4	Yes	sp
Mch.	*Purpuratum*			1.2–1.7	5 %	25–30	o	68.4–68.9	No	Ok
Mch.	*Bheemlicum*			0.8–1.0	1.5–8.5 %	30–35	PP	67	No	(sp)
Mch.	*Fluminis*			0.4–0.5	1.5–3 %	30–35	None	71.4	No	rh
Mch.	*Indicum*			0.8–1.0	1–4 %	30–35	None	67.1	CLH	sp
Mch.	*Litoris*			1–1.2	2–4 %	25–30	None	68.4	no	sp
Mch.	*Chrysaorae*			1–1.3	2–5 %	25–35	None	70.5	CLH	sp
Halochromatium		m	No	Rod	bchl a, vesicular internal membranes					
Hch.	*Salexigens*			2.0–2.5	8–11 %	20–30	B_{12}	64.6	Yes	sp

Hch.	*Glycolicum*			0.8–1.0	4–6 %	25–35	None	66.1–66.5	Yes	sp
Hch.	*Roseum*	No	GV	2.0–3.0	1.5–2.5 %	27	B_{12}	64	No	Ok
Thiohalocapsa		No	No	Sphere	bchl a, vesicular internal membranes					
Thc.	*Halophila*			1.5–2.5	4–8 %	20–30	B_{12}	65.9–66.6	Yes	Ok
Thc.	*Marina*			1.5–2.0	2 %	25–30	None	64.8	No	(Ok)
Rhabdochromatium		m	No	Rod	bchl a, vesicular internal membranes					
Rbc.	*Marinum*			1.5–1.7	1.5–5 %	30	None	60.4	No	ly
Thiorhodovibrio		m	No	Spiral	bchl a, vesicular internal membranes					
Trv.	*Winogradskyi*			1.2–1.4	2–3 %	33	None	61.0	Yes	(sp)
Isc.	*Buderi*			3.5–4.5	2–3 %	25–30	B_{12}	62.2–62.8	No	ra
Thiococcus		No	No	Sphere	bchl b, tubular internal membranes					
Tco.	*Pfennigii*			1.2–1.5	1–2 %	25	None	69.4–69.9	No	ts
Thioflavicoccus		m	No	Sphere	bchl b, tubular internal membranes					
Thioalkalicoccus		(m)	No	Sphere	bchl b, tubular internal membranes					
Tal.	*Limnaeus*			1.3–1.8	5 %	20–25	None	63.6–64.8	No	ts
Thiospirillum		m	No	Spiral	bchl a, vesicular internal membranes					
Tsp.	*Jenense*			2.5–4.5	None	20–25	B_{12}	45.5	No	rh,ly
Lamprobacter		m	GV	Rod	bchl a, vesicular internal membranes					
Lpb.	*Modestohalophilus*			2.0–2.5	1–2 %	25–27	B_{12}	64.0	Yes	Ok
Thiopedia		No	GV	Sphere	bchl a, vesicular internal membranes					
Tpd.	*Rosea*			2.0–2.5	None	23	None	62.5–63.5	No	Ok

Abbreviations: *m* motile, *GV* gas vesicle, *o* no information available, *ok* okenone, *sp* spirilloxanthin, *ra* rhodopinal, *rh* rhodopin, *ly* lycopene, *ts* tetrahydro-spirilloxanthin, *YE* yeast extract vitamins, *PP* pyridoxal phosphate, *n* niacin, *b* biotin, *p* pantothenate

Pigments

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 of *Chromatiaceae* are bacteriochlorophyll *a* or *b* and carotenoids of the spirilloxanthin, the okenone, or the rhodopinal groups. In the species with bacteriochlorophyll b, *Thiococcus pfennigii*, *Thioflavicoccus mobilis*, and *Thioalkalicoccus limnaeus*, tetrahydrospirilloxanthin and derivatives thereof occur. In general, the color of cultures of strains with carotenoids of the spirilloxanthin group appears 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 a 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 to 900 nm. Cultures of bacteria with bacteriochlorophyll *b* exhibit long wavelength absorption maxima at 980–1,200 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*g* from the internal membranes, which are used for measurement of the spectra. 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.

The DNA Base Composition

The DNA base ratio, expressed as the G+C content, has been found to span a large range (from 45.5 to 70.4 mol%) within the *Chromatiaceae*. Because the G+C content is a crude measure of genomic relatedness of bacteria, these values suggest enormous diversity within the *Chromatiaceae*. By and large, a quite narrow range of the G+C content is found within the established genera of the family (Guyoneaud et al. 1998; Imhoff et al. 1998). This is approx. 63–66 mol% in the cluster including species of *Thiocapsa, Thiolamprovum, Thiobaca, Lamprocystis*, and *Thiodictyon*, 67–71 mol% in *Marichromatium* species, 64–66 mol% in *Halochromatium* species, and 48–50 mol% in the *Chromatium* species. It is 61–64 mol% in *Thiocystis* (with the exception of strains of *Tcs. violacea*, 67.9 mol%), 60–64 mol% in *Allochromatium* (with the exception of strains of *Alc. vinosum*, 66.3 mol%, and *Alc. warmingii*, 55.1 mol%), and 62–67 mol% in *Thiorhodococcus* (with the exception of *Trc. kakinadensis*, 57.5 mol%). The cases of high intraspecies and intrageneric variation may require consideration in regard to the analytic results and/or the species assignment of the respective strains.

Lipids, Quinones, and Fatty Acids

Chemotaxonomic properties such as ring structure and the isoprenoid chain length of respiratory quinones and fatty acid composition of the cell membranes that have been found to be quite helpful to identify and classify new isolates of purple nonsulfur bacteria (Imhoff and Bias-Imhoff 1995; Hiraishi et al. 1984; Imhoff 1984b) are of low diagnostic value within the *Chromatiaceae*, which as far as analyzed all have MK-8 and Q-8 as major quinone components (Imhoff 1984b) and as major fatty acid C-18:1, C-16:1, and C-16:0 (Imhoff and Bias-Imhoff 1995). Polar lipids have been analyzed only in 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 (Imhoff, unpubl. results). 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 may reflect phylogenetic relations. More analytical data on *Chromatiaceae* are required to support this concept.

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 also use molecular hydrogen and thiosulfate as electron donors under reducing culture conditions and some can oxidize reduced iron ions (Widdel et al. 1993; Ehrenreich and Widdel 1994). Many species are strictly anaerobic and obligately phototrophic, and others are capable of chemolithoautotrophic or chemoorganoheterotrophic 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-β-hydroxybutyrate, elemental sulfur, and polyphosphate. A number of *Chromatiaceae* species require Vitamin B_{12} as growth factor, and a few species have additional growth requirements (*Table 9.1*).

We distinguish two major physiological groups of *Chromatiaceae*, versatile and specialized species, respectively.

1. The specialized species depend on strictly anoxic conditions and are obligately phototrophic. Sulfide is required; thiosulfate and hydrogen are not used as electron donors. Only acetate and pyruvate (or propionate) are photoassimilated in the presence of sulfide and CO_2. 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*.

2. 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, 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 proteins of reaction center and antenna non-covalently 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 *a* and a B1020 complex with bacteriochlorophyll *b* and mostly by additional peripheral antenna (B800–850 complexes and in a few cases also B800–820 complexes with bacteriochlorophyll *a*).

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 of *Chromatiaceae* 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 of the 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 intramembraneous 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 NADH, *Chromatiaceae* and other phototrophic purple bacteria require external electron donors such as reduced sulfur compounds or hydrogen. 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 towards 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 (Roelofson 1935; Gaffron 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 b-type (Gogotov 1978, 1984, 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 CO_2 and 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 on sulfide and thiosulfate, sulfur appears in the form of globules inside the 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 out of three proteins in *Allochromatium vinosum* and two proteins in *Thiocapsa roseopersicina* (Brune 1995b). 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 1968a, 1968b, 1974). Though fermentative growth under these conditions is very poor in *Chromatiaceae*, several species having these capabilities show improved survival in the absence of light and oxygen (Krasilnikova 1976; Krasilnikova et al. 1975, 1983; van Gemerden 1968a, b).

Enzymatic Reactions

Intermediates in enzymatic reactions of sulfide oxidation are sulfur and sulfite. While sulfur accumulates clearly visible in the cells, sulfite is not observed, possibly because of its cytoplasmic formation and immediate further oxidation to sulfate (Brune 1995a). 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, 1989; Brune 1989, 1995a).

Flavocytochrome c is present in several *Chromatiaceae* and 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 of periplasmic location (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.

A role of *sulfide quinone reductase*, which has been characterized from cyanobacteria and purple nonsulfur bacteria (Brune 1995a), in sulfide oxidation of *Chromatiaceae* has not been established so far.

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 (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.

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, 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), which in turn both can be oxidized by *Allochromatium vinosum*. A recent overview on the sulfur metabolism of purple sulfur bacteria is given by Frigaard and Dahl (2008).

Carbon Metabolism

In the *Chromatiaceae*, CO_2 is the most important carbon source. Under autotrophic growth conditions with CO_2 as sole carbon source, the Calvin cycle with ribulose bisphosphate 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_2 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 are used in the more versatile species of the *Chromatiaceae* (e.g., *Allochromatium vinosum*, *Thiocapsa roseopersicina*) than in the specialized *Chromatiaceae* (e.g., *Chromatium okenii, 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_2 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 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 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 aspect has received much attention (for reviews, see Hallenbeck 1987; Haselkorn 1986; Ludden and Roberts 1995; Madigan 1995; Vignais et al. 1985). Nitrogen metabolism has been much more intensively studied in purple nonsulfur bacteria compared to purple sulfur bacteria.

Isolation, Enrichment, and Maintenance Procedures

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, $CaSO_4$, 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 established (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.

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, it is recommended to adjust the pH to 7.2 and 7.4. 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 salt 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.

Illumination and light intensity are important selective factors for the development of phototrophic purple bacteria. 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, 1967). Two different illumination schemes with incandescent light are recommended here.

If continuous illumination at high light intensities of 1,000–2,000 lx and an incubation temperature of about 30 °C are 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 lx 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) or 4 h light and 8 h dark (van Gemerden 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 *b*, such as *Thiococcus pfennigii* (Eimhjellen 1970; Eimhjellen et al. 1967) and *Thioflavicoccus mobilis* (Imhoff and Pfennig 2001), is achieved with an infrared filter that transmits radiation only above 900–1,000 nm.

The concentration of sulfide is critical for a number of species that are inhibited by higher concentrations. Therefore, the sulfide concentrations should be kept as low as 1 mM or even less, so that the cultivation of sensitive forms is not excluded. High population densities can be achieved only by repeated addition of sulfide ("feeding") by using a neutralized sulfide solution. *Thiopedia rosea* is exceptional in being inhibited by sulfide concentrations already >0.6 mM and the addition of sodium dithionite (50 mg for 1 l) is required for cultivation of this bacterium (Pfennig and Trüper 1992).

The salinity and the mineral salt composition of the medium are 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, though the salinity of a natural habitat may not always be optimal for a particular isolate from this habitat. Therefore, use of enrichment media with different salinities may be useful for the isolation of a variety of species from the same sample. For marine isolates, NaCl concentration of 2–3 % is most appropriate. Some isolates also require increased concentrations of magnesium (e.g., 0.3 % $MgSO_4$ $7H_2O$) and sometimes also of calcium (Biebl and Pfennig 1978).

The incubation 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). Molecular approaches using *pufLM* gene sequences in the analysis of temperature-dependent enrichments have demonstrated the selective advantage of *Marichromatium gracile* at temperatures above 40 °C (Tank et al. 2011). This bacterium became most prominent in enrichments at elevated temperatures above 41 °C but was not detected at lower temperatures.

Specific approaches have been elaborated for the phototrophic purple bacteria on the basis of the *pufLM* genes (Tank et al. 2009) and were applied to selected habitats. The *pufLM* genes encode the light (L) and medium (M) subunits of the photosynthetic reaction center type II structural proteins of all phototrophic proteobacteria (purple sulfur bacteria, purple nonsulfur bacteria, as well as the aerobic phototrophic purple bacteria producing bacteriochlorophyll and forming a photosynthetic apparatus) and the phototrophic members of *Chloroflexi*. The primer-selected sequence stretch encompassed approx. 1,500 bases. This qualified the *pufLM* genes as a valuable tool for studies of environmental communities of phototrophic purple bacteria. Species recognition of these bacteria even in complex mixtures of environmental communities is now possible. In analogy with established rules for relationships of 16S rRNA gene sequence similarities, thresholds of 86 % and 95 % sequence similarity of the *pufLM* genes have been proposed for the distinction of genera and species of purple sulfur bacteria, respectively (Tank et al. 2009, 2011; Zeng and Jiao 2007).

Enrichment experiments along gradients of temperature (13–44 °C) and salinity (0–7.5 % NaCl) with samples from a Baltic Sea lagoon using the specific *pufLM* genetic approach (Tank et al. 2009, 2011) enabled resolution of the enrichmental communities at the species level. With the exception of three phylotypes (based on sequence similarity equivalent to species) found as single clones in the environmental sample, all were retrieved at least from one of the enrichments. In addition, six phylotypes of purple sulfur bacteria, among these three phylotypes most similar to the marine *Thiorhodococcus mannitoliphagus*, *Thiorhodococcus kakinadensis*, and *Marichromatium gracile*, were retrieved only after various enrichments, but were not seen in the original sample (Tank et al. 2011). These results demonstrated the relevance of temperature and salinity for the development and competition of individual species and strains. Most significant, *Marichromatium gracile* showed a clear competitive advantage at elevated temperatures <40 °C. They also proved the suitability of the applied media for the purple sulfur bacteria present in the sample and their reproduction in the enrichments. Quite interestingly, the community of *Chromatiaceae* inhabiting this brackish water

Baltic Sea lagoon was well adapted to elevated salt concentrations. Phylotypes assigned to the genera *Marichromatium*, *Allochromatium*, *Halochromatium*, *Thiorhodococcus*, *Thiocapsa*, *Thiorhodovibrio*, and *Thiohalocapsa* were identified, all of which required or tolerated the elevated salt concentrations in the enrichment cultures (Tank et al. 2011).

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_{12} (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 if the salt content is adequately adjusted.

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, due to the separation in or on agar, also these cells will grow out to small colonies and 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., it is recommended to use low temperatures and low light intensities as indicated above.

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. Medium 1 is suitable for almost all *Chromatiaceae* presently in laboratory culture, including those species that are most difficult to grow (e.g., *Thiopedia rosea, Thiospirillum jenense, Chromatium okenii*, and *Thiodictyon elegans*). This culture medium was published by Pfennig (1965), Pfennig and Lippert (1966), Trüper (1970), Eichler and Pfennig (1988), and Pfennig and Trüper (1992) with minor modifications. Here, the original trace element solution is replaced by a sulfate-free trace element solution SLA, which is used in the authors' lab for many years (Imhoff and Trüper 1977; Imhoff 1988b; Trüper and Imhoff 1981). In addition, a second, very similar culture medium is given (medium 2), which is easier to be prepared and which allows the cultivation of most of the common purple sulfur bacteria (Biebl and Pfennig 1978).

Medium 1

The medium (Medium 1, Modified from Eichler and Pfennig 1988) is prepared in a 5-l bottle with four openings at the top. Two openings for tubes are connected to tubings: (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 gastight screw caps. One of these openings is for the addition of sterile solutions and the other can serve as a gas outlet.

The composition of medium 1 as given below is for a total of 5 l of culture medium. The different solutions are prepared separately and mixed as indicated to obtain the final medium.

Solution 1

Distilled water	4,900 ml
KH_2PO_4	1.7 g
NH_4Cl	1.7 g
KCl	1.7 g
$MgSO_4 \cdot 7H_2O$	2.5 g
$CaCl_2 \cdot 2H_2O$	1.25 g

Solution 1 is autoclaved for 45 min at 121 °C in the 5-l bottle, together with a teflon-coated magnetic bar. It is cooled to room temperature under a N_2 atm with a positive pressure of 0.05–0.1 atm (a manometer for low pressures is required). The cold medium is saturated with CO_2 by magnetic stirring for 30 min under a CO_2 atm 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_2 gas or, better, a mixture of 95 % N_2 and 5 % CO_2 while the medium is magnetically stirred. For enrichment cultures or pure cultures

from marine or estuarine habitats, 100 g NaCl is added to solution 1 and the content of $MgSO_4 \cdot 7H_2O$ is increased to 15 g.

***Solution 2*: Vitamin B_{12} solution**
A sterile filtered stock solution containing 2 mg vitamin B_{12} in 100 ml distilled water is prepared and 5 ml is added to solution 1.

***Solution 3*: Sulfate-free trace element solution SLA (Imhoff and Trüper 1977)**

Distilled water	1 l
$FeCl_2 \cdot 4H_2O$	1,800 mg
$CoCl_2 \cdot 6H_2O$	250 mg
$NiCl_2 \cdot 6H_2O$	10 mg
$CuCl_2 \cdot 2H_2O$	10 mg
$MnCl_2 \cdot 4H_2O$	70 mg
$ZnCl_2$	100 mg
H_3BO_3	500 mg
$Na_2MoO_4 \cdot 2H_2O$	30 mg
$Na_2SeO_3 \cdot 5H_2O$	10 mg

The individual components are dissolved separately in a small volume of distilled water, the solutions are combined under mixing, the pH is adjusted with 1 N HCl to 2–3, and the final volume is adjusted to 1 l. 1 ml of this solution is added to 1 l of medium. Care should be taken to use fresh and not oxidized Fe-(II) chloride. The solution should be sterilized by filtration.

***Solution 4*: Na-bicarbonate solution**
A solution of 7.5 % Na-bicarbonate is prepared, saturated with CO_2, and autoclaved under a CO_2 atm in a tightly closed bottle, and 100 ml is added to solution 1.

***Solution 5*: Sodium sulfide solution**
A 10 % $Na_2S \cdot 9H_2O$ solution is prepared in a screw-cap bottle, and after replacement of the air by N_2, the bottle is tightly closed and autoclaved. 20 ml of the sterile solution is 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_2 (0.5 bar pressure) for approx. 40 min to pH 7.2. The medium is then immediately dispensed aseptically under pressure of N_2 (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 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 $Na_2S \cdot 9H_2O$ 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. In order 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 $Na_2S \cdot 9H_2O$ in 100 ml distilled water (a 0.15-M solution) in a 250-ml screw-cap bottle. After replacement of the air by N_2, 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 bar. The solution is brought to about pH 7.3 by dropwise addition of sterile 1 M H_2SO_4 on a magnetic stirrer. Attention has to be payed to slowly add the acid, because otherwise the sulfide solution becomes turbid due 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 is used for *Chromaticeae*. Before the addition, an equivalent amount of culture medium is aseptically removed from the bottle culture.

The much more advanced 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 tubing with a pinchcock to a test tube tightly closed by a screw cap (Siefert and Pfennig 1984). In a volume of 250 distilled water, 7.0 g $Na_2S \cdot 9H_2O$ and 2.65 g Na_2CO_3 are dissolved and autoclaved under CO_2 atm. The sterile solution is saturated with CO_2 under pressure (0.8 bar) until the pH is approx. 7.3. The solution is stored under CO_2 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 $Na_2S_2O_3 \cdot 5H_2O$ to a final volume of 100 ml distilled water). This solution is prepared in a 200-ml screw-cap bottle and autoclaved. 1 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 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 in order to enforce growth and colony size.

Medium 2

This medium (Medium 2, Modified from Biebl and Pfennig 1978) is similar in its composition to medium 1 but much easier to be prepared. Several solutions are dissolved separately and mixed in a 2-l 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 bar is put into the flask. The following solutions and amounts are for 1 l of culture medium.

Solution 1

Distilled water	950 ml
KH_2PO_4	1 g
NH_4Cl	0.5 g
$MgSO_4 \cdot 7H_2O$	0.4 g
$CaCl_2 \cdot 2H_2O$	0.05 g

For marine strains, 20 g NaCl is added to solution 1 and the amount of $MgSO_4 \cdot 7H_2O$ is increased to 3 g.

The solution is autoclaved in the cotton-plugged 2-l Erlenmeyer 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_{12} solution**

The solution is prepared as a sterile filtered stock solution containing 2 mg vitamin B_{12} in 100 ml distilled water and 1 ml is added to solution 1.

***Solution 3:* Trace element solution SL 12 (Pfennig and Trüper 1992)**

The salts of the following stock solution are dissolved in the order given, the pH is adjusted to 2–3 with HCl, the solution is sterilized, and 1 ml is added per liter medium.

Distilled water	1 l
Ethylene diamine tetraacetate-Na_2	3.0 g
$FeSO_4 \cdot 7H_2O$	1.1 g
H_3BO_3	300 mg
$CoCl_2 \cdot 6H_2O$	190 mg
$MnCl_2 \cdot 4H_2O$	50 mg
$ZnCl_2$	42 mg
$NiCl_2 \cdot 6H_2O$	24 mg
$Na_2MoO_4 \cdot 2H_2O$	18 mg
$CuCl_2 \cdot 2H_2O$	2 mg

Application is 1 ml per 1 l of medium.

***Solution 4:* Sodium bicarbonate solution**

A 5 % $NaHCO_3$ solution is prepared in distilled water and filter-sterilized and 30 ml is added to solution 1.

***Solution 5:* Sodium sulfide solution**

A freshly autoclaved 6 % solution of $Na_2S \cdot 9H_2O$ is prepared in distilled water and 6 ml is added to medium.

After mixing of solutions 1 through 5, the pH of the medium is adjusted with sterile 1 M H_2SO_4 or 1 M Na_2CO_3, solution to pH 7.2, and the volume brought to 1 l 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 1988b). 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 (1947) "Pasteur pipette" method 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 order to give rise to the growth of a large variety of phototrophic bacteria. In a modification of the method of Pfennig (Pfennig 1965; Trüper 1970; Imhoff 1988b), 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 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 one to three 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 is 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, they are sealed with a paraffin mixture (3 parts paraffin oil and 1 part paraffin) to prevent diffusion 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_2 and 5 % CO_2 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 200–1,000 lx.

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 in order to completely remove the liquid paraffin. Individual colonies are isolated from appropriate dilutions that show 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 GasPak 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 thioacetamide (depending on the size of the jar and the desired concentration) suspended with 1.0 ml of 0.2 N or 0.5 N HCl. The hydrogen sulfide is slowly released over a period of at least 1 week (Irgens 1983). Also included in the jars are a 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), which are then placed on top of the agar.

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 lx; 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 °C to +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 with 1 ml 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 with 1 ml neutral sulfide solution and are transferred after growth has started and they have formed elemental sulfur (milky appearance of the culture).

For long-term storage, preservation in liquid nitrogen is most 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 and approved over decades.

Ecology

Ecology of *Chromatiaceae*

Purple sulfur bacteria and in particular *Chromatiaceae* 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. In most cases, this development is hidden in the microscopic level and not visible to the naked eye. Positive enrichment cultures obtained from sediments or water samples containing hydrogen sulfide but not showing visible accumulations of colored bacteria demonstrate the presence of phototrophic purple bacteria in almost all shallow water sediments (e.g., Bavendamm 1924; Pfennig 1967; Imhoff 1988a, 2001). Quite often, colored blooms are formed by the mass development of purple sulfur bacteria that can be seen with the naked eye and naturally have attracted many scientists. Already, 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; Gorlenko et al. 1983; van Gemerden and Beeftink 1983; Madigan 1988; Lindholm 1987; Pfennig 1967, 1989b; Imhoff 1988a, 1992, 2001; van Gemerden and Mas 1995).

Though the species-specific analysis of environmental communities has been a major goal of ecological studies, this always has been quite problematic. All *microscopic analyses* were hampered by the lack of confidence into the identification of species by morphological criteria. The great advantage of all *culture-based approaches* was the possible delineation of physiological and other phenetic properties from the isolates, though the relevance of the isolated bacteria for the habitat always remained unclear. A breakthrough for *genetic approaches* for such ecological studies of *Chromatiaceae* was reached with the establishment of a specific functional genetic approach using structural genes of the photosynthetic apparatus (*pufLM* genes) and thus being specific for phototrophic bacteria (with reaction center type II) and the routing of this approach with a comprehensive database of sequences of pure culture isolates and established type species of *Chromatiaceae* (Tank et al. 2009, 2011; Thiel et al. 2010). Ultimately, the combination of culture-dependent and specific genetic aproaches is feasable now and makes possible the species-specific analysis of environmental communities including their response to changing environmental conditions as well as the analysis of relevant physiological properties (for discussion see Imhoff 2011).

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 countercurrent 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. Due to these processes, the sulfide horizon rises during the night and goes down 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 affinities to 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 (*Ectothiorhodospiraceae*, 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 Gemerden 1968a, b).

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 towards oxygen and flexible in their metabolism. Many purple sulfur bacteria are able to make use out of the oxygen and to 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 of ecological importance: (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 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 Gemerden 1990a, b; Schaub and van Gemerden 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 in the dark over prolonged time periods (see Imhoff 2001).

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 to conquer 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, 1980). Later, a *Didemnum* species revealed similar mass accumulations of this species (see Imhoff 1992), and it was isolated from the intestine 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 due 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 *Chromatiaceae* have bacteriochlorophyll *a*, with long wavelength absorption maxima between 800 and 900 nm. Only three species/genera are known that have bacteriochlorophyll *b*, with absorption maxima at 980–1,035 nm.

Light absorption in water masses and sediments follows completely different scenarios (see Imhoff 1992; Pfennig 1989b). In water, the potential light penetration is many meters, while in sediments due to 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 or films below a surface layer of cyanobacteria which measure fractions of a millimeter (Pfennig 1989b; Imhoff 1992). Bacteriochlorophyll absorption is of major importance in sediments, because infrared radiation penetrates particularly deep into sandy sediments (Hoffmann 1949), which favors bacteria that use photosynthetic pigments with absorption maxima in the far infrared, such as bacteriochlorophyll *b*. 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 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 by water, in particular above 800 nm. Therefore, the role of carotenoids (absorption maxima at 450–550 nm) in light harvesting increases with the water depth. Among the purple bacteria, in particular those with okenone as carotenoid have an advantage in deeper water layers because of the very efficient light absorption of this pigment.

Habitats of *Chromatiaceae*

In addition to the important and selective environmental factors described above (anoxic conditions, 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 of *Chromatiaceae* have moderate temperatures, but also thermal springs (Madigan 1986) and sea ice are inhabited by *Chromatiaceae* species (Petri and Imhoff 2001). Various species of the *Chromatiaceae* specifically inhabit different types of freshwater, marine, and hypersaline habitats. It should be noted that in many investigations, tentative identification of purple sulfur bacteria in natural samples was achieved by microscopic examination under consideration of characteristic morphological properties. Because of the limited confidence into these identifications, many species and genus designations in natural samples are quite problematic, unless isolates have been obtained and characterized. In the following selected view on habitats of *Chromatiaceae*, 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, in lakes, shallow ditches, ponds, and small stagnant water bodies in forests, botanical gardens, or up in 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 in the sediments or deeper parts of the water bodies. 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 nineteenth century (Ehrenberg 1838; Lankester 1873; Cohn 1875; Kützing 1883; Winogradsky 1888; Miyoshi 1897), much more numerous ones in the twentieth 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 in several meters depth at the chemocline and are not visible at the water surface (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; Düggeli 1924), the well-studied Belovod Lake (USSR, Kusnetzov 1970; Sorokin 1970), and Lake Cadagno (Switzerland; Bosshard et al. 2000) with blooms involving *Chromatium okenii*. Mixed populations of purple sulfur bacteria and green sulfur bacteria were found, e.g., in Suigetsu Lake and Kisarazu 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 either occur 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 (USA; 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 (the 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 areas 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 1993).

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), LakeMuliczne (Poland) with a *Thiopedia* bloom (Czeczuga 1968a), 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 H_2S/l (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 at 48–50 °C of *Thermochromatium tepidum* is exceptional. This moderately thermophilic purple sulfur bacterium was isolated from carbonaceous sinter in the Stygian Springs of Yellowstone Park (Madigan 1986).

Wastewater Ponds

Ananerobic 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 (Gloyna 1971; Holm and Vennes 1970; 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 *Thiolamprovum pedioforme* (Eichler and Pfennig 1986). In addition, *Thiocystis violacea* and *Allochromatium* species were 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 at rocky shores, in seawater pools and small puddles of salt marshes, in closed bays, and in large lagoons, but also in sediments of sandy beaches, of salt marshes, and of tidal flats and in muddy masses of decaying biomass of algae or sea weeds. At 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, they have been found and studied at the ocean coasts all over the world (see Pfennig 1967, 1989b; Pfennig and Trüper 1981; Imhoff 1988a, 1992, 2001; Trüper 1980; van Gemerden and Mas 1995 for reviews).

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 2001). Great metabolic versatility is of selective advantage at these habitats and the most versatile phototrophic purple bacteria frequently are among the dominant species here (Bavendamm 1924; Imhoff 1992, 2001; Molisch 1907; Trüper 1970). This versatility is reflected (1) in the ability to use different mechanisms 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 in marine coastal habitats. Other species frequently encountered are *Allochromatium warmingii*, *Thiocystis violascens*, *Allochromatium minutissimum*, *Thiocystis minor*, *Thiocapsa rosea*, *Marichromatium purpuratum*, and *Isochromatium buderi* (Imhoff 1988a, 1992, 2001; Pfennig 1989b; Trüper 1970, 1980).

Sediments at Marine Shorelines

Due 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. Their development is restricted to the sediment, whenever the production of sulfide is not sufficient to penetrate into the water. 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 Wadden Sea and of salt marshes.

An outstanding example is found in the microbial mats of the Great Sippewissett Salt Marsh (Cape Cod, MA, USA), 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 *b* and the bundles of tubular intracellular membranes in the coccoid cells. A second bacterium with bacteriochlorophyll *b* was isolated from this layer. It is a motile coccus and was described as a new species, *Thioflavicoccus mobilis* (Imhoff and Pfennig 2001). 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 *b* and to exhibit a number of unusual characteristics. This bacterium was described as the new species *Rhodospira trueperi* (Pfennig et al. 1997). Another new purple nonsulfur bacterium isolated from this habitat is *Roseospirillum parvum* (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 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, 1960); the Bay of Tunis (Heldt 1952; Stirn 1971) and Bietri Bay of the Ebrie Lagoon, Ivory Coast (Caumette 1984); the Etang du Prevost near Montpellier, France (Caumette 1986); lagoons at Messolonghi and Aitolikon in Greece (Hatzikakidis 1952, 1953); the Solar Lake on the Sinai Peninsula (Cohen et al. 1977); and Lake Mogilnoye on the Arctic island of Kildin (Isachenko 1914; Gorlenko et al. 1978).

A recent study on the communities of phototrophic purple bacteria in a coastal lagoon of the brackish water Baltic Sea deserves special attention, because this study used the specific genetic tools for anoxygenic photosynthetic bacteria with *pufLM* gene sequences and allowed an in-depth analysis of this

community (Tank et al. 2011), paralleled by culture-dependent investigation of the habitat. Quite interestingly, most phylotypes of this study could be clearly assigned to known genera. Altogether 14 out of 26 *pufLM* phylotypes were identified as *Chromatiaceae*. They affiliated to genera and species typically isolated from such habitats in previous studies, including *Marichromatium, Thiocystis, Thiorhodococcus, Allochromatium, Thiocapsa, and Thiorhodovibrio*, but also sequences related to moderately halophilic *Halochromatium and Thiohalocapsa* species, which were not commonly seen so far in brackish waters (Caumette et al. 1988, 1991, 1997; Imhoff 2001). As far as concluded from the genetic sequence analyses, the majority of the components of this community were new at the species level but known at the genus level.

The Black Sea

In contrast to estuarine or nearshore 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 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 2,240 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 approx. 10 years later, when the chemocline reached horizons (68–90 m) receiving light of minor intensities. At that time, large amounts of bacteriochlorophyll *e*, which are indicative of a bloom of brown-colored green sulfur bacteria, were detected (Repeta et al. 1989; Jørgensen et al. 1991; Overmann et al. 1992). Apparently, light intensities at the depth of the chemocline were insufficient to support the massive development of purple sulfur bacteria.

Sea Ice

Sea ice is generally regarded to be oxic though the existence of anoxic niches within the complex system of small brine channels within sea ice is quite likely and was concluded from the presence of various types of anaerobic and potentially anaerobic bacteria in the interior of sea ice from the southern Baltic Sea (Petri and Imhoff 2001). 16S rDNA analyses revealed the presence of bacteria related to *Rhabdochromatium* and *Thiorhodovibrio* within a distinct layer of this sea ice, which is indication for sea ice being a habitat of *Chromatiaceae* (Petri and Imhoff 2001).

Salt and Soda Lakes

Many habitats of phototrophic bacteria in the coastal zone 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. Though these waters have high fluctuations in salt concentrations, 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 1988a, 1992, 2001). Particular high fluctuation occurs in small splash water ponds at rocky coasts which have been found to contain phototrophic sulfur bacteria. Such habitats were studied in Japan (Taga 1967), Helgoland (Germany), and Yugoslavia (Imhoff 1988a).

Thalassohaline waters occur as natural evaporation pools of marine waters or as man-made evaporation ponds of marine salterns. A different type of hypersaline environments is found in various athalassohaline waters of inland salt water lakes. A few prominent examples of such lakes with largely different ionic composition are the Great Salt Lake (Utah), the Dead Sea (Israel), and the alkaline soda lakes of the Wadi Natrun in Egypt (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 1988a, 1992, 2001). Numerous isolates have been obtained and a number of new species of phototrophic purple sulfur bacteria have been described from marine salterns (Caumette 1993; Caumette et al. 1988, 1991, 1994), alkaline soda lakes in the Egyptian Wadi Natrun (Jannasch 1957; Imhoff and Trüper 1977, 1981; Imhoff et al. 1979), Russian soda lakes in Siberia and Mongolia (Bryantseva et al. 1999, 2000), and the Solar Lake (Cohen et al. 1977; Caumette et al. 1997; Imhoff 1983).

A number of remarkable salt lakes with different mineral salt compositions are found in the Chilean highlands representing extraordinary and extreme habitats with special conditions regarding salt concentration and composition, irradiation, and drastic diurnal changes. Like other hypersaline environments, lakes of the Salar de Atacama (Laguna Chaxa and Laguna Tebenquiche) exhibited the presence of extended purple-red-colored microbial mats in and on the surface of the lake sediments. A recent study using the extended *pufLM* primer system with approx. 1,500 nucleotide sequence length could resolve the phototrophic bacterial communities with great precision and due to the available extended database

allowed the correlation with sequences from almost all known *Chromatiaceae* species and to resolve new ones at the species level (Thiel et al. 2010). The study revealed a highly diverse and variable community of anoxygenic phototrophic bacteria from these lakes with representatives related to the type strains of the moderately and extremely halophilic purple sulfur bacteria *Halochromatium salexigens*, *Halochromatium glycolicum*, *Thiohalocapsa halophila*, *Ectothiorhodospira mobilis*, *Ectothiorhodospira variabilis*, and *Halorhodospira halophila* as "closest relatives" (Thiel et al. 2010). Evidence was also obtained for the presence of several phylotypes of BChl *b*-containing anoxygenic phototrophic bacteria distant to (<80 % sequence similarity) the genera *Thiococcus*, *Thioflavicoccus*, and *Thioalkalicoccus*, which form a distinct phylogenetic branch among the *Chromatiaceae* (Imhoff et al. 1998; Bryantseva et al. 2000; Tank et al. 2009). Most remarkable was the dominance and diversity of a novel, so far unknown lineage of *pufLM* containing Gammaproteobacteria, which was highly diverse and prevalent in different lakes of the Salar de Atacama (Thiel et al. 2010). In regard to the extraordinary situation of the habitats in the Chilean highlands with extreme climatic and environmental conditions and great geographic distance to all so far investigated habitats of phototrophic bacteria, it is not really surprising that most of the bacteria recognized by *pufLM* sequences in the two salt lakes represent new bacteria, more than two-thirds even at the genus level or higher taxonomic rank (Thiel et al. 2010; Imhoff 2011).

Application

Phototrophic purple sulfur bacteria have been and are being used in a number of biotechnological 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_{12} (Toohey 1971; Koppenhagen 1981; Koppenhagen et al. 1981) 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, 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_2S-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 oxyen 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 unregular periods of sunshine, because considerable efforts have to be made for artificial illumination. Under these circumstances, the high expenditure on energy and money considerably lowers the possible commercial acceptance of such processes.

Wastewater 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; 1965; Cooper et al. 1975), *Chromatiaceae* in municipal sewage (May and Stahl 1967), *Thiocapsa roseopersicina* and *Allochromatium vinosum* in potato waste (Holm and Vennes 1970), *Thiopedia rosea* in feedlot manure (Wenke and Vogt 1981), and *Thiocapsa roseopersicina* in sugar factory effluents (N. Pfennig and H.G. Trüper, unpublished observations).

An advanced system using phototrophic bacteria in the purification of municipal and industrial wastewater has been 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 due 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 have been used by different research groups and

with different purple bacteria to produce hydrogen (see Kumazawa and Mitsui 1982; 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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10 The Family *Colwelliaceae*

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Abstract

The family *Colwelliaceae* is part of the order *Alteromonadales* within the class *Gammaproteobacteria* and currently comprises the type genus *Colwellia* and the genus *Thalassomonas*. Collectively, *Colwelliaceae* encompasses at least 19 species. Both genera are strictly marine in terms of distribution and appear as curved to straight rod-shaped cells that form primarily nonpigmented colonies, possess a polar or subpolar flagellum, and are catalase and oxidase positive. Metabolism varies between the two genera, with *Colwellia* species being facultatively anaerobic and able to grow fermentatively and also by using at least nitrate as an electron acceptor. Most *Thalassomonas* species are instead strictly aerobic; however further study is required to confirm this. Both members of *Colwelliaceae* have attracted interest in terms of extremophilic environmental research and biotechnological investigations. The genus *Colwellia* contains several obligately psychrophilic (cold-requiring) and piezophilic (pressure-requiring) species that synthesize omega-3 polyunsaturated fatty acids while a number of *Thalassomonas* species possess potent agarolytic activity. The species *Colwellia psychrerythraea* represents a model for understanding how bacteria thrive at freezing temperatures.

Taxonomy, Historical, and Current Short Description of the Family *Colwelliaceae* Ivanova et al. 2004, 1773[VP]

Col.well.i′a.ce.ae. N.L. fem. n. *Colwellia*, type genus of the family; suff. *-aceae*, ending to denote a family; N.L. fem. pl. n. *Colwelliaceae*, the *Colwellia* family.

The family *Colwelliaceae* was first described by Ivanova and colleagues (2004) as part of an effort to create taxonomic harmony within a large clade of almost exclusively marine bacteria located within class *Gammaproteobacteria*. This clade now represents the order *Alteromonadales* (Bowman and McMeekin 2005) and consists of at least 22 genera as of late 2012. In addition to the genera *Colwellia* and *Thalassomonas*, the other members of the order include *Aesturaiibacter*, *Agarivorans*, *Algicola*, *Aliagarivorans*, *Alkalimonas*, *Alishewanella*, *Alteromonas*, *Bowmanella*, *Catenovulum*, *Ferrimonas*, *Glaciecola*, *Idiomarina*, *Moritella*, *Oceanisphaera*, *Paraferrimonas*, *Pseudoalteromonas*, *Psychromonas*, *Rheinheimera*, *Salinimonas*, and *Shewanella*. The order *Alteromonadales* is subdivided into several families; besides *Colwelliaceae* these families include *Alteromonadaceae*, *Ferrimonadaceae*, *Idiomarinaceae*, *Moritellaceae*, *Pseudoalteromonadaceae*, and *Shewanellaceae*. The description of new genera since 2004 prompts description of new families or perhaps more usefully, when more genome data becomes available, a taxonomic reappraisal of the entire order.

The genus *Colwellia* was first described by Deming et al. (1988) on the basis of 5S rRNA sequence data of two very psychrophilic strains including strain ATCC 27364^T (NRC1004) isolated from Flounder eggs collected near Trondheim, Norway, and barophilic strain BNL-1^T, collected from surface sediment of the Puerto Rico Trench at a depth of 7,410 m. Strain ATCC 27364^T, which possessed an unusual bright red prodigiosin-like pigment (D"Aoust and Gerber 1974) and produced a self-toxic growth response, was initially named by Dáoust and Kushner (1972) "*Vibrio psychroerythrus*." ATCC 27364^T and BNL-1^T were designated *Colwellia psychrerythraea*, the type species, and *Colwellia hadaliensis*, respectively, by Deming and colleagues (1988). Subsequently a further 10 species have since been added to the genus, deriving from a diversity of marine environments, and include *C. demingiae*, *C. hornerae*, *C. psychrotropica*, *C. rossensis*

E. Rosenberg et al. (eds.), *The Prokaryotes – Gammaproteobacteria*, DOI 10.1007/978-3-642-38922-1_230,

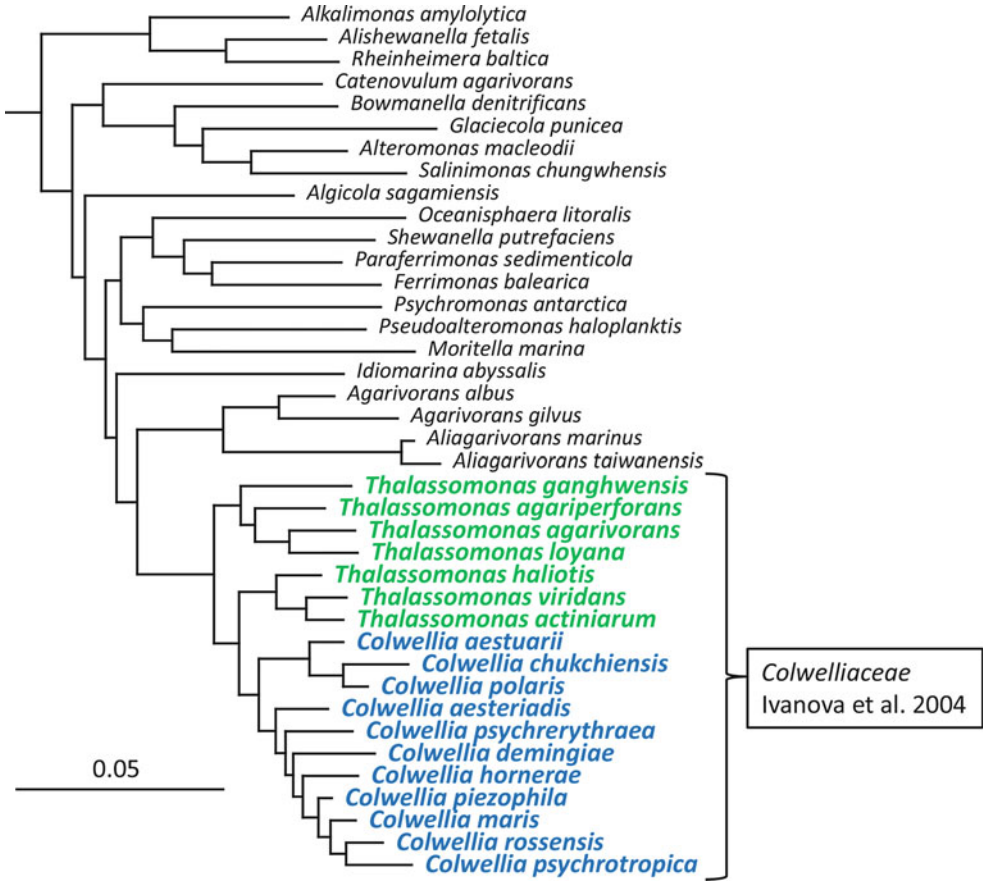

Fig. 10.1

16S rRNA gene sequence-based neighbor-joining tree (distances based on maximum likelihood algorithm) showing the position of members of the family *Colwelliaceae* (which are shown in colored type) within the order *Alteromonadales*. *Thermotoga maritima* and *Coprothermobacter platensis* represented out-groups. Sequences used come from the type strains of the following species (GenBank accession code): *Colwellia piezophila* (NR_024805), *Colwellia maris* (NR_024635), *Colwellia rossensis* (NR_025957), *Colwellia psychrotropica* (NR_026055), *Colwellia psychrerythraea* (NR_037047), *Colwellia aestuarii* (NR_043509), *Colwellia asteriadis* (EU599214), *Colwellia chukchiensis* (FJ889599), *Colwellia hornerae* (JN175346), *Colwellia demingiae* (U85845), *Colwellia polaris* (DQ007434), *Thalassomonas viridans* (NR_042048), *Thalassomonas haliotis* (NR_041662), *Thalassomonas actiniarum* (NR_041661), *Thalassomonas agariperforans* (HM237288), *Thalassomonas ganghwensis* (NR_025717), *Thalassomonas agarivorans* (NR_043649), *Thalassomonas loyana* (NR_043066), *Agarivorans albus* (NR_024788), *Agarivorans gilvus* (GQ200591), *Aliagarivorans marinus* (FJ167390), *Aliagarivorans taiwanensis* (FJ167391), *Algicola sagamiensis* (NR_027234), *Alishewanella fetalis* (AF144407), *Alkalimonas amylolytica* (AF250323), *Alteromonas macleodii* (Y18228), *Bowmanella denitrificans* (DQ343294), *Catenovulum agarivorans* (GQ262000), *Ferrimonas balearica* (CP002209), *Glaciecola punicea* (U85853), *Idiomarina abyssalis* (NR_024891), *Moritella marina* (AB038033), *Oceanisphaera litoralis* (AJ550470), *Paraferrimonas sedimenticola* (NR_041444), *Pseudoalteromonas haloplanktis* (X67024), *Psychromonas antarctica* (Y14697), *Rheinheimera baltica* (AJ441080), *Salinimonas chungwhensis* (AY553295), *Shewanella putrefaciens* (X81623)

(Bowman et al. 1998), *C. maris* (Yumoto et al. 1998), *C. piezophila* (Nogi et al. 2004), *C. aestuarii* (Jung et al. 2006), *C. polaris* (Zhang et al. 2008), *C. asteriadis* (Choi et al. 2010), and *C. chukchiensis* (Yu et al. 2011).

Thalassomonas the sister genus of *Colwellia* includes seven species as of late 2012. The type species, *Thalassomonas viridans*, was isolated from a Mediterranean sea oyster (Macián et al. 2001) and subsequent additional species also derive from a diverse range of strictly marine sites mainly located in temperate to tropical regions. These species include *T. ganghwensis* (Yi et al. 2004), *T. agarivorans* (Jean et al. 2006), *T. loyana* (Thompson et al. 2006), *T. actiniarum*, *T. haliotis* (Hosoya et al. 2009), and *T. agariperforans* (Park et al. 2011).

Molecular Analyses

Colwellia and *Thalassomonas* species cluster together and possess a maximum 16S rRNA gene sequence dissimilarity of approximately 7 %. *Thalassomonas* species in most 16S rRNA gene-based trees are not monophyletic but form two paraphyletic sub-lineages (*Fig. 10.1*). One sub-lineage

contains the species *T. viridans*, *T. actiniarum*, and *T. haliotis* while the second contains *T. ganghwensis*, *T. loyana*, *T. agarivorans*, and *T. agariperforans*. *Colwellia* is on the other hand clearly monophyletic with *C. chukchiensis*, *C. polaris*, and *C. aestuarii* forming a peripheral relatively distinct sub-lineage.

The next closest related genera to family *Colwelliaceae* include *Agarivorans* (Kurahashi and Yokota 2004) and *Aliagarivorans* (Jean et al. 2009), which currently are not affiliated with a family level taxon. Available data however does not lend convincing justification that either of these genera should be placed in *Colwelliaceae*, either on the basis of 16S rRNA gene-based phylogeny or on the basis of phenotypic data. Further data is required and as mentioned previously a more in-depth genome sequence data-based appraisal is required to further develop the higher level taxonomy amongst the members of order *Alteromonadales*.

Phenotypic Properties

Collectively, the species of family *Colwelliaceae* contains Gram-negative, rod- to curved rod-shaped cells that in almost all cases are motile via a single polar flagella, catalase, cytochrome *c* oxidase, and alkaline phosphatase positive. Most strains require both Na^+ and divalent cations (Mg^{2+} and/or Ca^{2+}) for growth while no strain has been isolated that grows at a temperature above 42 °C. Biochemical traits common amongst *Colwelliaceae* member species include the ability to reduce nitrate to nitrite (but not further), hydrolysis of aesculin, Tween 80, casein, L-tyrosine, and starch. No strains so far have been shown to form indole from L-tryptophan or produce arginine dihydrolase, lysine decarboxylase, or ornithine decarboxylase. The primary respiratory lipoquinone is ubiquinone-8 while the majority of phospholipid fatty acids are 14–18 carbon chain ($C_{14\text{-}18}$) monounsaturated and unsaturated types; branched fatty acids make up only a relatively small proportion of total fatty acids in most strains.

Genus *Colwellia* Deming et al. 1988, 328[AL]

Col.well′i.a. N.L. fem. dim. n. *Colwellia*, named in honor of the American microbiologist Professor Rita R. Colwell.

The type species of the genus *Colwellia* is *Colwellia psychrerythraea* (Deming et al. 1988). Given that 12 species in genus *Colwellia* have been described to date, the collective metadata (*Table 10.1*) means that a good concept of the basic phenotypic nature of the genus can be demonstrated. All *Colwellia* species are able to grow at low temperature; however the original concept that the genus being purely psychrophilic (Deming et al. 1988; Bowman et al. 1998) no longer can be said to be true since one species *C. aestuarii* was isolated from a temperate location and grows at temperatures up to 35 °C. The fact that cold-adapted species mingle with more warm temperate adapted species suggests psychrophiles are recently evolved (Franzmann 1996). Whether the predilection towards psychrophily in the genus *Colwellia* reflects a bias of isolation from cold marine sites is unknown; however it is reasonable to assume at least at this stage that *Colwellia* is by and large a highly cold-adapted lineage of bacteria, a rather rare feature amongst cultivated bacteria and archaea. The geographical distribution of *Colwellia* is broad but appears to be strictly marine (see Ecology section).

In comparison to the genus *Thalassomonas*, *Colwellia* species have lower temperature growth ranges and optima for growth and in general are slightly larger (range 1–5 × 0.4–1.0 μm) and are pronouncedly more curved in shape. *Colwellia* is also facultatively anaerobic and able to grow either by aerobic oxidation, fermentation of carbohydrates, and by anaerobic respiration with nitrate and other electron acceptors such as manganese (see Ecology section) but likely unable to use ferric iron as electron acceptor. Acetate and a variety of other simple compounds can serve as electron donors. Most species can form chitinases but so far none have been found that degrade agar. The species generally grow on a range of carbohydrates, organic acids, amino acids, and related compounds (*Table 10.1*). Overall, the data suggests *Colwellia* species have broad ranging biochemical versatility of moderate depth.

Two species of genus *Colwellia*, *C. hadaliensis* (Deming et al. 1988) and *C. piezophila* (Nogi et al. 2004), are obligately barophilic inhabiting hadal surface sediments of deep-sea trenches. Both require for growth high hydrostatic pressure with best growth occurring at 60–75 MPa at 10 °C, equivalent to depths that they were isolated from (6,000–8,000 m). Growing such strains requires highly specialized methods (see ISOLATION and MAINTENANCE section) and given the technicalities little data is available on these species in general. *C. hadaliensis* BNL-1^T appears to be no longer available for study.

The primary fatty acids vary considerably between *Colwellia* species as shown in *Table 10.2*. This is unusual since normally fatty acids are relatively similar between species of a given genus. To some extent the variation amongst *Colwellia* species reflects analysis methods but also likely reflects physiological and genetic adaptations specific to species. In five species (*C. aestuarii*, *C. asteriadis*, *C. chukchiensis*, *C. piezophila*, *C. polaris*) whole-cell fatty acids were analyzed that must have contained a substantial level of lipopolysaccharide-derived fatty acids, which tend to include shorter chain length (C_{10}–C_{14}) and largely hydroxylated types. The amounts of $C_{14:0}$, $C_{15:0}$, $C_{14:1}$, $C_{15:1}$, $C_{16:1}$, and $C_{17:1}$ isomers, $C_{16:0}$ iso, and the polyunsaturated fatty acids (PUFA) docosahexaenoic (DHA, $C_{22:6}$ ω3c) and eicosapentaenoic acids (EPA, $C_{20:5}$ ω3c) vary considerably between species suggesting additional biosynthetic genes are present in certain strains, especially the more psychrophilic species, all of which can form PUFA. Culture conditions likely also influence the quantitative distribution of fatty acids. The ability to form PUFA is a special, albeit species-dependent feature of genus *Colwellia* since the trait only occurs sporadically across the order *Alteromonadales*

Table 10.1
Phenotypic characteristics of *Colwellia* species

Phenotypic characteristic[a]	*Colwellia* species code[b]											
	1	2	3	4	5	6	7	8	9	10	11	12
Colony pigment	V[c]	–	–	–	–	–	–	–	–	–	–	–
Size (mm)	1.5–4.5 × 0.4–0.6	1.5–4.5 × 0.4–0.6	1.5–3.0 × 0.4–0.8	1.5–3.0 × 0.4–0.8	1.5–3.0 × 0.4–0.8	0.7–1.0 × 0.4–0.5	1.8–3.1 × 0.4–0.5	0.9–4.0 × 0.6–0.9	2.0–4.0 × 0.8–1.0	3.0–5.0 × 0.8	2.0–4.0 × 0.6–1.0	1.1–4.5 × 0.5–1.0
Gas vesicles	–	–	+	–	–	–	–	–	–	–	–	–
Isolation site	Fish eggs, sea ice	Sea ice	Sea ice/ seawater interface	Sea ice	Meromictic saline lake	Starfish skin	Tidal flat sediment	Sea ice	Deep-sea sediment (Japan Trench)	Deep-sea sediment (Puerto Rico Trench)	Seawater (off Hokkaido)	Seawater (Arctic Ocean)
Temperature growth range	<0–15	<0–15	<0–15	<0–20	<0–25	0–25	4–35	<0–25	<0–10	<0–10	<0–25	<4–30
Optimum temperature for growth (C)	10	10	10	10–15	15–20	15–20	25–30	20	5–10	5–10	20	25
Requires sea salts or divalent cations	+	+	+	+	+	–	–	–	+	+	–	+
Seawater salinity tolerance range (psu)	20–60	20–60	20–50	20–60	10–100	0–100	0–60	10–60			20–60	10–80
Optimum seawater salinity for growth (psu)	30	30–40	30	30–40	30–40	20–30	20–30	20–30	30–40	30–40	30–40	30–40
High hydrostatic pressure needed for growth	–	–	–	–	–	–	–	–	+	+	–	–
Optimum pressure for growth (MPa)									60	75		
Produce												
Arginine dihydrolase, indole from L-tryptophan	–	–	–	–	–			–				–
Lysine decarboxylase, ornithine decarboxylase	–	–	–	–	–			–	–		–	–
Urease	V	–	+	–	+		–	–				+
H_2S from L-cysteine									–			–
β-galactosidase (ONPG test)*	–	–	–	–	–	–	–	–				–
API ZYM kit enzymatic activity												
Alkaline phosphatase	+	+	+	+	+	+	+	+				+
Acid phosphatase						+	+	–				–
Esterase (C4), esterase (C8)						+	+	–				+
Esterase (C14)						–	–	–				–

Naphthol-AS-BI-phosphohydrolase						+	+	–				+
Trypsin, α-chymotrypsin						–	–	–				–
α-galactosidase, α-mannosidase, α-fucosidase, β-glucuronidase						–	–	–				–
β-glucosidase						–	+	–				–
N-acetyl-β-glucosaminidase						+	–	–				–
Hydrolysis of												
Aesculin	+	+	–	+	–	–	–	+				+
Tween 20, Tween 40, Tween 60							+				+	
Tween 80	+	–	–	+	+	–	+	+			+	+
Lecithin								–				
Xanthine, hypoxanthine							–					
Urate	–	–	–	–	+							
DNA	–	–	–	–	–						+	
L-tyrosine	V	–	–	–	+		–					
Gelatin	V	–	–	–	–	–	–	+	+		+	+
Casein	+	+	–	+	+	–	+	+	–		–	+
Starch	+	+	+	+	–	+	–	+	–		–	–
Chitin	+	+	+	–	+	+	–	–	–	+	–	–
Agar	–	–	–	–	–	–	–	–	–		–	–
Assimilates or metabolizes												
L-arabinose	–	–	+	–	–	–	+	+	–		–	–
D-fructose	–	–	+	–	–	+	–	+	–		–	–
D-galactose	–	–	+	–	–	–	+	+	–			+
D-glucose	+	–	+	–	–	–	+	+			+	+
D-mannose	–	–	–	–	–	–	+		–		–	–
L-rhamnose	–	–	–	+	–		+		–			–
DL-xylose	–	–	–	–	–		+	–	+		–	–
Cellobiose	V	–	–	–	–	–	+	+	–			–
D-lactose	–	–	–	–	–	–	+	–	–		–	–
D-maltose	+	–	–	–	–	–	+	+	+		–	–
D-melibiose	–	–	–	–	–	–	+	+			–	–
Sucrose	–	–	–	–	–	–	+	+	–		–	–
D-trehalose	–	–	–	–	–	–	+		–			–

Table 10.1 (continued)

Phenotypic characteristic[a]	*Colwellia* species code[b]											
D-raffinose	–	–	–	–	–		–		–		–	–
Glycogen	+	V	+	–	–							–
Adonitol	–	–	–	–	–							–
DL-arabitol	–	–	–	–	–							–
Myoinositol	–	–	–	–	–		–		–			–
D-mannitol	–	–	–	–	–		–	+	–			–
D-sorbitol	–	–	–	–	–		–	+	–			–
D-gluconate	–	–	+	–	–							–
N-acetyl-D-glucosamine	+	+	+	–	+							–
Glycerol	–	–	+	+	–			+	–		–	–
DL-lactate	V	+	–	+	+							
L-aconitate	–	–	–	–	–							
Oxaloacetate	+	+	–	+	+							
Citrate	–	+	+	+	–	–	–	–				+
Fumarate	+	+	+	+	+							
DL-β-hydroxybutyrate	+	–	+	–	+							
DL-malate	–	–	+	–	+	–	+	–				
2-oxoglutarate	–	–	–	–	+							
Pyruvate	+	+	+	+	+		–	+				
Succinate	+	+	+	+	+			–				
Acetate	+	+	+	+	+	+	–	+				
Propionate	V	+	–	+	–							
Butyrate	+	+	+	+	+							
Isobutyrate	–	V	–	–	+							
Valerate, caproate	–	+	+	+	+							

Isovalerate, nonanoate, adipate, pimelate	−	−	−	−	−							
Heptanoate, azelate	−	V	−	+	−							
Caprate	−	V	−	−	−							
Malonate	−	V	+	−	−							
Glutarate	−	+	−	+	−							
Caprylate	−	V	−	−	−							
L-alanine	−	V	+	−	+							
L-asparagine	−	+	+	−	+							
L-aspartate	−	V	+	−	+							
L-glutamate, L-proline	+	+	+	+	+		−					
L-phenylalanine	−	V	−	−	−							
L-serine	−	+	−	−	−							
L-threonine, putrescine	−	−	−	−	−							
L-tyrosine	V	−	−	−	+							
Gamma-aminobutyrate	−	+	+	+	+							
Hydroxy-L-proline	−	V	−	+	−							
DNA base G+C composition (mol%)	35–38	37	38	39	42	40	39	39	39	45–46	39	41

[a]All *Colwellia* species are Gram-negative; appear as straight to curved rodlike cells; are motile via a single polar flagellum, catalase, and cytochrome *c* oxidase positive; able to reduce of nitrate to nitrite; and have a facultatively anaerobic metabolism and can grow anaerobically via either fermentation of carbohydrates and by using nitrate as an electron acceptor. The optimum pH for growth is approximately that of seawater (pH 7–8)

[b]*Colwellia* species code: 1, *C. psychrerythraea*; 2, *C. demingiae*; 3, *C. rossensis*; 4, *C. hornerae*; 5, *C. psychrotropica*; 6, *C. asteriadis*; 7, *C. aestuarii*; 8, *C. polaris*; 9, *C. piezophila*; 10, *C. hadaliensis*; 11, *C. maris*, and 12, *C. chukchiensis*

[c]Abbreviations: + test positive, − test negative, *V* test result data varies between different strains of the species. A blank cell indicates no data is available

Table 10.2

Fatty acid profiles of the species of the genus *Colwellia*

Fatty acids[b]	*Colwellia* species code[a]										
	1	2	3	4	5	6	7	8	9	10	11
	% fatty acid composition										
$C_{10:0}$								1	2	2	2
$C_{10:0}$ 3-OH								TR	3		
$C_{11:0}$								TR		1	TR
$C_{11:0}$ 3-OH								4		5	1
$C_{12:0}$						1–2	2	3	TR	TR	TR
$C_{12:0}$ 3-OH							3	TR	3	3	2
$C_{12:1}$ 3-OH											
$C_{12:0}$ 3-OH iso						TR[c]			5	6	3
$C_{13:0}$								TR			
$C_{13:0}$ 3-OH/$C_{15:1}$ iso[d]								TR			
$C_{13:0}$ iso	TR	TR	TR	TR	TR						
$C_{14:0}$ 3-OH/$C_{16:1}$ iso[e]								TR			
$C_{14:0}$	5–8	7–8	5	3	TR	9		2	2	2	TR
$C_{14:1}$ ω7c	5–7	9–10	3	3	2	2	2				
$C_{14:1}$ ω5c	TR	TR	TR	TR	TR						
$C_{14:0}$ iso	TR	TR	TR	TR	TR						
$C_{15:1}$ ω8c	0–2	2–3	4	20	4	3	6	3	11	18	9
$C_{15:1}$ ω6c	0-TR			1.1	TR			TR			
$C_{15:0}$	2–11	TR-1	3	14	3	3	4	4	5		6
$C_{16:0}$	27–33	22–24	27	13	22	31–33	25	27	10	8	13
$C_{16:0}$ 3-OH								TR			
$C_{16:0}$ iso	0-TR			10				TR	10	6	7
$C_{16:1}$ ω9c	6–9	9–12	2	2			6	4	6	4	5
$C_{16:1}$ ω7c/$C_{15:0}$ 2-OH iso[f]	31–36	37–38	43	15	57	48–50	45	37	24	23	28
$C_{17:0}$	0–1	TR	TR	2	2		3	2	3	2	4
$C_{17:0}$ iso	TR	TR	TR	TR	TR						
$C_{17:1}$ ω8c	0–1	TR	TR	6	5		4		12	14	13
$C_{17:1}$ ω7c	TR	TR	TR	TR	TR						
$C_{17:1}$ ω6c	TR			2	TR						
$C_{18:0}$	TR-2	TR	TR	2	TR			TR		TR	TR
$C_{18:1}$ ω9c	0–2	TR	TR	1	TR			TR	TR	TR	TR
$C_{18:1}$ ω7c	TR-2	1–2	4		2			2	1		3
$C_{20:5}$ ω3c	0–2		TR		TR						
$C_{22:6}$ ω3c	5–8	3–4	2	1	1						

[a]*Colwellia* species code: 1, *C. psychrerythraea*; 2, *C. demingiae*; 3, *C. rossensis*; 4, *C. hornerae*; 5, *C. psychrotropica*; 6, *C. piezophila*; 7, *C. maris*; 8, *C. asteriadis*; 9, *C. aestuarii*; 10, *C. polaris*; and 11, *C. chukchiensis*. No data is available for *C. hadaliensis*

[b]Fatty acid nomenclature: C_n, carbon chain length; 2-OH or 3-OH, α- and β-hydroxy fatty acids; iso, iso-branched fatty acids; :*n*, number of double bonds present; ω*n*c, *cis*-isomer monounsaturated fatty acid with the bond located at the indicated number of carbon units from the methyl end of the molecule. In the case of polyunsaturated fatty acids, the first double bond is located at the third carbon unit from the methyl end

[c]TR, trace fatty acid making up <1 % of total analyzed fatty acids

[d]Summed feature 1 – the indicated fatty acids cannot be separated in the MIDI fatty acid analysis system

[e]Summed feature 2 of the MIDI system

[f]Summed feature 3 of the MIDI system. Based on more sophisticated separation and mass spectrometric confirmatory methods, the correctly identified fatty acid of this summed feature is $C_{16:1}$ ω7c. $C_{15:0}$ 2-OH iso was not detected in any strain even at trace levels. $C_{15:0}$ 2-OH iso is in general absent in all members of the order *Alteromonadales*

(e.g., also found in some *Shewanella*, *Psychromonas*, *Moritella* species) and in the related order *Vibrionales* (*Photobacterium*). PUFA synthesis also occurs in some marine members of the phylum *Bacteroidetes* (e.g., the genera *Psychroflexus*, *Aureospirillum*).

Genus *Thalassomonas* Macián et al. 2001, 1283

Tha.lass'.o.mo.nas. Gr. n. *thalassa*, the sea; Gr. n. *monas*, a unit; N.L. fem. n. *Thalassomonas*, a monad from the sea.

The type species of genus *Thalassomonas* is *Thalassomonas viridans* (Macián et al. 2001). *Thalassomonas* and its 7 species represent the warm climate relatives of genus *Colwellia* with most strains able to grow well at 30 °C but either grow poorly or not grow at 4 °C (Table 10.3). As such there is no evidence of true psychrophily (or barophily) within this genus based on the fairly random process of species descriptions. The species of *Thalassomonas* to date have been isolated from temperate to tropical ecosystems from a wide range of marine sources, including fauna and flora. The temperature dichotomy between *Colwellia* and *Thalassomonas* suggests that each genus diverged from an ancestor and subsequently expanded to populate essentially climactically different oceanic regions. Phenotypically, *Thalassomonas* is rather similar to *Colwellia* species in a broad sense as described in the introduction.

The phenotypic traits that consistently separate *Thalassomonas* from *Colwellia* are relatively few given the inherent variation possible between individual species. Overall, *Thalassomonas* spp. seem more consistently saccharolytic than *Colwellia* species with most species able to degrade a number of polysaccharides (Table 10.3); however they are non-fermentative. With the exception of *T. agariperforans*, most reports claim *Thalassomonas* species are strictly aerobic. *T. agariperforans* was found like *Colwellia* spp. to be able to anaerobically respire by using nitrate as electron acceptor. The testing of anaerobic growth in the genus has been cursory and further work is clearly needed to confirm if *Thalassomonas* like *Colwellia* is able to engage in anaerobic respiration. Other differential traits that can broadly separate *Thalassomonas* from *Colwellia* include the ability by some species to attack agar and alginate while most species also degrade extracellular DNA but not chitin.

The lipoquinones have been examined in most species via liquid chromatography and ubiquinone-8 (Q-8) predominates with low levels of Q-7 and Q-9 also present (Table 10.4). Similar to *Colwellia* species, fatty acid composition variation occurs between *Thalassomonas* species; however PUFA is absent. Shorter chain components (C_9 to C_{13}) are most likely associated with LPS given several are hydroxylated. In other respects fatty acid profiles are very analogous to that of *Colwellia* species (Table 10.2). Clearly, fatty acid analysis as a genus level diagnostic tool is not very useful given the species-level variation.

Enrichment, Isolation, and Maintenance Procedures

Isolation of *Colwellia* and *Thalassomonas* species is generally straightforward except for barophiles, which is a challenging, dangerous, and expensive enterprise. Fortunately, most species can be isolated from readily accessible marine locations and primary isolation and subsequent routine cultivation utilizes standard easily prepared marine media.

Colwellia species are common in sea ice; however distribution is extremely patchy (Bowman 2013). To improve one's chances of obtaining *Colwellia* spp., sea ice should ideally possess visible algal assemblages, appearing as olive to brown bands in the ice layer, especially at the base of the layer. Sea-ice cores are obtained using an ice auger or "jiffy" drill. The core is then sliced using an electric saw and ice pieces are melted in sterile seawater at 2–4 °C. Once melted the samples can either be directly plated onto marine agar or preincubated at 2–4 °C for 1–2 days in marine broth at a ratio of 1:10 to 1:100. Marine broth consists of 0.5 % w/v bacteriological peptone and 0.2 % w/v yeast extract in either 1,000 ml seawater or instead of seawater 35 g sea salts (purchased from aquarium supply company) in 1,000 ml distilled water. Raw seawater can be filtered and/or aged in the dark (and then autoclaved) as desired but has little any effect on cultivation success. Commercial sources of the medium include, for example, marine 2216 from Difco Laboratories. To create marine agar usually 1.5 % w/v agar is added to marine broth prior to autoclaving (15 min at 121 °C); however alternative solidifying agents could be used as desired (i.e., gellan gum). Sea-ice isolates are typically isolated, purified, and maintained on marine agar at 2–10 °C. *Colwellia* spp. from sea ice have relatively patchy growth and often slowly create crystalline precipitates in the medium.

Colwellia and *Thalassomonas* species have been successfully isolated from marine-sourced samples by simple direct plating and subsequent purification using on marine agar. This includes marine fauna (anemone, starfish, oyster, coral), with tissue or coral skeleton homogenized or ground up as appropriate before dilution in marine broth or buffer (e.g., 3 % NaCl in 10 mM Tris buffer at pH 8.0) and spread plating. Other media have been used including 1:5 to 1:10 diluted marine agar; PY broth/agar (0.3 % w/v polypeptone, 0.1 % yeast extract, 2.5 % w/v NaCl, 0.5 % w/v $MgCl_2.6H_2O$, pH 7.8; 1.5 % w/v agar); PYSE broth/agar (0.8 % w/v peptone, 0.3 % yeast extract, 3 % w/v NaCl, 0.07 % w/v KCl, 0.53 % w/v $MgSO_4.7H_2O$, 0.13 % w/v $CaSO_4.2H_2O$, 0.11 % w/v $MgCl_2.6H_2O$, pH 7.9; 1.5 % w/v agar); A1 broth/agar medium (1 % w/v soluble starch, 0.4 % w/v peptone, 0.2 % yeast extract, seawater, pH 8.1; 1.8 % agar); and MR2A, a marine version of R2A using seawater or water containing sea salts instead of distilled water. In the case of MR2A, it is best to make up this medium from individual components (0.05 % w/v proteose peptone, 0.05 % w/v casamino acids, 0.05 % w/v D-glucose, 0.05 % w/v soluble starch, 0.03 % w/v sodium pyruvate, 0.005 % w/v $MgSO_4.7H_2O$, in seawater or 3.5 % w/v/sea salts solution, approx. pH 8; 1.5 % w/v agar) and adding, if desired, the K_2HPO_4 (0.03 % w/v final

Table 10.3
Phenotypic characteristics of *Thalassomonas* species

Phenotypic characteristics	*T. viridans*	*T. haliotis*	*T. ganghwensis*	*T. loyana*	*T. agarivorans*	*T. agariperforans*
Colony pigment	Green-blue (diffusible)	Brown (diffusible)	Yellow	Cream	Off-white	Yellow-white
Size (mm)	1.5–2.0 × 0.8–1.0	1.0–2.0 × 0.5–0.7	1.5–2.3 × 0.5–0.8	1.0–2.0 × 0.5–0.8	1.4–2.2 × 0.4–0.7	1.5–3.0 × 0.8–1.0
Gas vesicle formation	–	–	–	–	–	–
Isolation site	Oyster	Abalone	Tidal mud	Diseased coral	Seawater	Marine sand
Temperature growth range	15–37	15–30	15–40	18–35	15–35	4–37
Optimum temperature for growth (C)	30	25	30–35	30	25–30	30
Requires sea salts or divalent cations	+	+	+	–	–	+
Seawater salinity tolerance range (psu)	20–40	20–40	10–80	10–80	10–40	0–60
Optimum seawater salinity for growth (psu)	30	30	30–40	30–40	30	20
Cytochrome *c* oxidase	+	+	+	–	+	+
Catalase	+	+	+	+	+	+
Strictly aerobic	+	+	+			–
Anaerobic growth (via nitrate respiration)	–		–			+
Nitrate reduction to nitrite	–	+	+	+	+	+
Produces						
Arginine dihydrolase, indole from L-tryptophan	–	–	–	–	–	–
Lysine decarboxylase, ornithine decarboxylase	–		–	–	–	–
Urease		–	–	–	–	–
H_2S from L-cysteine			–	–	–	–
API ZYM enzyme activities						
Alkaline phosphatase, naphthol-AS-BI-phosphohydrolase		+	+	+	+	+
Acid phosphatase		+	+	–	±	+
Esterase (C4)		+	+	+	+	–
Esterase (C8)		+	–	+	+	–
Esterase (C14)		–	–	–	–	–
Trypsin, α-chymotrypsin			–	–	–	–
α-galactosidase, β-glucosidase, α-mannosidase, α-fucosidase, β-glucuronidase		–	–	–	–	–
β-galactosidase	±	–	±	±	±	–
α-glucosidase		–	–	–	±	–
N-acetyl-β-glucosaminidase		–	–	–	+	–
Hydrolysis of						
Aesculin		+	+	+	+	+
Tween 20, Tween 40, Tween 60		+	+	+	+	+
Tween 80	±	+	+	+	±	+
Lecithin	+	–	–	–	–	
Xanthine, hypoxanthine			–	–	–	–
DNA	+	+	+	+	+	+
L-tyrosine, gelatin, casein	+	+	+	+	+	–
Starch	+	+	–	+	+	+
Chitin	–		–			
Agar	–		–	–	+	+
Alginate	–	–	–	+	+	

Table 10.3 (continued)

Phenotypic characteristics	*T. viridans*	*T. haliotis*	*T. ganghwensis*	*T. loyana*	*T. agarivorans*	*T. agariperforans*
Cellulose, xylan			−			+
Assimilates or metabolizes						
L-arabinose	−	−	−	−	−	−
D-fructose	+	−	−	−		
D-galactose	−	−	+	+	+	+
D-glucose, D-maltose	+	+	+	+	+	+
D-mannose, L-rhamnose, DL-xylose	−	−	−	−		
D-ribose	V	+	−	−	−	
Cellobiose	V	+	−	+	−	
D-lactose	+	−	−	−	+	
D-melibiose	−	+	−	−	−	
Sucrose	+	+		−		
D-trehalose		+	−	−	−	−
D-raffinose		−	−	−		
Glycogen	−	+	−	−	−	
Inulin		−	−	+		
Amygdalin	+	+	−	−	−	
Arbutin	+	−	−	−	−	
Salicin	+	−	−	−	−	
i-erythritol	−	−	−	−	−	
Myo-inositol, D-mannitol, D-sorbitol	−	−	−	−		
D-xylitol	−	+	−	−	−	
D-gluconate	−	−		−		
2-ketogluconate	+	+	+	+	−	
5-ketogluconate	−	+	+	−	−	
N-acetyl-D-glucosamine	−	+		+		
Glycerol	−	+	−	−	−	
Citrate	−	−	−	−	−	−
DL-malate	−	−	−	−	−	−
Succinate	V		+	−	−	−
Tartrate	−		+	−		
Formate			−	−	−	−
Acetate	−		+	+		
Caprate	+	−	−	−	−	
Benzoate			−	−	−	−
L-arginine	V		−	+		
L-asparagine, glycine	−		+	−		
L-glutamate			−	+	−	−
L-tyrosine	+		+	−		
L-ornithine	V		−	−		
DNA G+C composition (mol%)	48–49	50	42	39	41–43	44

[a]All *Thalassomonas* species are Gram-negative and appear as straight to curved rodlike cells; are motile via a single polar (or subpolar) flagellum, catalase, and cytochrome *c* oxidase positive; and can reduce nitrate to nitrite. Fermentation of carbohydrates has not been observed for any species of the genus so far. The optimum pH for growth is approximately that of seawater (pH 7–8)

[b]Abbreviations: + test positive, − test negative, *V* test result data varies between different strains of the species, ± test has conflicting data between reports. A blank cell indicates no data is available

Table 10.4

Fatty acid and lipoquinone composition of *Thalassomonas* species

Lipid components	*T. viridans*	*T. actiniarum*	*Thaliotis*	*T. ganghwensis*	*T. loyana*	*T. agarivorans*	*Tnn agariperforans*
Fatty acid[a]							
$C_{9:0}$	0-TR[b]						
$C_{10:0}$	TR			3–5		1–2	TR
$C_{10:0}$ 3-OH	TR			2			4
$C_{11:0}$	1–2			TR	TR	1–2	
$C_{11:0}$ 3-OH	3–4			2	2	TR-1.4	
$C_{11:0}$ 3-OH iso	TR						
$C_{12:0}$	1–2	2	2	TR	3	5–7	2
$C_{12:0}$ iso	0-TR						
$C_{12:0}$ 3-OH	5–6	2	2	3	7	2	TR
$C_{12:0}$ 3-OH iso	TR						
$C_{12:1}$ 3-OH							5
$C_{13:0}$	TR				TR	5–7	
$C_{13:0}$ 3-OH/$C_{15:1}$ iso[c]	1–2				TR	2–3	
$C_{13:0}$ iso						0–1	
$C_{14:0}$	2–3	2	3	TR	9–13	4–5	2
$C_{14:0}$ iso				TR	TR	1–2	
$C_{14:0}$ 3-OH/$C_{16:1}$ iso[d]				1		0–2	3
$C_{15:1}$ ω8c	4–6	TR	2	2	2	2–3	TR
$C_{15:1}$ ω6c	TR-1						
$C_{15:0}$	6–11	2	4	1	3	0–6	TR
$C_{15:0}$ iso	TR						
$C_{16:0}$	11–14	32	31	19–22	9	17–20	31
$C_{16:0}$ iso	TR			3–7		TR	
$C_{16:1}$ ω9c				4–5	5	2–4	
$C_{16:1}$ ω7c/$C_{15:0}$ 2-OH iso[e]	21–28	45	39	20–23	27–31	8–13	39
$C_{17:0}$	3–5	2	3	1	TR	6–11	TR
$C_{17:0}$ iso	TR						
$C_{17:1}$ ω8c	14–20	5	6	4–7	10–12	9–13	
$C_{17:1}$ω6c	TR						
$C_{18:0}$	TR	1	TR	1	TR	1–2	TR
$C_{18:1}$ ω9c	TR			2	3	2–3	
$C_{18:1}$ ω7c	3–6	6	3	11–19	9	4–5	14
$C_{20:1}$ ω7c	0-TR						
Lipoquinone type:							
Ubiquinone-7	1	6	1	0	2	2	
Ubiquinone-8	98	81	98	96	97	97	100
Ubiquinone-9	1	13	1	4	1	1	

[a]Fatty acid nomenclature: C_n, carbon chain length; 2-OH or 3-OH, α- and β-hydroxy fatty acids; iso, iso-branched fatty acids; :*n*, number of double bonds present; ω*n*c, *cis*-isomer monounsaturated fatty acid with the bond located at the indicated number of carbon units from the methyl end of the molecule. In the case of polyunsaturated fatty acids, the first double bond is located at the third carbon unit from the methyl end

[b]TR, trace fatty acid making up <1 % of total analyzed fatty acids

[c]Summed feature 1 – the indicated fatty acids cannot be separated in the MIDI fatty acid analysis system

[d]Summed feature 2 of the MIDI system

[e]Summed feature 3 of the MIDI system

MR2A medium concentration) separately after autoclaving to avoid precipitation occurring. It is likely these media can be used interchangeably for most *Colwelliaceae* species. The water temperature from where the sample was obtained dictates the incubation temperature subsequently used with psychrophiles growing well at 10–15 °C.

For long-term preservation most strains can be kept either (1) on marine agar slants at 4 °C to room temperature (depending on temperature preference of the strain), (2) cryopreserved in marine broth (or broth versions of the other medium described above) containing 15–20 % v/v glycerol at −80 °C or on cryopreservation beads, (3) as a suspension in sterile seawater at 10–20 °C. Long-term preservation such as lyophilization and freezing in liquid N_2 is possible (detailed protocols are described by DiFernando and Vreeland 2006). Some strains, such as the type strain of *C. psycherythraea* ATCC 27374^T, form autotoxic compounds or proteins. To maintain these strains, the seawater suspension option must be performed since these cultures completely inactivate within a few days on agar media.

For barophilic *Colwellia* species deep-sea samples, usually superficial benthic mud, obviously need to be collected by relatively sophisticated means using manned (such as the famous DSV *Alvin* or the *Shinkai* 6500) or remote-controlled submersibles (such as the *Mir*). Samples are held at constantly low temperature (0–2 °C) to offset pressure change. Samples are enriched or isolated in bags in pressure vessels made of stainless steel (i.e., SAE grade 304). The pressure bag isolation system, which employs low melting point agar (Kato et al. 1995; Kato 2006, 2011) has been used to successfully isolate barophiles of the order *Alteromonadales* including *Colwellia piezophila* (Nogi et al. 2004). To maintain oxygen "at high" pressure, the hydrofluorocarbon fluorinert FC-72 (3 M Inc.) saturated with oxygen can be added at 20 % (v/v). Barophiles when grown at higher temperature must have higher pressure applied; thus typically they are grown and maintained at low temperature (<4 °C). Because of the technical demands and safety issues in working with barophiles, few are available in culture except those that can grow at low temperature at atmospheric pressure.

Genome-Based and Genetic Studies

Only relatively limited genome studies and no genetic knockout or manipulation have been performed on members of the family *Colwelliaceae* so far. Most research has focussed at the protein level and cell product level (e.g., PUFA, exopolysaccharides). As of late 2012, only one representative of family *Colwelliaceae* sea-ice bacterium *Colwellia psychrerythraea* 34H has had its genome sequence determined (Methé et al. 2005). The genome of strain 34H is complete and has a size of 5,373,180 bp with a G+C of 37.9 mol% and contains 4,937 predicted protein coding genes, 85 tRNAs, 9 *rrna* (16S/23S/5S rRNA) operons, and 1 structural RNA gene. Strain 34H has attracted scientific interest since it dwells at constantly sub-zero temperatures in its native sea-ice habitat and the genome was the first obtained from an obligate psychrophile. As a result the 34H genome has proven very useful for understanding the types of traits that allow for life at freezing temperature. This includes detailed information on the amino acid composition of the 34H proteome that has lead to a flurry of publications on protein thermostability in recent years.

One important aspect of the psychrophilic lifestyle is membrane fluidity. At low temperature membrane lipids become increasingly rigid, and without alteration to the chemical makeup to maintain an optimal viscosity, the membrane can deform leading to its rupture, subsequent cell leakage, and death. Amongst various psychrophilic bacteria, one aspect of this homeoviscosity adaptation is the synthesis of PUFA (Russell and Nichols 2000). *C. psychrerythraea* strains can form both DHA and EPA (*Table 10.2*). Omega-3 PUFA is synthesized via polyketide synthases (Metz et al. 2001) and the *pfaABCD* gene cluster coding these enzymes in 34H is similar in conserved domain structure to those found in other gamma-proteobacteria (Shulse and Allen 2011).

Accumulating compatible solutes is extremely important for maintenance of protein stability since proteins constantly interact with water within the cell and are subject to denaturation processes from a number of sources (Wiggins 2008). Compatible solute accumulation is a fundamental basis for adaptation to low temperature whether the organism is cold adapted or not (e.g., Hoffmann and Bremer 2011), since low temperature directly compromises protein stability (Privalov 1990). On the basis of genome data, strain 34H has the ability to synthesize the major osmoprotectants betaine and glycine betaine either de novo or from imported choline. The 34H genome also includes sarcosine oxidase, which suggests betaine and glycine betaine also may serve as sources of carbon and energy, and likely these compounds are constantly turned over in the cell thus balancing cellular metabolic requirements under constant sub-zero temperatures.

The 34H genome also revealed the ability to synthesize polyhydroxyalkanoate granules and to also be able to synthesize and degrade cyanophycin (L-arginyl-poly-L-aspartic acid)-like polyamides. Cyanophycin is a natural nitrogen storage polymer often formed by cyanobacteria that has engendered biotechnological interest in recent years (Mooibroek et al. 2007). It was originally claimed that genes were present in 34H that suggested the potential to metabolize aromatic hydrocarbons; however further examination suggests these enzymes are related to catabolism of aromatic amino acids such as L-tyrosine. In general, based on accumulated taxonomic reports, members of the *Colwelliaceae* cannot degrade aromatic hydrocarbons; however the ability to use straight chain alkane hydrocarbons and grow on fatty acids is evident (see Ecology section).

The cold-active enzyme capacity of strain 34H has been extensively studied in order to understand protein thermostability and function at low temperature. Several enzymes of strain 34H involved in housekeeping processes, intermediary metabolism, and nutrient acquisition have been characterized by various approaches such as X-ray crystallography, including

phenylalanine hydroxylase (Leiros et al. 2007), a secreted highly cold-active aminopeptidase structurally similar to human bifunctional leukotriene A4 hydrolase (Huston et al. 2004; Bauvois et al. 2008), isocitrate dehydrogenases (Maki et al. 2006), cold shock (RNA chaperone) proteins (Moon et al. 2009), DEAD box RNA-dependent RNA helicases involved in ribosome assembly and stabilization (Cartier et al. 2010), DNA gyrase subunit A (Jung et al. 2010), and the Hsp60 molecular chaperone GroEL/GroES (Yamauchi et al. 2012). Similar studies have also been performed using a strain that is now the type strain of *Colwellia maris* (Watanabe and Takada 2004; Yoneta et al. 2004) as well as other uncharacterized *Colwellia* strains (Wang et al. 2005, 2006; Olivera et al. 2007). Few if any studies have tackled transcriptional regulation in *Colwellia* species; studies on heat shock however have revealed that RNA polymerase sigma subunits are involved in stress adaptation responses in *Colwellia* (Yamauchi et al. 2003, 2006).

Ecology

In terms of ecosystem function, *Colwelliaceae* are classic marine secondary producers with a broad-based marine distribution. Based on observational and functional studies, *Colwelliaceae* are collectively ubiquitous and are involved in decomposing organic material of intermediate complexity (hydrocarbons, lipids, proteins, polysaccharides) within pelagic zone particulates, sea ice, and within algal, faunal, and floral associations and engaged in anaerobic respiration and/or fermenting simple compounds within superficial marine sediments.

Colwellia and *Thalassomonas* species have been detected in numerous studies. The low-temperature association of *Colwellia* results in its ubiquitous presence in cold and polar marine ecosystems including the pelagic zone, sea ice, relic marine salinity meromictic lakes, epi-shelf lakes forming on ice shelves, and effectively the benthos of the entire ocean regardless of depth (Deming et al. 1988; Bowman et al. 1997; DeLong et al. 1997; Junge et al. 2002; Bowman and McCuaig 2003; Nogi et al. 2004; Zeng et al. 2005; Yu et al. 2006; Prabagaran et al. 2007; Collins et al. 2010; Veillette et al. 2011). Besides low-temperature ecosystems, *Colwelliaceae* have been found in many marine contexts including brines to preserve or process food (Abriouel et al. 2011), endocytic or epiphytic relations with dinoflagellates (Seibold et al. 2001; Wichels et al. 2004), marine biofilms (Gillan et al. 1998; Finnegan et al. 2011), faunal associations (Du et al. 2010), and aquaculture systems (McIntosh et al. 2008). The species *T. loyana* appear to be associated with coral disease (Thompson et al. 2006) and the use of bacteriophage specific against this species has been suggested as a control agent to reduce disease (Efrony et al. 2009). Algae-associated *Colwellia* and other epiphytic bacteria may produce enzymes that combat photooxidative stress for the algae (Hünken et al. 2008). The genome of *C. psychrerythraea* 34H was noted to contain several catalases and two classes of superoxide dismutase suggesting an active capacity to deal with reactive oxygen species (Methé et al. 2005).

Colwellia species thrive at sub-zero temperatures within the brine channels of sea ice (see review on sea-ice microbiology for more information Bowman 2013) where they are motile and divide at temperatures of −10 °C or less (Junge et al. 2003, 2006). In these environments *Colwellia* are infected by equally cold-adapted bacteriophages and thus are part of a psychrophilic microbial loop specially adapted to the highly dynamic and extreme sea-ice environment (Borriss et al. 2003; Wells and Deming 2006a, b). Metazoa within these loops may benefit from PUFA synthesized by *Colwellia* species since it is required for higher life-form neurological development. Metazoa are unable to synthesize EPA or DHA de novo and must acquire them in their diet (Nichols 2003). Highly cold-adapted enzymes still surprisingly active at 0 °C no doubt allow these bacteria to be successful in what would be considered a hostile environment (Huston et al. 2000; Yu et al. 2009). In sea ice exopolysaccharides secreted by *Colwellia* strains as well as other sea-ice dwelling species appear to be capable of influencing ice crystal formation. Accumulated evidence suggests polysaccharide formation is also crucial in controlling nutrient access and acting as a cryoprotectant during freezing in this ice brine environment (Marx et al. 2009; Ewert and Deming 2011; Krembs et al. 2011). *Colwellia* spp. also likely produce ice-binding proteins that allow them to actively colonize sea ice as it forms (Raymond et al. 2007). Dispersal of *Colwellia* species into the atmosphere by the wind was speculated as potentially impacting atmospheric ice formation and cloud formation via nucleation reactions due to these ice-affecting properties (Junge and Swanson 2008).

Studies using stable isotope probing showed unexpectedly that *Colwellia* species and other related bacteria within the order *Alteromonadales*, the family *Oceanospirillaceae*, and species within the genus *Arcobacter* of the class *Epsilonproteobacteria* are capable of manganese reduction while using acetate as an electron donor (Vandieken et al. 2012). The results suggest *Colwellia* (and perhaps also *Thalassomonas*) is involved in anaerobic respiration processes in surface sediments using nitrate and manganese as electron acceptors to oxidize simple organic compounds. This is consistent with *Colwelliaceae* being relatively abundant in cold surficial sediments.

Evidence suggests that *Colwellia* species can assimilate bicarbonate directly in the dark via anaplerotic fixation reactions in which tricarboxylic acid cycle intermediates are created via carboxylases including malic enzyme, pyruvate carboxylase, and pyruvate carboxykinase (Alonso-Sáez et al. 2010). It is uncertain how significant bicarbonate take-up is in terms of the bigger picture of oceanic CO_2 adsorption or what controls the uptake or indeed the physiological benefits. Metatranscriptomic studies suggest *Colwelliaceae* are highly responsive to bacterioplankton activity (Eilers et al. 2000; Stewart et al. 2012), which suggests *Colwelliaceae* and other heterotrophic bacteria may have influence in carbon budgets in marine ecosystems, especially locations that are highly productive.

Several studies indicate *Colwellia* species are able to degrade hydrocarbons, including straight chain alkanes, indicating strains may contain alkane monooxygenases and other similar

enzymes. This is consistent with *Colwellia* and *Thalassomonas* species being able to utilize various monocarboxylic and dicarboxylic compounds such as caprate and azelate (Tables 10.1 and 10.3). Oil contaminated sites or culture enrichments with moderate length alkanes as carbon sources have been shown to become enriched in members of the family *Colwelliaceae* (Powell et al. 2004; Brakstad et al. 2008; Schwermer et al. 2008; Korenblum et al. 2010; Baelum et al. 2012); with suggestions that degradation may also occur in anaerobic sites (Gittel et al. 2012).

Applications

Owing to the characteristic psychrophily of *Colwellia* species, their psychoactive enzymes have attracted interest ranging beyond the study of protein thermostability to application-oriented research (Wang et al. 2008; Kuddus and Ramteke 2012). An expression system that allows recombinant protein production in yeast could aid in studying specific protein products directly in assay screening (Seok et al. 2012). Potent agarolytic enzymes produced by *Thalassomonas* strains (Ohta et al. 2005) have also been studied in the context of biotechnological applicability. An interesting application of the latter is the utilization of *Thalassomonas* agarases to improve the medicinal potency of seaweed-derived polysaccharides (Hatada et al. 2006). These polysaccharides are used as a food (e.g., nori, laver) but also in Chinese traditional medicine and have been shown to have antioxidant properties, acting as scavengers of reactive oxygen species (Rocha de Souza et al. 2007). Agarolytic activity has been proposed to release sulfated galactan antioxidants from porphyran and other similar highly sulfated, complex polysaccharides from brown algae. *Colwellia* species capacity to produce PUFA has attracted interest. One goal is to reduce the reliance of fish oil in farmed fish production (e.g., salmon mariculture). Fish oil supplies contain needed EPA and DHA required for proper fish development and growth. Rotifers fed DHA/EPA-producing *Colwellia psychrerythraea* ACAM 605 and EPA-producing *Shewanella gelidimarina* ACAM 456^T accumulated these PUFA and thus could transfer nutriceutical lipids to fish larvae (Lewis et al. 1998). Bacterial sources of PUFA are however likely to be too low yielding and attention has turned to other sources of PUFA. However, cloning of PUFA synthesis genes into other organisms, such as plants, remains a potential. Other applications involving members of the *Colwelliaceae* include an interesting adaptation of *Colwellia psychrerythraea* 34H-derived DNA ligase gene *ligA* to develop temperature-sensitive versions of pathogens such as *Francisella tularensis* and *Salmonella enteritidis* that allow for larger delivery of live cellular vaccine that would be effective in inducing an immune response but mitigating the potential for disease via the vaccine itself (Duplantis et al. 2010). *Thalassomonas* and *Colwellia* species like its relative *Pseudoalteromonas* has a capacity for bioactive compound synthesis (reviewed Bowman 2007; Manilal et al. 2010); however studies have only been of limited scope so far.

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11 The Family *Coxiellaceae*

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Abstract
In this chapter, three genera of intracellarul paprasies, *Aquicella, Coxiella* and *Diplorickettsia*, within the family of *Coxiellaceae* will be briefly discussed.

The family *Coxiellaceae* contains three genera: *Aquicella*, *Coxiella*, and *Diplorickettsia*. They are all intracellular parasites with a broad distribution among different hosts ranging from insects to mammals.

Coxiella burnetii is the etiological agent of the disease Q fever (Kazar 2005). The microorganisms are maintained in insects, most likely in ticks that transmit them to domestic animals. Cattle, goats, and sheep are infected following tick bites causing an asymptomatic disease, although in pregnant cows, it can result in abortions. Human transmission is via an aerosol involving contaminated animal products. Infections are most common among livestock farmers, workers in the food industry who are exposed to contaminated meat or milk, and those involved in harvesting or processing wool. The majority of infections are asymptomatic; however, a significant fraction will progress to Q fever. The disease symptoms and outcome depend on the nature of the infection, i.e., whether it is acute or chronic. Acute Q fever has a flu-like manifestation with fever, cough, chills, and sweats, with an occasional neurological involvement. In infected pregnant women, there is a risk of miscarriage. In most cases, the infection resolves over a period of several weeks even without treatment, but the antibiotic doxycycline is routinely used in a regimen lasting over a year. Less common is the chronic form of the disease, which occurs in a minority of infected patients and is the consequence of delay in antibiotic therapy. The most severe form is endocarditis in individuals who are immunosuppressed or with previous heart damage, and the mortality rate can reach 50 %.

The ability of *C. burnetii* to cause human infections is directly linked to its intracellular life cycle (van Schaik et al. 2013). Following inhalation of the organisms, *C. burnetii* invade monocytes or macrophages, where they replicate in enclosed intracellular endocytic vesicles, which fuse with other lysosomal vesicles creating an acidic environment. Unlike other bacteria phagocytized and killed by these immune cells, *C. burnetii* can control its own pH homeostasis using several H+/Na+ transporters allowing them to replicate within the harsh acidic environment of the vesicle. The major virulence determinant of *C. burnetii* expressed during the intracellular growth is a secretion system (called the type 4 secretion system) capable of translocating over 100 effector proteins directly from the bacterial cytoplasm to the infected cell's cytosol, where they modify various host functions for the benefit of bacterial replication and avoidance of host defenses. While within the vesicles, *C. burnetii* also dampen the normal bactericidal activities of these cells; they also remain unrecognized by the host immune system by preventing the apoptosis of infected cells. Consequently, they are not killed and persist for prolonged time periods until, during acute infection, the immunocompetent host is capable of mobilizing essential host defense mechanisms. The flu-like disease symptoms of infected individuals are very likely due to the pathogen-induced unregulated production of systemic immunomodulators like cytokines and interferon. The infections are diagnosed by analyzing blood samples for the presence of antibodies to the organism or its DNA by molecular (PCR-based) detection methods.

Until recently, *C. burnetii* has been difficult to grow in the laboratory, and either animal hosts (rodents), embryonated eggs, or tissue culture cells are used for their propagation. They are Gram-negative coccobacilli, and when purified from the vacuoles of infected cells, they appear in two morphological forms as large and small cell variants. Complex media consisting of lysates of eukaryotic cells and a defined medium were developed allowing culturing of these organisms axenically (Omsland et al. 2009). Compared to other intracellular parasites, the genome of *C. burnetii* is relatively large and encodes proteins for most energy-generating pathways including glycolysis, the pentose phosphate pathway, and the TCA cycle (Seshadri et al. 2003). The organism has the ability to transport a limited number carbohydrates including glucose-6-phosphate. However, when growing in the protein-rich environment of the host cells, *C. burnetii* prefer to use amino acids imported from the vesicles as the source of carbon and energy. The genome also encodes functional purine and pyrimidine biosynthetic enzymes as well as those for various vitamins and cofactors but lacks genes for enzymes necessary to synthesize over half of its amino acids; these have to be imported by specific membrane transporters following the breakdown of proteins in the endocytic vesicles. The *C. burnetii* genome encodes enzymes required of the synthesis of fatty acids and phospholipids, but a substantial fraction of its membrane is derived from preformed lipids of the host.

Aquicella lusitana and *Aquicella siphonis* were isolated from two different freshwater reservoirs (Santos et al. 2003). They were successfully cultured on yeast extract containing media where charcoal was included. The organisms were Gram-negative, nonmotile, filamented rods. Both of the *Aquicella* species were also able to infect and grow in amoebae but not human cell lines.

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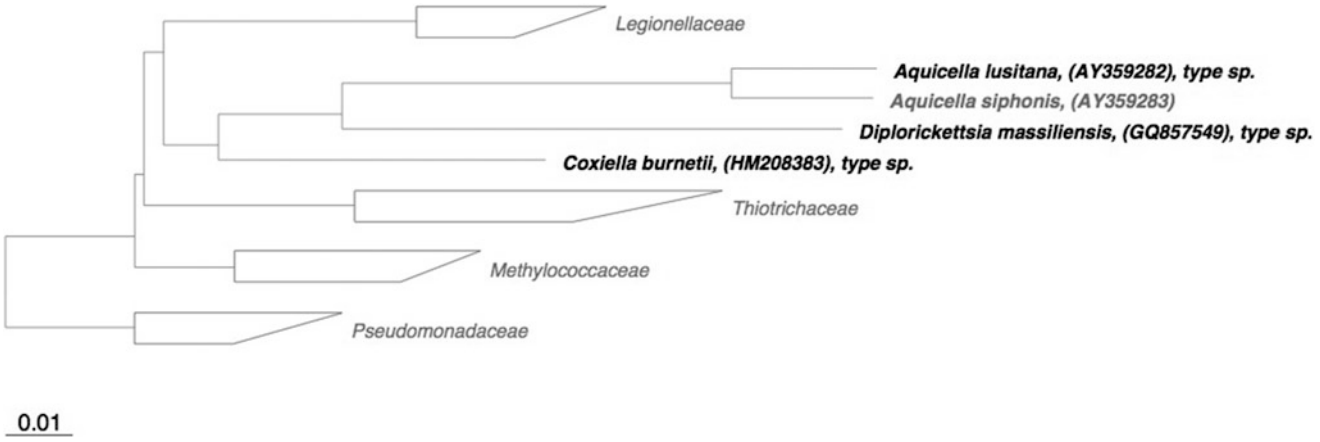

Fig. 11.1

The phylogeny of the family *Coxiellaceae* reconstructed from 16S rRNA gene sequences by Neighbor Joining method. Phylogenetic reconstruction of the family *Coxiellaceae* based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes-Cantor correction. The sequence datasets and alignments were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high quality type strain sequences proportionally distributed among the different bacterial and archaeal phyla. In addition, a 40 % maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. Scale bar indicates estimated sequence divergence

Ticks also harbor another intracellular pathogen, *Diplorickettsia massiliensis* (Mediannikov et al. 2010). In several instances, serology and DNA methods were used to demonstrate that this organism is a likely cause of tick-borne infections in humans. In infected cells, they appear as paired rods. They can be propagated in cultured mammalian cells of human and mouse origin but not axenically (*Fig. 11.1*).

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12 The Family *Ectothiorhodospiraceae*

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Abstract
The family *Ectothiorhodospiraceae*, first proposed in 1984, is affiliated with the *Gammaproteobacteria*. As of July 2012, it encompassed 15 genera: *Ectothiorhodospira* [type genus], *Alkalilimnicola*, *Alkalispirillum*, *Aquisalimonas*, *Arhodomonas*, *Ectothiorhodosinus*, *Halorhodospira*, *Methylonatrum*,

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Natronocella, *Nitrococcus*, *Thioalbus*, *Thioalkalivibrio*, *Thiohalomonas*, *Thiohalospira*, and *Thiorhodospira*, with a total of 38 species, including two that were considered later heterotypic synonyms of earlier published species. Physiologically the family is highly diverse. Four genera consist of anoxygenic phototrophs, four genera contain sulfur-oxidizing chemolithotrophs, one monospecific genus performs autotrophic nitrite oxidation, five genera contain aerobic chemoheterotrophs, and one monospecific genus is facultatively methylotrophic. Anaerobic growth using nitrate and other oxidized nitrogen compounds as electron acceptors occurs in some non-phototrophic members of the family. Many representatives are halophilic and/or alkaliphilic, and they can be found worldwide in saline, hypersaline, and alkaline aquatic and terrestrial environments.

Taxonomy: Historical and Current

Family *Ectothiorhodospiraceae* Imhoff 1984a, 339[VP]

Ec.to.thi.o.rho.do.spi.ra'ce.ae. N.L. fem. n. *Ectothiorhodospira*, type genus of the family; *-aceae*, ending to denote a family; N.L. fem. n. *Ectothiorhodospiraceae*, the *Ectothiorhodospira* family

Type genus: *Ectothiorhodospira*.

The mol% G+C of the DNA varies between 50.5 and 74.5.

The family *Ectothiorhodospiraceae*, consisting of Gram-negative bacteria, was circumscribed on the basis of phylogenetic analysis of 16S rRNA sequences (Imhoff 1984a, 2005a, 2006). The family is phenotypically, metabolically, and ecologically diverse. The family includes organisms that live photoautotrophically using reduced sulfur compounds as the electron donors, as photoheterotrophs, as chemolithoautotrophs on reduced sulfur compounds or nitrite, as chemoorganotrophs that oxidize simple carbon compounds with oxygen or nitrate as the electron acceptor, or as facultative methylotrophs.

The family was originally split off from the *Chromatiaceae* to encompass phototrophic representatives that contain bacteriochlorophyll *a* or *b* and carotenoids and preferably grow anaerobically in the light using reduced sulfur compounds as electron donors, oxidize sulfide to elemental sulfur which is deposited outside the cells, and form sulfate as the final oxidation product (e.g., genera *Ectothiorhodospira*, *Halorhodospira*). Based on their phylogenetic affiliation, different groups of sulfur- or nitrite-oxidizing chemolithotrophs and aerobic and facultatively anaerobic chemoheterotrophs were later classified within the family, so that today only 4 out of the 15 genera are anoxygenic phototrophs. A phenotypic property common to many members of the family is their requirement for alkaline and/or saline/hypersaline conditions for growth.

At the time of writing (July 2012), the family contained 15 genera with a total of 38 species whose names have standing in the nomenclature (Tables 12.1–12.6): *Ectothiorhodospira* [type genus] (9 species of which two are considered later heterotypic synonyms), *Alkalilimnicola* (2 species), *Alkalispirillum* (1 species), *Aquisalimonas* (1 species), *Arhodomonas* (1 species), *Ectothiorhodosinus* (1 species), *Halorhodospira* (4 species), *Methylonatrum* (1 species), *Natronocella* (1 species), *Nitrococcus* (1 species), *Thioalbus* (1 species), *Thioalkalivibrio* (10 species), *Thiohalomonas* (2 species), *Thiohalospira* (2 species), and *Thiorhodospira* (1 species).

Phylogenetic Structure of the Family and Its Genera

Phylogenetically the family *Ectothiorhodospiraceae* is affiliated with the *Gammaproteobacteria*, order *Chromatiales*. Figure 12.1 shows a neighbor joining tree of the type strains of the 38 species of the family *Ectothiorhodospiraceae*. It includes the two species of the genus *Thiohalomonas* (Sorokin et al. 2007c), a genus not previously assigned to the family but phylogenetically positioned within the group. Also shown are the positions of *Methylohalomonas lacus* and *Acidiferrobacter thiooxydans*, organisms previously assigned to the family but not closely affiliated with it (see below).

The different genera are well separated within the tree. It should be noted that the quality of the available 16S rRNA sequence of *Ectothiorhodospira mobilis* DSM 237, the type species of the type genus of the family, is poor, and resequencing is recommended. This strain is a neotype, as the original strain on which the description was based (Pelsh 1936) is no longer available (Trüper 1968).

Molecular taxonomic studies toward the classification of *Ectothiorhodospira* and related anoxygenic phototrophs started in the early 1970s with a comparative study of the G+C content of the DNA (Mandel et al. 1971), and the first 16S rRNA sequence studies, still based on the oligonucleotide cataloging method, were published by Stackebrandt et al. (1984). DNA-DNA and rRNA-DNA hybridization studies (Ivanova et al. 1985) and comparison of complete 16S rRNA gene sequences supported the reclassification of the extremely halophilic strains in the newly established genus *Halorhodospira* (Imhoff and Süling 1996). In-depth studies by Ventura and coworkers, based on total DNA restriction patterns, quinone composition, DNA-DNA reassociation, and ribotyping RFLP of 16S/23S rRNA genes, improved our understanding of the taxonomic relationships within the group (Ventura et al. 1993, 1999, 2000) and led to the proposal to consider *E. vacuolata* as a later heterotypic synonym of *E. shaposhnikovii* and *E. marismortui* as a later heterotypic synonym of *E. mobilis* (Ventura et al. 2000).

A comparative study of the *cbbL* gene (large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase) of the

Table 12.1

The genera classified within the family *Ectothiorhodospiraceae*, as of July 2012

Genus	Number of species	Type species	General properties
Phototrophs:			
Ectothiorhodospira [type genus]	9[a]	*Ectothiorhodospira mobilis*	Rod-shaped, vibrioid, or spiral-shaped cells, motile by a polar tuft of flagella. Contain lamellar stacks of intracellular photosynthetic membranes that are continuous with the cytoplasmic membrane. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids. Grow photoautotrophically under anoxic conditions with reduced sulfur compounds or hydrogen as electron donors. Sulfide is oxidized to sulfate, with elemental sulfur, deposited outside the cells, as intermediary product. Grow in saline and hypersaline environments. Some species are alkaliphilic
Ectothiorhodosinus	1	*Ectothiorhodosinus mongolicus*	Curved rods or semicircular nonmotile cells that contain bacteriochlorophyll *a* and carotenoids. Moderately halophilic and moderately alkaliphilic. Grow anaerobically as photoautotrophs or photoheterotrophs. The photosynthetic system is located on concentric lamellae lining the cell envelope. When grown on sulfide as an electron donor, globules of elemental sulfur as an intermediary product are deposited outside the cells
Halorhodospira	4	*Halorhodospira halophila*	Spirals or rod-shaped cells, motile by bipolar flagella, and containing lamellar stacks of internal photosynthetic membranes. Photosynthetic pigments are bacteriochlorophyll *a* or *b* and carotenoids. Reduced sulfur compounds serve as electron donors for photoautotrophic growth under anoxic conditions; photoheterotrophic growth is also possible. When grown on sulfide as an electron donor, globules of elemental sulfur as an intermediary product are deposited outside the cells. Moderately to extremely halophilic and extremely alkaliphilic
Thiorhodospira	1	*Thiorhodospira sibirica*	Vibrioid or spiral-shaped cells, motile by means of a monopolar tuft of flagella. Internal photosynthetic membranes are parallel lamellae piercing the cytoplasm lengthwise or underlying the cytoplasmic membrane. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids. Obligately phototrophic, strictly anaerobic, alkaliphilic, and slightly halophilic. During growth with sulfide as the electron donor, globules of elemental sulfur are found outside the cytoplasm, in the medium, attached to the cells, or in the periplasm. In the presence of sulfide, organic substances may be photoassimilated
Chemolithotrophs:			
Nitrococcus	1	*Nitrococcus mobilis*	Spherical cells, occurring singly or in pairs, motile by 1–2 flagella. Obligate lithoautotrophs that grow aerobically at 70–100 % seawater salinity and derive energy from the oxidation of nitrite to nitrate. Intracytoplasmic membranes occur as tubes randomly arranged throughout the cytoplasm
Thioalbus	1	*Thioalbus denitrificans*	Nonmotile, obligately chemolithoautotrophic and facultatively anaerobic bacteria that use reduced sulfur compounds as electron donors with oxygen or nitrate as electron acceptors. Slightly halophilic and neutrophilic
Thioalkalivibrio	9[b]	*Thioalkalivibrio versutus*	Curved rods, spirilla or coccoid cells, motile by 1–3 polar flagella or nonmotile. Obligate chemolithoautotrophs that oxidize sulfide, thiosulfate, sulfur and tetrathionate and fix CO_2 via the Calvin cycle. Sodium is required for growth, and many species are extremely halotolerant and alkaliphilic. Some strains deposit elemental sulfur in the periplasmic space
Thiohalomonas	2	*Thiohalomonas denitrificans*	Nonmotile obligately chemolithoautotrophic, facultatively anaerobic, moderately halophilic and neutrophilic rods or vibrios that use reduced sulfur compounds as electron donors with oxygen or nitrate as the electron acceptors
Thiohalospira	2	*Thiohalospira halophila*	Spiral-shaped motile, aerobic, obligately chemolithoautotrophic bacteria that oxidize inorganic sulfur compounds to sulfate. Tetrathionate is an intermediate of thiosulfate oxidation. The genus includes extremely halophilic, neutrophilic and moderately halo(alkali)philic representatives. Cellular fatty acids are dominated by saturated C_{16}-C_{18} species

Table 12.1 (continued)

Genus	Number of species	Type species	General properties
Chemoheterotrophs and facultative chemoheterotrophs:			
Alkalilimnicola	2	*Alkalilimnicola halodurans*	Short, straight, oval-shaped cells occurring singly or in pairs, and motile by means of a single polar flagellum. Aerobic, grows by respiration on organic acids and other organic compounds. Nitrate is reduced, but nitrite is not. Grows optimally under alkaline conditions. Moderately halophilic and extremely halotolerant
Alkalispirillum	1	*Alkalispirillum mobile*	Spiral-shaped cells, motile by means of a single polar flagellum. Alkaliphilic and slightly halophilic, growing aerobically using acetate, succinate, aspartate, or glucose as energy sources
Aquisalimonas	1	*Aquisalimonas asiatica*	Rod-shaped motile cells occurring singly, in pairs or in long chains. Aerobic, chemoorganotrophic, growing on sugars, organic acids, and other simple organic compounds. Moderately halophilic and alkalitolerant. Nitrate is reduced, but nitrite is not
Arhodomonas	1	*Arhodomonas aquaeolei*	Rod-shaped cells that occur singly or in pairs, motile by means of a single polar flagellum. Grow as chemoorganotrophs with oxygen or nitrate as electron acceptor. Mesophilic and halophilic. Sodium is required for growth
Natronocella	1	*Natronocella acetinitrilica*	Rod-shaped, yellow pigmented cells. Aerobic heterotrophs that can use aliphatic nitriles as carbon and energy source. Highly salt-tolerant and obligately alkaliphilic
Methylotrophs:			
Methylonatrum	1	*Methylonatrum kenyense*	Short rods that can grow as methylotrophs on methanol, but can also use ethanol and acetate. During methylotrophic growth CO_2 is fixed via the Calvin cycle. Moderately salt-tolerant and obligately alkaliphilic

[a]Including *E. marismortui* and *E. vacuolata*, considered as later heterotypic synonyms of *E. mobilis* and *E. shaposhnikovii*, respectively (Ventura et al. 2000)
[b]The description of a tenth species, *T. sulfidiphilus*, is in press (Sorokin et al. 2012)

haloalkaliphilic obligately autotrophic sulfur-oxidizing bacteria of the genus *Thioalkalivibrio* showed the existence of a number of clusters: (1) *T. thiocyanodenitrificans* and *T. denitrificans*; (2) *T. jannaschii*, *T. nitratis*, *T. versutus*, and *T. thiocyanoxidans*; and (3) *T. paradoxus* and *T. nitratireducens* (Tourova et al. 2005). These same clusters can be recognized in the 16S rRNA tree of *Fig. 12.1*. A more comprehensive study of 16S rRNA, *cbbl*, and *nifH* gene sequences showed the *cbbl* gene to be present in all strains examined (5 strains of *Ectothiorhodospira*, 3 *Halorhodospira*, 1 *Thiorhodospira*, 1 *Ectothiorhodosinus*, 9 *Thioalkalivibrio*, 4 *Alkalispirillum*, 2 *Alkalilimnicola*, 1 *Nitrococcus*, and 1 *Arhodomonas*) with the exception of the type strains of *Alkalispirillum mobile* and *Arhodomonas aquaeolei*. The *nifH* gene was found in all *Ectothiorhodospira*, *Halorhodospira*, and *Thiorhodospira* species, but not in *Ectothiorhodosinus*. Unexpectedly, *nifH* fragments were also recovered from *Alkalilimnicola halodurans*, an organism not reported to fix molecular nitrogen (Tourova et al. 2007).

The tree of *Fig. 12.1* also includes the two monospecific genera *Methylohalomonas* and *Acidiferrobacter*. *Methylohalomonas lacus* is an obligate methylotroph that oxidizes C_1 compounds as carbon and energy sources and uses the serine pathway for carbon assimilation (Sorokin et al. 2007b). *Acidiferrobacter thiooxydans* (earlier designated "*Thiobacillus ferrooxidans* m-1") is an acidophilic (pH optimum ~2, minimum 1.2), thermotolerant (growing up to 47 °C), facultatively anaerobic bacterium that grows by oxidation of Fe(II), pyrite, sulfide, sulfur, or tetrathionate (Hallberg et al. 2011). These two genera have been assigned to the family *Ectothiorhodospiraceae*, but as the tree shows, they are not closely affiliated with the group. Therefore they are not further discussed in this chapter.

Genome Analysis

At the time of writing (July 2012), information was available on the genome sequences of 6 members of the *Ectothiorhodospiraceae*, 4 of which being type strains (*Ectothiorhodospira haloalkaliphila*, *Halorhodospira halophila*, *Thioalkalivibrio sulfidiphilus*, *Alkalilimnicola ehrlichii*) (*Table 12.7*). Two additional sequenced strains are *Ectothiorhodospira* sp. PHS-1, isolated from a hot spring microbial mat on Paoha Island, Mono Lake, CA, and *Thioalkalivibrio* sp. K90mix, recovered from a mixture of sediment samples collected from different soda lakes of the Kulunda Steppe, Altai, Russia. Detailed publications exist on the genome sequences of *T. sulfidiphilus* HL-EbGr7^T and *Thioalkalivibrio* sp. K90mix (Muyzer et al. 2011a, b). The chromosomes are between 2.7 and 3.5 Mbp in length and contain between 2,791 and 3,319 protein-coding genes. *Thioalkalivibrio* sp. K90mix also contains a 240 kbp plasmid.

Prior to the publication of the genome sequence of *H. halophila*, detailed information was obtained for a number

Table 12.2

Comparison of selected characteristics of the members of the genus *Ectothiorhodospira*

	Ectothiorhodospira mobilis [a]	*E. haloalkaliphila* [b]	*E. magna* [c]	*E. marina* [b]	*E. marismortui* [d,i]	*E. salini* [e]	*E. shaposhnikovii* [f]	*E. vacuolata* [g,i]	*E. variabilis* [h]
Type strain	DSM 237[j]	ATCC 51935	VKM B-2537	DSM 241	DSM 4180	NBRC 105915	DSM 243	DSM 2111	VKM B-2479
Cell size (μm)	0.7–1.0 × 2.0–2.6 (young cells), 3.6–4.8 (spirals)	0.7–1.2 × 2.0–3.0	2.0–3.2 × 9.6–20	0.8–1.2 × 1.5–4.0	0.9–1.3 × 1.5–3.3	1.0–1.5 × 2.0–3.5	0.8–0.9 × 1.5–2.5	1.5 × 2–4	0.8–1.2 × 1.2–1.5
Cell shape	Weakly curved short spirals	Vibrios – short spirals	Spirals	Curved to slightly bent rods	Short curved rods	Vibrioid to spiral shaped	Rods to slightly bent vibrios	Rods	Ovals, rods or twisted spirals
Motility	Monopolar tuft of flagella	Monopolar tuft of flagella	Polar tuft of flagella	Polar tuft of flagella	Monopolar tuft of flagella	Polar flagella	Monopolar tuft of flagella	Monopolar tuft of flagella	Polar flagella
Gas vesicles	–	–	NR	–	–	NR	–	+	Variable
Color	Red	Red	Red – brownish red	Red	Red-purple	Reddish-brown	Red	Pink-red	Red-purple
Pigments	Bchl *a*, spirilloxanthin carotenoids	Bchl *a*, spirilloxanthin carotenoids	Bchl *a*, spirilloxanthin carotenoids	Bchl *a*, spirilloxanthin carotenoids	Bchl *a*, spirilloxanthin carotenoids	Bchl *a*, spirilloxanthin carotenoids	Bchl *a*, spirilloxanthin carotenoids	Bchl *a*, spirilloxanthin carotenoids	Bchl *a*, spirilloxanthin carotenoids
Internal membranes	Stacks of lamellar membranes	Stacks of lamellar membranes	Lamellae not in stacks, distributed freely in the cytoplasm	Stacks of lamellar membranes	Stacks of lamellar membranes	Lamellae parallel to the cytoplasmic membrane	Stacks of lamellar membranes	Stacks of lamellar membranes	Stacks of lamellar membranes
pH range for growth and optimum	Opt. 7.5–8.0	8.5–10.0	8.0–11.0 (Opt. 9.0–10.0)	Opt. 7.5–8.5	Opt. 7–8	7–10 (Opt. 7.5)	Opt. 8.0–8.5	Opt. 7.5–9.5	7.5–10 (Opt. 9.0–9.5)
Temperature range for growth and optimum (°C)	Opt. 25	Opt. 26–40	20–45 (Opt. 30–35)	Opt. 30–40	Opt. 35–45	Opt. 30	Opt. 30–35	Opt. 30–40	20–45 (Opt. 30–35)
Salinity range and optimum (% NaCl)	1–5 (Opt. 2–3)	2.5–15 (Opt. 5)	0–8 (Opt. 0.5–1.5)	0.5–10 (Opt. 2–6)	1–20 (Opt. 3–8)	0.5–12 (Opt. 5)	0–7 (Opt. 3)	0.5–10 (Opt. 1–6)	2–20 (Opt. 5–8)
Electron donors for photoautotrophic growth	Hydrogen, sulfide, thiosulfate (some strains)	Hydrogen, sulfide, thiosulfate	Hydrogen, sulfide, elemental sulfur	Hydrogen, sulfide, sulfite, elemental sulfur, thiosulfate	Hydrogen, sulfide, thiosulfate	–	Hydrogen, sulfide, sulfite, elemental sulfur, thiosulfate	Hydrogen, sulfide, elemental sulfur, thiosulfate	Sulfide, elemental sulfur, thiosulfate

Table 12.2 (continued)

	Ectothiorhodospira mobilis [a]	*E. haloalkaliphila* [b]	*E. magna* [c]	*E. marina* [b]	*E. marismortui* [d,i]	*E. salini* [e]	*E. shaposhnikovii* [f]	*E. vacuolata* [g,i]	*E. variabilis* [h]
Substrates for photoheterotrophic growth	Acetate, fumarate, malate, pyruvate, succinate Some strains: butyrate, fructose, glucose, lactate, propionate	Acetate, fumarate, pyruvate, malate, succinate	Acetate, fumarate, malate, succinate Weak growth on propionate, pyruvate	Acetate, fumarate, lactate, malate, propionate, pyruvate, succinate	Acetate, fumarate, malate, pyruvate, succinate Weak growth on glycerol, lactate, propionate	Acetate, malate, pyruvate	Butyrate, fructose, fumarate, lactate, malate, propionate, succinate	Acetate, fumarate, Propionate, malate, pyruvate, succinate Weak growth on fructose	Acetate, fumarate, lactate, malate, propionate, pyruvate, succinate
Examples of substrates not used for photoheterotrophic growth	Butyrate, ethanol, lactate, propanol	Ethanol, fructose, glucose, propanol	Butyrate, ethanol, formate, glucose, glycerol, lactate	Butyrate, ethanol, fructose, glucose, propanol	Butyrate, ethanol, formate, glucose, methanol,	Butyrate, fructose, fumarate, glucose, lactate, propionate, succinate	Ethanol, propanol	Butyrate, ethanol, glucose, lactate, propanol	Butyrate, ethanol, glycerol, malonate
Vitamin requirement	–[k]	–	–[k]	–	–	*p*-Aminobenzoate, pantothenate, pyridoxal phosphate	–	–	–
Sulfate assimilation	+	+	NR	+	–	–	+	–	–
Major fatty acids or cluster of fatty acids[l]	$C_{18:1}$, $C_{16::0}$, $C_{18:0}$ (V)	(VII)	$C_{18:1}$, $C_{16:1}$, $C_{16:0}$	NR	(V)	$C_{18:1\ \omega7c}$, $C_{16:0}$, $C_{19:0\ cyclo\ \omega8c}$, $C_{16:1}$	$C_{18:1}$, $C_{16::0}$, $C_{16:1}$ (VI)	(VI)	NR
Main respiratory quinones	Q-8 (+Q-7, MK-7)	Q-8, MK-7	Q-7, MK-7	Q-8, MK-7	Q-8, MK-7	MK-7, Q-7	Q-7 (+ MK-7)	Q-7 (+ MK-7)	NR
G + C content of DNA (mol%)	65.0–68.4	62.2–63.5	59.2	62.8	65	63	61.0–64.0	61.4–63.6	62.3–63.3
Sample source and site	Salt flats, Galapagos Islands	Alkaline salt and soda lakes	Soda lake, Transbaikal region, Russia	Coastal marine sediments	Hypersaline sulfur spring, Dead Sea, Israel	Solar saltern, India	Alkaline salt lakes, Jordan, Kenya	Alkaline salt lakes, Jordan, Kenya	Soda lake, Wadi Natrun, Egypt

Data taken from: [a]Trüper (1968); [b]Imhoff and Süling (1996); [c]Bryantseva et al. (2010); [d]Oren et al. (1989); [e]Ramana et al. (2010); [f]Cherni et al. (1969); [g]Imhoff et al. (1981); [h]Gorlenko et al. (2009)
Additional data on growth substrates are given by Imhoff (2005b, 2006) and in the original species descriptions
[i]*E. marismortui* and *E. vacuolata* were considered as later heterotypic synonyms of *E. mobilis* and *E. shaposhnikovii*, respectively (Ventura et al. 2000)
[j]Neotype strain, as the original isolate of Pelsh (1936) was lost
[k]No vitamin requirement was reported, but some strains may require vitamin B_{12}
[l]See Thiemann and Imhoff (1996) for the composition of Cluster V, VI, and VII
NR not reported, *Bchl* bacteriochlorophyll

Table 12.3

Comparison of selected characteristics of the members of the genera *Halorhodospira*, *Ectothiorhodosinus*, and *Thiorhodospira*

Character	*Halorhodospira halophila* [a,b]	*H. abdelmalekii* [b,c]	*H. halochloris* [b,d]	*H. neutriphila* [b,e]	*Ectothiorhodosinus mongolicus* [f]	*Thiorhodospira sibirica* [g]
Basonym	*Ectothiorhodospira halophila*	*Ectothiorhodospira abdelmalekii*	*Ectothiorhodospira halochloris*			
Type strain	DSM 244	DSM 2110	DSM 1059	DSM 15116	UNIQEM U217	ATCC 700588
Cell size (μm)	0.6–0.9 × 2–8	0.9–1.2 × 4–6	0.5–0.6 × 2.5–8.0	1–1.2 × 2–5	0.3–0.5 × 0.7–1.0	3–4 × 7–20
Cell shape	Spiral	Spiral	Spiral	Curved rods	Vibrio-shaped, semicircular or toroid	Vibrioid to spiral
Motility	Bipolar tuft of flagella	Bipolar tuft of flagella	Bipolar tuft of flagella	Polar tuft of flagella	–	Monopolar tuft of flagella
Color	Red-purple	Pale green to green	Pale green to green	Pinkish-red	Pink to brownish red	Brownish red to red
Pigments	Bchl *a*, c spirilloxanthin carotenoids	Bchl *b*, dihydroxylycopene diglucoside diesters	Bchl *b*, dihydroxylycopene diglucoside diesters	Bchl *a*, spirilloxanthin carotenoids	Bchl *a*, spirilloxanthin carotenoids	Bchl *a*, spirilloxanthin carotenoids
Internal membranes	Stacks of lamellar membranes	Stacks of lamellar membranes	Stacks of lamellar membranes	Stacks of lamellar membranes	Concentric lamellae lining the cell envelope	Parallel lamella piercing lengthways the cytoplasm or underlying the cytoplasmic membrane
pH range for growth and optimum	Opt. 8.5–9.0	Opt. 8.0–9.2	Opt. 8.1–9.1	6.0–8.5 (Opt. 6.8–7.0)	7.6–10.1 (Opt. 8.3–9.1)	7.5–10.5 (Opt. 9.0–9.5)
Temperature range for growth and optimum (°C)	Opt. 47	Opt. 30–40	Opt. 48	Opt. 30–35	Opt. 30–35	Opt. 25–30
Salinity range and optimum (% NaCl)	3–30 (Opt. 11–20)	5–30 (Opt. 14–16)	10–35 (Opt. 14–27)	6–30 (Opt. 9–12)	0.5–9 (Opt. 1–7); carbonate 0.1–1.2 M (Opt. 0.24 M)	5–80 g/l $NaHCO_3$ or 0–60 g/l NaCl + 5 g/l Na_2CO_3
Electron donors for photoautotrophic growth	Sulfide, sulfur, thiosulfate	Sulfide, sulfur	Sulfide, sulfur	Sulfide (poor), sulfur (poor)	Sulfide (poor); thiosulfate (only in presence of organic substrates)	Sulfide, sulfur
Substrates for photoheterotrophic growth	Acetate, fumarate, malate, propionate, pyruvate, succinate	Acetate, fumarate, pyruvate, succinate (poorly used: malate, propionate)	Acetate, fumatate, malate, propionate, pyruvate, succinate	Acetate, fumarate, propionate (some strains), pyruvate, succinate	Acetate, fumarate, lactate, malate, propionate, pyruvate, succinate (poorly used: fructose)	Acetate, fumarate, malate, propionate, pyruvate, succinate

Table 12.3 (continued)

Character	*Halorhodospira halophila* [a,b]	*H. abdelmalekii* [b,c]	*H. halochloris* [b,d]	*H. neutriphila* [b,e]	*Ectothiorhodosinus mongolicus* [f]	*Thiorhodospira sibirica* [g]
Examples of substrates not used for photoheterotrophic growth	Butyrate, ethanol, fructose, glucose	Butyrate, ethanol, formate, fructose, glucose, lactate, methanol	Butyrate, ethanol, formate, fructose, glucose, lactate, methanol	Lactate, malate	Butyrate, ethanol, glucose, valerate	Butyrate, ethanol, fructose, glucose, lactate
Requirement for growth factors	- (some strains are stimulated by vitamin B_{12})	–	–	–	–[h]	NR
Sulfate assimilation	–	–	+	–	NR	NR
Major fatty acids or cluster of fatty acids [i]	$C_{18:1}$, $C_{19:0\ cycl}$, $C_{18:0}$, $C_{16:0}$ (II)	$C_{18:1}$, $C_{16:0}$, $C_{19:0\ cycl}$ (III)	$C_{18:1}$, $C_{16:0}$, $C_{19:0\ cycl}$ (IV)	NR	NR	NR
Main respiratory quinones	MK-8 (+ Q-8, MK-5)	MK-4/5 (+ Q-8, MK-8)	MK-4/5 (+ Q-8, MK-8)	NR	Q-8	NR
G + C content of DNA (mol%)	66.5–69.7	63.3–63.8	50.5–52.9	74.1–74.5	57.5	56.0–57.4
Sample source and site	Salt lake, Oregon	Hypersaline soda lakes, Wadi Natrun, Egypt	Hypersaline soda lakes, Wadi Natrun, Egypt	Microbial mat, saltern evaporation pond, France	Steppe soda lake, Mongolia	Surface sediment, Lake Malyi Kasytui, Siberia

Data taken from: [a]Raymond and Sistrom (1967, 1969); [b]Imhoff (2005c); [c]Imhoff and Trüper (1981); [d]Imhoff and Trüper (1977); [e]Hirschler-Reá et al. (2003); [f]Gorlenko et al. (2004); [g]Bryantseva et al. (1999); Imhoff and Gorlenko (2005)

[h]No vitamin requirement was reported, but vitamin B_{12} was included in the growth medium

[i]See Thiemann and Imhoff (1996) for the composition of Cluster II, III, and IV

Additional data on growth substrates are given by and in the original species descriptions

NR not reported, *Bchl* bacteriochlorophyll

Table 12.4

Comparison of selected characteristics of the members of the genus *Thioalkalivibrio*

Character	*Thioalkalivibrio versutus* [a]	*T. denitrificans* [a]	*T. halophilus* [b]	*T. jannaschii* [c]	*T. nitratireducens* [d]	*T. nitratis* [a]	*T. paradoxus* [e]	*T. sulfidiphilus* [f]	*T. thiocyano-denitrificans* [g]	*T. thiocyan-oxidans* [e]
Type strain	CBS 100464	NCCB 10001	DSM 15791	DSM 14478	DSM 14787	NCCB 10002	DSM 13531	NCCB 100376	UNIQEM U226	DSM 13532
Cell size (μm)	NR	NR	0.3–0.4 × 1–2	0.3–0.4 × 1–2	0.8–2	NR	0.8–1 × 1.2–2	0.4 × 3–8	0.5–0.7 × 1.5–5	0.5–0.6 × 0.8–1.4
Cell shape	Curved rods or spirilla	Small curved rods	NR	Curved rods	Coccoid or barrel-shaped	Rods	Barrel-like rods	Slightly curved slender rods	Rods	Curved rods
Motility	Single polar flagellum	Single polar flagellum	Single polar flagellum	Single polar flagellum	–	NR	–	Single polar flagellum	Single polar flagellum	Single polar flagellum
Color	Yellow	–	Yellow	Yellow	–(Colonies become reddish with age)	Yellow	–	–	–	Yellow
pH range for growth and optimum	7.50–10.65 (Opt. 10.0–10.2)	7.50–10.65 (Opt. 10.0–10.2)	7.5–9.8 (Opt. 8.0–9.0)	7.5–10.5	8.0–10.5 (Opt. 9.5–10.0)	7.50–10.65 (Opt. 10.0–10.2)	9.5–10.2 (Opt. 9.8)	8.0–10.5 (Opt. 10)	Opt. 9.6–10	9.5–10.2 (Opt. 9.8)
Temperature range for growth and optimum (°C)	NR	NR	NR (Cultures grown at 30–35)	NR	NR	NR	NR	Opt. 35, grows up to 40–41	NR (Cultures grown at 30)	NR
Na^+ requirement and optimum (M)	NR	NR	Na^+ required (Opt. 2; up to 5 tolerated)	0.4–4	0.2–1.5 (Opt. 0.4–0.5)	NR	NR	0.2–1.5 (Opt. 0.4)	0.3–1.8	0.3–1 Some strains up to 4.3
Substrates for autotrophic growth	Sulfide, sulfur, sulfite, thiosulfate, trithionate, tetrathionate, pentathionate	Sulfide, thiosulfate	Sulfide, sulfur, thiosulfate, polysulfide, tetrathionate	Sulfide, sulfur, thiosulfate, polysulfide, tetrathionate	Sulfide, thiosulfate, polysulfide Slow growth on sulfur	Sulfide, thiosulfate	Sulfide, thiosulfate, polysulfide, thiocyananate, carbon disulfide Slow growth on sulfur	Sulfide, sulfur, thiosulfate, polysulfide, tetrathionate	Sulfide, sulfur, thiosulfate, polysulfide, tetrathionate, thiocyanate	Sulfide, sulfur, thiosulfate, polysulfide, tetrathionate, thiocyanate

Table 12.4 (continued)

Character	*Thioalkalivibrio versutus* [a]	*T. denitrificans* [a]	*T. halophilus* [b]	*T. jannaschii* [c]	*T. nitratireducens* [d]	*T. nitratis* [a]	*T. paradoxus* [e]	*T. sulfidiphilus* [f]	*T. thiocyano-denitrificans* [g]	*T. thiocyan-oxidans* [e]
Reduction of nitrate and other oxidized nitrogen compounds	NR	+ Grows well on thiosulfate + N_2O	NR	–	+ (to nitrite only)	+ (to nitrite only) No anaerobic growth with nitrate	–	–	+	–
Major fatty acids	NR	NR	NR	NR	NR	NR	NR	$C_{18:1\ \omega7}$, $C_{16:0}$, $_{C19\ cyc}$	NR	NR
Main respiratory quinone	Q-8	NR	NR	NR	NR	NR	NR	Q-8	NR	NR
G + C content of DNA (mol%)	63–65.6	62.3–65.0	65.1	63.7	64.8	61.3–62.1	65.6–66.4	63.5–65	63.1–63.7	66.2–66.9
Sample source	Soda lake, Siberia	Sediment, Lake Bogoria, Kenya	Hypersaline lake, Altai Steppe, Russia	Mono Lake, California	Sediment, Wadi Natrun, Egypt	Soda lake Nakuru, Kenya	Soda lakes, Egypt and Kenya	Sulfide-oxidizing Bioreactor; Lake Elmenteita, Kenya	Soda lakes, Egypt and Siberia	Soda lake, Kenya

Data taken from: [a]Sorokin et al. (2001a); [b]Banciu et al. (2004a); [c]Sorokin et al. (2002a); [d]Sorokin et al. (2003); [e]Sorokin et al. (2002b); [f]Sorokin et al. (2012); [g]Sorokin et al. (2004)
Additional data on growth substrates are given by Brenner et al. (2005) and in the original species descriptions
NR not reported

Table 12.5

Comparison of selected characteristics of the members of the genera *Thiohalomonas*, *Thiohalospira*, *Thioalbus*, and *Nitrococcus*

Character	*Thiohalomonas denitrificans* [a]	*Thiohalomonas nitratireducens* [a]	*Thiohalospira halophila* [b]	*Thiohalospira alkaliphila* [b]	*Thioalbus denitrificans* [c]	*Nitrococcus mobilis* [d]
Type strain	DSM 15841	DSM 16925	DSM 15071	DSM 17116	KCTC 5699	ATCC 25380
Cell size (μm)	0.4–0.5 × 2–6	0.5 × 1–5	0.4–0.5 × 2–8	0.4–0.5 × 2–4	0.5–0.8 × 1.2–1.5	1.5 × 1.8–3.5
Cell shape	Rods	Vibrios	Spirilla	Spirilla of variable length	Rods	Spheres
Motility	–	–	Single bipolar flagella	Single polar flagella	–	1–2 Subpolar flagella
Intracellular membranes	–	–	–	–	–	Tubular membranes randomly arranged in the cytoplasm
Pigmentation	–	–	Yellow membrane-bound pigment may be present	Yellow membrane-bound pigment	–	Yellowish to reddish because of cytochromes
pH range for growth and optimum	6.5–8.2 (Opt. 7.3–8.2)	Opt. 7.8–8.0	6.5–8.2 (Opt. 7.3–7.8)	7.0–10.2 (Opt. 8.5)	Opt. 7.0–7.5	Opt. 7.5–8.0
Temperature range for growth and optimum (°C)	NR (Cultures grown at 30)	NR (Cultures grown at 30)	Opt. 32–35	Opt. 35	10–40 (Opt. 28–32)	14–40 (Opt. 25–30)
Salinity range and optimum (% NaCl)	6–18 (Opt. 9–12)	6–15 (Opt. 6)	12–29 (Opt. 15–18)	3–24 (Opt. 12)	1–5 (Opt. 3)	70–100 % seawater
Substrates for chemolithoautotrophic growth	Sulfide, thiosulfate	Thiosulfate	Sulfide, sulfur, thiosulfate, tetrathionate	Sulfide, sulfur, thiosulfate, tetrathionate	Sulfide, sulfur, thiosulfate, tetrathionate	Nitrite
Major fatty acids	$C_{16:0}$, $C_{16:1\ \omega7}$, $C_{18:1\ \omega7}$	NR	10-methyl $C_{16:0}$, $C_{16:0}$, $C_{18:0}$	10-methyl $C_{16:0}$, $C_{16:0}$, $C_{18:0}$	$C_{16:0}$, $C_{16:1\ \omega7c}$, $C_{18:1\ \omega7c}$	NR
Main respiratory quinone	NR	NR	NR	NR	NR	NR
G + C content of DNA (mol%)	58–60	62.9	65.8–67.0	65.6	64.5	61.2
Sample source and site	Hypersaline lakes, solar salterns, Eurasia	Hypersaline lakes, Siberia	Hypersaline lakes, solar salterns, Eurasia	Sediment, hypersaline alkaline lakes, Wadi Natrun, Egypt	Marine sediment, South Korea	Ocean water

Data taken from: [a]Sorokin et al. (2007c); [b]Sorokin et al. (2008a); [c]Park et al. (2011); [d]Watson and Waterbury (1971); Spieck and Bock (2005)
Additional data on growth substrates are given in the original species descriptions
NR not reported

of its proteins from MALDI-TOF/TOF sequence analysis of 2-D PAGE-separated proteins (Samyn et al. 2006).

Phages

No bacteriophages infecting members of the *Ectothiorhodospiraceae* were yet described.

Phenotypic Analyses

The Properties of the Genera and Species of *Ectothiorhodospiraceae*

As all *Gammaproteobacteria*, the *Ectothiorhodospiraceae* possess a Gram-negative type of cell wall. Endospore formation was never observed. However, *Thioalkalivibrio versutus* was reported

Table 12.6

Comparison of selected characteristics of the members of the genera *Alkalilimnicola*, *Alkalispirillum*, *Aquisalimonas*, *Arhodomonas*, *Methylonatrum*, and *Natronocella*

Character	*Alkalilimnicola halodurans*[a]	*A. ehrlichii*[b]	*Alkalispirillum mobile*[c]	*Aquisalimonas asiatica*[d]	*Arhodomonas aquaeolei*[e]	*Methylonatrum kenyense*[f]	*Natronocella acetinitrilica*[g]
Type strain	DSM 13718	DSM 17681	DSM 12769	CCM 7368	ATCC 49307	DSM 15732	NCCB 100179[h]
Cell size (μm)	~1.5 × 2–6	0.3–0.5 × 1.5–2.5	~1 × ~3.7–4	0.7–0.9 × 2.0–10.0	0.8–1.0 × 2.0–2.5	0.5–0.7 × 1–1.2	0.4–0.5 × 1.5–4.0
Cell shape	Oval rods	Rods	Spirilla	Rods	Rods	Short rods	Rods
Motility	Single polar flagellum	Single polar flagellum	Single polar flagellum	+ (Mode of flagellation not reported)	Single polar flagellum	–	Single polar or subpolar flagellum
Pigmentation	–	–	–	–	–	–	Yellow – zeaxanthin and other carotenoids
pH range for growth and optimum	Opt. >8.5	7.3–10 (Opt. 9.3)	5.0–12.0 (Opt. 9.0–10.0)	6.0–10.8 (Opt. 7.5–8.5)	6–8 (Opt. 7)	8.3–10.5 (Opt. 10)	8–10.5 (Opt. 9.5–9.8)
Temperature range for growth and optimum (°C)	20–55 (Opt. 35)	13–40 (Opt. 30)	30–45 (Opt. 35–38)	20–50 (Opt. 37)	20–45 (Opt. 37)	NR	NR (Cultures grow at 30)
Salinity range and optimum (% NaCl)	0–28 (Opt. 3–8)	1.5–19 (Opt. 3)	0–25 (Opt. 6)	1–20 (Opt. 7–10)	6–20 (Opt. 15)	0.3–4 M total Na^+ (Opt. 0.5–1 M)	Up to 4 M total Na^+ (Opt. 0.6 M)
Substrates for aerobic metabolism	Short-chain fatty acids, alcohols and a few sugars, e.g., acetate, cellobiose, citrate, fructose, glucose, lactate, succinate	Acetate, lactate, malate[i], propionate, pyruvate, succinate. Hydrogen (chemoautotrophic growth)	Acetate, aspartate, glucose (slow growth), succinate	Acetate, glucose, glutamate, glycerol, maltose, proline, pyruvate, rhamnose, succinate, and many others	Acetate, butyrate, crotonate, ethanol, fumarate, gluconate, glutamate, glutamine, glycerol, isovalerate, lactate, propionate, pyruvate, succinate, valerate, xylose	Acetate, ethanol, formate, methanol	Acetonitrile, propionitrile
Substrates for anaerobic metabolism		Autotrophic growth on As(III), hydrogen, sulfide, or thiosulfate + nitrate; growth on formate + nitrate (but not with O_2)					
Examples of substrates not used		Alcohols, sugars			Fructose, glucose, mannose, methanol, ribose, sucrose	Dimethylamine, trimethylamine	

Table 12.6 (continued)

Character	*Alkalilimnicola halodurans*[a]	*A. ehrlichii*[b]	*Alkalispirillum mobile*[c]	*Aquisalimonas asiatica*[d]	*Arhodomonas aquaeolei*[e]	*Methylonatrum kenyense*[f]	*Natronocella acetinitrilica*[g]
Nitrate reduction	+	+	NR	+	+	NR	NR
Nitrite reduction	–	+	NR	–	–	NR	NR
Anaerobic growth on nitrate	+	+	NR	+	NR	NR	NR
Requirement for growth factors	Vitamins required; yeast extract and vitamins stimulate	NR	NR	NR	Biotin	NR[h]	NR[h]
Major fatty acids	$C_{18:1\ \omega7c}$, $C_{16:0}$, $C_{18:0}$	NR	$C_{18:1\ \omega7c}$, $C_{16:0}$, $C_{18:0}$, $C_{12:0}$	$C_{18:1\ \omega7c}$, $C_{16:0}$, $C_{12:0}$	$C_{16:0}$, $C_{18:1}$, $C_{19:0}$, $C_{16:1}$, $C_{18:0}$	$C_{18:1\ \omega7c}$, $C_{16:0}$	$C_{18:1\ \omega7c}$, $C_{16:0}$
Main respiratory quinone	NR	NR	NR	NR	NR	NR	NR
G+C content of DNA (mol%)	65.6	67.5	66.2	63.6–64.0	67	62–62.9	50.6–51.5
Sample source and site	Lake Natron, Tanzania	Mono Lake, California	Culture of *Halorhodospira halophila* from Summer Lake, Oregon	Alkaline saline lake, China	Petroleum reservoir production fluid, Oklahoma	Soda lakes, Altai, Russia	Alkaline soils and soda lakes

Data taken from: [a]Yakimov et al. (2001); [b]Hoeft et al. (2007); [c]Rijkenberg et al. (2001); [d]Márquez et al. (2007); [e]Adkins et al. (1993); Tanner and Imhoff (2005); [f]Sorokin et al. (2007b); [g]Sorokin et al. (2007a)
[h]In the effective publication the type strain NCCB 100179 was erroneously cited as NCCB 100123
[i]Malate is not used anaerobically in the presence of nitrate
[j]No vitamin requirement was reported, but vitamin B_{12} was included in the growth medium
Additional data on growth substrates are given by and in the original species descriptions
NR not reported

to form cyst-like resting cells that can survive heating for 15 min at 60–80 °C (Loiko et al. 2001). Elaborate intracellular membrane systems are present in the phototrophic species (genera *Ectothiorhodospira*, *Ectothiorhodosinus*, *Halorhodospira*, *Thiorhodospira*) (see, e.g., Remsen et al. 1968 for high-quality electron micrographs and the three-dimensional model by Wanner et al. (1986) based on such pictures) and the nitrite-oxidizing *Nitrococcus*. *Figure 12.2* shows the morphology and ultrastructure of a number of *Ectothiorhodospira* and *Halorhodospira* species.

Chemotaxonomically the group is quite diverse. While the types of polar lipids and fatty acids present in the membranes are generally characteristic of the *Gammaproteobacteria*, considerable variation exists among the genera and the species. Most phototrophic members contain a menaquinone in addition to ubiquinone. More detailed information on the lipids, fatty acids, and quinones present can be found in *Tables 12.2–12.6* and in the original species descriptions.

The Anoxygenic Phototrophic Genera *Ectothiorhodospira*, *Halorhodospira*, *Ectothiorhodosinus*, and *Thiorhodospira*

Genus *Ectothiorhodospira* Pelsh 1936, 120[AL]

Ec.to.thi.o.rho.do.spi'ra. Gr. prep. *ektos*, outside; Gr. n. *theion* (Latin transliteration *thium*), sulfur; Gr. n. *rhodon*, the rose; L. fem. n. *spira*, the spiral; N.L. fem. n. *Ectothiorhodospira*, spiral rose with sulfur outside.

Cells are rod shaped, vibrioid, or spiral shaped and motile by a polar tuft of flagella, multiply by binary fission, and may contain gas vesicles. In most species the internal photosynthetic membranes are arranged in lamellar stacks that are continuous with the cytoplasmic membrane. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids. Cells grow photoautotrophically under anoxic conditions with reduced sulfur compounds or hydrogen as electron donors

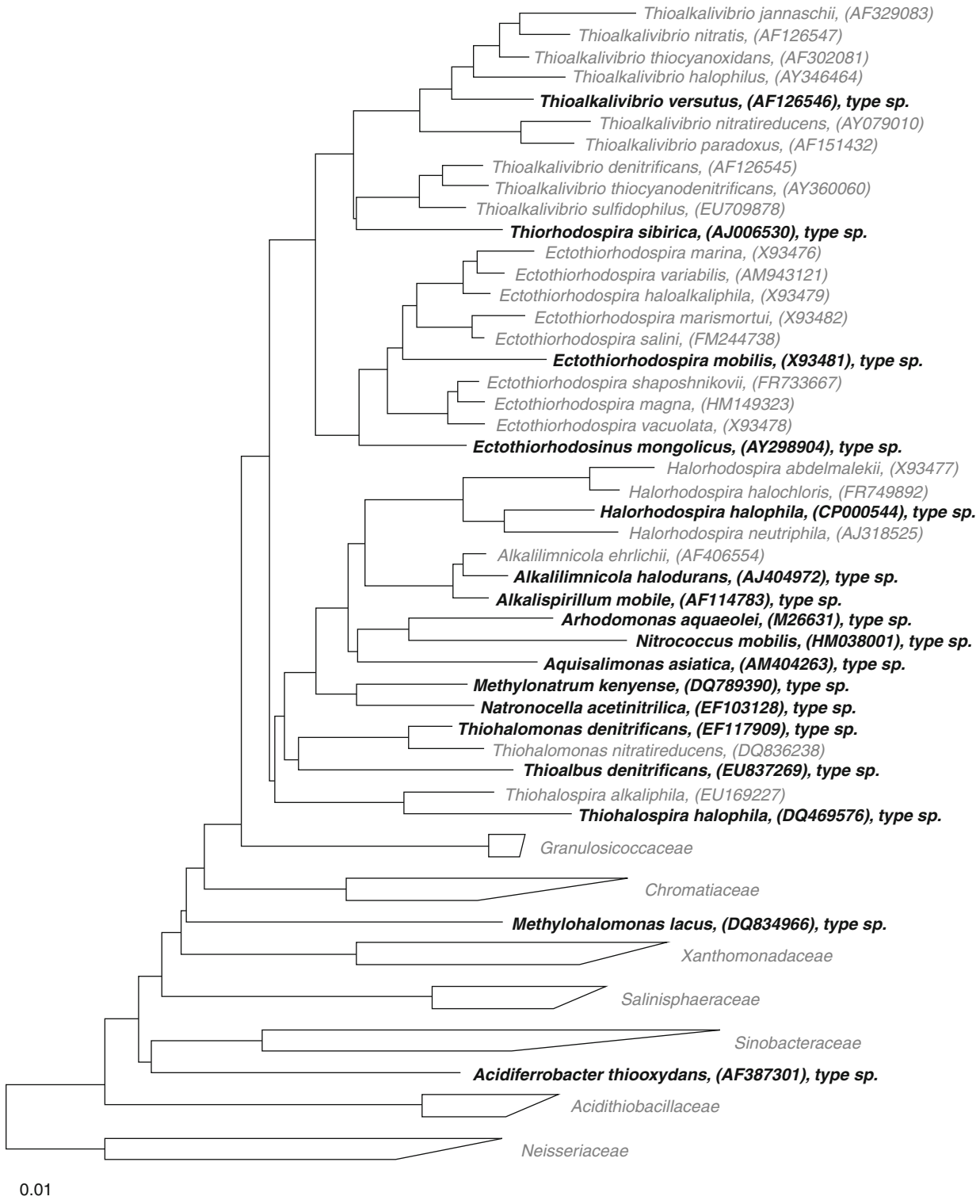

Fig. 12.1

Phylogenetic reconstruction of the family *Ectothiorhodospiraceae* based on the neighbor-joining algorithm with the Jukes-Cantor correction. Sequence dataset and alignments according to The All-Species Living Tree Project, release LTPs108 (Yarza et al. 2010). The tree topology was stabilized with the use of a representative set of 767 high-quality type strain sequences proportionally distributed among the different bacterial and archaeal phyla. In addition, a 40 % maximum frequency filter was applied to remove hypervariable positions from the alignment. Scale bar indicates estimated sequence divergence

Table 12.7

Properties of the sequenced genomes of members of the *Ectothiorhodospiraceae* (as of July 2012)

	Ectothiorhodospira sp. PHS-1 [a]	*Ectothiorhodospira haloalkaliphila* ATCC 51935^{T} [b]	*Halorhodospira halophila* SL1^{T} [c]	*Thioalkalivibrio* sp. K90mix [d]	*Thioalkalivibrio sulfidiphilus* HL-EbGr7^{T} [e]	*Alkalilimnicola ehrlichii* MLHE-1^{T} [f]
Accession number	NZ_AGBG 00000000	(high quality draft)	NC_008789	NC_013889 (chromosome) NC_013930 (plasmid)	NC_011901	NC_008340
Genome length (bp)	2,943,210	3,425,904	2,678,452	2,744,800 (chromosome) 240,256 (plasmid)	3,464,554	3,275,944
G + C content	63.7	63.0	68.0	65.5	65.1	67.5
Extrachromosomal elements	NR	NR	NR	1	0	0
Number of predicted genes	2,841	NR	2,493	2,942	3,366	2,940
Predicted protein-coding genes	2,791	3,169	NR	2,888	3,319	NR
Number of 16S rRNA genes	NR	2	2	3	3	2

[a]Data taken from: [a]Organism from a hot spring microbial mat, Paoha Island, Mono Lake, CA; incomplete draft version – http://mistdb.com/bacterial_genomes/summary/3034; [b]High-quality draft – http://genome.jgi-psf.org/ectha/ectha.info.html; http://genome.ornl.gov/microbial/ehal; [c]http://microbes.ucsc.edu/cgi-bin/hgGateway?db=haloHalo_SL1; See also Samyn et al. 2006;.[d]Muyzer et al. (2011a); [e]Muyzer et al. (2011b); [f]http://microbes.ucsc.edu/cgi-bin/hgGateway?db=alkaEhrl_MLHE_1

[T]type strain, *NR* not reported

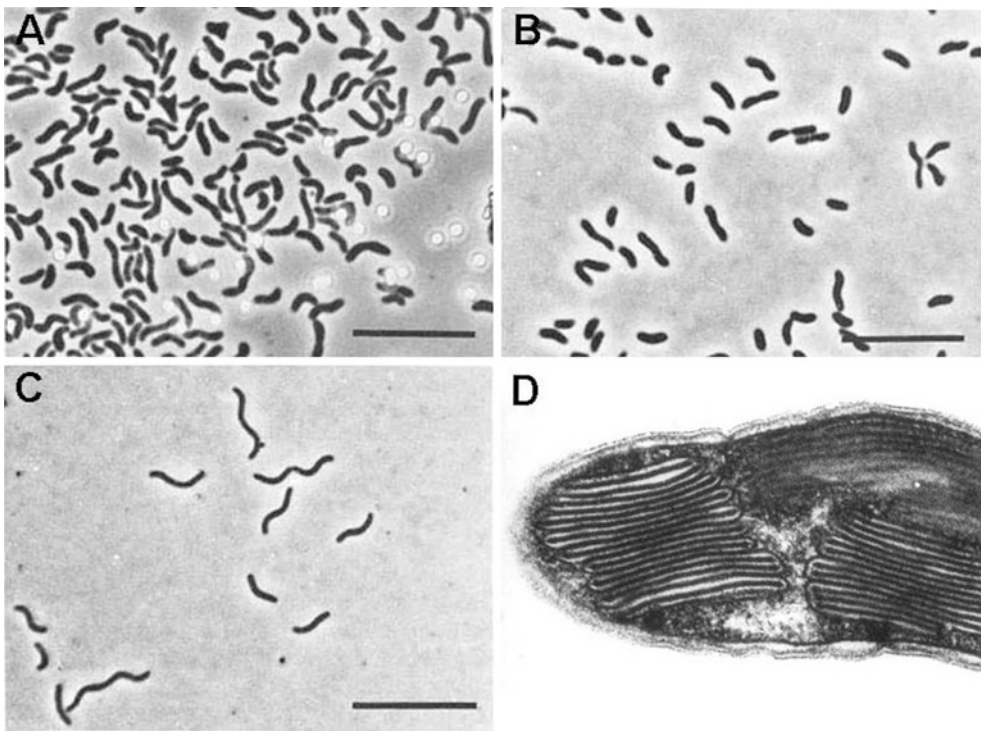

Fig. 12.2

The morphology of selected members of the genus *Ectothiorhodospira* and *Halorhodospira*: (A) *E. mobilis*, (B) *H. halophila*, (C) *H. halochloris*; Bar = 1.0 μm (from Imhoff 2006); (D) *E. mobilis*, stacks of membranes (from Kondratieva et al. 1992). Note the presence of sulfur globules in panel (A)

or photoheterotrophically with a limited number of organic compounds. Sulfide is oxidized to sulfate, with S^o, which is deposited outside the cells, as an intermediary product. Some species can grow microaerobically to aerobically in the dark. Sodium chloride is required for growth, which is dependent on saline and alkaline conditions. Compatible solutes may include glycine betaine, sucrose, and *N*α-carbamoyl-L-glutamine amide. Growth factors are not required by most species, but vitamin B_{12} enhances growth in some strains. Storage products are polysaccharides, poly-β-hydroxybutyrate, and polyphosphate. Most species live in marine and saline environments that contain sulfide, have slightly to extremely alkaline pH, and are exposed to light, such as estuaries, salt flats, salt lakes, and soda lakes.

The mol% G + C of the DNA is 59.2–68.4.

Type species: *Ectothiorhodospira mobilis*.

The genus *Ectothiorhodospira* currently contains 9 species: *E. haloalkaliphila*, *E. magna*, *E. marina*, *E. mobilis*, *E. salini*, *E. shaposhnikovii*, *E. variabilis*, *E. marismortui* (considered a later heterotypic synonym of *E. mobilis*), and *E. vacuolata* (considered a later heterotypic synonyms of *E. shaposhnikovii*).

The main features of the members of the genus are summarized in Table 12.2.

Additional comments:

- Most *Ectothiorhodospira* species may be primarily considered photoautotrophs with a potential to lead a photoheterotrophic mode of life. However, *E. salini*, isolated from anoxic sediment of a saltern in India, does not grow photoautotrophically and only thrives photoorganoheterotrophically and chemoorganoheterotrophically. Another reported unusual feature of this species is its requirement for growth factors: *p*-aminobenzoate, pantothenate, and pyridoxal phosphate (Ramana et al. 2010).
- Polar lipids are mainly phosphatidylglycerol, cardiolipin, phosphatidylcholine, and phosphatidylethanolamine. Detailed studies have been published on the polar lipid, fatty acid, and quinone composition of several species of the genus and the ways these features depend on salt concentration and other growth conditions (Asselineau and Trüper 1982; Imhoff 1984b; Imhoff and Thiemann 1991; Imhoff et al. 1982; Thiemann and Imhoff 1991, 1996). The chemical composition of the lipopolysaccharides of *E. mobilis* and *E. shaposhnikovii* was reported by Zahr et al. (1992).

Genus *Halorhodospira* Imhoff and Süling 1997, 915^{VP} (Validation list no. 62) (Effective Publication: Imhoff and Süling 1996, 112)

Ha.lo.rho.do.spi'ra. Gr. n. *hals*, *halos*, salt; Gr. n. *rhodon*, the rose; L. fem. n. *spira*, the spiral; N.L. fem. n. *Halorhodospira*, the spiral rose from salt lakes.

Cells are spirals or rod shaped and motile by bipolar flagella that multiply by binary fission. Internal photosynthetic membranes are lamellar stacks that are continuous with the cytoplasmic membrane. Photosynthetic pigments are bacteriochlorophyll *a* or *b* and carotenoids. Cells grow photoautotrophically under anoxic conditions with reduced sulfur compounds as electron donors or photoheterotrophically with a limited number of simple organic compounds. Sulfide is oxidized to sulfate, with S^o, which is deposited outside the cells as intermediary product. Growth is dependent on highly saline and alkaline conditions. At least 10 % (w/v) total salt concentration is required by all known species, some of which grow in saturated salt concentrations. Compatible solutes include glycine betaine, ectoine, and trehalose. Growth factors are not required. Storage products are polysaccharides, poly-β-hydroxybutyrate, and polyphosphate. Found in hypersaline and extremely saline environments with slightly to extremely alkaline pH (up to pH 11–12) that contain sulfide and are exposed to light, such as salt flats, salt lakes, and soda lakes.

The mol% G + C of the DNA is 50.5–74.5.

Type species: *Halorhodospira halophila*.

The genus *Halorhodospira* currently contains 4 species: *H. abdelmalekii*, *H. halochloris*, *H. halophila*, and *H. neutriphila*.

The main features of the members of the genus are summarized in Table 12.3.

Additional comments:

- The major polar lipids are phosphatidylglycerol, cardiolipin, and phosphatidylcholine. Phosphatidylethanolamine is absent in the green, bacteriochlorophyll *b*-containing *H. halochloris* and *H. abdelmalekii*. Detailed studies have been published on the polar lipid, fatty acid, and quinone composition of several species of the genus and the ways these features depend on salt concentration and other growth conditions (Asselineau and Trüper 1982; Imhoff 1984b; Imhoff and Thiemann 1991; Imhoff et al. 1982; Thiemann and Imhoff 1996). The chemical composition of the lipopolysaccharide of *H. halophila* was reported by Zahr et al. (1992).
- While also containing carotenoids of the spirilloxanthin series, *E. halophila* contains very little rhodopin as compared to *E. mobilis* and *E. shaposhnikovii* (Schmidt and Trüper 1971).

Genus *Ectothiorhodosinus* Gorlenko, Bryantseva, Panteleeva, Tourova, Kolganova, Makhneva, and Moskalenko 2007, 1371^{VP} (Validation list no. 116) (Effective Publication: Gorlenko, Bryantseva, Panteleeva, Tourova, Kolganova, Makhneva, and Moskalenko **2004**, 72)

Ec.to.thi.o.rho.do.si'nus. Gr. prep. *ektos*, outside; Gr. n. *theion* (Latin transliteration *thium*), sulfur; Gr. n. *rhodon*, the rose; L. masc. n. *sinus*, a bending, curve, fold; N.L. masc. n. *Ectothiorhodosinus*, red curved rod with sulfur outside.

Cells are vibrio shaped or semicircular and nonmotile. Cells contain bacteriochlorophyll *a* and carotenoids. The internal photosynthetic membranes have the form of concentric lamellae lining the cell wall. Cells are anaerobic, photolithoautotrophic, facultatively photolithoheterotrophic, or photoorganoheterotrophic. Sulfide is

utilized as an electron donor. Globules of elemental sulfur are formed as an intermediary product of sulfide oxidation and are deposited outside the cells. In the presence of sulfide and carbonates, organic compounds are photoassimilated. Depends on intermediate salinity and alkalinity for growth,

The mol% G + C of the DNA is 57.5.

Type species and currently sole species of the genus: *Ectothiorhodosinus mongolicus*.

The main features are summarized in Table 12.3.

Additional comment:

– The specific epithet *mongolicum* (sic) in the original species description (Gorlenko et al. 2004) was corrected to *mongolicus* at the time the name was validated.

Genus *Thiorhodospira* Bryantseva, Gorlenko, Kompantseva, Imhoff, Süling, and Mityushina 1999, 700[VP]

Thi.o.rho.do.spi'ra. Gr. n. *theion* (Latin transliteration *thium*), sulfur; Gr. n. *rhodon*, the rose; L. fem. n. *spira*, the spiral; N.L. fem. n. *Thiorhodospira*, the spiral rose with sulfur.

Cells are vibrioid or spiral shaped and motile by means of a monopolar flagellar tuft and multiply by binary fission. Internal photosynthetic membranes are parallel lamellae piercing the cytoplasm lengthwise or underlying the cytoplasmic membrane. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids. Cells are obligately phototrophic and strictly anaerobic. During photolithoautotrophic growth with sulfide as the electron donor, globules of S^o are formed outside the cytoplasm, in the medium, attached to the cells, or in the periplasm. The final oxidation product is sulfate. In the presence of sulfide, organic substances may be photoassimilated. Development is dependent on sodium salts in low concentrations and on alkaline conditions. Habitat: surface of sediments rich in organic matter and microbial mats from brackish soda lakes that contain hydrogen sulfide.

The mol% G + C of the DNA is 56.0–57.4.

Type species (and currently sole species) of the genus: *Thiorhodospira sibirica*.

The main features are summarized in Table 12.3.

The Chemolithotrophic Genera *Thioalkalivibrio*, *Thiohalomonas*, *Thiohalospira*, *Thioalbus*, and *Nitrococcus*

Genus *Thioalkalivibrio* Sorokin, Lysenko, Mityushina, Tourova, Jones, Rainey, Robertson, and Kuenen 2001, 578[VP]; Emend. Sorokin, Gorlenko, Tourova, Tsapin, Nealson, and Kuenen 2002a, 919; Emend. Banciu, Sorokin, Galinski, Muyzer, Kleerebezem, and Kuenen 2004b, 333

Thi.o.al.ka.li.vi'bri.o. Gr. n. *theion* (Latin transliteration *thium*), sulfur; N.L. n. *alkali* (from Arabic *al-qualyi*, the ashes of saltwort), soda ash; N.L. *vibrio*, to set in tremulous motion, move to and fro, vibrate; N.L. masc. n. *vibrio*, that which vibrates, and also a bacterial genus name *Vibrio*; N.L. masc. n. *Thioalkalivibrio*, sulfur alkaline vibrio.

Cells are curved or straight rods or spirilla with 1–3 polar flagella. Nonmotile coccoid species also occur. The cell wall is usually rippled. Cells possess carboxysomes. Cells are obligate chemolithoautotrophs that oxidize sulfide, thiosulfate, sulfur, and tetrathionate. The carbon assimilation proceeds via the Calvin cycle. They are halotolerant up to 1.2–1.5 M Na^+ and require at least 0.3 M Na^+. Most strains grow optimally in soda-rich media. Some strains depend on chloride for growth and can grow up to saturating concentrations of NaCl. They are obligately autotrophs, oxidizing reduced sulfur (sulfide, thiosulfate, elemental sulfur, sulfite, and polythionates). Some strains deposit globules of elemental sulfur in the periplasmic space. Young colonies are bright white from heavy sulfur deposition which is gradually converted to soluble polysulfide-like compounds. Polysulfide is oxidized completely to sulfate without intermediary sulfur formation. Carbon is assimilated via the Calvin cycle. Most species grow at pH 7.50–10.65 with an optimum at pH 10–10.2. The major ubiquinone is Q-8. All strains are halotolerant and able to grow in the presence of up to 1.2–1.5 total Na^+.

The mol% G + C of the DNA is 61.3–66.9.

Type species: *Thioalkalivibrio versutus*.

The genus *Thioalkalivibrio* as of July 2012 contains 9 species: *T. denitrificans*, *T. halophilus*, *T. jannaschii*, *T. nitratireducens*, *T. nitratis*, *T. paradoxus*, *T. thiocyanodenitrificans*, *T. thiocyanoxidans*, and *T. versutus*. The description of an additional species (*T. sulfidiphilus*) is currently in press (Sorokin et al. 2012). The main features of the members of the genus are summarized in Table 12.4.

Additional comments:

– Sorokin et al. (2001a) proposed the genus name *Thioalkalivibrio*. This name was corrected by the List Editor of *International Journal of Systematic and Evolutionary Microbiology* to *Thialkalivibrio* (Notification List, *International Journal of Systematic and Evolutionary Microbiology* 51, 796, 2001). However, based on the decision of the Judicial Commission of the International Committee on Systematics of Prokaryotes made in Paris in 2002, the recommendations governing the use of the connecting vowel should be overruled by the usage in chemistry and physics. Therefore the original spelling *Thioalkalivibrio* must be used.
– Cyanate was identified as an intermediate of thiocyanate oxidation by *T. thiocyanodenitrificans* (Sorokin et al. 2004).

Genus *Thiohalomonas* Sorokin, Tourova, Braker, and Muyzer 2007c, 1587[VP]

Thi.o.ha.lo.mo'nas. Gr. n. *theion* (Latin transliteration *thium*), sulfur; Gr. n. *hals*, *halos*, salt; L. fem. n. *monas*, a unit, a monad; N.L. fem. n. *Thiohalomonas*, salt (−tolerant), sulfur-utilizing monad.

Cells are nonmotile, obligately chemolithoautotrophic, facultatively anaerobic rods or vibrios. Reduced sulfur compounds are used as the electron donors with oxygen or nitrate as the electron acceptors. Cells are moderately halophilic and neutrophilic and found in hypersaline lakes and salterns.

The mol% G + C of the DNA is 58–62.9.

Type species: *Thiohalomonas denitrificans*.

The genus *Thiohalomonas* as of July 2012 contains 2 species: *T. denitrificans* and *T. nitratireducens*. The main features of the members of the genus are summarized in Table 12.5.

Genus *Thiohalospira* Sorokin, Tourova, Muyzer, and Kuenen 2008a, 1690[VP]

Thi.o.ha.lo.spi'ra. Gr. n. *theion* (Latin transliteration *thium*), sulfur; Gr. n. *hals*, *halos*, salt; L. fem. n. *spira*, the spiral; N.L. fem. n. *Thiohalospira*, halophilic sulfur spirillum.

Cells are obligately chemolithoautotrophic, aerobic, motile spirilla that oxidize inorganic sulfur compounds to sulfate. Tetrathionate is usually an intermediate of thiosulfate oxidation. The genus includes extremely halophilic, neutrophilic, and moderately halo(alkali)philic members. Cellular fatty acids are dominated by saturated C_{16}–C_{18} species.

The mol% G + C of the DNA is 65.6–67.

Type species: *Thiohalospira halophila*.

The genus *Thiohalospira* as of July 2012 contains 2 species: *T. alkaliphila* and *T. halophila*. The main features of the members of the genus are summarized in Table 12.5.

Genus *Thioalbus* Park, Pham, Jung, Kim, Kim, Roh, and Rhee 2011, 2048[VP]

Thi.o.al'bus. Gr. n. *theion* (Latin transliteration *thium*), sulfur; L. masc. adj. *albus*, white; N.L. masc. n. *Thioalbus* intended to mean a bacterium which oxidizes thiosulfate and whose colonies are white.

Cells are nonmotile, obligately chemolithoautotrophic, and facultatively anaerobic. Reduced sulfur compounds are used as the electron donors with oxygen or nitrate as the electron acceptors. Cells are slightly halophilic and neutrophilic. They are found in marine sediments.

The mol% G + C of the DNA is 64.5.

Type species and currently sole species of the genus: *Thioalbus denitrificans*.

The main features are summarized in Table 12.5.

Genus *Nitrococcus* Watson and Waterbury 1971, 224[AL]

Ni.tro.coc'cus. L. n. *nitrum*, native soda, natron, nitrate; N.L. masc. n. *coccus* (from Gr. masc. n. *kokkos*, grain, seed), coccus, sphere; N.L. masc. n. *Nitrococcus*, nitrate sphere.

Cells are spherical and 1.5 μm or more in diameter, occur singly or in pairs, are motile by 1–2 flagella, and reproduce by binary fission. Cells may occur in clumps embedded in a slime matrix. Intracytoplasmic membranes occur as tubes randomly arranged throughout the cytoplasm. Cells are obligate lithoautotrophs that derive energy from the oxidation of nitrite to nitrate with oxygen as the electron acceptor. Optimal growth is achieved in 70–100 % seawater enriched with nitrite and other inorganic salts. Organic growth factors are not required.

The mol% G+C of the DNA is 61.2.

Type species and currently sole species of the genus: *Nitrococcus mobilis*.

The main features are summarized in Table 12.5.

The Heterotrophic/Facultative Heterotrophic/Methylotrophic Genera *Alkalilimnicola*, *Alkalispirillum*, *Aquisalimonas*, *Arhodomonas*, *Methylonatrum*, and *Natronocella*

Genus *Alkalilimnicola* Yakimov, Giuliano, Chernikova, Gentile, Abraham, Lünsdorf, Timmis, and Golyshin 2001, 2142[VP]

Al.ka.li.lim.ni'co.la. N.L. n. *alkali* (from Arabic *al-qualyi*, the ashes of saltwort), soda ash; Gr. n. *limnos*, pool of standing water, lake; L. suffix *–cola* (from L. n. *incola*) a dweller, inhabitant; N.L. masc. n. *Alkalilimnicola*, a dweller of alkaline lakes.

Cells are short, straight, and oval shaped, occur singly or in pairs, and are motile by means of a single polar flagellum. Cells usually produce an extracellular matrix of non-fibrillar appearance. Metabolism is respiratory; nitrate is reduced but nitrite is not. Optimal growth is found under alkaline conditions (pH >8.5). Cells are moderately halophilic and extremely halotolerant. Poly-β-hydroxybutyrate is formed as storage product.

The mol% G + C of the DNA is 65.6–67.5.

Type species: *Alkalilimnicola halodurans*

The genus *Alkalilimnicola* currently contains 2 species: *A. ehrlichii* and *A. halodurans*.

The main features of the members of the genus are summarized in Table 12.6.

Additional comments:

- The original spelling *Alcalilimnicola* (Yakimov et al. 2001) was changed by the List Editor of *International Journal of Systematic and Evolutionary Microbiology* to *Alkalilimnicola* (Notification list, *International Journal of Systematic and Evolutionary Microbiology* 52, 5, 2002).
- *A. ehrlichii* can grow heterotrophically but also as a chemoautotroph, oxidizing arsenite to arsenate with nitrate as the electron acceptor which is reduced to nitrite. Autotrophic growth on hydrogen + nitrate is also possible. Aerobic chemoautotrophic growth could not be demonstrated (Oremland et al. 2002; Sorokin et al. 2006; Hoeft et al. 2007).

– *A. ehrlichii* oxidizes carbon monoxide under aerobic conditions, but the reaction does not support growth (Hoeft et al. 2007). Aerobic enrichments inoculated with material from soda lakes and with CO as the sole substrate (<5 % O_2 and <20 % CO in the gas phase) under extremely haloalkaline conditions yielded additional carboxydotrophic *Alkalilimnicola* strains (Sorokin et al. 2010).

Genus *Alkalispirillum* Rijkenberg, Kort, and Hellingwerf 2002, 1075 1076[VP] (Validation list no. 86) (Effective Publication: Rijkenberg, Kort, and Hellingwerf 2001, 374)

Al.ka.li.spi.ril'lum. N.L. n. *alkali* (from Arabic *al-qualyi*, the ashes of saltwort), soda ash; L. fem. n. *spira*, a spiral; N.L. dim. neut. n. *spirillum*, a small spiral; N.L. neut. n. *Alkalispirillum*, a small spiral living under alkaline conditions.

Cells are spiral shaped and motile by means of a single polar flagellum and grow aerobically under alkaline and saline conditions. They are chemoorganotrophic, using simple organic acids and other small organic substrates.

The mol% G + C of the DNA is 66.2.

Type species and currently sole species of the genus: *Alkalispirillum mobile*.

The main features are summarized in ❯ *Table 12.6*.

Genus *Aquisalimonas* Márquez, Carrasco, Xue, Ma, Cowan, Jones, Grant, and Ventosa 2007, 1140[VP]

A.qui.sa.li.mo'nas. L. n. *aqua*, water; L. n. *sal*, *salis*, salt; L. fem. n. *monas*, a unit, a monad; N.L. fem. n. *Aquisalimonas*, a bacterium living in salted water.

Cells are rod shaped and motile, occurring singly, in pairs, or in long chains. They are alkalitolerant and moderately halophilic; salt is required for growth. Nitrate is reduced, but nitrite is not.

The mol% G + C of the DNA is 63.6–64.0.

Type species (and currently sole species of the genus): *Aquisalimonas asiatica*.

The main features are summarized in ❯ *Table 12.6*.

Additional comment:

– The species description contains the statement: "Strictly aerobic. Grows anaerobically with nitrate" (Márquez et al. 2007). If so, the species may be considered to be facultatively anaerobic.

Genus *Arhodomonas* Adkins, Madigan, Mandelco, Woese, and Tanner 1993, 518[VP]

A.rho.do.mo'nas. Gr. pref. *a-*, not; Gr. n. *rhodon*, the rose; L. fem. n. *monas*, a unit, a monad; N.L. fem. n. *Arhodomonas*, a monad that is not rose colored.

Cells are nonencapsulated rods that occur singly or in pairs, are motile by means of a single polar flagellum, and multiply by binary fission. They are chemoorganotrophs with a respiratory metabolism that primarily utilize organic acids as carbon and energy source, using oxygen or nitrate as the electron acceptors. Cells are halophilic; sodium is required for growth.

The mol% G + C of the DNA is 67.

Type species (and currently sole species of the genus): *Arhodomonas aquaeolei*.

The main features are summarized in ❯ *Table 12.6*.

Genus *Methylonatrum* Sorokin, Trotsenko, Doronina, Tourova, Galinski, Kolganova, and Muyzer 2007b, 2768[VP]

Me.thy.lo.na'trum. N.L. n. *methylum* (from Fr. *méthyle*, coined from Gr. n. *methu*, wine and Gr. n. *hulê*, wood), methyl radical; N.L. pref. *methylo-*, pertaining to the methyl radical; N.L. neut. n. *natron* (arbitrarily derived from the Arabic n. *natrun* or *natron*) soda, sodium carbonate; N.L. neut. n. *Methylonatrum*, methyl group-utilizing soda (methyl group-loving bacterium).

Cells are short rods that can grow as methylotrophs on methanol or on larger carbon compounds (ethanol, acetate). The autotrophic Calvin cycle is used for carbon assimilation during methylotrophic growth. They are moderately salt tolerant and obligately alkaliphilic.

The mol% G + C of the DNA is 62–62.9.

Type species and currently sole species of the genus: *Methylonatrum kenyense*.

The main features are summarized in ❯ *Table 12.6*.

Genus *Natronocella* Sorokin, van Pelt, Tourova, Takaichi, and Muyzer 2007, 1372[VP] (Validation list no. 116) (Effective Publication: Sorokin, van Pelt, Tourova, Takaichi, and Muyzer 2007, 1163)

Na.tro.no.cel'la. N.L. n. *natron* (arbitrarily derived from the Arabic n. *natrun* or *natron*) soda, sodium carbonate; N.L. pref. *natrono-*, pertaining to soda; L. fem. n. *cella*, a room, a storeroom and in biology a cell; N.L. fem. n. *Natronocella*, a cell that can tolerate soda.

Cells are rod shaped with yellow pigmentation. They are obligately heterotrophic, can use aliphatic nitriles as carbon and energy source, and are highly salt tolerant and obligately alkaliphilic.

The mol% G + C of the DNA is 50.6–51.5.

Type species and currently sole species of the genus: *Natronocella acetinitrilica*.

The main features are summarized in ❯ *Table 12.6*.

Additional comment:

– Acetonitrile is degraded first to acetamide via the nitrile hydratase/amidase pathway in *N. acetinitrilica* (Sorokin et al. 2007a).

Isolation, Enrichment, and Maintenance Procedures

Phototrophic members of the family (genera *Ectothiorhodospira*, *Ectothiorhodosinus*, *Halorhodospira*, *Thiorhodospira*) can be enriched in the light (incandescent lamps) in liquid cultures and isolated in deep agar dilution series in anaerobic media of the appropriate salinity and pH, using sulfide as electron donor, with or without organic carbon sources such as acetate, succinate, and malate (Bryantseva et al. 1999; Grant et al. 1979; Imhoff 2006).

Most species of chemoautotrophic sulfur oxidizers (genera *Thioalkalivibrio*, *Thioalbus*, *Thiohalomonas*) have been recovered from enrichment cultures using thiosulfate, tetrathionate, or polysulfide as the electron donor in mineral media of the appropriate pH and salinity, either aerobically or anaerobically in the presence of nitrate (Banciu et al. 2004a; Park et al. 2011; Sorokin et al. 2001a, 2002a, 2006, 2007c, 2008a). *Thioalkalivibrio sulfidiphilus* was obtained from gradient cultures with an opposing gradient of O_2 and H_2S and isolated on agar plates with sulfide incubated under microoxic conditions (Sorokin et al. 2012).

Alkalilimnicola ehrlichii was isolated from an enrichment culture that contained As(III) as the electron donor and nitrate as the electron acceptor (Oremland et al. 2002). *Natronocella acetinitrilica* was enriched in medium with acetonitrile as the sole carbon source at pH 10 (Sorokin et al. 2007a).

Maintenance

The phototrophic members (genera *Ectothiorhodospira*, *Halorhodospira*, *Thiorhodospira*) can be maintained as liquid cultures in closed airtight screw-cap bottles at 4 °C in a refrigerator or even at room temperature in dim light for several months to years. Storage in agar tubes in the dark at 4–10 °C for up to a year is also possible. Species that have bacteriochlorophyll *b* (*H. abdelmalekii*, *H. halochloris*) lose viability relatively quickly and are relatively sensitive to prolonged storage, particularly with respect to oxygen and light. For long-term storage, preservation in liquid nitrogen is recommended in the presence of 5 % dimethyl sulfoxide as preservative (Imhoff 2005b, c, 2006; Imhoff and Gorlenko, 2005).

Cultures of *Thioalkalivibrio* can be stored at 4 °C for 2 months between transfers in liquid salts medium at pH 10 containing 4 mM $MgCl_2$ to stabilize the cell walls. Cells can also be stored in 10 % glycerol (v/v) at −80 °C (Brenner et al. 2005).

Liquid cultures of *Nitrococcus* can survive starvation for more than 1 year when kept at 17 °C. Nevertheless, cells should be transferred to fresh media every 4 months. For long-term preservation, storage in liquid nitrogen is recommended using a cryoprotective buffer containing sucrose and histidine (Spieck and Bock 2005).

Alkalilimnicola, *Alkalispirillum*, *Aquisalimonas*, and *Arhodomonas* strains are supplied by culture collections as freeze-dried cultures.

Physiological and Biochemical Features

The extremely halophilic and alkaliphilic *Halorhodospira halochloris* was used as a model organism to investigate the mechanism of osmotic adaptation and the use of organic osmotic "compatible" solutes. Glycine betaine was first identified as an osmotic solute in a prokaryote in *H. halochloris* (Galinski and Trüper 1982), and ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid), an osmotic solute now known to be widely used in the bacterial world, was first detected in this organism (Galinski et al. 1985). *H. halochloris* can in addition produce trehalose as an osmotic solute, particularly under nitrogen limitation (Galinski and Herzog 1990). Another novel osmotic solute, *N*α-carbamoyl-L-glutamine-1-amide, was first (and thus far only) detected in *Ectothiorhodospira marismortui* (Galinski and Oren 1991). The haloalkaliphilic, sulfur-oxidizing bacterium *Thioalkalivibrio versutus* also produces glycine betaine is as an osmotic solute (Banciu et al. 2004b).

A number of novel pigments have been discovered in members of the *Ectothiorhodospiraceae*. Bacteriochlorophyll *b* of the green *Halorhodospira* species *H. halochloris* and *H. abdelmalekii* is esterified with Δ-2,10-phytanediol, not with phytol as in *H. halophila* (Imhoff 2006). These green anoxygenic phototrophs also contain a novel class of carotenoids, dihydroxylycopene diglucoside diesters (Takaichi et al. 2001). Investigations on negative phototaxis in *H. halophila* led to the identificaton of the "photoactive yellow protein," a 14 kDa protein that carries a prosthetic group of 4-hydroxy-cinnamic acid (*Fig. 12.3a*), linked by a thioester bond to a cysteine residue in the protein (Hellingwerf et al. 2002; Sprenger et al. 1993). The molecule acts as a photosensor. Following absorption of a photon, it undergoes a complex photocycle.

Some of the non-phototrophic members of the *Ectothiorhodospiraceae* are colored yellow. The pigmentation can be due to the presence of zeaxanthin and other carotenoids, such as in *Natronocella acetinitrilica* (Sorokin et al. 2007a), or to the unusual yellow pigments natronochrome and chloronatronochrome, derivatives of a fully unsaturated fatty acid with a phenyl group, found in the natrono(alkali)philic sulfur-oxidizing bacterium *Thioalkalivibrio versutus* (*Fig. 12.3b, c*) (Takaichi et al. 2004).

Ecology

Phototrophic members of the *Ectothiorhodospiraceae* can be found both in shallow marine surface sediments and in alkaline and often hypersaline inland lakes (Imhoff 2006). *Ectothiorhodospira mobilis* and *E. magna* are examples of species adapted to life in the marine environment. It is interesting to note that *Ectothiorhodospira* species have also been recovered from marine sponges in the Adriatic Sea, together with anoxygenic phototrophic belonging to other taxonomic groups. Although these sponges live in aerobic surroundings, sulfate-reducing bacteria could also be isolated from them, and it is thus possible

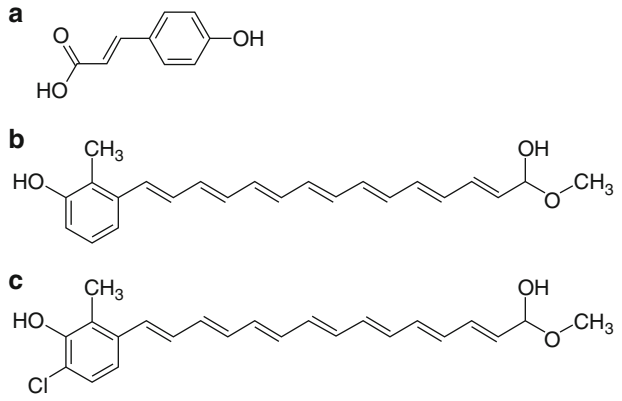

Fig. 12.3

The structure of 4-hydroxy-cinnamic acid, the prosthetic group of the photoactive yellow protein first detected in *Halorhodospira halophila* (Hellingwerf et al. 2002) (a) and the yellow pigments natronochrome (b) and chloronatronochrome (c) of *Thioalkalivibrio versutus* (Takaichi et al. 2004)

that locally, a cycle is active in which sulfate is reduced to sulfur and then reoxidized by phototrophic prokaryotes (Imhoff and Trüper 1976). Neutrophilic species such as *Ectothiorhodospira salini* and *Halorhodospira neutriphila* were recovered from hypersaline evaporation ponds of solar salterns, where they may be found within red layers in benthic microbial mats or within evaporitic gypsum crusts below one or more layers of cyanobacteria (Hirschler-Reá et al. 2003; Oren et al. 1995; Ramana et al. 2010; Ventura et al. 1988). *E. marismortui* was isolated from the outflow channel of a hypersaline sulfur spring on the shore of the Dead Sea (Oren et al. 1989). *Ectothiorhodospira* dominates the microbial consortium in a hot spring microbial mat at Mono Lake, CA, that displays arsenite-dependent photoautotrophy. Sequences of 16S rRNA genes related to *Alkalilimnicola ehrlichii* and of members of the *Bacteroidetes* were also found. Pure cultures of *Ectothiorhodospira* isolated from this ecosystem (see also the section on "Genome Analysis") did not oxidize arsenite under the conditions tested (Budinoff and Hollibaugh 2008).

Especially prominent is the development of alkaliphilic phototrophic members of the family in soda lakes such as the lakes of the Wadi Natrun, Egypt (Imhoff and Trüper 1977, 1981; Imhoff et al. 1978). At first it was assumed that the red coloration of the brines of the Wadi Natrun lakes can be mainly contributed to these organisms (Jannasch 1957). This is probably not the case: bacterioruberin carotenoids of the haloalkaliphilic Archaea (*Natronobacterium*, *Natronococcus*, and relatives) dominate in the absorption spectrum of the biomass collected from the brines. Still, 10^5–10^6 cells per ml of each *Halorhodospira halophila* and *H. halochloris* could be recovered from mud and water samples of the Wadi Natrun (Imhoff et al. 1979).

The haloalkaliphilic bacteria belonging to the *Ectothiorhodospiraceae* (genera *Thioalkalivibrio*, *Thiohalomonas*, *Thiohalospira*, *Thioalbus*) have all been described in the twenty-first century. Thanks to the in-depth studies of Sorokin and coworkers; we now have much information about this novel group of organisms and the functions they perform in soda lakes and in alkaline soils (Sorokin and Kuenen 2005), including their function in the nitrogen cycle as denitrifying organisms (Sorokin et al. 2001b). The current list of species belonging to the group only represents a small part of the true diversity. Fingerprinting by repetitive extragenic palindromic (REP)-PCR of 85 *Thioalkalivibrio* isolates from Mongolia, Kenya, California, Egypt, and Siberia yielded 56 different genotypes (Foti et al. 2006).

The true ecological niche of *Alkalispirillum mobile* is unknown. It was isolated from cultures of *Halorhodospira halophila* SL-1, a strain that originated from Summer Lake, Oregon. *Alkalispirillum* is an obligate aerobic heterotroph, and *Halorhodospira* was always grown in the absence of oxygen in mineral media. The nature of the interaction between the two organisms that enabled the survival of *A. mobile* remains unclear (Rijkenberg et al. 2001). Another heterotroph that may grow in association with an anoxygenic phototroph is *Alkalilimnicola halodurans*, isolated from sediments of the soda-depositing Lake Natron in the East African Rift Valley. It was isolated in tubes for the cultivation of anoxygenic phototrophic sulfur bacteria followed by plating on agar plates: nonpigmented colonies of *Alkalilimnicola* appeared among the purple ones. The organism can grow over a very wide range of NaCl concentration, from salt-free medium to 27 % (Yakimov et al. 2001).

Arhodomonas aquaeolei was isolated from a petroleum reservoir fluid in Oklahoma (Adkins et al. 1993), but it does not degrade hydrocarbons. Still, *Arhodomonas* spp. may be involved in the biodegradation of aromatic hydrocarbons. Enrichment cultures set-up with benzene and toluene and 14, 23, and 29 % NaCl and inoculated with sediment from the oil seeps of Rozel Point at the northern basin of Great Salt Lake, Utah, degraded the substrate within 1, 2, and 5 weeks, respectively. The cultures were dominated by *Gammaproteobacteria*, and 44–69 % of the 16S rRNA phylotypes recovered in gene libraries were affiliated with *Arhodomonas* (Sei and Fathepure 2009). The authors stated that they had from an oilfield isolated an *Arhodomonas* sp. that degrades benzene/toluene/xylene at high salinity.

Pathogenicity, Clinical Relevance

No members of the *Ectothiorhodospiraceae* are known to be pathogenic to humans, animals, or plants.

Sensitivity tests to different antibiotics have been reported for four species only: *Alkalilimnicola halodurans* (Yakimov et al. 2001), *Aquisalimonas asiatica* (Márquez et al. 2007), *Arhodomonas aquaeolei* (Adkins et al. 1993), and *Thioalkalivibrio sulfidiphilus* (Sorokin et al. 2012) (*Table 12.8*).

Application

For the removal of toxic sulfide from natural gas and from sulfide-containing gases generated during industrial processes,

Table 12.8
Sensitivity of four species of the family *Ectothiorhodospiraceae* to selected antibiotics and other antimicrobial compounds

	Alkalilimnicola halodurans [a]	*Aquisalimonas asiatica* [b]	*Arhodomonas aquaeolei* [c]	*Thioalkalivibrio sulfidiphilus* [d]
Ampicillin	–	NR	+	–
Bacitracin	NR	–	NR	NR
Carbenicillin	NR	NR	+	NR
Cephalotin	NR	–	NR	NR
Chloramphenicol	+	+	+	+
Erythromycin	NR	+	+	NR
Gentamycin	–	NR	–	NR
Kanamycin	–	–	NR	–
Nalidixic acid	–	NR	NR	NR
Neomycin	NR	–	–	NR
Penicillin	NR	–	+	NR
Rifampicin	NR	–	NR	–
Streptomycin	–	–	–	NR
Sulphomethoxazole	+	NR	NR	NR
Tetracycline	–	–	+	–
Trimethoprim	–	NR	NR	NR
Vancomycin	–	–	–	NR

[a]Yakimov et al. (2001); [b]Márquez et al. (2007); [c]Adkins et al. (1993); [d]Sorokin et al. (2012)
+ sensitive, – resistant, *NR* not reported

the first step is the scavenging of the sulfide in an alkaline medium. Therefore it is advantageous when further processing such as the oxidation of the sulfide to elemental sulfur can be performed at alkaline pH as well. For that purpose bacteria of the genus *Thioalkalivibrio* are the organisms of choice. Thus, a fed-batch bioreactor operating at pH 10 was designed to clean high-pressure natural gas and sour gas produced in the petrochemical industry. As inoculum served a mixture of pure cultures and enrichments containing haloalkaliphilic *Thioalkalivibrio* (*Ectothiorhodospiraceae*) and *Thioalkalimicrobium* (*Thiotrichales*, *Piscirickettsiaceae*) strains (van den Bosch et al. 2007).

A further exploitation of this principle is found in the "Thiopaq" process to remove sulfide from biogases. Here the H_2S from the gas phase is stripped into an alkaline solution, which is transferred to a bioreactor where obligately chemolithoautotrophic and extremely haloalkaliphilic sulfur oxidizers of the genus *Thioalkalivibrio* oxidize the sulfide to elemental sulfur, which can be further used as a fertilizer or as a fungicide. As inoculum served sediments of alkaline soda lakes in Mongolia, Siberia, and Egypt and pure and enrichment cultures of *Thioalkalivibrio* spp. (Sorokin et al. 2008b; Muyzer et al. 2011b). Depending on the pH at which the system is operated, the organisms that became dominant resembled *T. jannaschii* and *T. versutus* (pH 10) or the facultatively alkaliphilic *T. halophilus* (at lower pH) (Muyzer et al. 2011b). The recently described species *T. sulfidiphilus* was isolated using a dilution series in sulfide-oxygen gradient tubes, using material from such a bioreactor as the inoculum (Sorokin et al. 2012.). Its genome sequence has been published (Muyzer et al. 2011b).

The osmotic ("compatible") solute ectoine, first discovered in *Halorhodospira halochloris* (Galinski et al. 1985), is now widely used in a number of applications ranging from the stabilization of labile proteins and other biomolecules to cosmetics (Buenger and Driller 2004) and even inhalations for the treatment of lung disorders (Harishchandra et al. 2011). However, its industrial production is based on heterotrophic ectoine synthesizing bacteria such as *Halomonas* and not on members of the *Ectothiorhodospiraceae*.

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13 The Family *Enterobacteriaceae*

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Abstract
Enterobacteriaceae is a family of Gram-negative, facultatively anaerobic, non-spore-forming rods. Characteristics of this family include being motile, catalase positive, and oxidase negative; reduction of nitrate to nitrite; and acid production from glucose fermentation. However, there are also many exceptions. Currently, the family comprises 51 genera and 238 species. The number of species per genus ranges from 1 to 22. Twenty-two genera contain only one species, while seven genera have more than ten species. *Enterobacteriaceae* is closest to *Vibrionaceae* and *Pasteurellaceae* as sister clades with all members except for the genera

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Arsenophonus and *Thorsellia* being clustered together in one clade. Of the 30 genera with two or more species, 21 are likely to be monophyletic based on clustering on 16 rDNA sequence and other data. However, seven genera are likely to be polyphyletic requiring further reclassification. *Enterobacteriaceae* has been heavily sequenced from across the spectrum of the family diversity with 180 complete genomes covering 47 species and 21 genera. The genome size ranges from 422,434 bp, coding for just 362 ORFs, to 6,450,897 bp, coding for 5,909 ORFs. *Enterobacteriaceae* is ubiquitous in nature. Many species can exist as free living in diverse ecological niches, both terrestrial and aquatic environments, and some are associated with animals, plants, or insects only. Many are significant human, other animal, and/or plant pathogens causing a range of infections. There are numerous applications using members of *Enterobacteriaceae* including biocontrol in agriculture, production of numerous recombinant proteins and nonprotein products, control of infection diseases, anticancer agents, biowaste recycling, and bioremediation. Genome-based phylogeny and genomics are expected to further delineate the members of *Enterobacteriaceae* and refine the classification of the genera and species within this family.

Introduction

Enterobacteriaceae is a family of Gram-negative, facultatively anaerobic non-spore-forming rods. It is a member of the γ-class of Proteobacteria and the order *Enterobacteriales*. General characteristics of this family include being motile with the exception of *Arsenophonus, Biostraticola, Klebsiella, Moellerella, Obesumbacterium, Raoultella, Shimwellia, Tatumella*, and the endosymbionts (*Buchnera, Sodalis*, and *Wigglesworthia*); catalase positive; oxidase negative except *Plesiomonas*; and the use of the Embden–Meyerhof pathway for sugar metabolism and acid production from glucose fermentation. They are distinct from other family of Gram-negative rod-shaped bacteria based on cell geometry, flagellar arrangement, oxidase production, sodium requirements, as well as the presence of Enterobacterial common antigen (ECA) (Francino et al. 2006). However, the classic morphological and biochemical characterizations, which previously defined this family, are no longer unequivocal. The 16S rDNA and other gene sequences are needed to delineate members of this family (Francino et al. 2006).

The family of *Enterobacteriaceae* is large. We have attempted to give an overview of the family and cover each genus with phenotypic characteristics, phylogenetic relationship, and habitats. It is not possible to address many of the aspects of the family nor is possible to cover the depth that we wish to do. In preparation of this chapter, we have not attempted to cite all original references and will also have missed or have had to leave out many important works in relevant area.

Taxonomy, Historical and Current

Currently, there are 51 genera within the family *Enterobacteriaceae*, which are *Arsenophonus, Biostraticola, Brenneria, Buchnera, Budvicia, Buttiauxella, Cedecea, Citrobacter, Cosenzaea, Cronobacter, Dickeya, Edwardsiella, Enterobacter, Erwinia, Escherichia, Ewingella, Gibbsiella, Hafnia, Klebsiella, Kluyvera, Leclercia, Leminorella, Lonsdalea, Mangrovibacter, Moellerella, Morganella, Obesumbacterium, Pantoea, Pectobacterium, Phaseolibacter, Photorhabdus, Plesiomonas, Pragia, Proteus, Providencia, Rahnella, Raoultella, Saccharobacter, Salmonella, Samsonia, Serratia, Shigella, Shimwellia, Sodalis, Tatumella, Thorsellia, Trabulsiella, Wigglesworthia, Xenorhabdus, Yersinia, and Yokenella.*

Since last edition of this book, 10 new genera have been added to this family. Four new genera were established as a result of isolation of new organisms, *Thorsellia* in 2006 (Kampfer et al. 2006), *Biostraticola* in 2008 (Verbarg et al. 2008), *Gibbsiella* in 2010 (Brady et al. 2010a), and *Mangrovibacter* in 2010 (Rameshkumar et al. 2010). Six new genera were created by reclassification: *Cosenzaea* by transfer of *Proteus myxofaciens* (Giammanco et al. 2011), *Cronobacter* by transfer of *Enterobacter sakazakii* (Iversen et al. 2007, 2008), *Dickeya* by transfer of *Pectobacterium chrysanthemi* and *Brenneria paradisiaca* (Brady et al. 2012a; Samson et al. 2005), *Phaseolibacter* by transfer from the family *Pseudomonas* (Halpern et al. 2013), *Plesiomonas* by transfer from the family *Vibrionaceae*, *Shimwellia* by transfer of *Escherichia blattae* and *Obesumbacterium proteus* biogroup2 (Priest and Barker 2010), and *Lonsdalea* by transfer of *Brenneria quercina* (Brady et al. 2012a). One genus has been lost but the name still exists: *Calymmatobacterium* had one species *C. granulomatis*, which is now reclassified as *Klebsiella granulomatis* (Carter et al. 1999). Another genus, *Levinea*, has had no members since 1991. *Levinea* had two species, *L. amalonatica* and *L. malonatica*, which were reclassified as *Citrobacter amalonaticus* and *C. koseri* in 1982 and 1990, respectively. All genus and species are listed on the Euzeby list (http://www.bacterio.cict.fr), except *Gibbsiella papilionis* and *Pectobacterium aroidearum*, which have just been published.

The number of species per genus ranges from 1 to 22. Twenty-two genera contain only one species, while seven genera have more than 10 species. *Xenorhabdus* has the large number of species (Auch et al. 2010). The total number of species of the family stands at 238. The detail of species for each genus is listed in ❯ *Table 13.1* together with the type strain for each species. There are many new species appeared since last edition of this book with 15 new species in 2012 alone.

Phylogenetic Structure

The 16S rDNA phylogeny clusters *Enterobacteriaceae* with *Vibrionaceae* and *Pasteurellaceae* as sister clades. The neighbor-joining (NJ) tree is shown in ❯ *Fig. 13.1*. All members except for the genera *Arsenophonus* and *Thorsellia* are clustered together in one clade. Both *Arsenophonus* and *Thorsellia* are placed outside the family in between *Vibrionaceae* and *Pasteurellaceae*. In the

Table 13.1
Type strains of *Enterobacteriaceae* species

Species name	Accession no.	Type strain
Arsenophonus nasoniae	AY264674	SK14 = ATCC 49151 = DSM 15247 = LMG 12584
Biostraticola tofi	AM774412	BF36 = CIP109699 = DSM 19580
Brenneria alni	AJ233409	PVFi 20 = ATCC 700181 = CCUG 48887 = CIP 104916 = DSM 11811 = ICMP 12481 = NCPPB 3934
Brenneria goodwinii	JN544202	FRB 141 = R-43656 = BCC 845 = LMG 26270 = NCPPB 4484
Brenneria nigrifluens	U80203	ATCC 13028 = CCUG 48853 = CIP 105198 = CFBP 3616 = CFBP 4998 = DSM 30175 = LMG 2694 = NCPPB 564
Brenneria rubrifaciens	AJ233418	ATCC 29291 = CCUG 48854 = CFBP 3619 = CIP 105203 = DSM 4483 = ICMP 1915 = LMG 2709 = NCPPB 2020
Brenneria salicis	U80210	ATCC 15712 = CCUG 48855 = CFBP 802 = CIP 105204 = DSM 30166 = ICMP 1587 = LMG 2698 = NCPPB 447
Buchnera aphidicola		No type strain
Budvicia aquatica	AJ233407	Eb 13/82 = 20186 = 20186HG01 = ATCC 35567 = CIP 103240 = CNCTC 20186 = CNCTC 350 = DSM 5075 = LMG 8813
Budvicia diplopodorum	HE574451	D9 = CCM 7845 = DSM 21983
Buttiauxella agrestis	AJ233400	Gavini F-44 = ATCC 33320 = CDC 1176-81 = CIP 80.31 = CUETM 77-167 = DSM 4586 = JCM 1090 = LMG 7861 = NCTC 12119
Buttiauxella brennerae	AJ233401	S1/6-571 = serial (Müller et al.) n° 145 = ATCC 51605 = CCUG 35509 = CIP 106477 = DSM 9396
Buttiauxella ferragutiae	AJ233402	Serial (Müller et al.) n° 054 = ATCC 51602 = CDC 1180-81 = CIP 106355 = CUETM 78-31 = DSM 9390
Buttiauxella gaviniae	AJ233403	S1/1-984 = serial (Müller et al.) n° 062 = ATCC 51604 = CCUG 35508 = CIP 106356 = DSM 9393
Buttiauxella izardii	AJ233404	S3/2-161 = serial (Müller et al.) n° 151 = ATCC 51606 = CCUG 35510 = CIP 106357 = DSM 9397
Buttiauxella noackiae	AJ233405	NSW 11 = serial (Müller et al.) n° 170 = ATCC 51607 = CCUG 35511 = CIP 106358 = DSM 9401
Buttiauxella warmboldiae	AJ233406	NSW 326 = serial (Müller et al.) n° 182 = ATCC 51608 = CCUG 35512 = CIP 106359 = DSM 9404
Cedecea davisae	AF493976	005 = ATCC 33431 = CDC 3278-77 = CCUG 12370 = CIP 80.34 = DSM 4568 = JCM 1685 = LMG 7862
Cedecea lapagei	Not found	004 = ATCC 33432 = CDC 0485-76 = CCUG 12371 = CIP 80.35 = DSM 4587 = JCM 1684 = LMG 7863
Cedecea neterii	AB086230	002 of Grimont et al. = ATCC 33855 = CCUG 18763 = CDC 0621-75 = CIP 103241 = DSM 13693 = JCM 7582 = LMG 7864 = NCTC 12120
Citrobacter amalonaticus	FR870441	ATCC 25405 = CCUG 4860 = CECT 863 = CIP 82.89 = DSM 4593 = JCM 1661 = LMG 7873 = NCTC 10805
Citrobacter braakii	AF025368	CDC 80-58 = ATCC 51113 = CCUG 30792 = CIP 104554
Citrobacter farmeri	AF025371	ATCC 51112 = CCUG 30798 = CDC 2991-81 = CIP 104553
Citrobacter freundii	AJ233408	ATCC 8090 = CCUG 418 = CIP 57.32 = DSM 30039 = HAMBI 1695 = IFO (now NBRC) 12681 = JCM 1657 = LMG 3246 = NCAIM B.01468 = NCTC 9750 = NRRL B-2643
Citrobacter gillenii	AF025367	ATCC 51117 = CCUG 30796 = CDC 4693-86 = CIP 106783 = DSM 13694
Citrobacter koseri	HQ992945	ATCC 27028 = CCUG 4859 = CIP 105014 = CIP 82.87 = DSM 4595 = JCM 1658 = LMG 5519 = NCTC 10786
Citrobacter murliniae	AF025369	ATCC 51118 = CCUG 30797 = CDC 2970-59 = CIP 104556 = DSM 13695
Citrobacter rodentium	AF025363	ATCC 51116 = CCUG 30795 = CDC 1843-73 = CIP 104675
Citrobacter sedlakii	AF025364	ATCC 51115 = CCUG 30794 = CDC 4696-86 = CIP 105037
Citrobacter werkmanii	AF025373	ATCC 51114 = CCUG 30793 = CDC 876-58 = CIP 104555
Citrobacter youngae	AB273741	ATCC 29935 = CCUG 30791 = CDC 460-61 = CIP 105016

Table 13.1 (continued)

Species name	Accession no.	Type strain
Cosenzaea myxofaciens	DQ885259	ATCC 19692 = BCRC 12222 = CCRC 12222 = CCUG 18769 = CIP 106872 = DSM 4482 = JCM 1670 = LMG 7876 = NCIMB 13273
Cronobacter condimenti	FN539031	1330 = CECT 7863 = LMG 26250
Cronobacter dublinensis subsp. *dublinensis*	EF059892	DES187 = DSM 18705 = JCM 16467 = LMG 23823
Cronobacter dublinensis subsp. *lactaridi*	EF059838	E464 = DSM 18707 = JCM 16468 = LMG 23825
Cronobacter dublinensis subsp. *lausannensis*	EF059841	E515 = DSM 18706 = JCM 16469 = LMG 23824
Cronobacter malonaticus	EF059881	CDC 1058-77 = DSM 18702 = LMG 23826
Cronobacter muytjensii	EF059845	ATCC 51329 = CIP 103581
Cronobacter sakazakii	EF088379	ATCC 29544 = CCUG 14558 = CDC 4562-70 (78-067947) = CIP 103183 = DSM 4485 = JCM 1233 = LMG 5740 = NBRC 102416 = NCTC 11467
Cronobacter turicensis	EF059891	z3032 = DSM 18703 = LMG 23827
Cronobacter universalis	EF059877	CECT 7864 = LMG 26249 = NCTC 9529
Dickeya chrysanthemi	AJ233412	ATCC 11663 = CCUG 38766 = CFBP 2048 = CIP 82.99 = DSM 4610 = ICMP 5703 = LMG 2804 = NCAIM B.01392 = NCPPB 402
Dickeya dadantii	AF520707	Hayward B374 = CFBP 1269 = ICMP 1544 = NCPPB 898
Dickeya dianthicola	AF520708	CFBP 1200 = ICMP 6427 = LMG 2485 = NCPPB 453
Dickeya dieffenbachiae	AF520712	CFBP 2051 = ICMP 1568 = LMG 25992 = NCPPB 2976
Dickeya paradisiaca	Z96096	ATCC 33242 = CFBP 4178 = LMG 2542 = NCPPB 2511
Dickeya zeae	AF520711	CFBP 2052 = ICMP 5704 = LMG 2505 = NCPPB 2538
Edwardsiella hoshinae	AB050825	2-78 = ATCC 33379 = CIP 78.56 = DSM 13771 = JCM 1679 = NCTC 12121
Edwardsiella ictaluri	AB050826	SECFDL GA 77-52 = ATCC 33202 = CDC 1976-78 = CCUG 18764 = CIP 81.96 = DSM 13697 = JCM 1680 = JCM 16934 = NCTC 12122
Edwardsiella tarda	AB050827	ATCC 15947 = CCUG 1638 = CIP 78.61 = DSM 30052 = JCM 1656 = LMG 2793 = NCCB 73021 = NCTC 10396
Enterobacter aerogenes	AB004750	ATCC 13048 = CCUG 1429 = CIP 60.86 = DSM 30053 = HAMBI 101 = HAMBI 1898 = IFO (now NBRC) 13534 = JCM 1235 = LMG 2094 = NCAIM B.01467 = NCTC 10006
Enterobacter amnigenus	AB004749	ATCC 33072 = CCUG 14182 = CIP 103169 = CUETM 77-118 = DSM 4486 = HAMBI 1297 = JCM 1237 = LMG 2784 = NCTC 12124
Enterobacter arachidis	EU672801	Ah-143 = KCTC 22375 = NCIMB 14469 = KCTC 22375
Enterobacter asburiae	AB004744	1497-78 = ATCC 35953 = CCUG 25588 = CCUG 25714 = CIP 103358 = JCM 6051 = NCTC 12123
Enterobacter cancerogenus	Z96078	ATCC 33241 = CCUG 25231 = CFBP 4167 = CIP 103787 = ICMP 5706 = LMG 2693 = NCPPB 2176
Enterobacter cloacae subsp. *cloacae*	AJ251469	ATCC 13047 = CIP 60.85 = DSM 30054 = JCM 1232 = LMG 2783
Enterobacter cloacae subsp. *dissolvens*	Z96079	ATCC 23373 = CIP 105586 = JCM 6049 = LMG 2683
Enterobacter cowanii	AJ508303	888-76 = CCUG 45998 A = CCUG 45998 B = CIP 107300 = JCM 10956
Enterobacter gergoviae	AB004748	ATCC 33028 = CCUG 14557 = CDC 604-77 = CIP 76.1 = DSM 9245 = JCM 1234 = LMG 5739 = NCTC 11434
Enterobacter helveticus	DQ273688	513/05 = DSM 18396 = JCM 16470 = LMG 23732
Enterobacter hormaechei	AJ508302	0992-77 = ATCC 49162 = CCUG 27126 = CIP 103441
Enterobacter kobei	AJ508301	ATCC BAA-260 = CCUG 49023 = CIP 105566 = DSM 13645 = JCM 8580 = NIH 1485-79
Enterobacter ludwigii	AJ853891	EN-119 = CCUG 51323 = CCUG 51354 = CIP 108491 = DSM 16688
Enterobacter mori	EU721605	R18-2 = CGMCC 1.10322 = LMG 25706
Enterobacter nimipressuralis	Z96077	ATCC 9912 = CIP 104980 = ICMP 1577 = JCM 6050 = NCPPB 2045
Enterobacter oryzae	EF488759	Ola 51 = CGMCC 1.7012 = LMG 24251

Table 13.1 (continued)

Species name	Accession no.	Type strain
Enterobacter pulveris	DQ273684	601/05 = DSM 19144 = JCM 16471 = LMG 24057
Enterobacter pyrinus	AJ010486	ATCC 49851 = CCUG 48320 = CDC G6570 = CFBP 4168 = CIP 104019 = DSM 12410 = ICMP 12530 = KCTC 2520
Enterobacter radicincitans	AY563134	D5/23 = CCUG 50898 = CIP 108468 = DSM 16656
Enterobacter soli	GU814270	LF7 = ATCC BAA-2102 = LMG 25861
Enterobacter turicensis	DQ273681	508/05 = DSM 18397 = JCM 16472 = LMG 23730
Erwinia amylovora	AJ233410	ATCC 15580 = CFBP 1232 = CIP 82.82 = DSM 30165 = ICMP 1540 = IFO (now NBRC) 12687 = LMG 2024 = NCAIM B.01108 = NCPPB 683
Erwinia aphidicola	FN547376	X 001 = CIP 106296 = IAM 14479 = JCM 21238 = LMG 24877 = NBRC 102417
Erwinia billingiae	JN175337	Billing E63 = CIP 106121 = LMG 2613 = NCPPB 661
Erwinia mallotivora	AJ233414	ATCC 29573 = CFBP 2503 = CIP 105197 = DSM 4565 = ICMP 5705 = LMG 2708 = NCPPB 2851
Erwinia oleae	GU810925	DAPP-PG 531 = DSM 23398 = LMG 25322
Erwinia papayae	AY131237	CFBP 5189 = NCPPB 4294
Erwinia persicina	U80205	HK 204 = ATCC 35998 = CFBP 3622 = CIP 105199 = JCM 3704 = IAM 12843 = CDC 9108-82 = AJ 2716 = ICMP 12532 = LMG 11254 = NBRC 102418 = NCPPB 3774
Erwinia piriflorinigrans	GQ405202	CECT 7348 = CFBP 5888
Erwinia psidii	Not found	ATCC 49406 = CFBP 3627 = CIP 105200 = DSM 17597 = ICMP 8426 = LMG 7039 = NCPPB 3555 = PDDCC 8426
Erwinia pyrifoliae	EF122435	Ep16/96 = CFBP 4172 = CIP 106111 = DSM 12163
Erwinia rhapontici	AJ233417	ATCC 29283 = CFBP 3163 = CIP 105202 = DSM 4484 = ICMP 1582 = LMG 2688 = NCPPB 1578
Erwinia tasmaniensis	AM055716	Et1/99 = DSM 17950 = NCPPB 4357
Erwinia toletana	FR870447	A37 = ATCC 700880 = CECT 5263 = CFBP 6631
Erwinia tracheiphila	Y13250	ATCC 33245 = CFBP 2355 = CIP 105205 = ICMP 5845 = LMG 2707 = LMG 2906 = NCPPB 2452
Erwinia typographi	GU166291	Y1 = DSM 22678 = LMG 25347
Erwinia uzenensis	AB546198	YPPS 951 = YPPS951 = LMG 25843 = NCPPB 4475
Escherichia albertii	AJ508775	Albert 19982 = CCUG 46494 = JCM 17328 = LMG 20976
Escherichia coli	X80725	ATCC 11775 = CCUG 24 = CCUG 29300 = CIP 54.8 = DSM 30083 = JCM 1649 = LMG 2092 = NBRC 102203 = NCCB 54008 = NCTC 9001
Escherichia fergusonii	AF530475	ATCC 35469 = CDC 0568-73 = CIP 103357 = CIP 104947 = DSM 13698 = JCM 21226 = LMG 7866 = NBRC 102419 = NCTC 12128
Escherichia hermannii	JN175345	ATCC 33650 = CCUG 15714 = CDC 980-72 = CIP 103176 = CIP 104946 = DSM 4560 = HAMBI 1693 = JCM 1473 = LMG 7867 = NCTC 12129
Escherichia vulneris	AF530476	ATCC 33821 = CCUG 15715 = CDC 875-72 = CIP 103177 = DSM 4564 = HAMBI 1694 = JCM 1688 = LMG 7868 = NBRC 102420 = NCTC 12130
Ewingella americana	JN175329	ATCC 33852 = CCUG 14506 = CDC 1468-78 = CIP 81.94 = DSM 4580 = JCM 5911 = LMG 7869 = NCTC 12157
Gibbsiella dentisursi	AB566415	NUM 1720 = DSMZ 23818 = JCM 17201
Gibbsiella papilionis	JQ650257	LEN33 = KACC 16707 = JCM 18389
Gibbsiella quercinecans	GU562337	FRB 97 = LMG 25500 = NCPPB 4470
Hafnia alvei	M59155	ATCC 13337 = CCUG 41547 = CIP 57.31 = DSM 30163 = HAMBI 1279 = HAMBI 1876 = JCM 1666 = LMG 10392 = NCTC 8105 = NRRL B-4260
Hafnia paralvei	FM179943	ATCC 29927 = CDC 4510-73 = LMG 24706
Klebsiella alba	EF154517	CW-D 3 = LMG 24441 = KCTC 12878 = CCTCC AB 206144
Klebsiella granulomatis		No type strain
Klebsiella michiganensis	JQ070300	W14 = ATCC BAA-2403 = DSM 25444
Klebsiella oxytoca	AF129440	ATCC 13182 = CCUG 15717 = CIP 103434 = DSM 5175 = HAMBI 1301 = JCM 1665 = LMG 3055 = NBRC 102593

Table 13.1 (continued)

Species name	Accession no.	Type strain
Klebsiella pneumoniae subsp. *ozaenae*	AF130982	ATCC 11296 = CCUG 15938 = CIP 52.211 = JCM 1663 = LMG 3113 = NCTC 5050
Klebsiella pneumoniae subsp. *pneumoniae*	X87276	ATCC 13883 = CCUG 225 = CIP 82.91 = DSM 30104 = HAMBI 450 = IFO (now NBRC) 14940 = JCM 1662 = LMG 2095 = NCTC 9633
Klebsiella pneumoniae subsp. *rhinoscleromatis*	Y17657	ATCC 13884 = CCUG 417 = CIP 52.210 = JCM 1664 = LMG 3184 = NCTC 5046
Klebsiella singaporensis	AF250285	LX3 = DSM 16265 = JCM 12419
Klebsiella variicola	AJ783916	F2R9 = ATCC BAA-830 = CFNE 2004 = DSM 15968
Kluyvera ascorbata	AF008579	ATCC 33433 = CCUG 15716 = CDC 0648-74 = CIP 82.95 = DSM 4611 = JCM 21070 = LMG 7871 = NBRC 102466
Kluyvera cryocrescens	AF310218	12993 = ATCC 33435 = CCUG 18767 = CDC 2065-78 = CIP 82.96 = DSM 4588 = JCM 7580 = LMG 7859 = NBRC 102467
Kluyvera georgiana	AF047186	Serial (Müller et al.) n° 189 = ATCC 51603 = CCUG 35513 = CDC 2891-76 = CIP 106361 = DSM 9409 = JCM 16938
Kluyvera intermedia	AF310217	Gavini E 86 = ATCC 33110 = CCUG 14183 = CIP 79.27 = CUETM 77-130 = DSM 4581 = HAMBI 1299 = JCM 1238 = LMG 2785 = NBRC 102594 = NCTC 12125
Leclercia adecarboxylata	JN175338	ATCC 23216 = CIP 82.92 = DSM 5077 = HAMBI 1696 = JCM 1667 = LMG 2803 = NBRC 102595 = NCTC 13032
Leminorella grimontii	AJ233421	ATCC 33999 = CDC 1944-81 = CIP 103359 = DSM 5078 = JCM 5902 = LMG 7912 = NCTC 12152
Leminorella richardii	Not found	ATCC 33998 = CDC 0978-82 = CIP 103360 = DSM 14849 = JCM 5905 = LMG 7911 = NCTC
Lonsdalea quercina subsp. *britannica*	JF311442	LMG 26267 = NCPPB 4481
Lonsdalea quercina subsp. *iberica*	JF311441	LMG 26264 = NCPPB 4490
Lonsdalea quercina subsp. *populi*	JQ291575	NY060 = DSM 25466 = NCAIM B 02483
Lonsdalea quercina subsp. *quercina*	AJ233416	ATCC 29281 = CCUG 48867 = CFBP 3617 = CIP 105201 = DSM 4561 = ICMP 1845 = LMG 2724 = NCPPB 1852
Mangrovibacter plantisponsor	EF643377	MSSRF40 = DSM 19579 = LMG 24236
Moellerella wisconsensis	JN175344	2896-78 = ATCC 35017 = CIP 103034 = DSM 5076 = JCM 5895 = LMG 10145 = NCTC 12132
Morganella morganii subsp. *morganii*	AJ301681	ATCC 25830 = ATCC 8076H = CCUG 6328 = CIP 103763 = CIP A231 = DSM 30164 = JCM 1672 = LMG 7874 = NBRC 3848 = NCCB 73065 = NCTC 235
Morganella morganii subsp. *sibonii*	DQ358146	8103-85 = AB 2048 = ATCC 49948 = CCUG 30886 = CIP 103648 = DSM 14850 = JCM 16939
Morganella psychrotolerans	DQ358135	U2/3 = DSM 17886 = JCM 16473 = LMG 23374
Obesumbacterium proteus	AJ233422	ATCC 12841 = CCUG 2078 = CIP 82.93 = DSM 2777 = LMG 3054 = NCIB (now NCIMB) 8771 = VKM B-964
Pantoea agglomerans	AJ233423	ATCC 27155 = CCUG 539 = CDC 1461-67 = CFBP 3845 = CIP 57.51 = DSM 3493 = ICPB 3435 = ICMP 12534 = ICPB 3435 = JCM 1236 = LMG 1286 = NBRC 102470 = NCTC 9381
Pantoea allii	AY530795	BD 390 = LMG 24248
Pantoea ananatis	U80196	ATCC 33244 = CFBP 3612 = CIP 105207 = ICPB EA175 = LMG 2665 = NCPPB 1846 = PDDCC 1850
Pantoea anthophila	EF688010	BD 871 = LMG 2558 = NCPPB 1682
Pantoea brenneri	EU216735	BD 873 = CDC 3482-71 = LMG 5343
Pantoea calida	GQ367478	1400/07 = DSM 22759 = LMG 25383
Pantoea conspicua	EU216737	BD 805 = CDC 3527-71 = LMG 24534
Pantoea cypripedii	AJ233413	ATCC 29267 = CFBP 3613 = CIP 105195 = DSM 3873 = LMG 2655 = LMG 2657 = NCPPB 3004 = PDDCC 1591

Table 13.1 (continued)

Species name	Accession no.	Type strain
Pantoea deleyi	EF688011	R-31523 = BD 767 = BCC 109 = LMG 24200
Pantoea dispersa	DQ504305	ATCC 14589 = CCUG 25232 = CIP 103338 = DSM 30073 = LMG 2603
Pantoea eucalypti	EF688009	R-25678 = BD 769 = BCC 076 = LMG 24197
Pantoea eucrina	EU216736	BD 872 = CDC 1741-71 = LMG 5346 = LMG 2781
Pantoea gaviniae	GQ367483	A18/07 = DSM 22758 = LMG 25382
Pantoea rodasii	JF295053	BD 943 = BCC 581 = LMG 26273
Pantoea rwandensis	JF295055	BD 944 = BCC 571 = LMG 26275
Pantoea septica	EU216734	BD 874 = CDC 3123-70 = LMG 5345
Pantoea stewartii subsp. *indologenes*	JN175332	ATCC 51785 = CFBP 3614 = CIP 104006 = ICMP 77 = LMG 2632 = NCPPB 2280
Pantoea stewartii subsp. *stewartii*	U80208	ATCC 8199 = CFBP 2349 = CFBP 3167 = CIP 104005 = DSM 30176 = ICMP 257 = ICPB SS11 = IMET 11187 = LMG 2715 = NCPPB 2295 = NRRL B-794
Pantoea vagans	EF688012	R-21566 = BD 765 = BCC 105 = LMG 24199
Pantoea wallisii	JF295057	BD 946 = BCC 682 = LMG 26277
Pectobacterium aroidearum	JN600323	SCRI 109 = NCPPB 929 = LMG 2417 = ICMP 1522
Pectobacterium atrosepticum	Z96090	ATCC 33260 = CFBP 1526 = CIP 105192 = ICMP 1526 = LMG 2386 = NCPPB 549
Pectobacterium betavasculorum	U80198	ATCC 43762 = CFBP 1539 = CFBP 2122 = CIP 105193 = ICMP 4226 = LMG 2464 = LMG 2466 = NCPPB 2795
Pectobacterium cacticida	AJ223409	1-12 = Dye EH-3 = ATCC 49481 = CFBP 3628 = CIP 105191 = ICMP 1551-66 = ICMP 11136 = ICPB EC186 = LMG 17936 = NCPPB 3849
Pectobacterium carotovorum subsp. *carotovorum*	AJ233411	ATCC 15713 = CFBP 2046 = CIP 82.83 = DSM 30168 = HAMBI 1429 = ICMP 5702 = LMG 2404 = NCAIM B.01109 = NCPPB 312 = VKM B-1247
Pectobacterium carotovorum subsp. *odoriferum*	AJ223407	CFBP 1878 = CIP 103762 = ICMP 11533 = NCPPB 3839
Pectobacterium wasabiae	U80199	SR91 = ATCC 43316 = CFBP 3304 = CIP 105194 = ICMP 9121 = LMG 8404 = NCPPB 3701 = PDDCC 9121
Phaseolibacter flectens	AB021400	ATCC 12775 = CFBP 3281 = ICMP 745 = LMG 2187 = NCPPB 539
Photorhabdus asymbiotica subsp. *asymbiotica*	Z76755	3265-86 = ATCC 43950 = CIP 106331 = DSM 15149
Photorhabdus asymbiotica subsp. *australis*	AY280572	9802892 = ACM 5210 = CIP 108025
Photorhabdus luminescens subsp. *akhurstii*	AJ007359	FRG04 = CIP 105564 = DSM 15138
Photorhabdus luminescens subsp. *caribbeanensis*	EU930345	HG29 = CIP 109949 = DSM 22391
Photorhabdus luminescens subsp. *hainanensis*	EU930342	C8404 = CIP 109946 = DSM 22397
Photorhabdus luminescens subsp. *kayaii*	AJ560630	1121 = DSM 15194 = NCIMB 13951
Photorhabdus luminescens subsp. *kleinii*	HM072284	KMD37 = ATCC BAA-2104 = DSM 23513
Photorhabdus luminescens subsp. *laumondii*	AJ007404	TT01 = CIP 105565 = DSM 15139
Photorhabdus luminescens subsp. *luminescens*	X82248	Hb = ATCC 29999 = CIP 106429 = DSM 3368
Photorhabdus luminescens subsp. *noenieputensis*	JQ424880	AM7 = ATCC BAA-2407 = DSM 25462
Photorhabdus temperata subsp. *cinerea*	EU136626	3107 = DSM 19724 = NCAIM B 02271
Photorhabdus temperata subsp. *khanii*	AY278657	C1 = NC19 = CIP 109947 = DSM 3369

Table 13.1 (continued)

Species name	Accession no.	Type strain
Photorhabdus temperata subsp. *stackebrandtii*	EF467859	GPS11 = ATCC BAA-2077 = DSM 23271
Photorhabdus temperata subsp. *tasmaniensis*	EU930339	T327 = CIP 109948 = DSM 22387
Photorhabdus temperata subsp. *temperata*	AJ007405	XINach = CIP 105563
Photorhabdus temperata subsp. *thracensis*	AJ560634	39-8 = CIP 108426 = DSM 15199 = NCIMB 13952
Plesiomonas shigelloides	X60418	ATCC 14029 = CCUG 410 = CIP 63.5 = DSM 8224 = LMG 4242 = NCCB 80007 = NCTC 10360
Pragia fontium	AJ233424	DRL 20125 = HG16 = CNCTC Eb11/82 = ATCC 49100 = CCUG 18073 = CDC 963-84 = CIP 103791 = DSM 5563 = IP 20125 = LMG 7875
Proteus hauseri	FR733709	ATCC 700826 = CCUG 35386 = CDC 1732-80 = CIP 106868
Proteus mirabilis	DQ885256	ATCC 29906 = CCUG 26767 = CIP 103181 = DSM 4479 = JCM 1669 = LMG 3257 = NCTC 11938
Proteus penneri	DQ885258	ATCC 33519 = CCUG 15722 = CDC 1808-73 = CIP 103030 = DSM 4544 = JCM 3948 = NCTC 12737
Proteus vulgaris	DQ885257	ATCC 13315 = HAMBI 91 = IFO (now NBRC) 3851 = NCIB (now NCIMB) 4175 = NCTC 4175 = NRRL B-3405
Providencia alcalifaciens	AJ301684	ATCC 9886 = CCUG 6325 = CIP 82.90 = DSM 30120 = JCM 1673 = NCTC 10286
Providencia burhodogranariea	HM038004	B = ATCC BAA-1590 = DSM 19968 = JCM 16940
Providencia heimbachae	AM040490	MUA 2-110 = ATCC 35613 = CCUG 16446 = CDC 8025-83 = CIP 103031 = DSM 3591 = NCTC 12003
Providencia rettgeri	AM040492	ATCC 29944 = CCUG 14804 = CIP 103182 = DSM 4542 = JCM 1675 = LMG 3259 = NCTC 11801
Providencia rustigianii	AM040489	ATCC 33673 = CCUG 15723 = CDC 0132-68 = CIP 103032 = DSM 4541 = JCM 3953 = NCTC 11802
Providencia sneebia	HM038003	A = ATCC BAA-1589 = DSM 19967 = JCM 16941
Providencia stuartii	AF008581	ATCC 29914 = CCUG 14805 = CIP 104687 = DSM 4539 = LMG 3260 = NCTC 11800
Providencia vermicola	AM040495	OP1 = CIP 108829 = DSM 17385
Rahnella aquatilis	AJ233426	133 = ATCC 33071 = CCUG 14185 = CIP 78.65 = DSM 4594 = HAMBI 1280 = JCM 1683 = LMG 2794
Raoultella ornithinolytica	U78182	ATCC 31898 = CCUG 26769 = CIP 103576 = CIP 103364 = DSM 7464 = JCM 6096 = NIH 90-72
Raoultella planticola	AF129443	V-236 = ATCC 33531 = CCUG 15718 = CDC 4245-72 = CIP 100751 = DSM 3069 = IFO (now NBRC) 14939 = JCM 7251 = NCTC 12998
Raoultella terrigena	Y17658	Gavini L 84 = ATCC 33257 = CCUG 12372 Bb = CIP 80.7 = CUETM 77-176 = DSM 2687 = HAMBI 1302 = IFO (now NBRC) 14941 = JCM 1687 = LMG 3222 = NCTC 13038
Salmonella bongori	AF029227	ATCC 43975 = CCUG 30042 = CIP 82.33 = DSM 13772 = NCTC 12419
Salmonella enterica subsp. *arizonae*	AF008580	ATCC 13314 = CCUG 6322 = CIP 82.30 = DSM 9386 = NCTC 8297
Salmonella enterica subsp. *diarizonae*	EU014688	ATCC 43973 = CCUG 30040 = CIP 82.31 = DSM 14847 = NCTC 10060
Salmonella enterica subsp. *enterica*	AE006468	LT2 (serovar Typhimurium) = ATCC 43971 = CCUG 42060 = CIP 60.62 = NBRC 13245 = NCIMB 11450 = NCTC 12416
Salmonella enterica subsp. *houtenae*	EU014684	ATCC 43974 = CCUG 30041 = CIP 82.32 = DSM 9221 = NCTC 12418
Salmonella enterica subsp. *indica*	EU014680	K1240 = ATCC 43976 = CCUG 30038 = CIP 102501 = DSM 14848 = NCTC 12420
Salmonella enterica subsp. *salamae*	EU014685	ATCC 43972 = CCUG 30039 = CIP 8229 = DSM 9220 = NCTC 5773

Table 13.1 (continued)

Species name	Accession no.	Type strain
Salmonella subterranea	AY373829	FRCI = ATCC BAA-836 = DSM 16208
Samsonia erythrinae	AF273037	CFBP 5236 = ICMP 13937
Serratia entomophila	AJ233427	A1 = ATCC 43705 = CIP 102919 = DSM 12358
Serratia ficaria	AJ233428	4024 = ATCC 33105 = CIP 79.23 = DSM 4569 = ICPB 4050 = JCM 1241 = LMG 7881 = NBRC 102596 = NCTC 12148
Serratia fonticola	AJ233429	11 = ATCC 29844 = CCUG 14186 = CCUG 37824 = CIP 78.64 = DSM 4576 = HAMBI 1274 = JCM 1242 = LMG 7882 = NBRC 102597 = NCTC 12965
Serratia glossinae	FJ790328	C1 = CCUG 57457 = DSM 22080
Serratia grimesii	AJ233430	ATCC 14460 = CCUG 15721 = CIP 103361 = DSM 30063 = HAMBI 1284 = IFO (now NBRC) 13537 = JCM 5910 = LMG 7883 = NCTC 11543 = NRRL B-4271
Serratia liquefaciens	AJ306725	ATCC 27592 = CCUG 9285 = CIP 103238 = DSM 4487 = JCM 1245 = LMG 7884 = NCTC 12962
Serratia marcescens subsp. *marcescens*	AJ233431	ATCC 13880 = CCUG 1647 = CFBP 4226 = CIP 103235 = DSM 30121 = HAMBI 1286 = JCM 1239 = LMG 2792 = NBRC 102204 = NCTC 10211 = NRRL B-2544 = VKM B-1248
Serratia marcescens subsp. *sakuensis*	AB061685	KRED = CIP 107489 = JCM 11315
Serratia nematodiphila	EU036987	DZ0503SBS1 = CGMCC 1.6853 = KCTC 22130
Serratia odorifera	AJ233432	ATCC 33077 = CDC 1979-77 = CCUG 14508 = CIP 79.1 = DSM 4582 = JCM 1243 = NBRC 102598 = NCTC 11214
Serratia plymuthica	AJ233433	ATCC 183 = CCUG 14509 = CIP 103239 = DSM 4540 = JCM 1244 = LMG 7886 = NBRC 102599 = NCTC 12961
Serratia proteamaculans	AJ233434	ATCC 19323 = CCUG 14510 = CIP 103236 = DSM 4543 = ICMP 1724 = NCPPB 245
Serratia quinivorans	AJ233435	4364 = ATCC 33765 = CIP 103237 = CIP 81.95 = DSM 4597 = LMG 7887 = NCTC 11544
Serratia rubidaea	AB004751	ATCC 27593 = CCUG 9286 = CCUG 10981 = CIP 103234 = DSM 4480 = JCM 1240 = LMG 5019 = NBRC 103169 = NCTC 12971
Serratia symbiotica	GU394001	CWBI-2.3 = DSM 23270 = LMG 25624
Serratia ureilytica	AJ854062	NiVa 51 = CCUG 50595 = JCM 16474 = LMG 22860
Shigella boydii	Not found	ATCC 8700 = CCUG 49022 = CIP 82.50 = DSM 7532 = NCTC 12985
Shigella dysenteriae	X96966	ATCC 13313 = CIP 57.28 = NCTC 4837
Shigella flexneri	X96963	ATCC 29903 = CIP 82.48 = DSM 4782
Shigella sonnei	FR870445	ATCC 29930 = CECT 4887 = CIP 82.49 = DSM 5570 = NCTC 12984
Shimwellia blattae	FJ267520	ATCC 29907 = CDC 9005-74 = CIP 103175 = CIP 104942 = DSM 4481 = HAMBI 1692 = JCM 1650 = LMG 3030 = NCTC 12127
Shimwellia pseudoproteus	FJ267523	521 = DSM 3038 = LMG 24835 = NCIMB 14534
Sodalis glossinidius	M99060	M1 = DSM 16929 = NCIMB 13495
Tatumella citrea	DQ838096	SHS 2003 = BD 875 = ATCC 31623 = CCUG 30156 = CIP 105599 = DSM 13699 = JCM 8882 = LMG 22049
Tatumella morbirosei	EU344769	BD 878 = CMC6 = LMG 23360 = NCPPB 4036
Tatumella ptyseos	AJ233437	H36 = ATCC 33301 = CDC D6168 = CDC 9591-78 = CCUG 14188 = CIP 81.97 = DSM 5000 = LMG 7888 = NCTC 11468
Tatumella punctata	FJ756351	SHS 2006 = BD 876 = ATCC 31626 = CCUG 30159 = CIP 105598 = DSM 13700 = JCM 8885 = LMG 22050
Tatumella terrea	EF688007	SHS 2008 = BD 877 = ATCC 31628 = CCUG 30161 = CIP 105600 = DSM 13701 = JCM 8887 = LMG 22051
Thorsellia anophelis	AY837748	CCUG 49520 = CIP 108754
Trabulsiella guamensis	AY373830	ATCC 49490 = CDC 0370-85 = CIP 103637 = JCM 21227 = NBRC 103172
Trabulsiella odontotermitis	DQ453129	Eant 3-9 = BCRC 17577 = LMG 23580
Wigglesworthia glossinidius		No type strain
Xenorhabdus beddingii	AY278675	Q58/1 = Q58 = ATCC 49542 = DSM 4764 = UQM 2871
Xenorhabdus bovienii	AY278673	T228 = ATCC 35271 = DSM 4766 = UQM 2210

Table 13.1 (continued)

Species name	Accession no.	Type strain
Xenorhabdus budapestensis	AJ810293	DSM 16342 = NCIMB 14016
Xenorhabdus cabanillasii	AY521244	USTX62 = CIP 109066 = DSM 17905
Xenorhabdus doucetiae	DQ211709	FRM16 = CIP 109074 = DSM 17909
Xenorhabdus ehlersii	AJ810294	DSM 16337 = NCIMB 14018
Xenorhabdus griffiniae	DQ211710	ID10 = CIP 109073 = DSM 17911
Xenorhabdus hominickii	DQ211719	KE01 = CIP 109072 = DSM 17903
Xenorhabdus indica	AM040494	28 = CIP 108830 = DSM 17382
Xenorhabdus innexi	AJ810292	DSM 16336 = NCIMB 14017
Xenorhabdus japonica	D78008	SK-1 = IAM 14265 = JCM 21111
Xenorhabdus koppenhoeferi	DQ205450	USNJ01 = CIP 109199 = DSM 18168
Xenorhabdus kozodoii	DQ211716	SaV = CIP 109068 = DSM 17907
Xenorhabdus magdalenensis	HQ877464	DSM 24915 = IMI 397775
Xenorhabdus mauleonii	DQ211715	VC01 = CIP 109075 = DSM 17908
Xenorhabdus miraniensis	DQ211713	Q1 = CIP 109069 = DSM 17902
Xenorhabdus nematophila	AY278674	ATCC 19061 = CCUG 14189 = DSM 3370 = LMG 1036
Xenorhabdus poinarii	D78010	G1 = ATCC 35272 = CIP 103468 = DSM 4768 = UQM 2216
Xenorhabdus romanii	DQ211717	PR06-A = CIP 109070 = DSM 17910
Xenorhabdus stockiae	DQ202309	TH01 = CIP 109067 = DSM 17904
Xenorhabdus szentirmaii	AJ810295	DSM 16338 = NCIMB 14019
Xenorhabdus vietnamensis	DQ205447	VN01 = CIP 109945 = DSM 22392
Yersinia aldovae	AF366376	Al 19955 = ATCC 35236 = CCUG 18770 = CDC 669-83 = CIP 103162 = CNY 6005 = IP 6005 = JCM 5892
Yersinia aleksiciae	AJ627597	Y159 = WA758 = DSM 14987 = LMG 22254
Yersinia bercovieri	AF366377	WAIP 208 = ATCC 43970 = CCUG 26329 = CDC 2475-87 = CIP 103323 = CNY 7506
Yersinia enterocolitica subsp. *enterocolitica*	AF366378	ATCC 9610 = CCUG 11291 = CCUG 12369 = CIP 80.27 = DSM 4780 = JCM 7577 = LMG 7899 = NCTC 12982
Yersinia enterocolitica subsp. *palearctica*	FJ717344	Y11 = CIP 106945 = DSM 13030
Yersinia entomophaga	DQ400782	MH96 = MH-1 = ATCC BAA-1678 = DSM 22339
Yersinia frederiksenii	AF366379	6175 = ATCC 33641 = CCUG 11293 = CIP 80.29 = NCTC 11470
Yersinia intermedia	AF366380	3953 = Bottone 48 = Chester 48 = ATCC 29909 = CCUG 11292 = CIP 80.28 = JCM 7579 = NCTC 11469
Yersinia kristensenii	AF366381	105 = ATCC 33638 = CCUG 8241 = CCUG 11294 = CIP 80.30 = JCM 7576 = NCTC 11471
Yersinia massiliensis	EF179119	50640 = CCUG 53443 = CIP 109351
Yersinia mollaretii	AF366382	WAIP 204 = ATCC 43969 = CCUG 26331 = CDC 2465-87 = CIP 103324 = CNY 7263
Yersinia nurmii	FJ717338	APN3a-c = DSM 22296 = LMG 25213
Yersinia pekkanenii	GQ451990	ÅYV7.1KOH2 = DSM 22769 = LMG 25369
Yersinia pestis	AF366383	ATCC 19428 = CIP 80.26 = NCTC 5923
Yersinia pseudotuberculosis	AF366375	ATCC 29833 = CCUG 5855 = CIP 55.85 = DSM 8992 = JCM 1676 = NCTC 10275
Yersinia rohdei	AF366384	H271-36/78 = ATCC 43380 = CCUG 38833 = CDC 3022-85 = CIP 103163 = JCM 7376 = LMG 8454
Yersinia ruckeri	AF366385	ATCC 29473 = CCUG 14190 = CDC 2396-61 = CIP 82.80 = HAMBI 1298 = JCM 2429 = JCM 15110 = NCTC 12986
Yersinia similis	AM182404	Y228 = CCUG 52882 = LMG 23763
Yokenella regensburgei	AB519796	ATCC 49455 = BCRC (formerly CCRC) 12225 = CIP 105435 = JCM 2403 = NBRC 102600 = NCTC 11966 = NIH 725-83

All type strains names are available from the Euzeby list (http://www.bacterio.cict.fr) except *Gibbsiella papilionis* and *Photorhabdus luminescens* subsp. *noenieputensis*

maximum likelihood (ML) tree, both are placed at the root of tree.

The new genus *Plesiomonas* transferred from *Vibrionaceae* is placed close to the root of the NJ tree as the earliest diverged member of the family. Note that the following species, *Cedecea lapagei, Enterobacter taylorae, Erwinia psidii, Klebsiella granulomatis, Leminorella richardii, Pectobacterium carnegieana, Saccharobacter fermentatus, Shigella boydii, Buchnera aphidicola*, and *Wigglesworthia glossinidia*, are not on the 16S rDNA tree due to either no 16S rDNA sequence available (the first seven) or no type strain defined (the last two) and, thus, were not inadvertently omitted.

Of the 30 genera with two or more species, 18 (*Buttiauxella, Cedecea, Cronobacter, Dickeya, Edwardsiella, Gibbsiella, Hafnia, Kluyvera, Morganella, Photorhabdus, Proteus, Providencia, Raoultella, Shimwellia, Tatumella, Trabulsiella, Xenorhabdus*, and *Yersinia*) are clustered in a single cluster on the 16S rDNA tree and thus are likely to be monophyletic. The two *Salmonella* species are not grouped together on the 16S rDNA tree. *S. bongori* is well separated from *S. enterica*. However, *Salmonella* is known to be monophyletic based on housekeeping genes. We confirmed that the 16S rDNA sequence of *S. bongori* used is correct by comparison with the genome sequenced *S. bongori* strain. Thus, this is an anomaly. Note that *Salmonella subterranea* (Shelobolina et al. 2004) does not belong to the genus *Salmonella* (Grimont and Weill 2007). *Shigella* did not form a cluster and mixed with *E. coli*, which is correct as it is long known that *Shigella* strains are clones of *E. coli* (Pupo et al. 2000). The 16 *Erwinia* species are in fact clustered together but with two *Pantoea* species (*P. gaviniae* and *P. calida*) and one *Enterobacter* species (*E. turicensis*) inside the cluster. Thus, *Erwinia* is likely to be monophyletic and the three non-*Erwinia* species should be reclassified. However, other data would be needed to support this clustering. The six *Pectobacterium* species (an additional species has no 16S rDNA sequence) are clustered together, suggesting that this genus is monophyletic. However, *Pectobacterium aroidearum* is at the base of the cluster and is quite distantly related to the other five species. There are also three non-*Pectobacterium* species (*Samsonia erythrinae* and two *Brenneria spp.*) within the cluster. For the two *Leminorella* spp., only one has 16S rDNA sequence available.

Seven genera (*Brenneria, Citrobacter, Enterobacter, Escherichia, Klebsiella, Pantoea*, and *Serratia*) are likely to be polyphyletic. The five *Brenneria* species are separated into three lineages, with *B. goodwinii* and *B. rubrifaciens* together and *B. alni* and *B. nigrifluens* together. *B. salicis* falls inside *Lonsdalea* and closest to *L. quercine* subsp. *quercine*. Based on this relationship *B. salicis* is a species f *Lonsdalea*. The 11 *Citrobacter* spp. are divided into two well-separated clusters and two singletons. *C. koseri* and *C. gillenii* are grouped with *Trabulsiella* and *Enterobacter nimipressuralis*, respectively. The 21 species of *Enterobacter* are scattered across the 16S rDNA tree with only four clusters of four, three, two, and two species and 10 unclustered species. Thus, reclassification of the *Enterobacter* genus is much needed. For example, *Enterobacter pyrinus* is grouped with a *Pantoea* cluster at the root of the cluster and may be reclassified as *Pantoea* if other data supports the relationship. Of the five *Escherichia* species, three (*E. albertii, E. coli*, and *E. fergusonii*) are grouped together whereas two (*E. hermannii* and *E. vulneris*) grouped away from the other three species and may need reclassification. All but two *Klebsiella* species are grouped together, with *K. oxytoca* and *K. michiganensis* being outside the clade. *K. oxytoca* clustered with *Enterobacter gergoviae*, while *K. michiganensis* stands alone. *K. michiganensis* was reported in 2012. In the original publication (Saha et al. 2013), *K. michiganensis* clustered with *K. oxytoca* in both 16S and *rpoB* trees and was separated from the main *Klebsiella* cluster. This taxonomy assignment seemed robust but exposed the problem of not including enough other species and genera to ascertain the genus identity of a novel species. The 19 *Pantoea* species were clustered into four clusters with 11, four, two, and two species in each cluster. The four clusters are well separated. The 15 *Serratia* species are grouped into two clusters with seven species each and one singleton (*Serratia ureilytica*), which are well separated on the 16S tree.

The relationships of genus and species inferred depending on the algorithms used and whether any highly variable sites are filtered out. Thus, many of the branches shown on the 16S gene tree in ❯ *Fig. 13.1* are perhaps unreliable, and caution needs to be taken in interpretation of the species/genus relationships. However, quite a number of the 16S-based relationships are likely to be correct as they are supported by other sequence data. These include the close relationship of *Salmonella, Citrobacter* (one of the two clusters only), and *Escherichia*; the close relationship of *Xenorhabdus* with *Photorhabdus* as sister clades; the close relationship of *Erwinia* with *Pantoea;* and the localization of *Obesumbacterium* within *Hafnia*.

There are numerous multilocus sequence typing (MLST) studies with very few that contain sufficient genera to give a good overview at family level. MLST at genus level will be discussed in respective genus sections below. The studies with a good number of *Enterobacteriaceae* family members were those of using a single gene such as *gyrB* which included 11 genera and 14 species (Dauga 2002), *dnaJ* (Pham et al. 2007) which included 27 genera and 93 species and *oriC* which included 10 genera and 38 species (Roggenkamp 2007), or two genes (*tuf* and *atpD*) which included 31 genera and 78 species (Paradis et al. 2005). The *gyrB* tree supports the grouping together of *Morganella, Proteus*, and *Providencia* on the 16S rDNA tree, while the *tuf–atpD* tree only supports the grouping of *Proteus* with *Providencia*, with *Morganella* on a different branch. *oriC* locus showed high sequence divergence making it only useful to differentiate closely related species/genera. The grouping of *Salmonella, Citrobacter* (one cluster), and *Escherichia* seen on 16S rDNA tree is also reflected in the *dnaJ, oriC*, and *tuf–atpD* trees. The *tuf–atpD* gene tree supports *Plesiomonas* as a member of the family *Enterobacteriaceae* and also supports the close relationship of *Hafnia* with *Obesumbacterium*. An MLST study using four housekeeping genes (*fusA, leuS, pyrG*, and *rpoB*) showed that *Hafnia* and *Obesumbacterium* share 95% similarity (Priest and Barker 2010). With such a high similarity, they should be merged as a single genus. The *tuf–atpD* gene tree

Fig. 13.1

Phylogenetic reconstruction of the family *Enterobacteriaceae* based on 16S rDNA and created using the neighbor-joining algorithm with the Jukes–Cantor correction. The sequence datasets and alignments were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010, http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high-quality type strain sequences proportionally distributed among the different bacterial and

places the outlier *C. youngae* into one of the main *Citrobacter* clusters and also showed that most of the *Enterobacter* spp. were unclustered as seen in the 16S rDNA tree.

Sequence analyses of *atpD, carA*, and *recA* genes from two to seven plant-associated species, representing six genera (*Brenneria*, *Dickeya*, *Enterobacter*, *Erwinia*, *Pantoea* and *Pectobacterium*), showed that *Erwinia* and *Brenneria* are sister groups of *Pantoea* and *Pectobacterium*, respectively (Young and Park 2007). This relationship is largely consistent with the 16S rDNA data (Hauben et al. 1998), although in the latter with more species included, the *Brenneria* spp. fall inside the cluster of *Pectobacterium* spp.

Additionally, the Husnik et al. (2011) study on endosymbionts (see below) provides a tree of 31 recognized species using 69 genes which is a quite comprehensive genome tree of *Enterobacteriaceae*. The tree helps to resolve the uncertainty of the phylogenetic clustering of *Arsenophonus* which is placed outside *Enterobacteriaceae* on the 16S rDNA sequence tree. *Arsenophonus* was placed inside the family on the genome tree. Seven genera (*Cronobacter, Dickeya, Edwardsiella, Erwinia Pectobacterium, Photorhabdus*, and *Xenorhabdus*) that have two or more species included in the study are all grouped together by genus showing a single origin. The three *Pectobacterium* species (*P. carotovorum, P. atrosepticum*, and *P. wasabiae*) were grouped together as a sister clade of the *Dickeya* species (*D. dadantii* and *D. zeae*). This relationship is consistent with the 16S rDNA tree. However, the sister relationship of the four *Erwinia* spp. (*E. amylovora, E. billingiae, E. pyrifoliae*, and *E. tasmaniensis*) with *Pantoea ananatis* is different from that based on the 16S rDNA sequence, but is consistent with the full genome tree below, suggesting the 16S rDNA-based relationship is incorrect. Both the 16S rDNA tree and the genome tree show *Xenorhabdus* and *Photorhabdus* are sister clades and monophyletic. *C. koseri* is separate from the other *Citrobacter* species which is similar to the 16S RNA sequence-based relationship.

A whole genome phylogenetic tree of *Enterobacteriaceae* is available on the PATRIC server (http://www.patricbrc.org) (Gillespie et al. 2011). The tree included 1,031 *Enterobacteriaceae* genomes including 196 complete genomes and 835 genomes from whole genome shotgun sequencing, representing 27 genera. A reduced version of the tree is shown in ❯ *Fig. 13.2* with details of species removed to show relationships among the genera. *Yersinia* clusters with *Serratia* as sister clades, showing the same relationship as the 16S rDNA tree. The genome tree grouped *Citrobacter koseri, C. rodentium, C. youngae*, and *C. freundii* together in one lineage. However, *Salmonella* is placed between *C. rodentium* and other *Citrobacter spp.*, which conflicts with other gene trees. *Escherichia fergusonii, E. albertii*, and *E. coli* are clustered together and are consistent with other gene trees. *E. hermannii* is not grouped with the other three *Escherichia* species as is the case with 16S rDNA tree, but grouped with *Cronobacter* at the base of clade. However, there is no genome available for *Escherichia vulneris* to ascertain its phylogenetic position. There are also anomalies. *Buchnera* and *Wigglesworthia* are grouped together, which is in conflict with the Husnik et al. study (2011). *Shigella* represented by

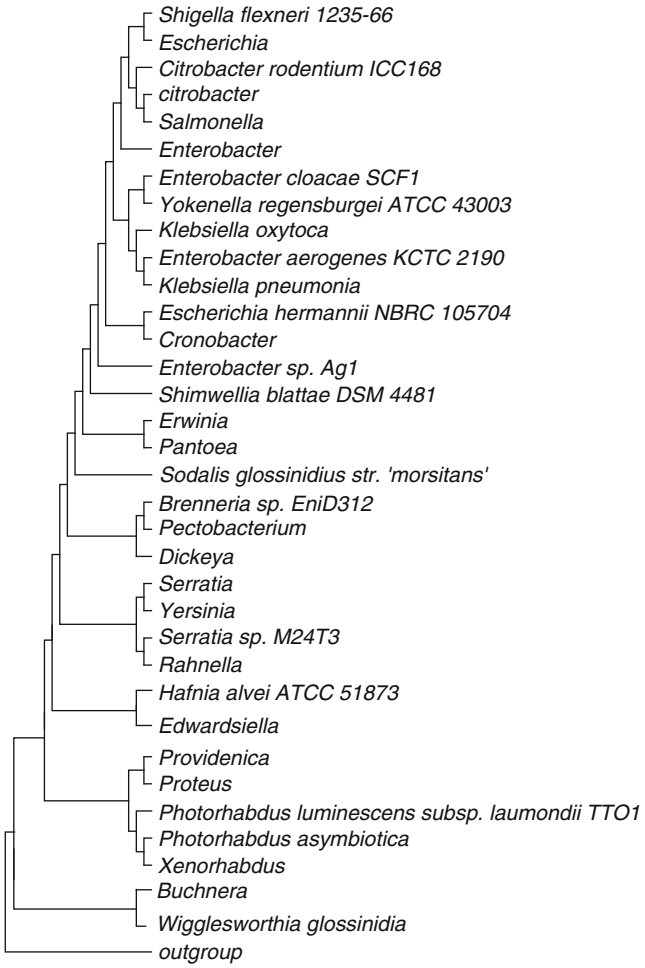

Fig. 13.2
Genome tree of *Enterobacteriaceae*. The tree was extracted from the whole genome phylogenetic tree available on the PATRIC server (http://www.patricbrc.org). Only genus name is shown if the node contains multiple genomes that are all from the same genus. Full species and strain name is shown if the node is a single genome

Fig. 13.1 (continued) archaeal phyla. Scale bar indicates estimated sequence divergence. Taxa are labeled by species name and in bold if it is the type species of the genus. In the abridged tree, terminal nodes marked by genus name only are those that contain collapsed branches comprising only species from that genus (not necessary all species of that genus). Nodes labeled with genus name plus "*sensu stricto*" are those containing the type species of the genus. Otherwise, they are labeled genus name plus "*sensu lato*" containing no type species of the genus. The type strain and GenBank accession numbers used for tree construction are listed in ❯ *Table 13.1*

a *S. flexneri* strain is placed outside the cluster of the three *Escherichia* species which is incorrect as *Shigella* strains are clones of *E. coli* (Pupo et al. 2000) and should be clustered with *E. coli*. The *Enterobacter* species, *E. asburiae, E. mori, E. cancerogenus, E. hormaechei*, and *E. cloacae*, are grouped together in one cluster. All except *E. cloacae* are clustered together on the 16S rDNA tree.

The phylogenetic relationships of endosymbionts have been studied using whole genome sequences which showed at least four independent origins (Husnik et al. 2011). Husnik et al. (2011) used 50 taxa of *Enterobacteriaceae* and 69 orthologous genes present in all taxa to determine the relationships of the endosymbionts. *Sodalis* and *Wigglesworthia* are grouped together in the same clade and shared the same origin as a sister clade to the *Edwardsiella* or *Dickeya/Pectobacterium* clades. *Arsenophonus* has an independent origin as a sister clade to *Proteus*, while *Buchnera* clusters as a sister clade to the *Erwinia/Pantoea* clade. The fourth independent clade contains only *Candidatus* endosymbionts.

Genomic Features

Enterobacteriaceae has been heavily sequenced from across the spectrum of the family diversity with 180 complete genomes covering 47 species and 21 genera, although many genomes are from the same species with vast majority of the genomes from *Escherichia* (in particular *E. coli*), *Salmonella*, and *Yersinia* (in particular *Y. pestis*). The 21 genera covered are *Buchnera, Citrobacter, Cronobacter, Dickeya, Edwardsiella, Enterobacter, Erwinia, Escherichia, Klebsiella, Pantoea, Pectobacterium, Photorhabdus, Proteus, Providencia, Rahnella, Salmonella, Serratia, Shigella, Sodalis, Wigglesworthia, Xenorhabdus*, and *Yersinia*.

The genome of *Enterobacteriaceae* comprises a single chromosome and the genome size varies enormously. The smallest genome is a *Buchnera aphidicola* genome of only 422,434 bp, coding for just 362 ORFs, whereas the largest belongs to *Klebsiella oxytoca* strain E718 of 6,450,897 bp, coding for 5,909 ORFs. The endosymbionts have smaller genomes as expected. The average genome size of *Buchnera aphidicola* based on 11 genomes is 603,090 bp with the largest size of 641,895 bp. The *Wigglesworthia glossinidia* genome averaged 711,269 bp, based on two strains sequenced. However, the *Sodalis glossinidius* genome is large at 4,292,502 bp but only encodes 2,516 genes. Considerable genome degradation occurred in *S. glossinidius* with over 900 pseudogenes. The non-endosymbiotic species within *Enterobacteriaceae* has a genome size over 3 Mb with an average of 4,938,615 bp and the smallest genome being that of *Edwardsiella tarda* strain FL6-60 of 3,728,801 bp encoding 3,256 CDS. Genome size variation within a species is just as large as between species. The 52 complete genomes of *E. coli* averaged 5,138,319 bp ranging from 4,557,041 to 5,855,531 bp. The G+C content of the endosymbionts ranges from 20% to 29%, differing dramatically from those of the non-endosymbionts which range from 38.9% to 59.7%. *S. glossinidius* is again an exception with a G+C content of 54.5%, typical of the non-endosymbiotic *Enterobacteriaceae*. Of the 180 complete genomes, 58% of the strains sequenced carry one to six plasmids.

Phenotypic Features of the Family

There are only a few characteristics of the family that differentiate it from other closely related families such as *Vibrionaceae*: glucose is fermented, cytochrome oxidase is negative, and nitrate is reduced to nitrite. However, there are also exceptions. Transferring of *Plesiomonas* into this family adds an exception to the common phenotype of oxidase negative as *Plesiomonas* is oxidase positive. Other exceptions include that certain genus (*Arsenophonus, Biostraticola, Brenneria, Lonsdalea, Photorhabdus, Saccharobacter, Samsonia, Sodalis*, and *Xenorhabdus*) or species of *Pantoea, Serratia, Trabulsiella*, and *Yersinia* cannot reduce nitrate. The endosymbionts lack many of the family features due to the substantial genome reduction (Akman et al. 2002; Burke and Moran 2011; Shigenobu et al. 2000; Toh et al. 2006). Phenotypic features of individual genus will be discussed separately in respective genus sections below. Note that common properties shared by most family members are not restated in the genus description, including being Gram-negative rod, facultative anaerobe, non-spore forming, catalase positive, oxidase negative, and capable of nitrate reduction, unless the property is an exception for the genus.

Isolation

Most clinically relevant or environmental members of *Enterobacteriaceae* can grow readily in blood or chocolate agar at 35–37°C without specific atmospheric requirement. Some require lower temperature for growth. Isolation from environments with mixed bacteria may require enrichment in selective media. For isolation of *Enterobacteriaceae* from food products for human consumption and the feeding of animals, as well as environmental samples in the area of food production and food handling, *Enterobacteriaceae* enrichment (EE) broth is a medium recommended by the ISO 21528-1:2004 standard for the detection and enumeration of *Enterobacteriaceae* by the most probable number method (Joosten et al. 2008; Weber et al. 2009). The EE broth contains pancreatic digest of gelatin and glucose as the nitrogen and energy sources and bile salts and Brilliant green as selective agents, which inhibit Gram-positive bacteria and most Gram-negative bacteria. However, some *Cronobacter* strains may not grow in EE broth (Joosten et al. 2008; Weber et al. 2009). For isolation of *Enterobacteriaceae* from clinical samples, three types of media may be used, nonselective media such as blood agar, selective/differential media such as MacConkey agar and Eosin methylene blue agar, or enrichment broths such as selenite broth and Gram-negative broth. The latter is particularly useful for recovery of *Salmonella* and *Shigella* from stool samples. *Klebsiella granulomatis* is an exception which requires specialized media for culture. It was cultured in

embryonated chicken eggs in 1943 (Anderson 1943), cell-free medium in 1962 (Goldberg 1962), and now in monocyte coculture system (Kharsany et al. 1997). Plant *Enterobacteriaceae* pathogens can readily grow on media for other *Enterobacteriaceae* and selective media are also available for some species (Goszczynska et al. 2006; Lee and Yu 2006). The reader is referred to Winn et al. (2006) for medically important organisms and Schaad et al. (2001) for plant pathogens. *Photorhabdus* spp., *Xenorhabdus* spp., and *Serratia symbiotica*, although forming a symbiotic relationship with its respective host, can be grown in vitro without much difficulty. However, endosymbionts generally is difficult or not possible to grow in vitro. *Sodalis glossinidius* can grow in vitro but requires special medium. *S. glossinidius* either can be cultured using C6/36 mosquito (*Aedes albopictus*) feeder cell culture or can be grown axenically on Mitsuhashi–Maramorosch (MM) agar supplemented with heat-inactivated fetal calf serum, fresh horse blood, or catalase (Dale and Maudlin 1999). Optimal growth can be achieved by growing under microaerophilic conditions (<10% oxygen) at 25 °C. *Wigglesworthia glossinidia* cannot exist as free living but remains metabolically active when maintained under transient tissue culture conditions for 72 h (Aksoy 1995). Similarly, *Buchnera* cannot grow outside its aphid host (Perez-Brocal et al. 2006; Shigenobu et al. 2000; Tamas et al. 2002; van Ham et al. 2003). Molecular analysis of *Buchnera* can be achieved by preparing DNA using acetone preservation (Fukatsu 1999).

Habitats

Enterobacteriaceae is ubiquitous in nature. Many species can exist as free living in diverse ecological niches, both terrestrial and aquatic environments, and some are often associated with animal, plants, or insects. For convenience, the organisms are broadly categorized into three types: (1) those that can cause human infections or primarily associated with human/animals and the environment, (2) those that are associated with plants or plant pathogens and the environment, and (3) those that are insects associated or endosymbionts. The demarcation of plant and human pathogens is not clear-cut as quite few genera can cause infections in both. Some species of plant-associated pathogens (e.g., *Pantoea agglomerans*) and insect pathogens (e.g., *Photorhabdus luminescens*) can cause infections in humans as an accidental host. Some human pathogens such as *Salmonella* and *E. coli* O157:H7 can also colonize or invade plants, and outbreaks have been associated with consumption of raw plant produce which may be a result of bacteria inside the plant tissue rather than at the surface (Tyler and Triplett 2008). There are 29 genera in category 1 (*Budvicia, Buttiauxella, Cedecea, Citrobacter, Cronobacter, Edwardsiella, Enterobacter, Escherichia, Ewingella, Hafnia, Klebsiella, Kluyvera, Leclercia, Leminorella, Moellerella, Morganella, Plesiomonas, Pragia, Proteus, Providencia, Rahnella, Raoultella, Salmonella, Serratia, Shigella, Tatumella, Trabulsiella, Yersinia*, and *Yokenella*), nine genera in category 2 (*Brenneria, Dickeya, Erwinia, Lonsdalea, Mangrovibacter, Pantoea, Pectobacterium, Saccharobacter*, and *Samsonia*), and seven genera in category 3 (*Arsenophonus, Buchnera, Photorhabdus, Sodalis, Thorsellia, Wigglesworthia*, and *Xenorhabdus*). *Biostraticola* seems to be an environment only inhabitant. The habitats of *Cosenzaea, Gibbsiella, Obesumbacterium*, and *Shimwellia* are unclear since there have been few isolations. Habitats of individual genus are discussed further in the sections below.

Antibiotic Resistance of Medically Important Genera

Members of *Enterobacteriaceae* are becoming increasingly resistant to currently available antibiotics (Marsik and Nambiar 2011; Paterson 2006; Tzouvelekis et al. 2012). In particular, resistance to β-lactams and fluoroquinolones is widespread among the *Enterobacteriaceae* and has emerged as major challenges as these antibiotics are important drug classes used to treat infections caused by *Enterobacteriaceae*. The newer β-lactamases encountered in *Enterobacteriaceae* include AmpC β-lactamases, extended-spectrum β-lactamases (ESBLs) (e.g., cefotaxime first isolated at Munich [CTX-M] types), and carbapenemases (e.g., *Klebsiella pneumoniae* carbapenemase [KPC] types and the metallo-β-lactamases [MBLs] and the oxacillinase types [OXA]) (Marsik and Nambiar 2011; Paterson 2006; Tzouvelekis et al. 2012).

AmpC β-lactamases, conferring resistant to most cephalosporins and monobactams, are normally chromosomally encoded and constitutively expressed in *E. coli* and inducible in *Enterobacter, C. freundii, Morganella, Providencia*, and *Serratia*. Plasmid-encoded AmpC has been found in *E. coli* and *Klebsiella* (Denton 2007).

ESBLs confer resistance to penicillins, cephalosporins, and monobactams. The common ESBLs are of TEM, SHV, and CTX-M types and are widely distributed among *Enterobacteriaceae*. They are most often present in *E. coli* and *K. pneumonia* and also in *P. mirabilis* and *Enterobacter aerogenes*. CTX-M family ESBLs are closely related to chromosomal β-lactamases found naturally in another member of *Enterobacteriaceae*, *Kluyvera* spp., with CTX-M-2 sharing 99% homology with that of *Kluyvera ascorbata* (Pitout 2008). CTX-M-producing organisms show co-resistance to trimethoprim–sulfamethoxazole, tetracycline, gentamicin, and ciprofloxacin. CTX-M, in particular CTX-M-15, is distributed globally.

KPC β-lactamases are widespread in *Enterobacteriaceae* and has been found in *Klebsiella, E. coli, Serratia, Enterobacter* spp., *Citrobacter freundii, Salmonella*, and *Raoultella*. They confer resistance to the penicillins, carbapenems, cephalosporins, and monobactams but are inhibited by commercialized β-lactamase inhibitors, such as clavulanic acid and tazobactam (Pitout 2008). MBLs include imipenem active IMP, verona integron-encoded MBL (VIM), and NDM-1 types (Tzouvelekis et al. 2012). MBLs of VIM and IMP types have been found in *Klebsiella, E. coli, S flexneri, S. marcescens, Morganella morganii, Proteus mirabilis, Proteus vulgaris, Providencia rettgeri*, and

Citrobacter. The most recent addition to the MBLs is NDM-1 which was first described in 2009 (Yong et al. 2009) and is now found in nearly all clinical species of *Enterobacteriaceae* and in many parts of the world (Walsh and Toleman 2011). The $bla_{\text{NDM-1}}$ can be carried on the chromosome and also on numerous different plasmids. Some $bla_{\text{NDM-1}}$ plasmids contain up to 14 other antibiotic resistance genes, which means that spread of NDM-1 also confers the recipient bacterium of multidrug resistance (Walsh and Toleman 2011). The OXA-type carbapenemases are less frequent in *Enterobacteriaceae* (Tzouvelekis et al. 2012). OXA-48 (first emerged in *K. pneumoniae*) has been found in *E. coli* and *C. freundii* (Tzouvelekis et al. 2012).

Quinolone resistance in *Enterobacteriaceae* is usually due to chromosomal mutations in DNA gyrase and topoisomerase IV. However, plasmid-mediated quinolone resistance has been found widely present including *E. coli, Klebsiella, Enterobacter, C freundii*, and *Providencia stuartii* and are associated with the presence of *qnr* genes, *aac*(6′)-Ib-cr encoding a variant aminoglycoside acetyltransferase, or *qepA* which encodes an efflux pump (Denton 2007; Strahilevitz et al. 2009). Quinolone resistance genes are often co-carried with ESBL genes (Strahilevitz et al. 2009).

Pathogenicity

Enterobacteriaceae is generally considered an enteric bacterium living in the gut of animals and does not cause disease. However, *Enterobacteriaceae* causes a range of diseases in humans/other animals and plants, with some genera/species causing disease in both as discussed above. Many of these pathogenic forms are known to have arisen multiple times through acquisition of virulence factors which are encoded by pathogenicity islands, plasmids and prophages, and are mobile. The best known and best studied is *E. coli* with multiple pathogenic types including enteropathogenic, enterohemorrhagic, enteroinvasive, enteroaggregative, and extraintestinal *E. coli* (Croxen and Finlay 2010). Each pathogenic type has been shown to have multiple independent lineages (Wirth et al. 2006).

Numerous virulence factors have been identified in *Enterobacteriaceae* (Chen et al. 2012; Croxen and Finlay 2010; Schmidt and Hensel 2004; Toth et al. 2006). A common theme of pathogenicity of the *Enterobacteriaceae* is that many virulence factors are encoded on pathogenicity islands which can be transferred intra- and inter-*Enterobacteriaceae* and similar virulence mechanisms are used across species and genera, and across human/animal and plant pathogens (Hacker and Kaper 2000; Schmidt and Hensel 2004). The common virulence factors include the flagella, LPS, ECA, fimbriae/adhesins, iron acquisition, and protein secretion systems. The LPS, ECA, and flagella are most likely to be present in the ancestral *Enterobacteriaceae* since they are widespread in the family. These are also present in nonpathogenic forms and are thus not virulence factors *per se*. However, they are known to be involved in virulence in both animal and plant pathogens (Schmidt and Hensel 2004).

Fimbriae/adhesins mediate adherence to epithelial cells and extracellular matrix proteins (Soto and Hultgren 1999). A wide array of fimbriae can found in *Enterobacteriaceae*. The chaperone/usher assembly class of fimbriae is the most diverse and is divided into six major phylogenetic clades and nine subclades (Nuccio et al. 2007). The fimbriae from *Enterobacteriaceae* are distributed among the six clades and nine subclades with γ1 and κ fimbriae being all from *Enterobacteriaceae*. A recent study of *Salmonella* fimbrial gene cluster by analyzing 90 *Salmonella* genomes found that there are 11.8 fimbrial gene clusters per genome encompassing all but one of chaperone/usher fimbrial clades and subclades (Yue et al. 2012). Fimbriae may be associated with tissue tropism due to receptor specificity, for example, binding to uroepithelial cells (Wright and Hultgren 2006), or host specificity (Kisiela et al. 2012; Yue et al. 2012). Inter-genera transfer of fimbrial operons also occurs. The *mrk* fimbrial operon sequences of 17 *K. pneumoniae* and nine *E. coli* strains were found to cluster together in two clades with one containing 16 *K. pneumoniae* and seven *E. coli* strains while the other consisting of one *K. pneumoniae* and two *E. coli* strains (Ong et al. 2010).

Iron is essential for virulence (Perry 1993; Ratledge and Dover 2000). There are multiple iron acquisition systems or strategies used by *Enterobacteriaceae*. The most common siderophores in *Enterobacteriaceae* are enterobactin and aerobactin which are found in both animal and plant pathogens (Toth et al. 2006). Possession of an iron acquisition system can enhance virulence. The high-pathogenicity island (HPI), first discovered in *Yersinia*, encodes genes for the synthesis of the siderophore yersiniabactin and is widely distributed in *Enterobacteriaceae* such as *Escherichia coli, Klebsiella, Citrobacter, Salmonella*, and *Enterobacter hormaechei* (Carniel 2001; Paauw et al. 2010). HPI has been associated with higher virulence (Carniel 2001).

There are eight known secretion systems (Desvaux et al. 2009) with all except T7SS being present in *Enterobacteriaceae*. Both T1SS and T2SS are widely present in *Enterobacteriaceae* as conserved secretion systems in Gram-negative bacteria (Douzi et al. 2012; Schmidt and Hensel 2004; Toth et al. 2006). They are involved in secreting many enzymes and toxins (Toth et al. 2006), some of which are directly responsible for disease symptoms.

The T3SS plays a very important role in interaction with the hosts by translocating various effector proteins into the host cells which functions in colonization, invasion, cytotoxicity, and subversion of the host defense system (Coburn et al. 2007). There may be multiple T3SSs in the genome delivering unique sets of different effectors. *Salmonella* carries two T3SSs with one facilitating the entry into host cells, while the other playing an important role in its survival inside the host cell (Malik-Kale et al. 2011). Studies show that the T3SSs in *Enterobacteriaceae* were gained from outside the family by lateral gene transfer. The T3SS of plant pathogens is closer to those of plant pathogens outside of the family than to those of animal pathogens within the family (Naum et al. 2009). The T3SS has been horizontally

transferred across species within the family as well. The best example is the locus of enterocyte effacement (LEE) PAI, which was first discovered in enteropathogenic and enterohemorrhagic *E. coli* encoding a T3SS and its effectors (McDaniel et al. 1995). The LEE has not only been transferred within *E. coli* multiple times (Jores et al. 2004), but also across species and genera. It has been found in *Citrobacter rodentium* which is widely used as a model to study enteropathogenicity (Deng et al. 2001) and has also been found in *Escherichia albertii* (Hyma et al. 2005) and *Salmonella enterica* serovar Sofia (Chandry et al. 2012). The T3SS from plant pathogens has also been inferred to have undergone transfer within the family (Naum et al. 2009).

The T4SS is involved in conjugation and DNA transfer. T4SS is usually present on plasmids but is found as a cluster on the chromosome in a *Pectobacterium carotovorum* strain (Toth et al. 2006). The T5SSs are the autotransporters which perform a wide variety of functions including adhesion, autoaggregation, invasion, biofilm formation, and cytotoxicity, and many of which may be present in the same genome (Henderson et al. 2004). The *E. coli* genomes were found to encode up to 12 autotransporters in an analysis of 28 genomes (Wells et al. 2010). The T6SS is used to target either eukaryotic cells or competitor bacteria. It has been found in *Citrobacter* (Bai et al. 2012), *Cronobacter* (Joseph et al. 2012), *E. coli* (Zhou et al. 2012), *Edwardsiella* (Yang et al. 2012), *Erwinia* (De Maayer et al. 2011), *Pantoea* (De Maayer et al. 2011), *Salmonella* (Blondel et al. 2010), and *Serratia* (English et al. 2012) and is probably quite widely present in *Enterobacteriaceae*. The T6SS found in a *Citrobacter freundii* strain was more closely related to that of a *Salmonella* strain than that of *C. rodentium* (Bai et al. 2012). The extracellular nucleation–precipitation pathway involved in secretion of curli in *E. coli* and *Salmonella* (Stathopoulos et al. 2000) was proposed as T8SS (Desvaux et al. 2009) but much less is known.

Applications

There are numerous applications using members of *Enterobacteriaceae*. Applications for biocontrol of insects in agriculture and production of numerous recombinant proteins and non-protein products are already on the market or have been in use for decades (Goldberg 1962; Shigenobu et al. 2000). Many new applications hold great promise such as biofuel production, biosurfactant production, biowaste recycling, and bioremediation of contamination by insecticides, explosives, radioactive materials, and heavy metals.

E. coli has been a commonly used host for recombinant protein production (Tamas et al. 2002). Metabolic engineering of *E. coli* has expanded its capacity to produce novel products. For example, metabolic engineering of biofuel-synthesis pathways in *E. coli* may make it practical to use *E. coli* for biofuel production (van Ham et al. 2003).

Salmonella can be attenuated to deliver antigens against multiple bacterial infections (Anderson 1943; Kharsany et al. 1997). It has been experimentally tested as a multivalent vaccine against *E. coli, Helicobacter pylori, Shigella dysenteriae, Y. pestis*, and *Mycobacterium tuberculosis* (Anderson 1943). *S. enterica* serovar Typhimurium (STM) has been extensively exploited for their potential as anticancer agent (Fukatsu 1999; Perez-Brocal et al. 2006). STM can target tumor tissues with a high specificity and has broad solid tissue targets including melanoma, lung, colon, breast, renal, hepatic, and prostate tumors. STM is attracted by compounds released by tumor cells to the tumor tissue, establishes infection and multiplies in the tumor tissue. It selectively grows in tumor tissues at approximately 1,000-fold over normal tissues. It exerts multiple effects on therapy. STM can be engineered relatively easily to deliver therapeutic compounds. STM infection activates antitumor host cell proteins and attracts immune cells to the tumors that help tumor regression.

A recent study exploited the symbiotic relationship of *Pantoea agglomerans* with mosquitoes as a novel means to fight malaria infection. *P. agglomerans* lives in the midgut of the mosquitoes where *Plasmodium* development also occurs. *P. agglomerans* was engineered to deliver two anti-*Plasmodium* effector proteins, scorpine, a potent antiplasmodial peptide, and (EPIP)4, four copies of *Plasmodium* enolase–plasminogen interaction peptide that prevents plasminogen binding to the ookinete surface. It was shown that these engineered *P. agglomerans* inhibited oocyst formation of the malaria parasite *Plasmodium falciparum* by up to 98% and also led to a reduction of 80–84 % in the proportion of infected mosquitoes (Roggenkamp 2007).

Two genera, *Photorhabdus* and *Xenorhabdus*, carried by nematodes Heterorhabditids and Steinernematids in their alimentary canals, respectively, have been employed in a number of ways for agricultural use (Akman et al. 2002; Shigenobu et al. 2000; Toh et al. 2006). These nematodes are entomopathogenic to other nematodes and a range of insect pests and are used as biocontrol agent (Forst et al. 1997). The biocontrol market is worth hundreds of millions of dollars (Shigenobu et al. 2000). The entomopathogenic effect is actually exerted through the symbiotic bacteria which infect and kill the insects with bacterial toxins (Forst et al. 1997). *Photorhabdus* and *Xenorhabdus* produce an array of toxins (ffrench-Constant et al. 2007; Forst et al. 1997). These toxins as extracts or cell suspensions can be applied to foliage for insect control (Munson et al. 1991). Additionally, the genes encoding the toxins can also be used to create transgenic plants for crop protection (ffrench-Constant and Bowen 2000).

Serratia has great potential for bioremediation. A diverse range of *Serratia* strains has been isolated that can degrade a range of compounds including diazinon, nicosulfuron, tributyl phosphate, dibenzofuran, hexachlorobutadiene, polycyclic aromatic hydrocarbons, methyl parathion, diesel, beta-cypermethrin (Abo-Amer 2011; Castellani and Chalmers 1919; Hauser 1885; Hickman-Brenner et al. 1985; Pandey et al. 2012; Toth et al. 2012; Waldee 1945; Winslow et al. 1920; Yaping et al. 1990; Yarza et al. 2010; Zhang et al. 2010, 2012); reduce molybdenum (Rahman et al. 2009); and absorb radioactive materials

uranium and radium (Zakeri et al. 2010) and heavy metals, zinc, chromium, cadmium, and lead (Cristani et al. 2012; Mondaca et al. 2002; Srivastava and Thakur 2012). *Serratia* can also utilize biowastes such as lignin and shellfish wastes (Hickman-Brenner et al. 1985; Wang et al. 2010).

Description of Individual Genus

Arsenophonus Gherna et al. 1991

The genus *Arsenophonus* (Ar.se. no. pho.' nus. Gr. n. *amen*, a male; Gr. suffix *phonus*, slayer; N. L. masc. n. *Arsenophonus*, male killer) was formally assigned in 1991 (Gherna et al. 1991) and *A. nasoniae* is the only species within this genus. It is a nonmotile, long rod (0.40–0.57 μm wide by 6.9–10.0 μm long) which is occasionally filamentous in young cultures. Colonies appear mucoid, gray-white, round, and convex with entire edges. Growth can be achieved in temperature between 15 °C and 35 °C (optimum 30 °C) and pH 6.2–8.7 (optimum pH 7.4–8.0). It is unable to utilize ammonium sulfate, potassium nitrate, complete defined amino acid mixtures, or acid-hydrolyzed peptones as nitrogen sources, but instead can use enzymatically digested proteins. It can use glucose, fructose, and sucrose as carbon sources, but weak growth is observed with cellobiose, maltose, trehalose, and D-xylose. Acid is produced from D-glucose, fructose, and sucrose. It is unable to utilize adonitol, L-arabinose, dulcitol, glycerol, *i*-inositol, lactose, D-mannitol, and raffinose. It is positive for gelatin hydrolysis and catalase but negative for nitrate reduction, Voges–Proskauer, methyl red, indole, H_2S production, oxidase, *o*-nitrophenyl-β-D-galactopyranoside, arginine dihydrolase, lysine, and ornithine decarboxylases and urease.

Arsenophonus is widely distributed in arthropods of medical, veterinary, and agricultural importance. *A. nasoniae* infects *Nasonia vitripennis*, a parasitic wasp that attacks filth flies and kills male embryos produced by an infected female. *Arsenophonus* can also be a plant pathogen (Bressan et al. 2012). It has been obtained as pure culture from the hippoboscid louse fly *Pseudolynchia canariensis* (Dale et al. 2006). *A. nasoniae* was isolated from parasitoids (Taylor et al. 2011) and ticks (Mediannikov et al. 2012) and, thus, is not restricted to *Nasonia* (Taylor et al. 2011).

Biostraticola Verbarg et al. 2008

The genus *Biostraticola* (Bi.o.stra.ti' co.la; Gr. N *bios* life, L. Neut. N. *stratum* layer, L. Masc. Suffix n. *–cola* [derived from *incola*] inhabitant, N.L. masc. N. *Biostraticola* inhabitant of a biofilm) is a nonmotile rod (1–1.3 × 3–4 μm) (Verbarg et al. 2008). It is unable to reduce nitrate. A wide range of carbohydrates can be fermented, but no gas is produced from glucose. Major fatty acid methyl esters are $C_{16:0}$ and $C_{16:1\omega7}$. Colonies on Reasoner's 2A agar (R2A) appear whitish with up to 3.5 mm in diameter upon growth at 25 °C for 24 h. Growth occurs between 5 °C and 30 °C (optimum 25–30 °C), pH 5.0–9.2 (optimum 8.2–9.2), and NaCl up to 3 %. It is positive for aminopeptidase and β-galactosidase and can utilize malonate and hydrolyze starch, but is negative for methyl red, Voges–Proskauer, indole, and growth on Simmons citrate, and unable to hydrolyze Tween 80, esculin and casein; does not have gelatinase, lecithinase, or urease; and cannot degrade tyrosine or utilize acetate.

Biostraticola tofi is the only species in the genus of *Biostraticola*. The type strain BF36T was first isolated from biofilm of a tufa deposit from the hard water creek Westerhöfer Bach, near Westerhof, Lower Saxony, Germany (Verbarg et al. 2008).

Brenneria Hauben et al. 1998, Brady et al. 2012 emend

Brenneria is a genus named in honor of the American bacteriologist Don J. Brenner which was proposed by Hauben et al. (1998). *Brenneria* is motile by means of peritrichous flagella. The bacterium is 1.3–3.0 × 0.5–1.0 μm with rounded ends, occurring singly or rarely in pairs. Growth is optimum at 27–30 °C (maximum 40 °C). *Brenneria* is negative for arginine dihydrolase, ornithine and lysine decarboxylases, amylases, citrate utilization, nitrate reduction, H_2S production, urease, indole, and gelatinase. Acid is produced from D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, D-mannitol, *N*-acetylglucosamine, and sucrose, but not from adonitol or dulcitol. Major fatty acids are $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, and $C_{17:0}$.

Currently, there are five *Brenneria* species: *B. alni*, *B. goodwinii*, *B. nigrifluens*, *B. rubrifaciens*, and *B. salicis*. Two species have moved out of this genus. *Brenneria quercina* and *B. paradisiaca* were transferred to *Lonsdalea* and *Dickeya*, respectively. *B. alni* causes bark canker of alder (*Alnus*) (Surico et al. 1996). *B. goodwinii* have been isolated from oak exhibiting symptoms of Acute Oak Decline (Denman et al. 2012). *B. nigrifluens* strains cause bark canker on Persian walnut (*Juglans regia*) (Wilson et al. 1957). *B. rubrifaciens* causes necrosis on Persian walnut (*Juglans regia*) (Wilson et al. 1967). *B. salicis* resides as an endophyte in wood and causes watermark disease on willow (*Salix* spp.) (Maes et al. 2009). The biochemical characteristics that can distinguish these species are summarized in *Table 13.2*.

Buchnera Munson et al. 1991

Buchnera (Buch.ne'ra. M.L. fem. n. *Buchnera*, named in honor of Paul Buchner, a German biologist who made extensive contributions to the study of endosymbiosis) is round or slightly oval shaped (2–5 μm in diameter) and lacks flagella. It is an obligate intracellular symbiont of the greenbug aphid, *Schizaphis graminum*, and cannot be cultivated outside the host. Under Rule 18a of the *International Code of Nomenclature of Bacteria*, when a species cannot be maintained in culture,

Table 13.2
Phenotypic characters that differentiate *Brenneria* species

Acid from	*B. alni* (n = 5)	*B. goodwinii* (n = 9)	*B. nigrifluens* (n = 5)	*B. rubrifaciens* (n = 5)	*B. salicis* (n = 3)
L-Arabinose	+	+	+	+	–
Amygdalin	+	+	–	–	–
D-Galactose	+	+	–	–	–
Gentiobiose	–	+	+	–	–
Inositol	–	+	+	–	–
Melibiose	–	+	+	–	–
Potassium gluconate	–	d	–	–	+
D-Raffinose	–	+	+	–	+
D-Sorbitol	–	+	+	–	–
D-Trehalose	+	+	+	–	–
D-Turanose	+	+	–	–	–
D-Xylose	+	d	+	–	–

Adopted from Denman et al. (2012)
+: 90–100 % strains positive in 1–2 days; –: negative; *d*: 11–89 % strains positive in 1–4 days

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). *B. aphidicola* is the sole species of the genus and is found in the mycetocytes of *S. graminum*. The symbiotic association of *Buchnera* with aphids was probably developed 150–200 million years ago (Buades et al. 1999).

Budvicia Bouvet et al. 1985, Lang et al. 2012 emend

Budvicia (Bud. vi' ci. A. L. Fem. N. *Budvicia* derived from Budvicium, the Latin name of the city České Budějovice) is a nonencapsulated, straight rod (0.8 × 2.5–3.0 μm), and motile with peritrichous flagella (when grown at 22 °C) (Bouvet et al. 1985; Lang et al. 2012). Small colonies are observed on nutrient agar. It grows between 4 °C and 36 °C (may or may not grow at 37 °C), does not grow in KCN medium, may or may not produce H_2S, may or may not hydrolyze urea and *o*-Nitrophenyl-β-D-galactopyranoside, and is unable to grow in Simmons citrate or Trabulsi acetate. It does not produce indole, nor deaminate tryptophan and phenylalanine, nor decarboxylate lysine, arginine, and ornithine, nor hydrolyze esculin. Voges–Proskauer test is negative and acid is not produced from maltose, D-mannose, glycerol, and trehalose. It does not produce extra cellular enzymes (lipase, Tween esterase, amylase, and deoxyribonuclease). The whole cell fatty acid profile is dominated by $C_{16:0}$, $C_{16:1\omega\,7c}$, cyclo-$C_{17:0}$, $C_{18:1\omega7c}$, and $C_{14:0}$ (Lang et al. 2012).

There are two species in the genus of *Budvicia*: *B. aquatica* (Bouvet et al. 1985) and *B. diplopodorum* (Lang et al. 2012). *B. aquatica* prefers low temperature (motility more frequent at 22 °C and no growth at 42 °C) and can grow in 0–4 % NaCl (slow growth at 6 % NaCl). Typically, colonies are small in size (0.1 < 1 mm on agar at 37 °C in 24 h) and appear translucent with smooth and entire edges. *B. diplopodorum* forms cream, colored, shiny, translucent, convex colonies on nutrient agar or R2A with 0.5 mm diameter in size after two days at 28 °C and becomes slimy after prolonged incubation. The main differences between the two species include absence of urease, gas from glucose, and growth in the presence of 2 % NaCl in *B. diplopodorum*. *B. diplopodorum* is also unable to produce acid from D-arabinose, D-arabitol, 2-ketogluconate, 4-ketogluconate, D-xylose, and D-lactose. In contrast, *B. aquatica* cannot produce acid from dulcitol and D-tagatose (Lang et al. 2012). *B. aquatica* was first detected in inflow surface water from a wastewater plant in České Budějovice, Czechoslovakia (Schubert and Groeger-Sohn 1998). *B. aquatica* was isolated from blood and urine samples from an immunocompromised 85-year-old female patient following exposure to the aftermath of Hurricane Katrina (Corbin et al. 2007). *B. diplopodorum* was first isolated from the gut of millipede, *Cylindroiulus fulviceps* (Diplopoda) (Lang et al. 2012).

Buttiauxella Ferragut et al. 1981

Buttiauxella (N.L. fem. dim. n. *Buttiauxella*, named in honor of the French microbiologist René Buttiaux) was first described by Ferragut et al. (1981). There are seven species in this genus: *B. agrestis, B. brennerae, B. izardii, B. warmboldiae, B. noackiae, B. ferragutiae*, and *B. gaviniae*. The last three were previously recognized as enteric groups 59, 63, and 64, respectively. The colonies are 1–2 mm in diameter after 1 day and 2–3 mm after 2 days of incubation. They grow well at 30–36 °C and poor to

Table 13.3
Phenotypic characters that differentiate *Buttiauxella* species

Characteristic	*B. agrestis*	*B. brennerae*	*B. ferragutiae*	*B. gaviniae*	*B. izardii*	*B. noackiae*	*B. warmboldiae*
N-Acetyl-L-glutamine	v	+	+	v	+	v	+
Arabinose	v	−	−	v	+	v	+
Arginine dihydrolase	−	v	−	+	−	+	+
Citrate	v	v	−	v	+	+	−
Fucose	v	−	−	v	v	v	+
Glycerol	v	−	−	−	−	−	−
myo-Inositol	−	−	−	−	−	−	+
KCN	v	+	+	v	+	+	−
5-Ketogluconate	v	+	−	v	v	v	−
Lactose	+	+	−	v	+	−	−
Lysine decarboxylase	−	−	+	−	−	−	−
Malonate	v	+	−	+	+	v	+
Maltitol	v	+	+	v	v	v	−
Melibiose	v	v	v	v	v	−	−
Ornithine decarboxylase	+	v	+	−	+	v	−
Palatinose	v	+	+	+	v	+	−
Raffinose	v	v	v	−	−	−	−
D-Sorbitol	v	−	+	v	−	−	−

Adapted from De Baere et al. (2002), Ferragut et al. (1981), and Muller et al. (1996)
v variable, + positive, − negative

moderate at 42 °C except *B. brennerae* which is unable to grow at 42 °C. Biochemical characteristics that are useful to differentiate *Buttiauxella* species are listed in Table 13.3. *Buttiauxella* has been isolated from slugs, snails, earthworms, drinking water, and soil (Janda 2006; Muller et al. 1996) and foods including raw salmon, beef meat, and milk (Charrier et al. 2006; Ercolini et al. 2009; He et al. 2009; Mace et al. 2012). *B. gaviniae* was isolated from a urine sample of a patient suffering from urine bladder pathology and neurological problems (De Baere et al. 2002).

Cedecea Grimont et al. 1981

Cedecea (N.L. fem. n. *Cedecea*) was originally named by Grimont et al. (1981) encompassing 17 clinical strains initially named CDC enteric group 15. *Cedecea* is motile and nonencapsulated. It is positive for Voges–Proskauer, *o*-nitrophenyl-*β*-D-galactopyranoside, lipase, and esculin hydrolysis and negative for lysine decarboxylase, indole, H_2S, urease, phenylalanine deaminase, gelatinase, deoxyribonuclease, chitinase, amylase, and polygalacturonase. It produces gas from glucose and acid from D-arabitol, D-cellobiose, maltose, D-mannitol, mannose, salicin, and trehalose but not from adonitol, L-arabinose, dulcitol, or L-rhamnose (Grimont et al. 1981). There are three species in the genus, *C. davisae*, *C. neterii*, and *C. lapagei*, which can be differentiated using sorbitol, sucrose, or xylose fermentation. *C. lapagei* and *C. neterii* are negative and positive for all three, respectively, whereas *C. davisae* is negative for sorbitol but positive for sucrose and xylose.

Cedecea has been isolated from a range of sites from human infections. *Cedecea* was most commonly recovered from blood and sputum samples. Akinosoglou et al. (2012) reviewed reports of *Cedecea* infections from 1980 onwards and found 16 cases. The majority of the cases (9/16) were due to *C. davisae*. Many of the patients were immunocompromised or had multiple comorbidities. It has also been isolated from fruit flies and vegetables (Janda 2006).

Citrobacter Werkman and Gillen 1932

The genus *Citrobacter* was first designated in 1932 by Werkman and Gillen (1932) and is motile by peritrichous flagella. It is negative for Voges–Proskauer, phenylalanine deaminase, and lysine decarboxylase but positive for indole and methyl red tests and can utilize citrate as a sole carbon source. There are 11 species: *C. freundii*, *C. koseri* (synonym *C. diversus*), *C. amalonaticus*, *C. farmeri*, *C. youngae*, *C. braakii*, *C. werkmanii*, *C. sedlakii*, *C. rodentium*, *C. gillenii*, and *C. murliniae*. The biochemical tests that can differentiate *Citrobacter* species are summarized in Table 13.4.

Table 13.4

Phenotypic characters that differentiate *Citrobacter* species

Characteristic	*C. freundii*	*C. koseri*	*C. amalonaticus*	*C. youngae*	*C. braakii*	*C. werkmanii*	*C. sedlakii*	*C. farmeri*	*C. rodentium*	*C. gillenii*	*C. murliniae*
Indole	(–)	+	+	(–)	v	–	+	+	–	–	+
Citrate (Simmons)	+	+	+	+	(+)	+	+	v	v	+	+
H_2S production	v	–	(–)	(+)	v	+	–	–	–	(+)	v
Urease	v	v	+	v	v	+	+	v	+	–	v
Arginine deaminase	+	+	+	+	+	+	+	+	–	(+)	(+)
Ornithine decarboxylase	(–)	+	+	–	(+)	–	+	+	+	–	–
Motility	(+)	+	+	+	v	+	+	+	v	(+)	+
KCN	+	–	+	+	+	+	+	+	–	+	+
Malonate	(–)	+	–	–	–	+	+	–	+	+	–
D-Glucose (gas)	(+)	+	(+)	(+)	(+)	+	+	+	+	+	+
Acid produced from											
Lactose	+	(+)	+	(+)	(+)	v	+	+	+	(+)	+
Sucrose	v	v	–	v	(–)	–	–	+	–	(–)	v
Dulcitol	v	v	–	v	v	–	+	–	–	–	+
Salicin	(–)	(+)	+	–	–	–	v	+	+	v	v
Raffinose	(+)	–	–	–	v	–	–	+	–	(–)	(–)
Cellobiose	(+)	+	+	v	+	(+)	+	+	+	(+)	+
α-CH_3-glucoside	v	(+)	(–)	–	v	–	–	+	–	–	–
Esculin	(–)	v	v	–	–	–	v	v	(+)	v	+
Melibiose	+	–	–	–	+	–	+	+	–	(+)	v
Glycerol	+	+	v	+	+	+	(+)	v	v	(+)	+
Sodium acetate	(+)	+	+	(+)	v	+	(+)	+	v	(–)	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+
ONPG	+	+	+	+	(+)	+	+	+	+	+	+

Adapted from Brenner et al. (1999)

–: 0–10 % positive; (–): 10–20 % positive; *v*: 20–80 % positive; (+): 80–90 % positive; +: 90–100 % positive

Of the 11 *Citrobacter* species, *C. rodentium* is more a "host-restricted" pathogen and has been isolated from laboratory mice and gerbils and has not been isolated from human infections. *C. rodentium* has been used as a model for studying enteropathogenicity of *E. coli* (Mundy et al. 2005). Other *Citrobacter* species are commensal inhabitants of the intestinal tract of humans and other animals. They have also been recovered from water, sewage, and soil, presumably due to contamination of the environment by fecal shedding. *Citrobacter spp.* are opportunistic pathogens of humans and have been associated with a range of infections including urinary tract infections, gastroenteritis, would infections, pneumonia, brain abscesses, septicemia, meningitis, and endocarditis, in particular in neonates and immunocompromised hosts (Borenshtein and Schauer 2006; Doran 1999). *C. koseri* (*diversus*) is best known as the cause of sepsis and meningitis leading to brain abscesses (Lin et al. 2011; Martinez-Lage et al. 2010; Vaz Marecos et al. 2012).

Cosenzaea Giammanco et al. 2011

Cosenzaea (Co.sen.za'e.a. N.L. fem.n. *Cosenzaea*, named after Benjamin J. Cosenza, the microbiologist who first described this bacterium as *Proteus myxofaciens* in 1966) was a reclassification from *Proteus myxofaciens* and *C. myxofaciens* is the only species in this genus (Giammanco et al. 2011). The cell size is 0.4–0.8 × 1.0–3.0 μm. It is motile by peritrichous flagella, grows optimally at 37 °C, forms thin uniform film when grown on solid media, and is hemolytic on blood agar. Slime can be produced at 25 °C in trypticase soy broth. It is positive for urea hydrolysis, Voges–Proskauer, Simmons citrate test, phenylalanine and tryptophan oxidative deamination, H_2S production after 3–4 days of incubation, gelatin hydrolysis at 22 °C, growth in KCN, and acid production from D-glucose, maltose, glycerol, and α-CH_2-glucoside. It is negative for indole production, lysine and ornithine decarboxylase, arginine dihydrolase, tyrosine decomposition, and malonate utilization. It was first isolated from larvae of the gypsy moth *Porthetria dispar* and described by Cosenza and Podgwaite (1966), and there are other reports of its isolations.

Cronobacter Iversen et al. 2008

Cronobacter was created as a genus from transfer of *Enterobacter sakazakii* (Iversen et al. 2007, 2008). *Cronobacter* is motile. It is positive for citrate utilization, esculin and arginine hydrolysis, and Voges–Proskauer test but negative for methyl red test, H_2S production, urea hydrolysis, lysine decarboxylation, and β-D-glucuronidase. Growth occurs at 6–45 °C, pH 5–10, and NaCl concentration of up to 7 % (w/v). It produces acid from D-glucose, D-sucrose, D-raffinose, D-melibiose, D-cellobiose, D-mannitol, D-mannose, L-rhamnose, L-arabinose, D-xylose, D-trehalose, galacturonate, and D-maltose. There are seven species (*C. condimenti, C. dublinensis, C. malonaticus, C. muytjensii, C. sakazakii, C. turicensis*, and *C. universalis*) and *C. dublinensis* has three subspecies (subsp. *dublinensis*, subsp. *lausannensis*, and subsp. *lactaridi*). The phenotypic characters differentiating *Cronobacter* species and *C. dublinensis* subspecies are summarized in ❯ *Table 13.5*. *C. condimenti* is the least biochemically active.

Cronobacter is an environmental organism and has been isolated from a variety of foods (Grimont and Grimont 2006). It has emerged as a pathogen in recent years. *Cronobacter* was isolated in powdered infant formula due to contamination of the manufacturing environment (Yan et al. 2012). It is an opportunistic pathogen that can cause necrotizing enterocolitis, bacteremia, and meningitis, predominantly in neonates (Yan et al. 2012). Mortality of infants with *Cronobacter* bacteremia or meningitis was up to 40 % (Yan et al. 2012). *Cronobacter* can also infect adults, in particular immunocompromised patients (Tsai et al. 2013). The four adult cases reported from Taiwan (Tsai et al. 2013) were not associated with infant formula and the source of infection was unclear. Plant material may be the natural habitat for *Cronobacter* species (Schmid et al. 2009).

Dickeya Samson et al. 2005

Dickeya (Dic.ke'ya. N.L. fem. n. *Dickeya* named in honor of the American phytopathologist Robert S. Dickey, for his contribution to research on the *Erwinia chrysanthemi* complex) is a rod (0.5–1.0 × 1.0–3.0 μm) with rounded ends, which occur mostly in singles or in pairs, but sometimes in chains (Samson et al. 2005). It is motile by means of peritrichous flagella and ferments glucose, hydrolyzes pectin, produces indole, and grows at 36 °C. *Dickeya* can utilize L-arabinose, *myo*-inositol, D-malate, malonate, D-mannose, mucate, saccharate, and meso-tartrate, but not D-trehalose, methyl-α-glucoside, D-arabitol, or sorbitol (Samson et al. 2005). There are five species of *Dickeya*: *D. chrysanthemi, D. dadantii, D. dianthicola, D. paradisiaca*, and *D. zeae*. The differential biochemical characteristics between species are summarized in ❯ *Table 13.6*.

D. dadantii has two subspecies: subsp. *dadantii* and subsp. *dieffenbachiae*. *D. dadantii* subsp. *dieffenbachiae* was initially described as a separate species of *Dickeya* (Samson et al. 2005). However, analyses of 16S rDNA gene and partial sequences of *gyrB*, *rpoB*, *infB*, and *atpD* genes showed that there is a close relationship between *D. dadantii* and *D. dieffenbachiae* (Brady et al. 2012a). Nevertheless, the two species can be differentiated phenotypically based on lactose, D-melibiose, and D-raffinose utilizations (all negative in *D. dieffenbachiae*), and hence, it was placed as a subspecies within *D. dadantii*.

Dickeya spp. were transfers of *Erwinia* or *Pectobacterium* species or biovars. 16S rDNA sequence grouped them together in one clade and has a monophyletic origin. This is also supported by *recA* sequence data (Parkinson et al. 2009). *Dickeya* has been isolated from soft rot and wilt of a range of plants (Cating and Palmateer 2011; Grenier et al. 2006; Ma et al. 2007; Palacio-Bielsa et al. 2007; Pu et al. 2012; Sławiak et al. 2009).

Table 13.5

Phenotypic characters for differentiating *Cronobacter* species

Characteristic	*C. condimenti*	*C. dublinensis*			*C. malonaticus*	*C. muytjensii*	*C. sakazakii*	*C. turicensis*	*C. universalis*
		subsp. *dublinensis*	subsp. *lactaridi*	subsp. *lausannensis*					
Motility	–	+	+	+	v	+	+	+	v
Carbon utilization									
Dulcitol	–	–	–	–	–	+	–	+	+
Indole	+	+	+	v	–	+	–	–	–
Malonate	+	+	–	–	+	+	–	v	+
10-Methyl α-D-glucopyranoside	+	+	+	+	+	–	+	+	+
Melezitose	–	+	–	–	–	–	–	+	+
Turanose	–	+	v	–	+	v	+	+	–
Inositol	–	+	+	–	v	+	v	+	+
Lactulose	–	+	+	–	+	+	+	+	+
Putrescine	–	+	+	v	v	+	+	+	–
cis-Aconitate	–	+	+	+	+	+	+	+	v
trans-Aconitate	–	+	+	+	+	+	–	–	–
4-Aminobutyrate	–	+	+	+	+	v	+	+	–
Maltitol	–	+	+	–	+	–	+	+	+
Palatinose	–	+	+	+	+	v	+	+	v

Adapted from Joseph et al. (2012)

v variable, + positive, – negative

Table 13.6
Phenotypic characters that differentiate six *Dickeya* spp.

Characteristic	*D. dadantii* subsp. *dadantii* and *D. zeae*	*D. dadantii* subsp. *dieffenbachiae*	*D. chrysanthemi*		*D. dianthicola*	*D. paradisiaca*
			bv. *parthenii*	bv. *chrysanthemi*		
D-Arabinose	+	+	–	–	–	+
D-Tartrate	–	–	d (25)	–	+	+
Inulin	–	–	–	+	d (88)	–
Lactose	+	–	d (75)	d (20)	–	d (17)
Growth at 39 °C	+	+	+	+	–	d (83)
cis-Aconitate	+	d (80)	–	d (20)	–	–
D-Melibiose, D-raffinose	+	–	+	+	d (44)	d (83)
5-Keto-D-gluconate	–	d (20)	–	–	–	+
Mannitol	+	+	+	+	+	–
Lecithin	+	+	+	+	+	–
Arginine dihydrolase	d (15)	–	–	+	d (69)	–
meso-Tartrate	+	+	d (75)	–	+	+
myo-Inositol	+	+	+	d (80)	+	–
Casein	+	d (80)	d (75)	+	d (75)	–

Adapted from Samson et al. (2005)
+: 90–100 % of strains positive; –: 90–100 % of strains negative; *d* (*n*): percentage of positive strains

Edwardsiella Ewing and McWhorter 1965

The genus *Edwardsiella* was named in honor of P.R. Edwards (a CDC microbiologist) (Ewing et al. 1965) and consists of three species: *E. tarda* (Ewing et al. 1965), *E. hoshinae* (Grimont et al. 1980), and *E. ictaluri* (Hawke et al. 1981). *E. tarda* is positive for H_2S production, indole, methyl red, and lysine and ornithine decarboxylases. Gas is produced from glucose, while most carbohydrate or sugar alcohols are not fermented. There are also atypical *E. tarda* strains which ferment L-arabinose, D-mannitol, and sucrose but unable to reduce tetrathionate (Grimont et al. 1980). Sucrose-positive strains have also been described by Walton et al. (1993) and Leung (1996). *E. hoshinae* produces acid from D-mannitol, sucrose, D-trehalose, and salicin and is positive for malonate test. *E. ictaluri* produces gas from glucose at 25 °C but not at 37 °C and does not produce indole. *E. ictaluri* does not have proteases, lipases, esterases, pectinase, collagenase, alginase, chitinase, and hyaluronidase and does not produce H_2S (Waltman et al. 1986).

Edwardsiella is found in aquatic environments and is a fish pathogen and an opportunistic human pathogen (Abbott and Janda 2006; Janda and Abbott 1993). *E. ictaluri* is exclusively a catfish pathogen causing enteric septicemia (Abbott and Janda 2006; Mohanty and Sahoo 2007). *E. tarda* has a wide host range and has been isolated from fish, reptiles, amphibians, chickens, other warm-blooded animals, and humans (Abbott and Janda 2006). *E. tarda* causes edwardsiellosis in freshwater and marine fishes, characterized by systemic hemorrhagic septicemia, internal abscesses, and skin lesions (Mohanty and Sahoo 2007). It also causes human infections, presenting most frequently as gastroenteritis (Janda and Abbott 1993). Extraintestinal infections can also occur which include meningitis, soft tissue infections, bacteremia, septicemia, cholecystitis, endocarditis, and osteomyelitis (Golub et al. 2010; Hashavya et al. 2011; Nelson et al. 2009; Ota et al. 2011; Wang et al. 2005a).

Enterobacter Hormaeche and Edwards 1960

Enterobacter was first described by Hormaeche and Edwards (Hormaeche and Edwards 1960). It can grow readily on any media. Acid and gas can be produced from glucose and lactose. Carbon dioxide is produced twice as much as hydrogen from glucose. Most species are negative for methyl red test and positive for Voges–Proskauer. It is negative for H_2S production and phenylalanine deaminase.

The genus *Enterobacter* is large, with 20 species: *E. aerogenes, E. amnigenus, E. arachidis, E. asburiae, E. cancerogenus, E. cloacae, E. cowanii, E. gergoviae, E. helveticus, E. hormaechei, E. kobei, E. ludwigii, E. mori, E. nimipressuralis, E. oryzae, E. pulveris, E. pyrinus, E. radicincitans, E. soli*, and *E. turicensis*. Biochemical characteristics that are useful for differentiation between species are summarized in ◉ *Table 13.7*. *E. cloacae* is divided into two subspecies, subsp. *cloacae* and subsp. *dissolvens*. *E. hormaechei* is divided into three subspecies, subsp. *oharae*, subsp. *hormaechei*, and subsp. *steigerwaltii* (Hoffmann et al. 2005b). However,

Table 13.7

Phenotypic characters for differentiating *Enterobacter* species

Characteristic	*E. aerogenes*	*E. amnigenus*	*E. arachidis*	*E. asburiae*	*E. cancerogenus*	*E. cloacae* subsp. *cloacae*	*E. cloacae* subsp. *dissolvens*	*E. cowanii*	*E. gergoviae*	*E. helveticus*	*E. hormaechei*	*E. kobei*	*E. ludwigii*	*E. mori*	*E. nimipressuralis*	*E. oryzae*	*E. pulveris*	*E. pyrinus*	*E. radicincitans*	*E. solis*	*E. turicensis*
Aesculin hydrolysis	+	+	−	+	+	v	+	+	+	+	−	−	(−)	+	+	−	+	+	+	ND	+
Arginine dihydrolase	−	v	+	v	+	+	+	−	−	−	v	+	+	+	(−)	+	−	−	−	+	−
Lysine decarboxylase	+	−	−	−	−	−	−	−	+	−	−	−	−	+	−	+	−	+	−	−	−
Methyl red test	−	v	−	+	−	−	−	−	−	+	−	−	−	−	−	+	+	−	−	ND	+
Motility	+	+	+	v	+	+	(−)	+	+	+	v	(+)	+	+	−	+	+	v	+	+	+
Ornithine decarboxylase	+	v	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	−
Voges–Proskauer reaction	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	(+)	−
Utilization of:																					
1-O-Methyl-α-galactopyranoside	(+)	+	+	(+)	−	+	+	+	+	+	−	+	+	v	−	ND	+	−	+	ND	+
Citrate	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−	+	+	−
D-Arabitol	+	−	+	−	−	v	−	−	+	−	(−)	−	−	+	−	−	+	−	+	ND	−
D-Sorbitol	+	v	+	+	−	+	+	+	−	−	(−)	+	+	+	+	+	−	−	+	+	−
L-Fucose	−	−	+	−	+	−	v	−	v	−	+	−	v	V	−	+	−	−	v	ND	−
Melibiose	+	+	−	−	−	(+)	+	+	+	+	(−)	+	+	+	+	+	+	−	−	+	+
Mucate	+	v	−	v	+	v	+	+	−	+	+	v	+	+	(+)	+	+	−	−	ND	+
Putrescine	v	−	−	(−)	+	+	+	−	v	+	−	v	−	+	−	−	−	+	−	ND	−
Sucrose	+	v	+	+	−	+	+	+	+	−	+	+	+	+	−	+	+	+	+	ND	−
α-L-Rhamnose	+	+	+	(+)	+	+	(+)	+	+	+	+	+	+	+	+	−	+	+	+	+	+

Adapted from Peng et al. (2009), Madhaiyan et al. (2010), Manter et al. (2011), and Zhu et al. (2011)

−: 0–10 % positive; (−): 10–20 % positive; *v*: 20–80 % positive; (+): 80–90 % positive; +: 90–100 % positive; *ND*: not determined

sequencing of *hsp60* and *rpoB* cannot distinguish the *E. hormaechei* subspecies (Paauw et al. 2008).

The genus is polyphyletic based on 16S rDNA sequence with 14 lineages scattered across the 16S rDNA tree. Unfortunately, there are no additional gene sequence data to allow a good resolution of the phylogeny of this genus. Hoffmann and Roggenkamp (2003) used *hsp60*, *rpoB*, and *hemB* to analyze strains from the *E. cloacae* complex which includes *E. asburiae*, *E. cloacae*, *E. hormaechei*, *E. kobei*, *E. ludwigii*, and *E. nimipressuralis* and found 12 genetic clusters. A further MLST study of the complex using seven housekeeping genes (*hsp60*, *rpoB*, *fusA*, *gyrB*, *leuS*, *pyrG*, and *rplB*) found that the six species were divided into two distinct clades, with one clade containing *E. hormaechei* only and the other clade, which is more heterogeneous, containing the remaining species of the complex.

Enterobacter can be found in soil, water, sewage, vegetable and fruits, plants (Egamberdieva et al. 2008; Kampfer et al. 2005; Madhaiyan et al. 2010; Stephan et al. 2007, 2008), and terrestrial and aquatic environments (Halda-Alija et al. 2001). Some species including *E. radicincitans*, *E. oryzae*, *and E. ludwigii* are plant-associated growth-promoting bacteria (de Melo Pereira et al. 2012; Kampfer et al. 2005; Pawlicki-Jullian et al. 2010; Peng et al. 2009), while others such as *E. mori* are plant pathogens. *E. soli* which can degrade lignin was isolated from soil (Manter et al. 2011).

Enterobacter can be isolated from the intestinal tracts of humans and other animals as commensals but are also significant human pathogens (Hoffmann et al. 2005a, c). *Enterobacter spp.* is the fourth most common cause of Gram-negative bloodstream infection (Al-Hasan et al. 2011). The *E. cloacae* complex is among the most common *Enterobacter* spp. causing nosocomial bloodstream infection with *E. cloacae* and *E. hormaechei* being the most frequently isolated (Mezzatesta et al. 2012). The incidence rate of *Enterobacter* spp. blood stream infections was found to be increasing in a population-based study in the USA (Al-Hasan et al. 2011). There is also an associated increase of antibiotic resistance (Al-Hasan et al. 2011; D'Agata 2004).

Erwinia Winslow et al. 1920, Hauben et al. 1998 emend

Erwinia (N.L. fem. n. *Erwinia*, named after Erwin F. Smith) consists of Gram-negative rods (0.5–1.0 × 1.0–3.0 μm) which can occur singly, in pairs, or in chains and motile by means of peritrichous flagella (Winslow et al. 1920). *Erwinia* does not produce indole or oxidize gluconate. It does not have arginine dihydrolase, caseinase, phenylalanine deaminase, and urease (Hauben et al. 1998). There are currently 16 species within the genus. Biochemical characteristics that differentiate the species are summarized in ❯ *Table 13.8*.

Many pathogenic and epiphytic *Erwinia* spp. have been isolated from apples and pears. *E. piriflorinigrans* was isolated from necrotic pear blossoms in Valencia, Spain (Lopez et al. 2011; Wensing et al. 2012); *E. amylovora, E. pyrifoliae*, as well as the related epiphytes *E. tasmaniensis* and *E. billingiae* have been isolated from apple and pear flowers in North America, Korea, Australia, and England (Billing and Baker 1963; Geider et al. 2006; Kim et al. 1999); and *E. uzenensis* was first isolated from black lesions on shoots of European pear trees (*Pyrus communis* L.) in an orchard in Japan (Matsuura et al. 2012). *E. oleae* and *E. toletana* were obtained in Italy from olive knots caused by *Pseudomonas savastanoi* (Moretti et al. 2011; Rojas et al. 2004). *E. papayae* has been associated with bacterial canker of papaya which emerged during the 1980s in different islands of the Caribbean (Gardan et al. 2004). *Erwinia* can also be isolated from insects; *E. aphidicola* and *E. typographi* were isolated from the gut of the pea aphid (Harada et al. 1997) and adult bark beetles (Skrodenytee-Arbaciauskiene et al. 2012), respectively.

Escherichia Castellani and Chalmers 1919

Escherichia (N.L. fem. n. *Escherichia*) was named in honor of Theodor Escherich, who first isolated the type species of the genus (Castellani and Chalmers 1919). The genus contains five species*: E. albertii, E. coli, E. fergusonii, E. hermannii*, and *E. vulneris. E. albertii* is a new member since last edition of this book. The genus *Escherichia* is polyphyletic due to *E. hermannii* and *E. vulneris* which are grouped outside the main cluster of the three species, *E. albertii, E. coli*, and *E. fergusonii*, in the 16S rDNA, *gapA and ompA* gene trees (Lawrence et al. 1991), and genome tree (only *E. hermannii* genome available). *E. vulneris* itself is also polyphyletic based on the *gapA* and *ompA* gene sequences with one of the three strains grouping with *Klebsiella pneumoniae* (Lawrence et al. 1991). Clearly, *E. hermannii* and *E. vulneris* need to be reclassified to eliminate this polyphyletic situation of the genus *Escherichia*. The key biochemic properties differentiating the species are listed in ❯ *Table 13.9*.

Among the five species of *Escherichia*, *E. coli* is well known as a common inhabitant of the intestinal tract of humans and other animals. *E. coli* is also a major pathogen to humans and other animals causing intestinal and extraintestinal infections (Croxen and Finlay 2010). *E. coli* has been involved in infections of humans of virtually all body systems and tissues. Pathogenic *E. coli* can be distinguished into several pathogenic classes based on mode of pathogenesis including enteropathogenic, enterohemorrhagic, enterotoxigenic, enteroaggregative, enteroinvasive, and extraintestinal pathogenic *E. coli* (EPEC, EHEC, ETEC, EAEC, EIEC, and ExPEC). EHEC is a subset of Shiga toxin *E. coli* (STEC). A new pathogenic form emerged recently with the German outbreak of an O104:H4 strain that is a Shiga toxin-producing enteroaggregative *E. coli* (Mellmann et al. 2011). *E. coli* is present in the environment as a fecal contamination and may also be a long-term inhabitant as environmental *E. coli* was found to be distinctive (Walk et al. 2009).

E. albertii is a relatively new member and is an emerging human pathogen (Abbott et al. 2003; Huys et al. 2003; Hyma et al. 2005; Ooka et al. 2012, 2013). It has been isolated from wild and domestic birds (Oaks et al. 2010) and detected in drinking

Table 13.8

Phenotypic characters for differentiating *Erwinia* species

Characteristic	*E. amylovora*	*E. aphidicola*	*E. billingiae*	*E. mallotivora*	*E. oleae*	*E. papayae*	*E. persicina*	*E. piriflorinigrans*	*E. psidii*	*E. pyrifoliae*	*E. rhapontici*	*E. tasmaniensis*	*E. toletana*	*E. tracheiphila*	*E. typographi*	*E. uzenensis*
Voges–Proskauer	+	+	+	+	v	ND	+	+	ND	w	+	+	–	w	–	+
Gelatinase	+	–	–	–	v	ND	ND	+	ND	–	–	–	–	–	–	+
Utilization of:																
Arabitol	–	–	+	–	+	ND	–	–	ND	–	–	–	+	–	–	ND
Cellobiose	–	+	–	–	–	ND	+	ND	ND	–	+	–	+	–	–	ND
Glycerol	–	+	–	–	–	+	+	+	+	+	+	–	–	–	–	+
Inositol	–	+	–	–	–	ND	+	+	ND	+	+	–	+	–	–	+
Maltose	–	+	+	–	–	–	+	ND	–	–	+	–	+	–	–	+
Mannitol	–	+	+	+	+	–	+	+	+	+	+	+	+	–	+	+
Mannose	–	+	+	+	+	+	+	ND	+	–	+	–	–	–	+	+
Melibiose	–	+	+	–	–	–	+	+	–	–	+	w	+	–	–	+
Raffinose	–	+	–	–	–	–	v	+	–	–	+	–	–	–	–	ND
Rhamnose	–	+	+	–	+	–	+	+	+	–	+	–	–	–	–	ND
Salicin	–	+	+	–	+	ND	+	ND	ND	ND	+	–	+	–	–	ND
Sucrose	+	+	–	+	–	+	+	+	+	+	+	+	–	+	–	+
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+

Adapted from Hao et al. (1990), Kim et al. (1999), Gardan et al. (2004), Moretti et al. (2011), Skrodenytee-Arbaciauskiene et al. (2012), and Matsuura et al. (2012)

v variable, *ND* not determined, + positive, – negative

Table 13.9
Phenotypic characters for differentiating *Escherichia* species

Characteristic	*E. albertii*	*E. coli*	*E. fergusonii*	*E. hermannii*	*E. vulneris*
Indole	–	+	+	+	–
Lysine	+	+	+	–	+
Ornithine	+	(+)	+	+	–
Growth in KCN	–	–	–	+	–
Fermentation of					
Lactose	–	+	–	(–)	–
D-Mannitol	+	+	+	+	+
Adonitol	–	–	+	–	–
D-Sorbitol	–	+	–	–	–
Raffinose	–	(+)	–	(–)	+
L-Rhamnose	–	(+)	+	+	+
D-Xylose	–	+	+	+	+
Cellobiose	–	–	+	+	+
D-Arabitol	–	–	+	–	–
Utilization of acetate	+	+	+	(+)	(–)

Adapted from Huys et al. (2003)
+: 85 % of strains positive; –: 85 % of strains negative; (+): 50–85 % of strains positive; (–) 50–85 % of strains negative

water (Felfoldi et al. 2010). Most of the *E. albertii* infections are sporadic, but a gastroenteritis outbreak has been reported in Japan (Ooka et al. 2012, 2013).

E. fergusonii has been isolated from blood, gallbladder, wound, urine, and diarrheal stools (Bain and Green 1999; Farmer et al. 1985b; Funke et al. 1993; Lagace-Wiens et al. 2010; Lai et al. 2011; Savini et al. 2008). It has also been isolated from farm animals (Forgetta et al. 2012; Hariharan et al. 2007; Oh et al. 2012; Rayamajhi et al. 2011; Wragg et al. 2009). There are few reports of *E. hermannii* infections. It has been isolated from wound, blood, cerebrospinal fluid, conjunctiva, and a persistent apical periodontitis lesion (Brenner et al. 1982a; Dahl et al. 2002; Ginsberg and Daum 1987; Pien et al. 1985; Poulou et al. 2008; Yamanaka et al. 2010). It has also been isolated from the environment (Hernandez et al. 1998). *E. vulneris* has been associated with wound infections, osteomyelitis, intravenous catheter-associated bacteremia, urosepsis, meningitis, and peritonitis (Arslan et al. 2008; Awsare and Lillo 1991; Brenner et al. 1982b; Horii et al. 2001; Jepsen et al. 1997; Kilani et al. 2008; Levine and Goldberg 1994; Mohanty et al. 2005; Spaulding and Rothman 1996). There is no report of isolation of *E. vulneris* from nonhuman sources.

Ewingella Grimont et al. 1983

Ewingella was established as a genus in 1983 by Grimont et al. (1983) to organisms formerly belonging to enteric group 40. Cells are 1.0–1.8 μm in length × 0.6–0.7 μm in width. Growth can occur between 15 °C and 37 °C and not 40 °C. *Ewingella* can grow in peptone water containing 8 % NaCl at 30 °C. *Ewingella* produces acid from glucose but not from L-arabinose, melibiose, raffinose, D-sorbitol, or sucrose. It is also negative for deoxyribonuclease, lysine, ornithine, and arginine decarboxylases; unable to utilize malonate; and positive for Voges–Proskauer. *E. americana* is the only species within this genus.

E. americana has been isolated from mollusks and mushrooms (Inglis and Peberdy 1996; Janda 2006; Muller et al. 1995; Reyes et al. 2004). *E. americana* has been reported to cause a range of human infections and has been isolated from the blood, sputum, conjunctiva, wounds, peritoneal fluid, and synovial fluid (Hassan et al. 2012; Pound et al. 2007). It was more frequently isolated from immunocompromised patients (Hassan et al. 2012; Pound et al. 2007).

Gibbsiella Brady et al. 2010

Gibbsiella (Gibb.si'el.la. N.L. fem. N. *Gibbsiella* named to honor British forest pathologist John N. Gibbs) can be short (0.9 × 1–1.5 μm) or long (1.1–1.5 × 3.0–6.0 μm) rods (Brady et al. 2010a). It does not have flagella but has very fine fimbriae. On nutrient agar, the colonies appear white to cream, round, convex, and smooth with entire edges. Growth is between 10 °C and 40 °C. It has a positive β-galactosidase activity, can utilize citrate, but is negative for H_2S, urease, indole, acetoin, gelatinase, arginine dihydrolase, lysine and ornithine decarboxylases, and tryptophan deaminase. It can produce acid from glycerol,

L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, D-mannitol, D-sorbitol, α-methyl-D-glucoside, *N*-acetyl-D-glucosamine, arbutin, esculin, ferric citrate, salicin, D-maltose, D-melibiose, D-saccharose, D-trehalose, D-raffinose, D-turanose, and D-arabitol. The major fatty acid components are $C_{14:0}$, $C_{16:0}$, and cyclo-$C_{17:0}$. The genus contains three species: *G. quercinecans*, *G. dentisursi*, and *G.papilionis*. The differentiating biochemical properties are acid production from inositol in *G. quercinecans* and negative reaction to D-arabinose in *G. dentisursi*. In addition, *G. dentisursi* produces sucrose-derived exopolysaccharides like *Streptococcus mutans* (Saito et al. 2012). *G. papilionis* is unable to produce acid from D-lactose, inositol, starch, and L-fucose and has cysteine arylamidase (Kim et al. 2012a).

Gibbsiella appears to have diverse habitats. *G. quercinecans* was isolated from oak trees displaying symptoms of extensive stem bleeding called Acute Oak Decline from Britain and Spain (Brady et al. 2010a). The type strain was isolated from sessile oak (*Quercus petraea*) in Hoddesdon Park Wood, Hertfordshire, England. *G. dentisursi* was isolated from the bear oral cavity (Saito et al. 2012). *G. papilionis* was isolated from the intestinal tract of a butterfly (*Mycalesis gotama*) (Kim et al. 2012a).

Hafnia Moller 1954

The genus *Hafnia* was first described by Moller (Moller 1954). This genus is typically positive for Voges–Proskauer, methyl red, and lysine and ornithine decarboxylases but negative for arginine dihydrolases. *Hafnia* can grow at 4–44 °C and in media containing 2–5 % of NaCl and pH 4.9–8.25 (Greipsson and Priest 1983). On selective media (MacConkey, Hektoen enteric, eosin methylene blue, and xylose–lysine–deoxycholate agars), it typically appears as large, smooth, convex, translucent colonies of 2–3 mm in diameter with an entire edge, although some may appear irregularly shaped (Janda and Abbott 2006).

There are two species in the genus: *H. alvei* and *H. paralvei*. *H. paralvei* was an elevation to species status of the *Hafnia alvei* hybridization group HG2 (Huys et al. 2010). The two species are distinctive but closely related on the 16S rDNA gene tree. Phenotypic tests that differentiate *H. alvei* from *H. paralvei* in most of the cases include malonate assimilation, esculin hydrolysis, and fermentation of D-arabinose and salicin (Janda and Abbott 2006). Another differential property is β-galactosidase activity (positive for *H. alvei* and negative for *H. paralvei*) (Huys et al. 2010).

Hafnia has been isolated from a variety of sources (Janda and Abbott 2006). The reader is referred to the comprehensive review by Janda and Abbott (Janda and Abbott 2006). *Hafnia* is a common inhabitant of the gastrointestinal tracts of animals, in particular mammals. It has also been recovered from food products including meat and vegetables. The reference to *H. alvei* in earlier publications should be aware of two changes in classification. Some *H. alvei* strains may actually be *E. albertii* (Janda and Abbott 2006). Based on the study of Okada and Gordon and by inference of its genetic group (HG2), *H. paralvei* were predominantly associated with freshwater fish, but both species were equally represented in mammals and reptiles they sampled (Okada and Gordon 2003). *Hafnia* can cause a range of human infections including bacteremia, gastroenteritis, and respiratory tract infections and is a rare cause of other extraintestinal infections (Janda and Abbott 2006). It is also pathogenic to other animals including chickens and fish (Janda and Abbott 2006).

Table 13.10
Biochemical characteristics that differentiate *Klebsiella* species

Characteristic	*K. alba*	*K. michiganensis*	*K. oxytoca*	*K. pneumoniae* subsp. *ozaenae*	*K. pneumoniae* subsp. *pneumoniae*	*K. pneumoniae* subsp. *rhinoscleromatis*	*K. singaporensis*
Indole	(+)	+	+	–	–	–	–
Gas from lactose at 44.5 °C	+	–	–	+	+	+	–
Growth at 10 °C	+	+	+	–	–	–	+
Methyl red	–	–	–	+	–	+	–
Voges–Proskauer	+	+	+	–	+	–	+
Utilization of							
Palatinose	ND	ND	+	–	+	–	–
L-Sorbose	+	ND	+	–	–	–	–
Urease	+	–	+	–	+	–	+

Adapted from Li et al. (2004), Xu et al. (2010), and Saha et al. (2013)
ND not determined, + positive, – negative

Klebsiella Trevisan 1885, Carter et al. 1999 emend

Klebsiella (N.L. fem. dim. n. *Klebsiella*) was named after Edwin Klebs (1834–1913), a German bacteriologist by Trevisan (1885). It consists of capsulated cells of 0.3–1.0 × 0.6–6.0 μm and can occur singly, in pairs, or short chains and nonmotile. It can utilize citrate and glucose as sole carbon source but not L-sorbose. Acid and gas is produced from glucose fermentation and most strains produce 2,3-butanediol as a major end product of fermentation. It is positive for catalase. The genus consists of five species (*K. alba*, *K. granulomatis*, *K. michiganensis*, *K. oxytoca*, and *K. pneumoniae*) and three subspecies within *K. pneumoniae* (subsp. *ozaenae*, *pneumoniae*, and *rhinoscleromatis*). *K. alba* (Xu et al. 2010) and *K. michiganensis* (Saha et al. 2013) are recent additions. The biochemical characteristics that differentiate *Klebsiella* species, except *K. variicola*, are summarized in *Table 13.10*.

Phylogenetic analyses of *Klebsiella* spp. have been performed using 16S rDNA (Hauben et al. 1998), *infB* (Hedegaard et al. 1999), *rpoB* (Mollet et al. 1997), *groE* (Harada and Ishikawa 1997), and *gyrB* (Dauga 2002), all of which suggest that *Klebsiella* is polyphyletic. Based on *gyrB* and *infB* sequence data, *Escherichia*, *Salmonella*, *Klebsiella*, *Enterobacter*, and *Citrobacter* form a single group (Dauga 2002; Hedegaard et al. 1999). *K. granulomatis*, the causative agent of donovanosis, was a reclassification of *Calymmatobacterium granulomatis* based on 16S rDNA gene sequence (Carter et al. 1999) which is also supported by *rpoB* gene sequence (Drancourt et al. 2001). There is no type strain for this species due to difficulties in the storage of the culture.

Klebsiella spp. can be found in a variety of sources including soil, water, plants, humans, and other animals (Brisse et al. 2006). Note that four *Klebsiella* species (*K. terrigena*, *K. ornithinolytica*, *K. planticola*, and *K. trevisanii*) have been reclassified to other genus and description of sources of isolation of *Klebsiella* in earlier literature includes these species. *Klebsiella* can be isolated from human intestinal tract and to a less extent from the nasopharynx and a range of human infections. *Klebsiella* is a significant cause of both nosocomial and community-acquired infections. Among the *Klebsiella* spp., *K. pneumonia* is the most common species in causing urinary tract infections, pneumonia, septicemias, and soft tissue infections (Keynan and Rubinstein 2007; Podschun and Ullmann 1998). An invasive syndrome caused by *K. pneumonia* that causes liver abscesses has been increasingly reported in Asia in recent years (Siu et al. 2012). A major challenge is antibiotics resistance (Keynan and Rubinstein 2007). *K. granulomatis* is a human-only pathogen causing granuloma inguinale (donovanosis) as a sexual transmitted infection and is a neglected disease and endemic in some areas with limited medical capacities (Lagergard et al. 2011). *Klebsiella* also causes serious infections in animals. *K. pneumonia* is known to cause endometritis and infertility in the mare and mastitis in cattle (Brisse et al. 2006; Samper and Tibary 2006; Zadoks et al. 2011).

K. alba was first isolated from a heavily polluted soil sample in Jiangsu Province, China (Xu et al. 2010). *K. michiganensis* was isolated from a toothbrush holder during a study which investigated microbial hot spots in a residential household in Michigan, USA (Saha et al. 2013). *K. singaporensis* was isolated from soil during screening for efficient isomaltulose-producing bacterial strains from sucrose (Li et al. 2004). *K. variicola* was first isolated from inside banana tissues, leaves, stems, as well as banana plantlets obtained from tissue culture by Rosenblueth et al. (2004).

Kluyvera Farmer et al. 1981

Kluyvera (N.L. fem. n. *Kluyvera* in honor of the Dutch microbiologist A.J. Kluyver) was first named by Asai et al. (1956) to include polarly flagellated bacteria which produced large amounts of a-ketoglutaric acid and later isolates formerly

Table 13.11
Phenotypic characters that differentiate *Kluyvera* species

Characteristic	*K. cochleae*/*K. intermedius*	*K. georgiana*	*K. ascorbata*	*K. cryocrescens*
Indole	–	+	+	+
Utilization of malonate	+	–	+	–
Voges–Proskauer	+	–	–	–
Lysine decarboxylase	–	+	+	–
Fermentation of				
Dulcitol	+	+	–	–
Glycerol	+G	w	w	+G
D-Sorbitol	+	–	–	–
Ascorbate	+	+	+	–
Growth on CIN	+	+	+	–

Adapted from Pavan et al. (2005)
+*G* gas is produced, *w* weak, *CIN* cefsulodin/irgasan/novobiocin, + positive, – negative

included in enteric group 8 (Farmer et al. 1981). Acid and usually gas as well as large amounts of α-ketoglutaric acid are produced from glucose and other carbohydrates. There are three species: *K. ascorbata*, *K. cochleae*, and *K. georgiana*. These species grow optimally at 30–36 °C with moderate to good growth at 42 °C and the colonies are 1–2 mm in diameter after 2 days of incubation on nutrient agar. *K. cryocrescens* can grow and ferment glucose at 5 °C. *K. ascorbata* produces acid from ascorbate (Farmer et al. 1981).

K. georgiana can be differentiated from *K. cochleae* based on the 3-phenyl-propionate and *m*-coumarate reactions. *K. cochleae* is metabolically least active among the four species and can be differentiated from other species since it is indole negative (Muller et al. 1996). However, a combination of tests is required to differentiate *K. georgiana* from *K. ascorbata* and *K. cryocrescens*. Biochemical characteristics that differentiate *Kluyvera* species are summarized in ❯ *Table 13.11*.

Kluyvera has been isolated from sewage, water, soil samples, vegetables, mollusks, and Egyptian fruit-bat (Han et al. 2010; Janda 2006). *Kluyvera* is now recognized as an important human pathogen although it was initially thought to be a benign saprophytic colonizer of the human respiratory, gastrointestinal, and urinary tracts (Carter et al. 2008; Darling et al. 2005; Janda 2006; Sarria et al. 2001). It can produce a wide spectrum of clinically significant disease ranging from soft tissue infections to sepsis with multi-organ failure (Carter et al. 2008; Darling et al. 2005; Sarria et al. 2001).

Leclercia Tamura et al. 1986

Leclercia was created as a genus in 1986 by transfer of *Escherichia adecarboxylata* (Tamura et al. 1986). The sole species of this genus is *L. adecarboxylata*, which was first described by Leclerc in 1962. It was initially designated as enteric group 41 and given the name *Escherichia adecarboxylata*. It is motile with peritrichous flagella and mesophilic. *L. adecarboxylata* forms colonies similar *to E. coli* in media such as MacConkey, deoxycholate-lactose, and eosin methylene blue agars. It gives positive reactions for both indole and methyl red while negative for Voges–Proskauer, citrate utilization, lysine and ornithine decarboxylases, and arginine dihydrolase. *L. adecarboxylata* can be differentiated from *E. coli* by its ability to assimilate malonate and ferment arabitol and cellobiose and inability to decarboxylate lysine. It can grow in the presence of KCN and can produce gas from glucose and acid from D-cellobiose, D-lactose, melibiose, L-rhamnose, adonitol, D-arabitol, dulcitol, and salicin. It is negative for H_2S production, phenylalanine deaminase, gelatinase, DNase, Tween 80 hydrolysis, and acid production from myo-inositol and alpha-methyl-D-glucoside. Variations in fermentation ability of D-raffinose, D-sucrose, and D-sorbitol are observed between strains (Tamura et al. 1986).

Leclercia has been isolated from food, drinking water, and other environmental sources (Janda 2006). It has been isolated from human infections including from human sputum, blood, urine, fecal, and wounds (Dalamaga et al. 2009; de Baere et al. 2001; Hess et al. 2008; Janda 2006; Tam and Nayak 2012).

Leminorella Hickman-Brenner et al. 1985

Leminorella (Lĕ-mean-nohr-rel'-la) was previously referred to as enteric group 57 and the name is derived from the surname of Leon Le Minor (Hickman-Brenner et al. 1985). It produces H_2S, acid from L-arabinose and D-xylose, and is positive for tyrosine clearing test. *Leminorella* is similar to *Proteus* in its ability to produce H_2S, tyrosinase activity, and inability to ferment D-mannose but is readily differentiated from *Proteus* by the absence of urea and phenylalanine deaminase reactions. The genus contains two species, *L. grimontii* and *L. richardii*, which can be differentiated based on methyl red, gas from glucose, or acid from dulcitol with the former being positive.

Leminorella has been isolated from human clinical specimens, but its clinical significance was unclear (Janda 2006). A retrospective study found *Leminorella* as clinically relevant bacteria causing primarily urinary tract infection, surgical site and soft tissue infection, secondary peritonitis, bacteremia, and lower respiratory tract infections (Blekher et al. 2000). Almost all the cases have comorbidities. There was one recent report of a spontaneous peritonitis caused by *L. grimontii* in a 63-year-old man with cirrhosis (Dalamaga et al. 2006). There is no report of *Leminorella* isolated from other sources.

Lonsdalea Brady et al. 2012

Lonsdalea (Lons.da'le.a. N.L. fem. n. *Lonsdalea* named in honor of David Lonsdale for his contributions to British forest pathology) is a new genus created by transfer of *Brenneria quercina* (Brady et al. 2012a). It is a short rod (0.5–1.0 × 1.0–2.0 μm) which occurs singly, in pairs, or in groups and motile by peritrichous flagella. It grows optimally at 28–30 °C and colonies appear white to cream on tryptone soya agar, round, convex, and smooth with entire edges. It is negative for β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, H_2S production, urease, indole, gelatinase, and reduction of nitrate. It is able to utilize citrate and produce acid from *N*-acetylglucosamine, arbutin, D-fructose, D-glucose, D-mannose, methyl α-D-glucopyranoside, D-ribose, salicin, sucrose, and turanose. Major fatty acids are $C_{14:0}$, $C_{16:0}$, $C_{18:1\ \omega\ 7c}$, $C_{17:10\ cyclo}$, and summed features 2 (iso-$C_{16:1}$ and/or $C_{14:0}$ 3-OH) and 3 ($C_{16:1\omega7c}$ and/or iso-$C_{15:0}$ 2-OH).

The sole species belonging to this genus is *L. quercina*, which was described earlier by Hildebrand and Schroth (1967) and Hauben et al. (1998) as *Brenneria quercina*. It is further divided into four subspecies, subsp. *quercina*, subsp. *iberica*, subsp. *britannica* (Brady et al. 2012a), and subsp. *populi* (Toth et al. 2012). Biochemical characteristics

Table 13.12
Phenotypic characters for differentiating *Lonsdalea quercina* subspecies

	Lonsdalea quercina subsp.			
Characteristic	*quercina*	*iberica*	*britannica*	*populi*
Citrate utilization	+	+	+	+
Voges–Proskauer	v	+	–	+
Acid from				
Glycerol	–	+	+	+
L-Arabinose	–	–	–	–
D-Galactose	+	+	–	+
Amygdalin	–	–	–	+
D-Cellobiose	–	–	–	+
D-Trehalose	–	–	+	+
D-Turanose	+	+	+	–

Adopted from Toth et al. (2012) , + positive, – negative

that differentiate these subspecies are summarized in Table 13.12.

Lonsdalea is a plant pathogen and has been associated with disease in oak trees. *L. quercina* was initially isolated from oak displaying symptoms of drippy nut disease of *Quercus* sp. in the USA and has been isolated from *Quercus robur* in Britain and from Mediterranean oak trees associated with bark canker in Spain (Biosca et al. 2003; Brady et al. 2012a).

Mangrovibacter Rameshkumar et al. 2010

Mangrovibacter (Man.gro'vi.bac'ter. N.L. n. *mangrovum* mangrove; N.L. masc. n. *bacter* rod; N; L. Masc. n. *Mangrovibacter* mangrove rod) is motile with peritrichous flagella (Rameshkumar et al. 2010). The cell size is 1.0 × 1.2–4.0 μm. Major fatty acids are $C_{16:0}$, $C_{18:1\omega7c}$, and summed feature 3 ($C_{16:1\omega7c}$ and/or iso-$C_{15:0}$ 2-OH). It is phenotypically similar to the genus *Cronobacter* (Rameshkumar et al. 2010).

M. plantisponsor is the sole species of the genus. It grows at 28°C in two days and appears as circular, smooth, creamy white, and 1–2 mm in diameter. It can grow at 15–40 °C (optimal 28–30 °C) and 0–8 % NaCl. It can utilize citrate. It does not have urease, lysine decarboxylase, tryptophan deaminase, indole production, hydrogen sulfide production, denitrification, and hydrolysis of Tween 80, DNA, starch, casein, and gelatin. Acid is produced from sucrose, raffinose, cellobiose, arabinose, sorbitol, glycerol, ribose, D-xylose, sucrose, galactose, glucose, fructose, mannose, rhamnose, mannitol, methyl α-D-glucoside, *N*-acetylglucosamine, arbutin, maltose, melibiose, gentiobiose, trehalose, L-fucose, D-arabitol, L-arabinose, gluconate, and 6-ketogluconate but not from erythritol, D-arabinose, 5-ketogluconate, adonitol, L-xylose, methyl β-D-xyloside, sorbose, dulcitol, inositol, methyl α-D-mannoside, lactose, inulin, melezitose, starch, glucogen, xylitol, turanose, D-lyxose, D-tagatose, D-fucose, and L-arabitol. It can utilize gluconate, 5-ketogluconate, and malate as carbon sources but cannot utilize phenylacetate, caprate, or adipate (Rameshkumar et al. 2010). *M. plantisponsor* can fix nitrogen and was isolated from roots of mangrove-associated wild rice (*Porteresia coarctata* Tateoka) (Rameshkumar et al. 2010).

Moellerella Hickman-Brenner et al. 1984

Moellerella (Mo-ler-ehl'-ah. L. fem. named after a Danish microbiologist, Vagn Møller) is established for the enteric group 46 (Böttger et al. 1987). It is nonmotile. The colony appearance is similar to that of *E. coli* on MacConkey and eosin methylene blue agars where they produce deep pink and dark colonies with green metallic sheen, respectively (Hickman-Brenner et al. 1884). *M. wisconsensis* is the only species of this genus. Generally, it can be differentiated from other members of *Enterobacteriaceae* based on being negative for indole test, Voges–Proskauer, H_2S production, urea, phenylalanine deaminase, lysine and ornithine decarboxylases, arginine dihydrolase, absence of gas production from D-glucose, and absence of acid production from trehalose. In contrast, it is positive for methyl red, Simmons citrate, and acid production from lactose and raffinose.

Moellerella has a wide distribution and has been isolated from animals including from a wild raccoon, a goat, water, and food (Casalinuovo and Musarella 2009; Sandfort et al. 2002; Stock et al. 2003). *Moellerella* has been isolated from human feces. However, its enteropathogenic role in causing diarrhea is unclear (Janda 2006; Stock et al. 2003). Other clinical isolations include those from the blood and gall bladder, bronchial aspirates, and peritoneum exudates (Aller et al. 2009; Cardentey-Reyes et al. 2009; Janda 2006; Stock et al. 2003). *Moellerella* seems to be an infrequent human pathogen.

Morganella Fulton 1943

The genus *Morganella* was first proposed by Fulton in 1943 (Fulton 1943). *Morganella* is positive for indole, salicin fermentation, acid and gas production from galactose, glucose and glycerol (slowly), and levulose and maltose, but negative for H_2S production and proteolytic activity and lactose and sucrose fermentation.

There are two species in this genus, *M. morganii* and *M. psychrotolerans* (Emborg et al. 2006). *M. morganii* is further divided into two subspecies and seven biogroups; *M. morganii* subsp. *morganii* containing biogroups A, B, C, and D and subsp. *sibonii* containing biogroups E, F, and G. *M. psychrotolerans* is psychrotolerant and able to grow at 2–35 °C and 77 % of the isolates can grow at 0 °C. They can tolerate up to 7.5 % of NaCl and pH 4.6–9.2. *M. psychrotolerans* can be differentiated from *M. morganii* with fermentation of D-galactose.

M. morganii has been isolated from feces of healthy humans and other animals (Manos and Belas 2006). *M. morganii* causes a range of human infections including sepsis, liver, renal and tubo-ovarian abscesses, skin and soft tissue infections, bacteremia, and pericarditis (Abdalla et al. 2006; Chang et al. 2011; Chen and Lin 2012; Chou et al. 2009; Falagas et al. 2006; Gautam et al. 2003; Ghosh et al. 2009; Golubic-Cepulic et al. 2004; Hakyemez et al. 2012; Lee and Liu 2006; Osanai et al. 2008; Sinha et al. 2006; Tsai and Chang 2002; Tsanaktsidis et al. 2003; Wang et al. 2005b; Yang et al. 2006). It is also a pathogen to other animals including fatal infections in chickens and pneumonia in a rabbit and a piglet (Ono et al. 2001; Roels et al. 2007; Zhao et al. 2012). *M. psychrotolerans* was isolated from seafood (Emborg et al. 2006).

Obesumbacterium Shimwell 1963

Obesumbacterium (L. neut. adj. *obesum*, fat; L. neut. n. *bacterium*, a rod; N.L. neut. n. *Obesumbacterium*, a fat, rod-shaped bacterium) was established as a genus in 1963 by Shimwell (Shimwell 1963). The sole species of the genus is *Obesumbacterium proteus*. There had been two hybridization groups within *O. proteus*. The HG groups were well separated on 16S rDNA and MLST gene (*fusA*, *leuS*, *pyrG*, and *rpoB*) trees. *O. proteus* HG2 has been transferred to genus *Shimwellia* as *S. pseudoproteus*. *O. proteus* HG1 remains as *O. proteus*. *O. proteus* is closely related to *H. alvei* by 16S rDNA gene sequence and MLST of the four genes and should be reclassified as a species of *Hafnia* as discussed above.

Obesumbacterium is pleomorphic from short plump rods to long bacilli (0.8–2.0 to 1.5–100 μm) and nonmotile. *O. proteus* is a slow grower on most common media (Janda 2006). *O. proteus* produces acid from the fermentation of D-glucose and D-mannose and is lysine decarboxylase-positive. *O. proteus* is a common brewery contaminant and has been associated with brewer yeast and brewing process (Priest and Barker 2010). *O. proteus* has not been associated with human disease, but has been isolated from the intestines of fish (Navarrete et al. 2010; Skrodenyte-Arbaciauskiene et al. 2006) as well as edible snails (Charrier et al. 2006).

Pantoea Gavini et al. 1989, Mergaert et al. 1993 emend

Pantoea (Gr. adj. pantoios, of all sorts or sources; N.L. fem. n. *Pantoea*, [bacteria] from diverse geographical and ecological sources) was originally named by Gavini et al. (1989) to group two closely related taxa, DNA hybridization groups 14,589 and 27,155, which were originally placed in the *Erwinia herbicola–Enterobacter agglomerans* complex. *Pantoea* is a non-capsulated straight rod (0.5–1.0 × 1.0–3.0 μm) and motile by peritrichous flagella. Colonies are smooth, translucent, and convex with entire edge and can be yellow, beige, or nonpigmented. Optimum growth temperature is 28–30 °C. It is positive for glucose dehydrogenase, gluconate dehydrogenase, and Voges–Proskauer test. It is negative for lysine and ornithine decarboxylase, urease, pectinase, H_2S production, and indole. Acid is produced from L-arabinose, L-rhamnose, *N*-acetylglucosamine, D-xylose, D-ribose, maltose, D-galactose, D-mannose, D-fructose, trehalose, and D-mannitol.

There are 19 species in the genus: *P. agglomerans, P. allii, P. ananatis, P. anthophila, P. brenneri, P. calida, P. conspicua, P. cypripedii, P. deleyi, P. dispersa, P. eucalypti, P. eucrina, P. gaviniae, P. rodasii, P. rwandensis, P. septica, P. stewartii, P. vagans*, and *P. wallisii*. Some biochemical characteristics that are most useful to differentiate *Pantoea* species from each other are summarized in ❯ *Table 13.13*. *P. stewartii* is further divided into two subspecies: subsp. *indologenes* and subsp. *stewartii*. *P. stewartii* subsp. *stewartii* differs from subsp. *indologenes* by its inability to produce indole, utilize citrate, and grow on *cis*-aconitate and forms acid from glycerol, D-arabitol, cellobiose, maltose, lactose, arbutin, and salicin.

The 19 *Pantoea* species were divided into four clusters with 11, 4, 2, and 2 species in each cluster by 16S rDNA sequence as described above (❯ *Fig. 13.1*). Deletoile et al. (2009) performed a MLST study using six housekeeping genes (*fusA*, *gyrB*, *leuS*, *pyrG*, *rplB*, and *rpoB*) on 36 *Pantoea* strains and showed that four *Pantoea* spp. (*P. agglomerans, P. ananatis, P. stewartii, and P. dispersa*) were grouped together, but *P. agglomerans* appeared to be well separated from the other species with more than 7 % divergence from *P. ananatis* and *P. stewartii*. The study also identified three *Pantoea* spp. that were closer to *Tatumella* and have now been reassigned to that genus, demonstrating the importance of MLST in *Pantoea* species classification. Brady et al. (2012b) used MLST of four genes (*gyrB, rpoB, infB*, and *atpD*) to divide 18 *Pantoea* species studied into three major groups. Group I consisted of *P. agglomerans*, *P. eucalypti*, *P. vagans*, *P. anthophila*, *P. deleyi*, *P. brenneri*, *P. conspicua*, *P. allii*, *P. ananatis*, and *P. stewartii*; group II consisted of *P. rodasii*, *P. rwandensis*, *P. wallisii*, *P. eucrina*, *P. dispersa*, and *P. cypripedii*; and group III consisted of *P. gaviniae*, *P. calida*, and *P. septica*. Based on the 16S rDNA sequence, all MLST group I spp. belong to the largest 16S rDNA tree cluster, while the MLST group II cluster is divided into two 16S rDNA clusters with *P. rodasii* and *P. rwandensis* in one cluster and *P. wallisii*, *P. eucrina*, *P. dispersa*, and *P. cypripedii* in another. The two clusters are well separated on the 16S rDNA tree. MLST group III is also separated into two with *P. gaviniae* and *P. calida* together as one cluster, and *P. septica* is grouped within the largest *Pantoea* 16S rDNA cluster. Thus, the MLST phylogeny showed good consistency with the 16S rDNA tree in the overall clustering of most but not all species.

Pantoea can be isolated from diverse ecological niches. Many are plant pathogens but some also can promote plant growth (Castagno et al. 2011; Feng et al. 2006; Kuklinsky-Sobral et al. 2004; Loiret et al. 2009; Sergeeva et al. 2007). *P. ananatis* causes disease in a wide range of economically important agricultural crops and forest tree species worldwide (Coutinho and Venter 2009). The symptoms can vary from leaf blotches and spots, dieback, and stalk, fruit, and bulb rot, depending on the host

Table 13.13
Phenotypic characters that can distinguish *Pantoea* species

Characteristic	*P. agglomerans*	*P. allii*	*P. ananatis*	*P. anthophila*	*P. brenneri*	*P. calida*	*P. conspicua*	*P. cypripedii*	*P. deleyi*	*P. dispersa*	*P. eucalypti*	*P. eucrina*	*P. gaviniae*	*P. rodasii*	*P. rwandensis*	*P. septica*	*P. stewartii* subsp.*indologenes*	*P. stewartii* subsp.*stewartii*	*P. vagans*	*P. wallisii*
Acid from																				
Glycerol	(d)	+	+	ND	d	+	–	+	+	(d)	d	–	+	+	+	d	d	–	d	+
Dulcitol	–	–	–	ND	d	–	+	–	–	–	–	–	–	–	+	–	–	–	–	+
Lactose	d	+	+	–	d	+	+	–	–	+	(+)	d	+	–	–	d	+	–	(+)	–
Sucrose	+	+	+	+	ND	ND	–	+	+	+	+	+	+	+	–	d	ND	ND	+	–
Raffinose	(d)	+	+	ND	d	ND	–	–	–	–	–	–	+	–	–	–	+	+	–	–
Utilization of:																				
Adonitol	–	+	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–
i-Erythritol	–	–	–	–	–	–	–	–	–	+	–	d	–	+	+	–	–	–	–	–
Lactose	–	+	+	–	(d)	+	+	+	–	–	+	–	+	d	–	d	+	d	–	–
Lactulose	–	+	+	–	d	+	–	–	–	–	–	–	+	–	–	d	+	d	–	–
L-Fucose	–	–	–	–	+	–	+	–	–	–	–	–	–	–	+	d	ND	ND	–	+
Melibiose	–	+	+	ND	–	+	–	+	–	–	–	–	+	d	+	d	+	+	–	–
D-Psicose	+	ND	+	ND	ND	ND	ND	+	+	(d)	(d)	ND	ND	+	+	ND	ND	ND	–	–
Quinic acid	–	+	(+)	ND	–	–	–	+	–	–	–	–	–	+	+	–	ND	ND	–	d
D-Serine	–	–	–	ND	ND	ND	ND	(+)	–	–	–	ND	ND	d	d	ND	ND	ND	–	–
Sucrose	(+)	+	+	+	+	+	–	+	+	+	+	+	+	+	–	d	ND	ND	+	–
Xylitol	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	d	ND	ND	–	–

Data is obtained from Brady et al. (2009, 2010b, 2011, 2012b)
+: 90–100 % strains positive in 1–2 days; (+): 90–100 % strains positive in 1–4 days; –: negative; *d*: 11–89 % strains positive in 1–4 days; *(d)*: 11–89 % strains positive in 3–4 days; and *ND*: not determined

Table 13.14
Phenotypic characters for differentiating *Pectobacterium* species

Characteristic	*P. aroidearum*	*P. carotovorum* subsp. *carotovorum*	*P. carotovorum* subsp. *odoriferum*	*P. atrosepticum*	*P. betavasculorum*	*P. wasabiae*	*P. cacticida*
Growth at 37 °C	+	+	+	–	+	–	+
Reducing substance from sucrose	–	–	–	+	+	–	–
Acid from α-methylglucoside	–	–	–	+	+	–	–
Gas from D-Glucose	–	–	–	–	–	–	
Utilization of							
D-Alanine	w	–	–	–	–	–	ND
D-Arabitol	–	w	+	–	–	–	–
D-Cellobiose	+	+	+	+	–	+	–
D-Galactonic acid lactone	w	–	–	+	–	+	ND
D-Glucosaminic acid	w	–	–	–	–	w	ND
Glucose-1-phosphate	–	+	–	–	–	–	ND
Glycogen	w	–	–	–	–	–	–
Glycyl-L-aspartic acid	+	–	w	–	–	–	ND
α-Ketoglutaric acid	+	–	–	–	–	–	ND
D,L-Lactic acid	+	–	–	+	+	+	ND
Maltose	w	w	+	–	+	w	–
D-Melibiose	+	+	+	+	–	–	–
D-Raffinose	+	+	+	+	–	–	–
D-Serine	+	–	–	–	–	–	ND
Tween 40, 80	+	–	+	+	w	w	ND
Xylitol	w	–	w	–	–	–	–

Adapted from Alcorn et al. (1991) and Nabhan et al. (2012)
w weak, *ND* not determined, + positive, – negative

infected. *P. vagans*, *P. eucalypti*, *P. deleyi*, and *P. anthophila* were first isolated from eucalyptus leaves and shoots showing symptoms of blight and dieback collected in Uganda, Uruguay, and Argentina as well as maize displaying brown stalk rot symptoms in South Africa (Brady et al. 2009). *P. allii* was isolated from onion seeds in South Africa and from an onion plant exhibiting center rot symptoms in the USA (Brady et al. 2011). *P. rodasii*, *P. rwandensis*, and *P. wallisii* were first isolated from Eucalyptus seedlings showing symptoms of bacterial blight and dieback in Colombia, Rwanda, and South Africa (Brady et al. 2012b).

Some *Pantoea* species are human pathogens. *P. agglomerans* which was classified as *Enterobacter agglomerans* previously is a known human pathogen and seems to be the main *Pantoea* sp. that causes human infections and has now been isolated from nosocomial infections, sepsis, and eye infections (Boszczowski et al. 2012; Cruz et al. 2007; Lee et al. 2010). *P. ananatis* has been associated with corneal infiltrate in an agricultural worker following ocular injury with a rice husk (Manoharan et al. 2012). *Pantoea* has also been isolated from foods. *P. gaviniae* and *P. calida* were first isolated from powdered infant formula and infant formula production environment (Popp et al. 2010).

Pectobacterium Waldee 1945, Hauben et al. 1998 emend

Pectobacterium (N.L. suff. pecto, from Gr. adj. pêktos, curdled, congealed, pertaining to pectin; L. neut. n. bacterium, a small rod; N.L. neut. n. *Pectobacterium*) was first described by Waldee in 1945 (Hauben et al. 1998; Waldee 1945) as a pectolytic bacterium. Cells are 0.5–1.0 × 1.0–3.0 μm in size which occur either singly, in pairs, or in chains. It is motile by peritrichous flagella and is positive for esculin hydrolysis, but negative for arginine, lysine or ornithine decarboxylases, tryptophan deaminase, and urea and starch hydrolysis. Acid is produced from fructose, galactose, glucose, mannose, *N*-acetylglucosamine, ribose, rhamnose, salicin, and sucrose but not from adonitol, arabitol, lyxose, β-methylglucoside, sorbose, starch, and tagatose. The major fatty acids include $C_{12:0}$, $C_{14:0}$, $C_{15:0}$,

$C_{16:0}$, $C_{17:1\omega8c}$, and $C_{17:0}$. There are six species, *P. aroidearum*, *P. atrosepticum, P. betavasculorum, P. wasabiae, P. carotovorum*, and *P. cacticida*, one of which, *P. carotovorum*, contains two subspecies, subsp. *odoriferum* and subsp. *carotovorum*. Biochemical characteristics that are useful to differentiate *Pectobacterium* species are summarized in Table 13.14.

Pectobacterium is a phytopathogen and can cause soft rots, necroses, and wilts of plants (Janda 2006; Ma et al. 2007). *P. atrosepticum, P. carotovorum* subsp. *brasiliensis, P. carotovorum* subsp. *carotovorum*, and *P. wasabiae* were detected in potato stems with blackleg symptoms using species- and subspecies-specific PCR (De Boer et al. 2012). *P. atrosepticum* was most commonly present but was the sole *Pectobacterium* sp. detected in only 52 % of the diseased stems. *P. wasabiae* was most frequently present in combination with *P. atrosepticum* and was the sole *Pectobacterium* sp. detected in 13 % of diseased stems.

Photorhabdus Boemare et al. 1993

Boemare et al. (1993) proposed the genus *Photorhabdus* to include bacterial symbiont of entomopathogenic nematodes, *Heterorhabditis*, which had previously been assigned to the genus *Xenorhabdus* as *X. luminescens. Photorhabdus* is motile by peritrichous flagella. The cell size is 0.5–2 × 2–10 μm. It can produce bioluminescent of which intensity may vary within and between isolates. It is positive for gelatinase and lipase, but negative for oxidase, *o*-nitrophenyl-β-D-galactopyranoside, Voges–Proskauer test, arginine dihydrolase, and lysine and ornithine decarboxylase tests. Acid is produced from glucose, fructose, mannose, and *N*-acetylglucosamine. Variable acid production is observed from glycerol, ribose, and maltose. Succinate, fumarate, L-tyrosine, L-glutamate, and glucosamine can be utilized as the sole carbon source. Optimum temperature is 25 °C and some cannot grow at 37 °C.

Photorhabdus can be easily distinguished from other members of *Enterobacteriaceae* based on the following properties: bioluminescence, a negative nitrate reductase reaction, and annular hemolysis on sheep blood agar (Fischer-Le Saux et al. 1999).

Colony morphology may vary. There are two phases of its growth cycle. Phase one corresponds to the infective stage of the nematode host; and phase two is only observed during in vitro growth. The colonies from phase one culture are highly mucoid and can absorb dye, and the cultures can produce agar-diffusible antimicrobial compounds and lecithinase. Most of them have protein inclusion bodies and are more bioluminescent and lipolytic than those in phase two. In contrast, phase two cells are not mucoid and do not absorb dye, and lecithinase may be detected. Phase one cultures can become phase two during the stationary period of in vitro cultures, but the change from phase two to phase one has not been demonstrated. The differences of these two phases are reviewed by Forst et al. (1997).

There are three recognized species: *P. luminescens*, *P. temperata*, and *P. asymbiotica*. There are no biochemical properties that uniquely define the species for differentiation. The initially defined properties that distinguish the species have become variable properties within a species when more strains are characterized. *P. luminescens* is further divided into nine subspecies: subsp. *carribeanensis*, subsp. *hainanensis* (Tailliez et al. 2010), subsp. *kleinii* (An and Grewal 2011), subsp. *akhurstii*, subsp. *laumondii*, subsp. *luminescens* (Fischer-Le Saux et al. 1999), subsp. *kayaii*, subsp. *thracensis* (Hazir et al. 2004), and subsp. *noenieputensis* (Ferreira et al. 2012). Biochemical characteristics distinguishing the subspecies are found in Table 13.15.

P. temperata is further differentiated into six subspecies, including subsp. *temperata* (Fischer-Le Saux et al. 1999), subsp. *khanii*, subsp. *tasmaniensis*, subsp. *thracensis* (Tailliez et al. 2010), and subsp. *stackebrandtii* (An and Grewal 2010). Biochemical characteristics distinguishing the subspecies are found in Table 13.16.

P. asymbiotica is divided into two subspecies, subsp. *asymbiotica* and subsp. *australis* based on the analyses of *gyrB* and 16S rDNA genes (Akhurst et al. 2004). *P. asymbiotica* subsp. *asymbiotica* is positive for urease and can hydrolyze esculin; acid is produced from trehalose and esculin but variably from gluconate and is negative for DNase and positive for Tween 60 and Tween 80 esterases. *P. asymbiotica* subsp. *australis* differs in that it can grow at a maximum of 40 °C, is positive for DNase, is mostly negative for esculin hydrolysis, and has no urease. Acid is produced from gluconate and variably from esculin but not from trehalose. It is mostly negative for Tween 60 and Tween 80 esterases.

The relationships of the three species have been analyzed using single or multiple housekeeping genes (Akhurst et al. 2004; Peat et al. 2010; Tailliez et al. 2010). Tailliez et al. (2010) performed MLST using four housekeeping genes, *recA*, *gyrB*, *dnaN*, and *gltX*, to determine the relationships of the three species and subspecies. The three species are well separated with *P. luminescens* as a sister clade to *P. temperata* and *P. asymbiotica* diverged first. The MLST sequences also separated the subspecies within each species. In contrast, 16S rDNA sequence can give erroneous species or subspecies relationships as lateral transfer of the 16S rDNA gene seems to have occurred within *Photorhabdus*. A strain that was typed by MLST as *P. luminescens* subsp. *luminescens* is close to *P. asymbiotica* subsp. *asymbiotica* by 16S rDNA sequence. Similarly, two stains belonged to *P. luminescens* subsp. *thracensis* by 16S rDNA sequence but were classified as *P. temperate* by MLST.

Photorhabdus is associated with or pathogenic to nematodes. *Photorhabdus* spp. colonizes the intestines of *Heterorhabditis* spp., which carry them into susceptible insects that are then killed and degraded for nutrients (reviewed by Clarke 2008). Kuwata et al. (2008) showed that *P. asymbiotica* is mutualistically associated with Japanese heterorhabditid entomopathogenic nematodes. *Photorhabdus* can cause human infections (Farmer et al. 1989; Gerrard et al. 2003, 2004; Peel et al. 1999). *P. luminescens* can cause both localized soft tissue and disseminated infections (Farmer et al. 1989; Peel et al. 1999). *P. asymbiotica* is an emergent human pathogen (Gerrard et al. 2004) and has been recovered from patients in both USA and Australia (Weissfeld et al. 2005; Wilkinson et al. 2010).

Table 13.15

Phenotypic characters that differentiate *Photorhabdus luminescens* subspecies

Characteristic	*P. luminescence* subsp.							
	akhurstii	*caribbeanensis*	*hainanensis*	*kayaii*	*kleinii*	*laumondii*	*luminescens*	*noenieputensis*
Upper treshold temperature for growth	37–40	38–40	39–40	37–38	35	35–37	38–40	37
Pigmentation	+	v	–	v(+)	+	+	+	+
Dnase	v(+)	w	–	v(+)		v(+)	+	–
Arginine dihydrolase	–	v	–	v(–)	–	–	–	w
Simmon's citrate	v(+)	+	+	v(+)	ND	v(+)	+	+
Urease	v(+)	+	–	v(+)	+	+	v	+
Indole production	v(+)	v	+	v(+)	–	v	+	–
Voges–Proskauer	–	–	–	v(–)	ND	v(–)	–	+
Esculin hydrolysis	+	+	+	v	ND	v(+)	v	+
Acid production from								
Glycerol	+	+	+	v	ND	+	+	+
Ribose	+	+	+	v(+)	ND	v(+)	v	+
Adonitol	–	–	–	–	ND	–	–	w
Glucose	+	+	+	v(+)	ND	+	+	+
Fructose	+	+	+	v	ND	v(+)	+	+
Mannose	+	+	+	v(+)	ND	v(+)	+	+
Inositol	v(+)	w	–	v(–)	ND	v(–)	+	+
Mannitol	v(+)	+	+	–	ND	v(–)	v	–
Sorbitol	–	–	–	–	ND	–	–	w
N-acetyl glucosamine	v	+	+	v	ND	v(+)	+	+
Esculin	+	+	+	v(+)	ND	v(+)	v	+
Salicin	–	–	–	v(–)	ND	v(–)	–	w
Maltose	+	+	+	v(–)	ND	+	v	+
Trehalose	v(+)	v	+	v(–)	ND	v(+)	w	+
Xylitol	–	–	–	–	ND	–	–	w
L-Fucose	v(+)	+	+	–	ND	–	w	w
5-Ketogluconate	v(–)	v	+	v(–)	ND	v(–)	w	–
Assimilation of								
Inositol	+	+	+	v(+)	ND	+	+	+
Mannitol	+	+	+	–	–	–	+	–
N-acetyl glucosamine	+	–	+	+	ND	+	+	+
Esculin	+	v	+	+	ND	+	v	+
D-Trehalose	v(+)	+	–	v(+)	ND	v(+)	+	+
Xylitol	v(–)	–	–	–	ND	–	–	+
L-Fucose	v	v	–	–	ND	–	–	–
Gluconate	+	+	+	+	ND	v(+)	+	+
Caprate	v	–	–	v(–)	ND	v(–)	+	–
L-Malate	v(+)	+	–	v(+)	ND	v(+)	+	–
Citrate	v(+)	+	–	+	–	+	+	+

Adapted from Ferreira et al. (2012) and An and Grewal (2011)

+: 90 % of strains positive; *v*(+): 50–89 % of strains positive; *v*(–): 11–49 % of strains positive; –: 0–10 % of strains positive; *v*: variable; *w*: weak positive; *ND*: not determined

Table 13.16
Phenotypic characters that differentiate *Photorhabdus temperata* subspecies

	P. temperata subsp.					
Characteristic	*cinerea*	*khanii*	*stackebrandtii*	*tasmaniensis*	*temperata*	*thracensis*
Arginine dihydrolase	ND	+	+	ND	–	–
β-Galactosidase	ND	–	–	ND	–	ND
Gelatinase	ND	+	+	ND	+	ND
Tryptophan deaminase	–	v	v	–	–	ND
Urease	–	+	v	–	v	w
Indole	–	–	–	+	–	–
Citrate (Simmons)	+	v	v	–	v	v
Acid is produced from						
Arabinose	ND	–	–	–	–	ND
Amygdalin	ND	v	v	–	–	ND
Glucose	ND	+	+	+	+	+
Inositol	+	+	+	–	v	–
Mannitol	–	–	–	–	v	–

Adapted from An and Grewal (2010) and Tailliez et al. (2010)
w weak, *v* variable, *ND* not determined, + positive, – negative

Phaseolibacter Halpern et al. 2013

Phaseolibacter (Pha.se.o.li.bac'ter.L. n. phaseolus, a kind of bean with an edible pod, French beans; N. L. masc. N. bacter, a rod; N. L. masc. N. *Phaseolibacter*) is motile with one or two polar flagella, and the cell size is 0.5–0.8 × 1.2–2.3 μm (Halpern et al. 2013). It is able grow at 4–44 °C with 0–60 % sucrose (optimum 10–25 %). Colonies grown on LB or R2A agar for 48 h are 1 mm in diameter. The size is bigger (3–5 mm in diameter) and colonies appear smooth, mucoid, and grayish white in color if the media are supplied with sucrose. It is positive for Voges–Proskauer test. D-glucose, sucrose, and D-melibiose are fermented, while D-mannitol, inositol, sorbitol, rhamnose, and amygdalin are not. It is negative for arginine dihydrolase, lysine and ornithine decarboxylases, H_2S production, indole, gelatinase, urease, and citrate utilization. The major fatty acids are $C_{16:0}$, summed feature 2 ($C_{14:0\ 3}$-OH, $C_{16:1}$ iso I) and summed feature 3 ($C_{16:1\omega7c}$ and/or $C_{15:0}$ iso 2-OH). *P. flectens* is the only species belonging to this genus. This genus was transferred from *Pseudomonas* as strains belonging to this genus shares up to 96.6 % sequence similarities with species in *Enterobacteriaceae* while only 84.7 % to *Pseudomonas aeruginosa* (Halpern et al. 2013). *P. flectens* was first isolated from *Phaseolus vulgaris* (Halpern et al. 2013) and described as the pathogen of the pods of the French bean in Australia by Johnson (1956) and since then no other isolation has been reported.

Plesiomonas Habs and Schubert 1962

The first description of *Plesiomonas* was given by Ferguson and Henderson in 1947 who named it Paracolon C27 (Ferguson and Henderson 1947). The bacterium was described as being Gram-negative, late-lactose fermenting which reacted with *Shigella sonnei* phase I antiserum, but was positive for indole and motile with polar flagella. Schmid et al. (1954) proposed four different biotypes, based on the fermentation of dulcitol, lactose, and salicin. This bacterium underwent renaming several times (Eddy and Carpenter 1964). The genus *Plesiomonas* was only named in 1962 based on the recommendations by Habs and Schubert (Habs and Schubert 1962) and placed within the family *Vibrionaceae*. *Plesiomonas* is phylogenetically more related to the genus *Proteus* (MacDonell et al. 1986; Martinez-Murcia et al. 1992; Ruimy et al. 1994) and is now in the family *Enterobacteriaceae*. It is the only oxidase-positive member of *Enterobacteriaceae*. The sole member of the genus is *P. shigelloides*.

Most strains are motile with lophotrichous flagella, although lateral flagella with a shorter wavelength may be produced by young cultures (Inoue et al. 1991). Inclusion bodies have been detected at early stage of growth which may be composed of polyphosphates, phosphorous, potassium, magnesium, and silicon (Ogawa and Amano 1987; Pastian and Bromel 1984). *P. shigelloides* grows *at* 38–39 °C and at pH 5–8 (Farmer et al. 2006).

P. shigelloides is ubiquitous in surface waters and soil and can cause gastroenteritis and extraintestinal infections (Brenden et al. 1988). It has been reported that *P. shigelloides* co-infects with rotavirus to cause diarrhea (Escobar et al. 2012). Infection may have been quite common in regions of poor hygiene and may have played an ecological role in reducing *S. sonnei* infections. *P. shigelloides* shares the same O antigen as *S. sonnei*. Infection by the former may have provided protection against the latter based on epidemiological evidence that *S. sonnei* increases in regions where living standards are improved and is more common in developed countries than developing nations (Sack et al. 1994).

Pragia Aldová et al. 1988

Pragia (Pra'gi. a. L. fem. n. *Pragia* of Prague, the city in which strains of this genus were identified) is motile with peritrichous flagella (at 22–37 °C) (Aldova et al. 1988). It can oxidize gluconate, produce H_2S, utilize citrate, but is negative for Voges–Proskauer and tyrosine clearing tests. *P. fontium* is the only species within the genus. *P. fontium* strains can grow at 4–37 °C but not at 42 °C. Colonies are approximately 0.5 mm in size on nutrient agar. *P. fontium* is lactose-negative and non-haemolytic. Some strains may produce a *Shigella*-like odor when grown on nutrient agar (Aldova et al. 1988). Most strains are positive for methyl red test but negative for indole, urea hydrolysis, lysine and ornithine decarboxylases, arginine dihydrolase, *o*-nitrophenyl-β-D-galactopyranoside, sodium acetate, and Jordan tartrate. Acid is produced from glucose and galactose but not from other sugars.

All 18 *P. fontium* strains used for initially defining the genus and species were isolated in Czechoslovakia, one isolated from drinking water in Frýdek–Místek area, while 17 others (16 water isolates and 1 stool isolate from a healthy woman) were isolated between 1982 and1986 at the Regional Hygiene Station at České Budějovice (Aldova et al. 1988). There are no recent reports of isolation of organisms from this genus, except a report in 2000 of 28 strains, 18 of which were isolated in Ukraine and 10 in the Czech Republic which was published in a Ukrainian journal (Pokhyl 2000) and details cannot be accessed.

Proteus Hauser 1885

The genus *Proteus* was first named by Gustav Hauser in 1885 to describe shape-shifting bacteria, which were isolated from putrefied meat (Hauser 1885). The name *Proteus* was thought to be given due to the swarming nature of the organisms. Members of this genus are short rods which may vary in length and motile. They grow optimally at 37 °C. They are positive for H_2S production, acid production from glucose and D-xylose, phenylalanine deaminase, tyrosine utilization, growth in KCN, and both urea and gelatin hydrolyses, but negative for Voges–Proskauer, lysine decarboxylase, and arginine dihydrolase (O'Hara et al. 2000a). There are currently four species within this genus, *P. mirabilis, P. penneri, P. vulgaris*, and *P. hauseri* (O'Hara et al. 2000a; b; Rustigian and Stuart 1945). Additionally, three genomospecies (Abdalla et al. 2006; Abo-Amer 2011; Achtman et al. 1999) which are originally within *P. vulgaris* may gain species status in the future. Biochemical characteristics that may be used to differentiate *Proteus* species is summarized in ❯ *Table 13.17*. However, most of these characteristics are variable within a species with few absolute.

Proteus spp. are widespread in the environment and have been isolated from the intestinal tract of mammals, birds and reptiles (Manos and Belas 2006). *P. mirabilis* and *P. vulgaris* are part of the normal flora of the human gastrointestinal tract. Both can cause urinary tract infections in humans, with *P. mirabilis* more common than *P. vulgaris* (Manos and Belas 2006; O'Hara et al. 2000a). *P. mirabilis* is a significant nosocomial pathogen. It can also cause other infections including bacteremia, neonatal meningoencephalitis, empyema, osteomyelitis, and endocarditis (Kalra et al. 2011; O'Hara et al. 2000a). It is also implicated in rheumatoid arthritis with *Proteus* urinary tract infections as a triggering factor (Ebringer and Rashid 2006). Much less is known of *P. hauseri* and *P. penneri. P. penneri* may have been inadvertently identified as *P. mirabilis* as both are indole negative, which differentiates *P. mirabilis* from *P. vulgaris* (Kishore 2012). Of 61 indole-negative *Proteus* isolates tested by Kishore (2012), eight were *P. penneri* with four from urine, three from abdominal drain-fluid, and one from diabetic foot ulcer (Kishore 2012). An intensive care unit *P. penneri* outbreak was reported (Kaistha et al. 2011). In that outbreak, *P. penneri* was isolated from tracheobronchial secretions from 10 patients and from the abdominal drainage fluid from one patient. Additionally, two and one patient had *P. penneri* recovered from urine and blood, respectively.

Providencia Ewing 1962

The genus *Providencia* was established by Ewing (1962) and was described to be composed of motile bacteria which possess characteristics of *Enterobacteriaceae*. Phenylalanine is rapidly deaminated, H_2S is not produced, gas can be produced from sugar fermentation, Voges–Proskauer is negative, urea is not hydrolyzed, and lactose is not fermented (Owen et al. 1987).

There are eight species in this genus: *P. alcalifaciens*, *P. burhodogranariea*, *P. heimbachae*, *P. rettgeri*, *P. rustigianii*, *P. sneebia*, *P. stuartii*, and *P. vermicola* (Ewing 1962; Juneja and Lazzaro 2009; Somvanshi et al. 2006b). *P. rettgeri* was a reclassification from *Proteus rettgeri* (Brenner et al. 1978) and *P. rustigianii* was a renaming of *P. alcalifaciens* biogroup 3 (Hickman-Brenner et al. 1983). Biochemical characteristics that can differentiate these species are summarized in ❯ *Table 13.18*.

Providencia spp. have been isolated from a wide variety of environments, including waste water and animal sources ranging from insects to humans. They have also been isolated from human stools as part of natural flora, although *P. alcalifaciens*, *P. rettgeri*, and *P. stuartii* may cause diarrhea (Guth and Perrella 1996; O'Hara et al. 2000a; Yoh et al. 2005). They have also been recovered from urine, throat, perineum, axilla, stool, blood, burns patients, ocular infections, and wound specimens (Dedeic-Ljubovic and Hukic 2009; Koreishi et al. 2006; O'Hara et al. 2000a; Unverdi et al. 2011). *P. stuartii* and *P. rettgeri* have been associated with urinary tract infections in hospitalized and nursing home patients with long-term indwelling urinary catheters (Dedeic-Ljubovic and Hukic 2009; O'Hara et al. 2000a). *P. stuartii* has been associated with an outbreak in an intensive care unit (Zavascki et al. 2012). *P. sneebia* and *P. burhodogranariea* were isolated from wild fruit fly (*Drosophila melanogaster*) (Juneja and Lazzaro 2009). *P. sneebia* is highly pathogenic to *D. melanogaster* (Galac and Lazzaro 2011). *P. vermicola* has been isolated from nematodes (Park et al. 2011; Somvanshi et al. 2006b).

Table 13.17

Phenotypic characters that differentiate *Proteus* species and genomospecies

Characteristic	Percentage positive at 48 h			
	P. mirabilis	*P. penneri*	*P. vulgaris*	*P. hauseri*
Indole production	2	0	100	100
Citrate (Simmons)	65	4	29	0
Hydrogen sulfide (on TSI Agar)	98	32	57	50
Motility		89	57	100
Gelatin hydrolysis (22 °C)	90	56	57	100
D-Glucose				
Gas production	96	46	86	0
Acid production from				
Glycerol	70	40	29	0
Lactose	2	9	0	0
Maltose	0	96	100	100
a-Methyl-D-glucoside	0	81	86	50
Raffinose	1	9	0	0
L-Rhamnose	1	0	0	0
Salicin	0	0	100	0
Sucrose	15	96	100	100
Trehalose	98	62	0	0
Tartrate (Jordan)	87	89	14	0
Aesculin hydrolysis	0	0	100	0
Acetate utilization	20	12	14	0
Lipase (corn oil)	92	35	14	0
Nitrate reduction	95	12	100	0
ONPG	0	80	57	100
DNase (25 °C)	50	10	0	0

Adopted from O'Hara et al. (2000a, b)
ND not determined

Table 13.18

Phenotypic characters that differentiate *Providencia* species

Characteristic	*P. alcalifaciens*	*P. burhodogranariea*	*P. heimbachae*	*P. rettgeri*	*P. rustigianii*	*P. sneebia*	*P. stuartii*	*P. vermicola*
Utilization of								
D-Adonitol	+	+	+	+	–	–	–	+
D-Arabitol	–	+	+	+	–	+	–	+
L-Arabitol	–	–	+	+	–	–	–	+
Esculin	–	–	–	+	–	+	–	–
D-Galactose	–	–	+	+	+	–	+	+
Inositol	–	+	+	+	–	–	+	+
2-Ketogluconate	–	+	+	+	–	–	–	+
Maltose	–	–	+	–	–	–	–	–
D-Mannitol	–	+	–	+	–	+	–	+
Trehalose	–	+	–	–	–	+	–	–
Xylitol	–	–	–	–	–	–	+	–

Adapted from Juneja and Lazzaro (2009), + positive, – negative

Rahnella Izard et al. 1979

The genus *Rahnella* was proposed by Izard et al. (1979) and contained one species *R. aquatilis*. However, there are at least three genomospecies (GS1–3) (Brenner et al. 1998). *R. aquatilis* is positive for ONPG and motile at 25 °C (nonmotile at 36 °C), but negative for H_2S production, lysine, and ornithine decarboxylases and arginine dihydrolase (Farmer et al. 1985a). It is weakly positive for phenylalanine deaminase and does not produce a yellow pigment (Farmer et al. 1985a). The majority of *Rahnella* strains produce acid from L-arabinose, D-glucose, lactose, D-mannitol, salicin, cellobiose, maltose, D-mannose, melibiose, L-rhamnose, D-xylose, and sucrose (Brenner et al. 1998).

Rahnella is widely distributed in nature and has been isolated from water, soil, and plant roots (Janda 2006). *R. aquatilis* has been rarely linked to human disease, most commonly in immunosuppressed patients (Gaitan and Bronze 2010). A case of intravenous catheter-associated *R aquatilis* infection leading to septic shock has been reported recently (Gaitan and Bronze 2010).

Raoultella Drancourt et al. 2001

Raoultella (Ra.oul.tel'la. M.L. dim. suffix *tella*; M.L. fem. n. *Raoultella* named after the French bacteriologist Didier Raoult) is a nonmotile and capsulated rod (Drancourt et al. 2001). *Raoultella* can grow at 10 °C and is able to use citrate and glucose as a carbon source and produces acid and gas from glucose, and most strains produce 2,3-butanediol from glucose fermentation and are positive for Voges–Proskauer test.

Raoultella was created in 2001 from reclassification of three *Klebsiella* species, *K. ornithinolytica*, *K. terrigena*, and *K. planticola* (Drancourt et al. 2001), as *R. ornithinolytica*, *R. terrigena*, and *R. planticola*, respectively. The biochemical characteristics that can differentiate the three species are summarized in *Table 13.19*.

Raoultella can be isolated from soil, water, vegetables, and fish (Bagley 1985; Podschun et al. 2001; Saha et al. 2011; Yu et al. 2011). *R. planticola* is more frequently isolated than the other two species in the aquatic environment based on the study by Podschun et al. (2001), with 27 of 208 (13 %) water samples collected from streams, lakes, and the Baltic Sea in Germany being positive for *R. planticola*, whereas neither *R. ornithinolytica* nor *R. terrigena* was detected. *Raoultella* has been isolated from a range of human clinical specimens including biliary tract infections (pancreatitis cholecystitis and cholangitis), soft tissue infections including necrotizing fasciitis and surgical wound infection, bacteremia, urinary tract infections, and enteric fever-like syndrome and fever of unknown origin (Alves et al. 2007; Hadano et al. 2012; Kim et al. 2012b; Mau and Ross 2010; Monnet and Freney 1994; Morais et al. 2009; Mori et al. 1989; O' Connell et al. 2010; Olson et al. 2012; Podschun et al. 1998; Podschun and Ullmann 1992; Sener et al. 2011; Solak et al. 2011; Teo et al. 2012; Westbrook et al. 2000; Wolcott and Dowd 2010; Yokota et al. 2012). These infections are caused by either *R. planticola* or *R. ornithinolytica*. A case of sepsis due to *R. terrigena* was reported (Shaikh and Morgan 2011).

Table 13.19
Phenotypic characters that differentiate *Raoultella* species

Test	*R. planticola*	*R. terrigena*	*R. ornithinolytica*
Indole	–	+	–
Ornithine decarboxylase	+	+	–
Utilization of			
4-Aminobutyrate	–	+	–
Benzoate	–	–	+
m-Coumarate	–	+	–
D-Glucosamine	–	–	+
Histamine	–	–	+
5-Ketogluconate	–	–	+
D-Melezitose	–	–	+
Phenylacetate	–	–	+
Putrescine	–	–	+
Quinate	–	–	+
D-Tagatose	+	+	+
L-Tartrate	+	+	+
D-Turanose	–	–	+

Adapted from Drancourt et al. (2001)

Saccharobacter Yaping et al. 1990

Saccharobacter (Sac. cha. ro. bac'ter. L. n. *saccharum*, sugar; M. L. n. *bacter*, a rod; M. L. masc. n. *Saccharobacter*, a sugar rod) is a small rod (0.5–0.9 × 1.0–1.9 μm), motile with peritrichous flagella, and grows at 36–46 °C (Yaping et al. 1990). The colonies on glucose-yeast extract agar are opaque and milky white, nonpigmented, smooth, and low convex with entire edges. It is indole negative but both methyl red and Voges–Proskauer positive. Citrate can be utilized and glucose can be fermented into ethanol, CO_2, and small amounts of acids but no H_2. Glucose is likely to be degraded by the Embden–Meyerhof–Parnas pathway. This is different from the other members of the family of *Enterobacteriaceae* in that the end products of fermentation are mixed acids or 2,3-butanediol. It can ferment D-fructose, sucrose, maltose, D-xylose, L-sorbose, trehalose, L-arabinose, D-galactose, D-mannitol, esculin, L-rhamnose, melibiose, and starch but not dulcitol, *myo*-inositol, raffinose, and gluconate. Lactose is only fermented after long (9 days) incubation. Nitrogen sources include ammonium sulfate, yeast extract, urea, phenylalanine, glutamine, and tryptone but not sodium glutamate. It is unable to hydrolyze gelatin or produce H_2S and negative in urease, nitrate reduction, and Moeller tests for decarboxylase. It is positive in β-galactosidase and arginine dihydrolase and for growth in 0.5 % KCN. It can grow in up to 35 % glucose and 6 % NaCl.

Table 13.20
Phenotypic characters that differentiate *Salmoenella enterica* subspecies and *Salmonella bongori*

Species	*S. enterica*						*S. bongori*
Subspecies	*enterica*	*salamae*	*arizonae*	*diarizonae*	*houtenae*	*indica*	
Characteristic							
Dulcitol	+	+	−	−	−	v	+
ONPG (2 h)	−	−	+	+	−	v	+
Malonate	−	+	+	+	−	−	−
Gelatinase	−	+	+	+	+	+	−
Sorbitol	+	+	+	+	+	−	+
Growth with KCN	−	−	−	−	+	−	+
L(+)-Tartrate	+	−	−	−	−	−	−
Galacturonate	−	+	−	+	+	+	+
γ-Glutamyltransferase	+*	+	−	+	+	+	+
β-Glucuronidase	v	v	−	+	−	v	−
Mucate	+	+	+	− (70 %)	−	+	+
Salicin	−	−	−	−	+	−	−
Lactose	−	−	− (75 %)	+ (75 %)	−	v	−
Lysed by phage O1	+	+	−	+	−	+	v

Adopted from Grimont and Weill (2007)
+: 90 % or more positive reactions; −: 90 % or more negative reactions; *v*: variable; *: Typhimurium d, Dublin –

S. fermentatus is the only species within the genus and was first isolated from squeezed leaf juice of agave in Wuhan, China. There is no 16S rDNA sequence available and no further report of *Saccharobacter* isolation since the original and the only report.

Salmonella Lignieres 1900

Salmonella (N.L. fem. dim. n. *Salmonella*, named in honor of D.E. Salmon, an American bacteriologist) is motile with peritrichous flagella. There are only two species with this genus: *S. enterica* and *S. bongori* (Tindall et al. 2005). Note that there is a species called *Salmonella subterranea* which does not belong to the genus *Salmonella* and remain genus-less and should be renamed to avoid confusion.

S. enterica is further divided into seven subspecies (I, II, IIIa, IIIb, IV, VI, and VII). Subspecies I, II, IIIa, IIIb, IV, and VI have been given subspecies names, *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*, respectively. Subspecies V is given species status as *S. bongori* which is now more commonly recognized. Subspecies VII exists based on genetic analysis and no name was given (Boyd et al. 1996). Biochemical characteristics that can differentiate between *S. enterica* subspecies and *S. bongori* are summarized in *Table 13.20*.

Salmonella is identified by serotyping with more than 2,400 serovars (Grimont and Weill 2007). These serovars were initially designated by Latin binomial species names which are now retained as the serovar names. For example, the old *S. typhimurium* is now serovar Typhimurium.

S. enterica lives in a wide range of hosts, from reptiles to birds and mammals. Subspecies I is generally isolated from warm-blooded animals including birds and mammals and also the most diverse with nearly 1,500 serovars, while other subspecies are isolated from cold-blooded animals. Subspecies I serovars cause the majority (99 %) of salmonellosis in humans and farm animals, although most are not pathogenic in their natural hosts (Wray and Wray 2000). Some serovars have adapted to become specialized clones causing human infections (Kingsley and Baumler 2000). The most distinctive is enteric fever in humans, caused by serovars Typhi, Paratyphi A, Paratyphi B, Paratyphi C, and Sendai (Selander et al. 1996). All except serovars Paratyphi A and Sendai are not closely related to serovar Typhi, having no known close relative among the common serovars (Kidgell et al. 2002). The capacity to cause enteric fever has been independently acquired several times. Several serovars have adapted to specific farm animals (Uzzau et al. 2000). Serovar Choleraesuis is generally believed to have adapted to pigs. It causes swine paratyphoid, but can also be isolated from human infections (Chiu et al. 2004). Serovars Gallinarum and Pullorum are poultry-specific pathogens (Chappell et al. 2009). Serovar Dublin is believed to have adapted to cattle, in which it causes systemic and enteric disease (Nielsen 2013); however, it is also frequently isolated from sheep (Liebana et al. 2002).

Samsonia Sutra et al. 2001

Samsonia (Sam.so'ni.a. M.L. fem. N. *Samsonia* after the French phytobacteriologist Régine Samson) is motile with peritrichous

flagella (Sutra et al. 2001). Cells have rounded ends. It is β-galactosidase positive and indole negative, produces acetoin but not H_2S, and does not have arginine dihydrolase, lysine and ornithine decarboxylases, urease, and tryptophan deaminase. No gas is produced from glucose fermentation and it does not reduce nitrate. Esculin can be hydrolyzed but not gelatin, Tween 80, casein, or lecithin. Citrate and malonate cannot be utilized.

S. erythrinae is the only species within this genus. Colonies on YBGA medium appear white to light beige, circular, convex, and glistening with regular edges and have a diameter of 2–5 mm after 48 h incubation at 28 °C (maximum temperature of 39 °C). Acids are produced from amygdalin, arabinose, inositol, mannitol, rhamnose, and saccharose but not from arabitol, erythritol, inulin, lactose, melibiose, α-methylglucoside, raffinose, and sorbitol. It was first isolated from diseased erythrina (*Erythrina* sp.) trees in Martinique (French West Indies) (Sutra et al. 2001) and there are no further reports of its isolation.

Serratia Bizio 1823

In 1823, Bizio described the red-pigmented bacteria observed on polenta and named it *Serratia marcescens* (Breed and Breed 1924). *Serratia* can grow on minimal medium without the addition of growth factors. Acid is produced from maltose, salicin, and trehalose. ONPG is hydrolyzed. There are 15 species currently recognized: *S. entomophila, S. ficaria, S. fonticola, S. glossinae, S. grimesii, S. liquefaciens, S. marcescens, S. nematodiphila, S. odorifera, S. plymuthica, S. proteamaculans, S. quinivorans, S. rubidaea, S. symbiotica*, and *S. ureilytica. S. marcescens* is divided into two subspecies subsp. *marcescens* and subsp. *sakuensis. Serratia* was first recognized by its non-diffusible red pigment, referred to as prodigiosin (Williams et al. 1956). However, most species are not pigmented. Those that produce pigment include *S. nematodiphila*, both subspecies of *S. marcescens*, *S. rubidaea*, *S. plymuthica*, and *S. fonticola. Serratia* are non-spore forming, except *S. marcescens* subsp. *sakuensis*. One species, *S. ureilytica*, has been described as a urea-dissolving species (Bhadra et al. 2005). Biochemical characteristics that can differentiate these species are summarized in *Table 13.21*.

Serratia can exist as free living or as a symbiont of plants and animals. *Serratia* species can often be found from water sources; *S. fonticola* (Gavini et al. 1979) and *S. ureilytica* (Bhadra et al. 2005) were first isolated from water, while *S. marcescens* subsp. *sakuensis* was from a domestic wastewater treatment tank (Ajithkumar et al. 2003). *S. ficaria* was originally isolated from figs, caprifigs, and fig wasps collected in California and Tunisia as well as from a small black ant in France (Grimont et al. 1979). It can also be found from soil, surfaces of plants, and midgut of invertebrates. *S. symbiotica* is an endosymbiont of black bean aphid *Aphis fabae* (Sabri et al. 2011), *S. nematodiphila* was isolated from the intestine of nematode *Heterorhabditidoides chongmingensis* (Zhang et al. 2009), while *S. glossinae* was from the midgut of the tsetse fly *Glossina palpalis gambiensis* (Geiger et al. 2010).

Shigella Castellani and Chalmers 1919

Shigella was named after the Japanese bacteriologist K. Shiga who first discovered the dysentery bacillus (Ewing 1949). There are four species of *Shigella: S. flexneri, S. boydii, S. dysenteriae*, and *S. sonnei*. The four *Shigella* species actually belong to *Escherichia coli* (Pupo et al. 2000), and there is no genetic basis to separate into four species. However, the genus and species status remain to avoid confusion in medical microbiology. MLST studies have shown that there were seven lineages of *Shigella* within *E. coli*, except *S. boydii* serotype 13 which is outside *E. coli* and belongs to the new species *E. albertii* (Hyma et al. 2005). *Shigella* and enteroinvasive *E. coli* (EIEC) share the same mode of pathogenesis.

There are only a few biochemical properties that are useful for separating *Shigella* species, including utilization of mannitol and ornithine decarboxylation (Farmer and Kelly 1991). *S. sonnei* is positive for both while *S. dysenteriae* is negative for both. *S. flexneri* and *S. boydii* are positive for mannitol but negative for ornithine (Ewing 1986). No biochemical traits can differentiate between *S. flexneri* and S. *boydii* and serology is required for their differentiation to species level. The classic biochemical properties used to differentiate *Shigella* from *E. coli* are the lack of lysine decarboxylase, non-fermentation of lactose and non-motility (Ewing 1986). EIEC may share all these biochemical properties with *Shigella* and additional biochemical tests, such as utilization of L-serine, D-xylose and/or sodium acetate, and mucate fermentation are required (Ewing 1986). Alternatively, biochemical tests including salicin fermentation, aesculin hydrolysis, and the combined positivity of gas from D-glucose and indole production can be used (van den Beld and Reubsaet 2012). EIEC isolates may be positive for one or more of the tests but *Shigella* is generally negative (Doyle and Padhye 1989). *Shigella* is a human-only pathogen. It can naturally infect captive primates leading to shigellosis. However, there is no evidence that shigellosis naturally occurs in wild primates (Germani and Sansonetti 2006).

Shimwellia Priest and Barker 2010

Shimwellia (Shim.wel'li.a. N.L. fem. n. *Shimwellia* named after J. L. Shimwell who first isolated the bacterium) is nonmotile and negative for growth in KCN and H_2S production (Priest and Barker 2010). Currently, this genus contains two species: *S. pseudoproteus* which is a reclassification of *O. proteus* biogroup 2 and *S. blattae* which was reclassified from *Escherichia blattae* (Priest and Barker 2010).

S. pseudoproteus follows the description for *O. proteus* biogroup 2. The cells appear pleomorphic on initial isolation and it prefers to grow near 30 °C and between pH 4.5–8.0. After growing on nutrient agar for 48 h, the colonies are small, circular, and entire, with 1.0–1.5 mm in diameter, convex elevation, and smooth glassy surface. It produces acid but not gas from glucose, rhamnose, and trehalose. No acid is produced from lactose, mannitol, salicin, and many other sugars. It is unable

Table 13.21
Phenotypic characters that differentiate *Serratia* species

Characteristic	*S. entomophila*	*S. ficaria*	*S. fonticola*	*S. glossinae*	*S. grimesii*	*S. liquefaciens*	*S. marcescens* subsp. *marcescens*	*S. marcescens* subsp. *sakuensis*	*S. nematodiphila*	*S. odorifera*	*S. plymuthica*	*S. proteamaculans*	*S. quinivorans*	*S. rubidaea*	*S. symbotica*	*S. ureilytica*
Urease	–	–	–	+	–	–	–	–	–	–	–	–	–	–	ND	+
Catalase	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole	–	–	–	–	–	–	–	–	–	+	–	–	–	–	ND	–
Acetoin	+	+	–	+	–	+	+	ND	–	+	–	+	+	–	ND	+
Utilization of																
L-Lysine	–	–	+	+	+	+	+	ND	+	+	–	+	+	–	–	+
L-Ornithine	–	–	+	+	+	+	+	–	+	+	–	+	+	–	–	+
Citrate	+	+	+	+	–	+	+	ND	+	+	+	+	–	+	–	+
Gelatin	+	+	–	–	–	–	+	ND	ND	+	–	–	+	+	–	+
Pyruvate	+	+	–	+	–	+	+	ND	–	+	–	+	+	–	ND	+
Inositol	+	–	+	+	+	+	+	ND	+	+	–	+	+	+	ND	+
D-Sorbitol	–	+	+	+	+	+	+	+	+	+	+	–	–	–	ND	+
L-Rhamnose	–	+	+	–	–	–	–	–	ND	+	–	–	+	–	ND	–
Sucrose	+	+	–	–	+	+	+	+	+	+	+	+	+	+	ND	+
Melibiose	–	+	+	+	+	+	+	–	+	+	+	–	+	+	ND	–
Amygdalin	+	+	+	+	+	+	+	ND	+	+	+	+	–	+	ND	+
L-Arabinose	–	+	+	+	+	+	+	–	ND	+	+	+	+	+	ND	–
Arbutin	+	+	+	+	+	+	–	ND	ND	+	+	+	–	+	ND	+
Cellobiose	W	W	–	–	+	–	–	ND	+	+	+	–	+	+	ND	+
Dextrin	–	–	–	W	–	–	–	ND	+	W	–	W	+	–	ND	+
Aesculin	+	+	+	+	+	+	+	ND	ND	+	+	+	–	+	+	+
D-Galactose	+	+	+	+	+	+	–	ND	+	+	+	+	+	+	ND	+
Glycerol	+	–	+	+	+	+	+	ND	ND	–	–	+	+	+	–	+
Inulin	–	+	–	–	–	–	–	ND	+	–	–	–	W	–	ND	+
Maltose	+	+	+	+	+	+	+	ND	+	+	+	–	–	+	–	+
Melezitose	–	W	–	–	–	+	–	ND	+	–	+	–	–	–	ND	–
Raffinose	–	–	+	+	+	W	–	–	+	–	+	–	+	+	ND	–
Starch	–	–	–	–	+	–	–	–	+	–	–	–	–	–	ND	–
D-Xylose	–	+	+	+	+	+	–	–	+	+	+	+	+	+	ND	–

Adapted from Sabri et al. (2011), Zhang et al. (2009), and Geiger et al. (2010)
w weak, *ND* not determined, + positive, – negative

to utilize malonate and negative for arginine dihydrolase, lysine decarboxylase, and Voges–Proskauer test.

S. blattae has the same description as those outlined by Burgess et al. (1973). Colonies grown on nutrient agar for 48 h are 1–5 mm diameter in size, circular, smooth, glossy, and creamy in color with a convex elevation and entire edge. It can produce acid and gas from glucose and acid from L-arabinose, glycerol, D-mannose, L-rhamnose, and D-xylose. It is positive for methyl red and lysine and ornithine decarboxylases but negative for arginine dihydrolase and Voges–Proskauer.

The habitat of *Shimwellia* is unclear. There are no further reports of isolations of either species after the initial isolation reports. *S. pseudoproteus* was isolated from ale brewery yeast (Priest and Barker 2010), while *S. blattae* was from a cockroach (Burgess et al. 1973).

Sodalis Dale and Maudlin 1999

Sodalis (So'da.lis. M.L. masc. N. *sodalis* a companion) is microaerophilic, nonmotile, and filamentous with cell size of 2–12 × 1–1.5 μm (Dale and Maudlin 1999). It can grow at 25–30 °C; the optimum atmospheric conditions are 5 % oxygen and carbon dioxide. It is negative for catalase, DNase, gelatinase, urease, nitrate reductase, indole production, hippurate hydrolysis, arginine dihydrolase, lysine, phenylalanine, and ornithine decarboxylases and starch hydrolysis. It can produce α-galactosidase and β-*N*-acetylglucosaminidase but not α-fucosidase, β-galactosidase, α- and β-glucosidase, β-glucuronidase, α-mannosidase, and β-xylosidase. It produces high level of lactic acid from *N*-acetyl-D-glucosamine and raffinose, while weak acid is produced from glucose, glycol chitosan, mannitol, and sorbitol. The only member of *Sodalis* is *S. glossinidius* which was first isolated from the midgut, fat body, and hemolymph of the tsetse fly *Glossina morsitans morsitans* (Dale and Maudlin 1999). *S. glossinidius* is known to live exclusively in endosymbiosis with tsetse flies (*Glossina*) and is one of the few insect bacterial endosymbionts that have been successfully cultured in vitro (Matthew et al. 2005).

Tatumella Hollis et al. 1981, Brady et al. 2010 emend

Tatumella (Ta.tum.ell'la. N.L. fem. dim. N. *Tatumella* named in honor of Harvey Tatum, an American bacteriologist) is non-capsulated, small in size (0.6–1.2 × 0.9–3.0 μm), and nonmotile at 36 °C (Brady et al. 2010c; Hollis et al. 1981). The colonies are nonpigmented but sometimes may appear pale beige to pale orange. It is catalase positive (weak and slow) and positive for glucose, gluconate and 2-ketogluconate dehydrogenases, Voges–Proskauer, methyl red, and Simmons citrate, but negative for urease and gelatinase, H_2S production, lysine and ornithine decarboxylases, tryptophan deaminase, KCN test, lipase, and DNase. Acid is produced from L-arabinose, D-galactose, D-glucose, glycerol, D-mannose, melibiose, D-ribose, and trehalose but not from amygdalin, dulcitol, erythritol, glutarate, glycogen, histamine, *myo*-inositol, methyl α-D-glucoside, propionate, L-rhamnose, sorbitol, or L-sorbose.

Tatumella consists of five species *T. citrea*, *T. ptyseos*, *T. punctata*, *T. terrea*, and *T. morbirosei*. *T. ptyseos* was the first described species for a group of clinical strains formally referred to as "EF-9" (eugenic fermenter) isolated in North and South America between 1960 and 1980 (Hollis et al. 1981). *T. citrea*, *T. punctata*, and *T. terrea* were previously described to belong to the genus *Pantoea* (*P. citrea*, *P. punctata*, and *P. terrea*, respectively), and *T. morbirosei* was previously included in the species *P. terrea* (Brady et al. 2010c). Biochemical characteristics that can differentiate the species of *Tatumella* are summarized in Table 13.22.

Tatumella has been isolated from fruits, soil, and human infections (Brady et al. 2010c; Hollis et al. 1981). The former two sources are associated with *T. citrea*, *T. punctata*, and *T. terrea*, while the last source was associated with *T. ptyseos* which has been isolated from the respiratory tract and blood (Hollis et al. 1981). *T. ptyseos* has also been detected in grapes (Nisiotou et al. 2011) and pineapples as a causative agent of pink disease (Marín-Cevada et al. 2010). *Tatumella* has also been detected from tank water samples (Al-Bahry et al. 2011).

Thorsellia Kämpfer et al. 2006

Thorsellia (Thor.sel.'li.a. N.L. fem. N. *Thorsellia* named in honor of Walborg Thorsell, a mosquito repellent research pioneer in Sweden) is motile (Kampfer et al. 2006). 0Growth can be observed at 15–45 °C (optimum 30 °C) and the generation time is 100 min. The major fatty acids are $C_{16:0}$, $C_{18:1\omega7c}$, and $C_{14:0}$. *Thorsellia* is positive for acid production from glucose, lactose, sucrose, D-mannitol, dulcitol, salicin, adonitol, inositol, rhamnose, maltose, trehalose, cellobiose, D-arabitol, and D-mannose and esculin hydrolysis. No acid is produced from sorbitol, L-arabinose, raffinose, D-xylose, methyl-D-glucoside, erythritol, and melibiose. *T. anophelis* is the only species belonging to the genus.

Thorsellia was first isolated from the midgut of *Anopheles gambiae* mosquitoes (Kampfer et al. 2006) and has also been detected on the surface microlayer (water) of rice paddies in Kenya (Briones et al. 2008). *Thorsellia* may have well adapted to the midgut environment because of its ability to utilize blood to enhance growth and its tolerance to the midgut alkaline conditions (Briones et al. 2008).

Trabulsiella McWhorter et al. 1991

Trabulsiella (Trah bool see ehl'lah) was derived from the surname of L. R. Trabulsi, a Brazilian bacteriologist, and is motile and nonpigmented and positive for lysine and ornithine decarboxylases, arginine dihydrolase, and ONPG test but negative for indole production (McWhorter et al. 1991). Acid is produced from the fermentation of glucose, arabinose, mannitol, rhamnose, and sorbitol.

This genus contains two species, *T. guamensis* and *T. odontotermitis*. *T. guamensis* was previously classified as CDC enteric group 90, based on its biochemical similarity to *Salmonella* subgroups 4 and 5. *T. odontotermitis* can be differentiated from *T. guamensis* based on H_2S production, nitrate reduction, and Voges–Proskauer test for which *T. odontotermitis* is negative for the first two and positive for the latter.

T. odontotermitis was isolated from the gut of the termite *Odontotermes formosanus* Shiraki (Chou et al. 2007).

Table 13.22
Biochemical reactions of the five *Tatumella* species

Characteristic	*T. ptyseos*	*T. citrea*	*T. morbirosei*	*T. punctata*	*T. terrea*
Motility at 36 °C	–	–	–	–	+
Arginine dihydrolase	–	+	w	+	–
Phenylalanine deaminase	+	–	+	–	–
Acid from					
Starch	–	–	+		
D-mannitol	–	+	+	–	–
Raffinose	–	–	–	–	–
Sucrose	+	–	–	+	–
Utilization of				+	+
Adonitol	–	–	+	–	–
L-Arabinose	–	+	+	–	–
Dextrin	–	+	+	–	–
Erythritol	–	+	+	–	–
Formic acid	–	+	w	–	+
Gentiobiose	–	w	–	+	–
Lactulose	–	+	–	–	–
Pyruvic acid methyl ester	–	w	w	–	+
Quinic acid	+	–	–	–	–
L-Tartrate	+	–	–	–	–
Trigonelline	–	–	+	–	–

Adapted from Brady et al. (2010c)
w weak reaction

T. guamensis has been isolated from vacuum cleaner dust, wheat flour, soil, and human feces (McWhorter et al. 1991). There is no evidence that it actually causes diarrhea in humans (McWhorter et al. 1991).

Wigglesworthia Aksoy 1995

Wigglesworthia (Wigg.les.worth'i.a. M.L. fem. n. *Wigglesworthia*, named in honor of the parasitologist V. B. Wigglesworth) (Aksoy 1995) is a nonmotile short rod (1–2 μm in length) which resides in the cytoplasm of the specialized epithelial cells called mycetocytes. Bacterial cells within mycetomes are 4–5 μm in length. It is an obligate endosymbiont although it can be kept for at least 72 h under transient tissue culture conditions. *W. glossinidia* is the only species within the genus and is a primary endosymbiont of tsetse flies (Aksoy 1995; Dale and Welburn 2001; Haines et al. 2002; Symula et al. 2011). There is no type strain for *W. glossinidia*.

Xenorhabdus Thomas and Poinar 1979, Akhurst 1983 emend

Xenorhabdus was first described by Thomas and Poinar (1979) and is a large rod (0.8–2.0 × 4.0–10.0 μm) and motile by peritrichous flagella. *Xenorhabdus* is symbiotically associated with insect-pathogenic nematodes of the genus *Steinernema*. It is unable to reduce nitrate; catalase negative and absent in lysine and ornithine decarboxylases, arginine dihydrolase, phenylalanine deaminase, and urease; and negative for indole and H_2S production and Voges–Proskauer and methyl red tests. Currently there are 22 species recognized. Most species can grow at temperatures of 35–42 °C with a few requiring temperatures of <35 °C. The biochemical characteristics differentiating these species are summarized in *Table 13.23*.

The phylogenetic relationships of the 22 species have been examined using four housekeeping genes (*recA*, *gyrB*, *dnaN*, and *gltX*) (Tailliez et al. 2010, 2012). They were divided into four clades. Clade I included eight species: *X. doucetiae, X. ehlersii, X. griffiniae, X. japonica, X. kozodoii, X. magdalenensis, X. poinarii, X. romanii*, and *X. vietnamensis*. Clade II covered seven species (*X. beddingii, X. hominickii, X. koppenhoeferi, X. mauleonii, X. miraniensis, X. nematophila*, and *X. szentirmaii*) and was not fully resolved except that two pairs of species are clustered together, *X. nematophila* with *X. koppenhoeferi* and *X. beddingii* with *X. miraniensis*. Clade III contained only one species, *X. bovienii*. Clade IV covered five species: *X. budapestensis, X. cabanillasii, X. indica, X. innexi*, and *X. stockiae*. The capacity to grow at temperatures >35 °C was found to be an ancestral character and was lost independently by

Table 13.23

Phenotypic characters that differentiate *Xenorhabdus* species

Characteristic	*X. beddingii*	*X. boviennii*	*X. budapestensis*	*X. cabanillasii*	*X. doucetiae*	*X. ehlersii*	*X. griffiniae*	*X. hominickii*	*X. indica*	*X. innexi*	*X. japonica*	*X. koppenhoeferi*	*X. kozodoii*	*X. magdalenensis*	*X. mauleonii*	*X. miraniensis*	*X. nematophila*	*X. poinarii*	*X. romanii*	*X. stockiae*	*X. szentirmaii*	*X. vietnamensis*
Max growth temperature (°C)	39	32–33	37	39–40	40–42	38–40	39	33–35	39–41	40	34	33	40–41	34	40	38	34–35	40	37	39	37–39	37
Pigmentation	+	+	–	v	+	v(+)	–	+	–	v	–	+	+	–	+	+	–	+	+	+	v(–)	+
DNase	+	+	+	+	v	v(–)	+	+	+	+	+	+	–	+	+	–	+	v(–)	–	+	+	+
Simmon's Citrate	+	v(–)	–	–	–	v(–)	+	–	–	v	–	–	v	–	+	+	v(–)	v(–)	–	–	v(+)	–
Aesculin hydrolysis	+	–	v	–	v	v(+)	+	+	v	+	–	–	v	–	+	+	v(–)	v(–)	–	+	+	+
Lecithinase	+	v(+)	v	v	+	v(+)	+	+	v	+	–	+	+	–	+	+	v(+)	–	–	+	–	+
Acid production from																						
Ribose	+	v(–)	–	–	v	v(+)	+	v(–)	–	+	+	–	+	–	+	+	v(–)	–	–	+	v(–)	–
Glucose	+	+	+	+	v	+	–	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	v(+)	+	v	v	v(+)	+	+	+	+	+	–	–	–	+	+	+	v(+)	+	+	v(+)	+
Mannose	+	+	+	+	v	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	v(+)	+
Inositol	–	v(–)	+	v	v	–	–	v(–)	+	v	–	–	–	–	+	–	v(–)	–	w	+	v(+)	–
sorbitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	v(+)	–
N-Acetylglucosamine	+	+	v	+	v	v(+)	–	v(+)	v	+	+	–	+	–	+	+	+	+	+	+	v(+)	+
Aesculin	+	–	–	–	v	v(+)	+	+	v	–	–	–	+	+	+	+	–	v(–)	–	+	+	+
Maltose	+	v(+)	–	v	v	v(+)	–	v(+)	–	–	+	+	+	+	+	+	v(+)	+	+	+	+	+
Trealose	+	v(+)	–	–	+	–	–	v(–)	–	v	–	–	+	+	+	+	v(+)	v(+)	+	–	–	+
Gluconate	+	v(–)	–	–	–	–	–	–	–	+	–	–	+	–	+	w	–	–	w	+	–	–
5-Ketogluconate	+	v(–)	+	v	–	v(+)	w	+	+	+	+	+	–	–	w	w	v(+)	v(–)	w	w	–	+
Assimilation of																						
Glycerol	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+

Table 13.23 (continued)

Characteristic	*X. beddingii*	*X. boviennii*	*X. budapestensis*	*X. cabanillasii*	*X. doucetiae*	*X. ehlersii*	*X. griffiniae*	*X. hominickii*	*X. indica*	*X. innexi*	*X. japonica*	*X. koppenhoeferi*	*X. kozodoii*	*X. magdalenensis*	*X. mauleonii*	*X. miraniensis*	*X. nematophila*	*X. poinarii*	*X. romanii*	*X. stockiae*	*X. szentirmaii*	*X. vietnamensis*
Ribose	+	v(+)	−	−	v	v(−)	−	v(−)	−	+	−	−	+	−	+	+	−	−	−	+	v(+)	−
Glucose	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+
Fructose	+	v(+)	+	v	v	v(−)	−	v(+)	+	+	+	−	−	−	+	+	+	v(+)	+	v(+)	−	+
D-Mannose	+	+	+	+	v	v(+)	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+
Inositol	−	−	−	−	+	−	w	v(−)	+	+	+	−	−	−	+	w	v(+)	−	+	+	+	w
N-Acetylglucosamine	+	+	v	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+
Aesculin	+	−	v	−	v	v(+)	+	+	v	v	−	−	v	+	+	+	−	v(+)	−	+	+	+
Maltose	+	+	v	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	v	+	v(+)	+	+	v	+	+	−	+	+	+	+	+	+	+	−	v(+)	+
D-Gluconate	+	+	v	−	+	v(+)	+	+	+	+	w	−	v	−	+	+	v(−)	+	+	+	+	+

Adopted from Tailliez et al. (2012)

+: more than 90 % positive; *v*(+): 50–89 % positive; *v*(−): 11–49 % positive; −: less than 10 % positive; *v*: variable; *w*: weak

a few species, *X. bovienii* (III), *X. hominickii*, *X. koppenhoeferi* and *X. nematophila* (II), and *X. japonica* (I). The species demarcation was found to be at a cutoff of 97 % nucleotide identity based on the concatenated sequence of the four genes (Tailliez et al. 2012).

Xenorhabdus has not been isolated in free-living form from soil or water. *Xenorhabdus* is carried in the intestine of nematodes (Boemare and Akhurst 2006). *X. indica* was isolated from a recently described nematode species, *Steinernema thermophilum* (Somvanshi et al. 2006a). *X. magdalenensis* was isolated from the nematode *Steinernema australe* (Tailliez et al. 2012). *Xenorhabdus* seems not to be a human pathogen. The earlier reports of *Xenorhabdus* human infections were due to *X. luminescens* which is now *Photorhabdus luminescens*.

Yersinia van Loghem 1944

Yersinia was named after the French bacteriologist A. J. E. Yersin who discovered the plague bacillus in 1894 (Van Loghem 1944). Most species of the genus are motile (at 25 °C, nonmotile at 37 °C) and urease positive and grow optimally at 22–25 °C. Acid but not gas is produced from glucose. *Yersinia* comprises 16 species, including *Y. aldovae*, *Y. aleksiciae*, *Y. bercovieri*, *Y. enterocolitica*, *Y. entomophaga*, *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii*, *Y. massiliensis*, *Y. mollaretii*, *Y. nurmii*, *Y. pekkanenii*, *Y. pestis*, *Y. pseudotuberculosis*, *Y. ruckeri*, *Y. similis*, and *Y. rohdei*. Neubauer et al. (2000) proposed *Y. enterocolitica* to be separated into two subspecies, subsp. *enterocolitica* and subsp. *palearctica*. Biochemical characteristics useful for differentiating *Yersinia* species are summarized in *Table 13.24*.

Kotetisvili et al. (2005) used MLST based on four genes, *glnA*, *gyrB*, *recA*, and *hsp60*, to analyze 58 strains representing 11 *Yersinia* species. For species with multiple strains, *Y. bercovieri*, *Y. enterocolitica*, *Y. intermedia*, *Y. pestis*, *Y. pseudotuberculosis*, and *Y. rohdei* were shown to be grouped together according to species showing single origin of the respective species. However, *Y. frederiksenii* and *Y. kristensenii* are not grouped by species, both of which have three independent lineages with one major cluster, suggesting multiple independent origins. The strains not falling into the major clades of these species should be considered for reclassification. *Y. ruckeri* was shown to be the most distantly related species within the genus (Kotetishvili et al. 2005). *Y. pestis* is known to be a clone of *Y. pseudotuberculosis* (Achtman et al. 1999).

Laukkanen-Ninios et al. (2011) analyzed seven gene fragments (*glnA*, *thrA*, *tmk*, *trpE*, *adk*, *argA*, and *aroA*) from 387 *Y. pseudotuberculosis* isolates and 31 isolates of other *Yersinia* species and showed that they were divided into four populations. Three of the populations corresponded to a species (*Y. pseudotuberculosis sensu stricto* (s.s.), *Y. pestis*, and *Y. similis*). The fourth population is yet to be given a species name and was referred to in that study with a vernacular name, Korean group.

Yersinia occupies a diverse range of niches. Three *Yersinia spp.*, *Y. enterocolitica*, *Y. pseudotuberculosis*, *and Y. pestis*, are significant pathogens for mammals including humans. However, the others, although generally treated as nonpathogenic, do have some pathogenic potential (Carniel 2003; Sulakvelidze 2000). *Y. pseudotuberculosis* and *Y. enterocolitica* are food and waterborne pathogens that cause enterocolitis in humans. *Y. pestis*, the causative agent of plague, is transmitted primarily by fleas and has been responsible for devastating epidemics throughout history. *Y. enterocolitica* is widely distributed in nature in aquatic environment and animals (Bottone 1997). Pigs are a major reservoir for human pathogenic strains of *Y. enterocolitica* (Fredriksson-Ahomaa et al. 2006). *Y. pseudotuberculosis* has been isolated mainly from warm-blooded animals like pigs, rodents, and birds and is also present in the environment (Fukushima et al. 2001). Contaminated water is also an important reservoir for *Y. pseudotuberculosis* (Fukushima et al. 2001).

Y. entomophaga was first isolated from diseased larvae of the New Zealand grass grub, *Costelytra zealandica* (Hurst et al. 2011). *Y. nurmii* was designated to three strains isolated from broiler meat packaged under modified temperature (Murros-Kontiainen et al. 2011a). *Y. pekkanenii* was first described to include three strains isolated from water, soil, and lettuce samples (Murros-Kontiainen et al. 2011b). *Y. ruckeri* is a fish pathogen and the causative agent of enteric red mouth disease of salmonids, mainly rainbow trout (Furones et al. 1993). *Y. massiliensis* was first isolated from fresh water in Marseilles, France (Merhej et al. 2008).

Yokenella Kosako et al. 1984

The name *Yokenella* was derived from the Japanese abbreviation "Yoken" for the National Institute of Health, Tokyo, and a modern Latin feminine noun formed by adding the ending "-ella." *Yokenella* is motile with peritrichous flagella and nonpigmented and grows at 37 °C but not 4 °C. It is negative for Voges–Proskauer and esculin hydrolysis. Acid and gas are produced from glucose and other carbohydrates. It can utilize citrate but not malonate, grows in KCN, and produces lysine and ornithine decarboxylases as well as β-galactosidase. L-arabinose, cellobiose, levulose, melibiose, and L-rhamnose can all be fermented. *Y. regensburgei* is the only species of this genus. *Y. regensburgei* has been isolated from well water, reptiles, salads, and the intestinal tract of insects and has also been isolated from human clinical samples (Abbott and Janda 1994; Fajardo Olivares et al. 2005; Kosako et al. 1984; Lo et al. 2011; Stock et al. 2004). However, there are very few cases of human *Yokenella* infections (Lo et al. 2011).

Concluding Remarks and Future Prospects

There has been a sharp increase of the number of genera and species in the family of *Enterobacteriaceae* since last edition of this book, and there is no doubt more species will be discovered. New approaches are needed to keep up to date the expansion of the family. The large number of genera/species made it hard or

Table 13.24
Phenotypic characters that differentiate *Yersinia* species

Characteristic	*Y. nurmii*	*Y. ruckeri*	*Y. mollaretii*	*Y. aldovae*	*Y. bercovieri*	*Y. intermedia*	*Y.rohdei*	*Y. enterocolitica* subsp. *enterocolitica*	*Y. enterocolitica* subsp. *palearctica*	*Y. aleksiciae*	*Y. frederiksenii*	*Y. kristensenii*	*Y. pestis*	*Y. pseudotuberculosis*	*Y. similis*	*Y. massiliensis*	*Y. entomophaga*	*Y. pekkanenii*
Cellobiose	w	–	+	–	+	+	+	+	+	+	+	+	–	–	–	+	+	+
Citrate	+	–	+	+	–	+	+	–	–	–	+	–	–	–	+	+	+	–
D-Xylose	–	–	+	+	+	+	+	+	–	+	+	+	+	+	+	+	–	+
Esculin	–	–	w	–	–	+	–	–	–	–	+	–	+	+	+	+	–	–
Glycerol	+	+	+	+	w	+	+	+	+	+	+	+	–	w	+	+	+	–
Indole	–	–	–	–	–	+	–	+	–	–	+	+	–	–	–	+	–	–
Inositol	–	–	w	+	–	+	–	w	+	w	+	w	–	–	–	+	–	–
Lactose	w	–	w	–	–	–	w	–	–	–	–	+	–	–	–	–	+	w
L-Arabinose	–	–	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+
Lysine	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Melibiose	–	–	–	–	–	+	+	–	–	–	–	–	–	+	–	–	+	–
ONPG	+	+	+	–	+	+	+	+	+	+	+	+	–	+	+	+	ND	+
Ornithine	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–
Raffinose	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	+	–
Rhamnose	–	–	–	+	–	+	–	–	–	–	+	–	–	w	+	–	–	–
Salicin	–	–	w	–	w	+	w	–	–	w	+	+	–	+	w	+	–	–
Sorbitol	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	–
Sorbose	–	–	+	–	–	+	–	+	+	–	+	+	–	–	–	+	–	–
Sucrose	+	–	+	–	+	+	+	+	+	–	+	–	–	–	–	w	+	–
Urea	–	–	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	+
Voges–Proskauer	+	–	–	+	–	+	–	–	–	–	+	–	–	–	–	–	ND	–

Adapted from Hurst et al. (2011) and Murros-Kontiainen et al. (2011a, 2011b)
w weak, *ND* not determined, + positive, – negative

nearly impossible to do a comprehensive review of the family *Enterobacteriaceae* without a community-based effort. Much has to be left out of this chapter such as antibiotic sensitivity of a species/genus, DNA–DNA hybridization data, and detailed genome data.

The 16S rDNA sequence has provided a remarkable taxonomy of *Enterobacteriaceae* at genus level with many relationships consistent with genome data. However, 16S rDNA sequence is clearly inadequate at species level due to mostly insufficient phylogenetic signal and to a much lesser or unknown extent of horizontal transfer (recombination) of the 16S rRNA gene. We made no attempt to add bootstrap values to the 16S rDNA tree, and it is very much doubtful that any of the nodes will be supported with high values. Therefore, interpretation of the relationships depicted in *Fig. 13.1* requires caution and should be done in conjunction with other data.

Interestingly, the genome trees also presented some anomalies. We clearly need more data than 16S rDNA, but selective use of the genome data is also critical to deduce the correct phylogeny of the members of *Enterobacteriaceae*. This aspect is illustrated by the phylogenetic position of the endosymbionts which has been shown to have derived multiple times within *Enterobacteriaceae* (Husnik et al. 2011). The study illustrated the need for more data as well as appropriate algorithms for phylogenetic inference (Philippe and Roure 2011).

Much reclassification of the *Enterobacteriaceae* family members has occurred in the past few years. This effort has reduced the number of polyphyletic genera. However, there are still many more to deal with. At least seven genera are polyphyletic. There are also cases where strains within a species do not share a single origin such as *Yersinia frederiksenii* and *Y. kristensenii*. The genus *Enterobacter* requires special attention as its species are scattered across the 16S rDNA tree. This leads to difficulty to establish the phenotypic and genetic identity of the genus. The genus *Shigella* is also a historical relic we have to face as *Shigella* genetically belongs to *E. coli* (Pupo et al. 2000). The wide adoption of the current nomenclature for *Salmonella* that replaced the old scheme of hundreds of species names gives us the encouragement that a reclassification of *Shigella* will be acceptable to the scientific community without compromising its medical significance.

A consensus of genus, species, and subspecies concept and classification criteria must be established for *Enterobacteriaceae* and for bacteria in general, although it has been very difficult so far. There have been much debate on species concept (Konstantinidis et al. 2006; Rossello-Mora 2005), but there has been very little debate on the status of genus and subspecies. A minimum requirement for a genus would at least be that a genus is a monophyletic group of species. Unfortunately, there were several cases (e.g., *Klebsiella michiganensis, Pantoea rodasii and P. rwandensis*) that a recent addition of a new species made the genus polyphyletic or containing even more independent lineages. An interesting case is *Salmonella subterranea*; it is genus-less as it does not belong to *Salmonella*. There are also several changes related to subspecies in *Enterobacteriaceae*. Subspecies should only be given to those that bear significant biological meaning. For example, *Salmonella enterica* subspecies I (subsp. *enterica*) is found mostly in warm-blooded animals, different from the other subspecies which are more commonly found in cold-blooded animals (Selander et al. 1996). The division correlates with niche distinction and adaptation/expansion. Many of the new subspecies added presented no obvious insight into their ecological distinctiveness or biological significance, although we do not argue for an ecological subspecies definition.

There is a clear need to further develop genome-based classification of genus/species within *Enterobacteriaceae*. This does not mean that we need the full genome but we would need to define a minimum set of genes to obtain an accurate inference of the relationships of the species and genera. Recombination within and between genera will affect the use of any genes. An effort to identify a set of genes present in most if not all of the *Enterobacteriaceae* may fulfill this need. Recent studies suggest that the traditional DNA–DNA hybridization may be replaced by digital DNA–DNA hybridization using genome sequence (Auch et al. 2010; Konstantinidis and Tiedje 2005). Other algorithms making use of genome data have also been developed to define a species (Auch et al. 2010; Konstantinidis et al. 2006). We are hopeful in the next edition of this book that genome-based phylogeny, genome content difference, ecological distinction, and phenotypic differentiation will delineate the members of *Enterobacteriaceae* with less phylogenetic uncertainty and more biological understanding.

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14 The Family *Francisellaceae*

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Abstract
The family *Francisellaceae* Sjöstedt (The proteobacteria, part B. *Bergey's Manual of Systematic Bacteriology*. Springer-Verlag, New York, 2005, pp. 200–210), most closely related to *Caedibacter taeniospiralis and Fangia hongkongensis*, within the Gammaproteobacteria, comprises the single type genus *Francisella* Olsufjev (J Hyg Epidemiol Microbiol Immunol, 14:67–74, 1970). The genus consists of small (0.7–1.7 μm), nonmotile, Gram-negative (staining faintly), aerobic coccobacilli, which may or may not require additional cysteine (or cystine) for culture, weakly catalase positive, and most (but not all) produce H_2S when cultured in cysteine-containing media. Members of the *Francisella* grow with entire, slightly convex pale white or gray, semitranslucent, mucoid colonies. Incubation time and temperature is dependent on species and strain, but they are relatively slowly growing. Many are capable of facultative intracellular growth. The *Francisella* have a trans-global distribution, although most isolates have been recovered in the Northern Hemisphere. The type species is *F. tularensis*, which is further divided into four subspecies, i.e., *tularensis*, *holarctica*, *mediasiatica*, and *novicida*. Subspecies *tularensis* and *holarctica* commonly called biotypes A and B, respectively, can in turn be further divided into several subpopulations based on genetic analysis. *F. tularensis* causes the disease tularemia in mammalian species and represents a potential category A bioterror weapon. Several members of the genus, e.g., *F. noatunensis* (subspecies *noatunensis* and *orientalis*) *and F. halioticida*, are highly virulent pathogens of fish and molluscs. These species can be readily distinguished from the remainder of the genus by their lower optimal and cardinal growth temperatures. Despite the relatively small number of described species, an increasing body of evidence exists for the existence of a large and diverse environmental population of as-yet undescribed *Francisella* species.

The *Francisellaceae*

N.L. fem. n. *Francisella*, type genus of the family; suff. *-aceae*, ending to denote family; N.L. fem. pl. n. *Francisellaceae*, the *Francisella* family (Sjöstedt 2005).

Taxonomy, Historical, and Current

The genus *Francisella*, named after Edward Francis, an American bacteriologist who extensively studied the relationship between tularemia and its etiological agent, currently contains four validly published species, further subdivided into six subspecies, (see *Table 14.1*) of which the type and most studied species is *F. tularensis*, a highly infectious bacterium causing the disease tularemia in mammals, including humans and a potential

E. Rosenberg et al. (eds.), *The Prokaryotes – Gammaproteobacteria*, DOI 10.1007/978-3-642-38922-1_236,

Table 14.1
Recognised species within the genus Francisella

Validly published name	Heterotypic synonyms
Francisella tularensis	
Francisella tularensis subsp. *tularensis*	
Francisella tularensis subsp. *holarctica*	
Francisella tularensis subsp. *mediasiatica*	
Francisella tularensis subsp. *novicida*	*F. novicida*
Francisella philomiragia	*F. philomiragia* subsp. *philomiragia*
Francisella noatunensis	
Francisella noatunensis subsp. *noatunensis*	*F. piscicida, F. philomiragia* subsp. *noatunensis*
Francisella noatunensis subsp. *orientalis*	
Francisella hispaniensis	
Francisella halioticida	
Francisella guangzhouensis	

Ref: www.bacterio.net

category A bioterror weapon (Rotz et al. 2002). Originally termed *Bacterium tularense* (McCoy and Chapin 1912), *F. tularensis* was subsequently proposed to represent both a member of the *Pasteurella* (Bergey et al. 1923) and *Brucella* (Topley and Wilson 1929) prior to the establishment of the genus *Francisella* (Dorofeev 1947). *F. tularensis* was then subsequently divided into subspecies *tularensis* (Olsufjev and Meshcheryakova 1983), subspecies *holarctica* (Olsufjev and Meshcheryakova 1983; Olsufjev et al. 1959), and subspecies *mediasiatica* (Aikimbaev 1966; Olsufjev and Meshcheryakova 1983; Olsufjev and Meshcheryakova 1982). *Francisella novicida* (Olsufjev et al. 1959), isolated from water, originally described as *Pasteurella novicida* (Larson et al. 1955) and proposed to represent a further subspecies of *F. tularensis* (Sjöstedt 2005), was not validated as such until Huber et al. (2010). Inclusion of *F. novicida* within *F. tularensis* has been the subject of discussion within the *Francisella* research community (Johansson et al. 2010a; Busse et al. 2010).

The genus *Francisella* can be separated into two major lineages, i.e., the *F. tularensis* lineage and a rather more diverse clade comprising the animal – and opportunistic human – pathogen first described as *Yersinia philomiragia* (Jensen et al. 1969), subsequently *F. philomiragia* (Wenger et al. 1989), along with diverse environmental isolates and pathogens of poikilothermic organisms. The first fish pathogenic species characterized was the agent of francisellosis in Atlantic cod (*Gadus morhua*) which was originally considered to represent a subspecies of *F. philomiragia*, i.e., subspecies *noatunensis* (Mikalsen et al. 2007). Shortly after, Ottem et al. (2007a) proposed establishment of a new species, *F. piscicida*, based on examination of a single isolate also from diseased Atlantic cod. As 16S rDNA sequences for *F. philomiragia* subsp. *noatunensis* and *F. piscicida* were 100 % similar, it was considered that they may represent heterotypic synonyms (IJSEM 2008). This was subsequently proven to be the case with both Ottem et al. (2009) and Mikalsen and Colquhoun (2009) proposing elevation of *F. philomiragia* subsp. *noatunensis* to *F. noatunensis*. In separate studies, *Francisella* strains (including a strain common to both studies) isolated from tilapia and three-lined grunt were proposed to represent (1) a subspecies of *F. noatunensis* (subsp. *orientalis*) (Ottem et al. 2009) and (2) an independent species, *F. asiatica* (Mikalsen and Colquhoun 2009). As the latter proposal is not, due to the rule on prior publication, considered validly published, it cannot be correctly used. A single isolate first isolated from farmed Atlantic salmon in Chile described by Birkbeck et al. (2007) has also been established to represent the only non-North Atlantic isolation of *F. noatunensis* subsp. *noatunensis* (Sjödin et al. 2012). Further recent additions to the genus include *F. hispaniensis* (Huber et al. 2010) isolated from human blood, *F. halioticida* (Brevik et al. 2011a) a pathogen of the abalone (*Haliotis gigantea*), and an until recently uncultured endosymbiont of the ciliate *Euplotes raikovi* proposed to represent *Candidatus* F. noatunensis subsp. endociliophora (Schrallhammer et al. 2011).

The Phylogenetic Structure of the Genus *Francisella*

On analysis of 16S rDNA sequences, the *Francisella* cluster (*Fig. 14.1*) within the gamma-subgroup of proteobacteria with their closest neighbors represented by *Fangia hongkongensis*, isolated from the outlet of a seawater sand filter (Lau et al. 2007), and *Caedibacter taeniospiralis*, an obligate bacterial endosymbiont of *Paramecium tetraurelia* (Beier et al. 2002). *Francisella* species are more distantly related to the fish pathogen *Piscirickettsia salmonis* and members of the *Legionellaceae*, including *Legionella pneumophila* (Larsson et al. 2005).

At the genus level 16S rDNA-based phylogeny (*Fig. 14.2*) demonstrates that most members can be grouped into one of the two major sister clades, previously designated clades I and II (Sjödin et al. 2012). Several branches are, however, poorly supported with low bootstrap frequencies in 16S rDNA analysis, which may in part reflect the relatively small genetic distances between taxa. While less comprehensive, inferred phylogenetic network analysis of whole-genome sequences indicates a substantial number of conflicting phylogenetic signals within the genus (*Fig. 14.3*), suggestive of recombination, which also may contribute to the lack of robustness of the 16S rDNA-based phylogeny.

Clade I includes *F. tularensis*, *F. hispaniensis*, and organisms thought to represent endosymbionts of arthropods, i.e., *Francisella*-like endosymbionts. *F. tularensis* subspp. *tularensis*, *holarctica*, and *mediasiatica* represent a single distinct monophyletic group, while a polyphyletic pattern is observed for *F. tularensis* strains classified as subsp. *novicida* or *novicida*-like. Within the latter group, some strains cluster with *F. tularensis*, while other organisms are monophyletic

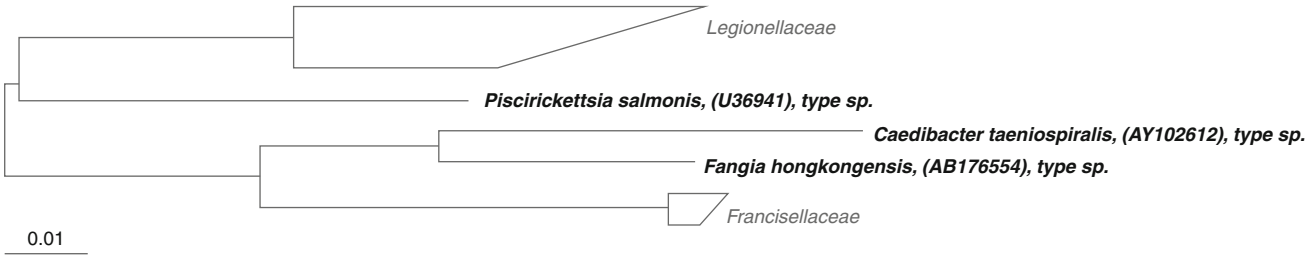

Fig. 14.1

Phylogenetic reconstruction of the family *Francisellaceae* based on 16S rDNA and created using the neighbor-joining algorithm with the Jukes-Cantor correction. The sequence datasets and alignments were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010, http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high-quality type strain sequences proportionally distributed among the different bacterial and archaeal phyla. In addition, a 40 % maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. Scale bar indicates estimated sequence divergence

with *F. hispaniensis*. Polyphyly for *F. tularensis* subsp. *novicida* and *novicida*-like organisms was also indicated in a previous genome-based study (Sjödin et al. 2010) suggesting that the taxonomic classification of this group should be revisited. A strongly supported monophyletic group formed by *Wolbachia persica* and *Francisella*-like endosymbionts is found deeply rooted within clade I. It has long been argued that *Wolbachia persica* should be reclassified as a *Francisella* species (Forsman et al. 1994; Noda et al. 1997; Niebylski et al. 1997).

Phylogenetic relationships within *F. tularensis* are shown in Fig. 14.4, inferred from whole-genome sequences. In contrast to the *novicida* group, the evolutionary processes within the pathogenic *F. tularensis* strains are typified by a limited or complete lack of recombination, resulting in extremely low levels of homoplastic genetic variation (Larsson et al. 2009; Sjödin et al. 2012). The more virulent subspecies *F. tularensis* subspp. *tularensis*, *mediasiatica*, and *holarctica* form a sister clade to most strains classified as subsp. *novicida* or *novicida*-like. Of the more virulent *F. tularensis* subspecies, *tularensis* and *mediasiatica* are monophyletic and form a sister clade to subsp. *holarctica* (Fig. 14.4a). Subspecies *tularensis* is divided in two distinctly separate subgroups, clades AI and AII, originally identified using multilocus VNTR analysis (Johansson et al. 2004a), with low overall genetic diversity (Fig. 14.4d). Both AI strains have been further delineated using pulsed-field gel electrophoresis (PFGE) into genetic clusters AIa and AIb (also denoted A-east and A-west), and AIIa and AIIb (Kugeler et al. 2009; Staples et al. 2006). It is not at present known how these clusters correspond to nucleotide-based phylogenies. *F. tularensis* subsp. *holarctica* can also be subdivided into four major subpopulations (Fig. 14.4b). Most strains fall into one of the three subpopulations (BI, BII, and BIV) which share a comparatively recent ancestry, whereas strains within the B.V clade, representing biovar japonica (Jellison 1974), are phylogenetically more deeply rooted. As is also true for subsp. *mediasiatica*, the genomic diversity of members within B.V is poorly understood due to the limited availability of genomic sequences. Most of the members classified as subsp. *novicida* or *novicida*-like form a clade which is more diverse than other *F. tularensis* taxa (Fig. 14.4c).

Clade II includes the species *F. noatunensis*, *F. philomiragia*, and *F. halioticida* and additional clinical and environmental *Francisella* organisms. The monophyly of *F. philomiragia* and *F. noatunensis* subsp. *noatunensis* and subsp. *orientalis* is indicated in the 16S-based rRNA gene tree (Fig. 14.2). The exact phylogenetic positions of these taxa remain, however, indeterminate in relation to other members of clade II, including the recently discovered taxa *Candidatus* F. noatunensis subsp. endociliophora (Schrallhammer et al. 2011), *F. halioticida* (Brevik et al. 2011a), and the clinical human isolate *Francisella* TX077308 (Siddaramappa et al. 2012), due to the weakly supported 16S rDNA topology. The ambiguous relationships within clade II are further indicated by the extensive network structure observed for *F. philomiragia* and *F. noatunensis* spp. *noatunensis* and *orientalis* in the network phylogeny (Fig. 14.3). Comparative analysis of *Francisella* core genes indicated, however, that members of *F. noatunensis* subp. *noatunensis* were more closely related to *F. philomiragia* than subsp. *F. noatunensis* subsp. *orientalis* (Sjödin et al. 2012b).

Several recently discovered *Francisella* taxa are found to diverge prior to the division of clades I and II. Two human clinical *Francisella* isolates, diverging outside the major clades, form a tight cluster and have been proposed to represent a novel *Francisella* species (Kugeler et al. 2008). As for clade II taxa, the phylogenetic position of these isolates remains unclear as *sdh*A, and 16S analyses have proposed their contrasting placement in either clade I or as deeply rooted taxa similar to that observed in Fig. 14.2. Several isolates obtained from air-conditioning systems (Qu et al. 2009), subsequently *designated* Francisella guangzhouensis (Qu et al. 2013) appear to have diverged prior to the two major clades.

Genomics

Since publication of the first *Francisella* genome (*F. tularensis* subsp. *tularensis* SCHU S4) in 2005 (Larsson et al. 2005),

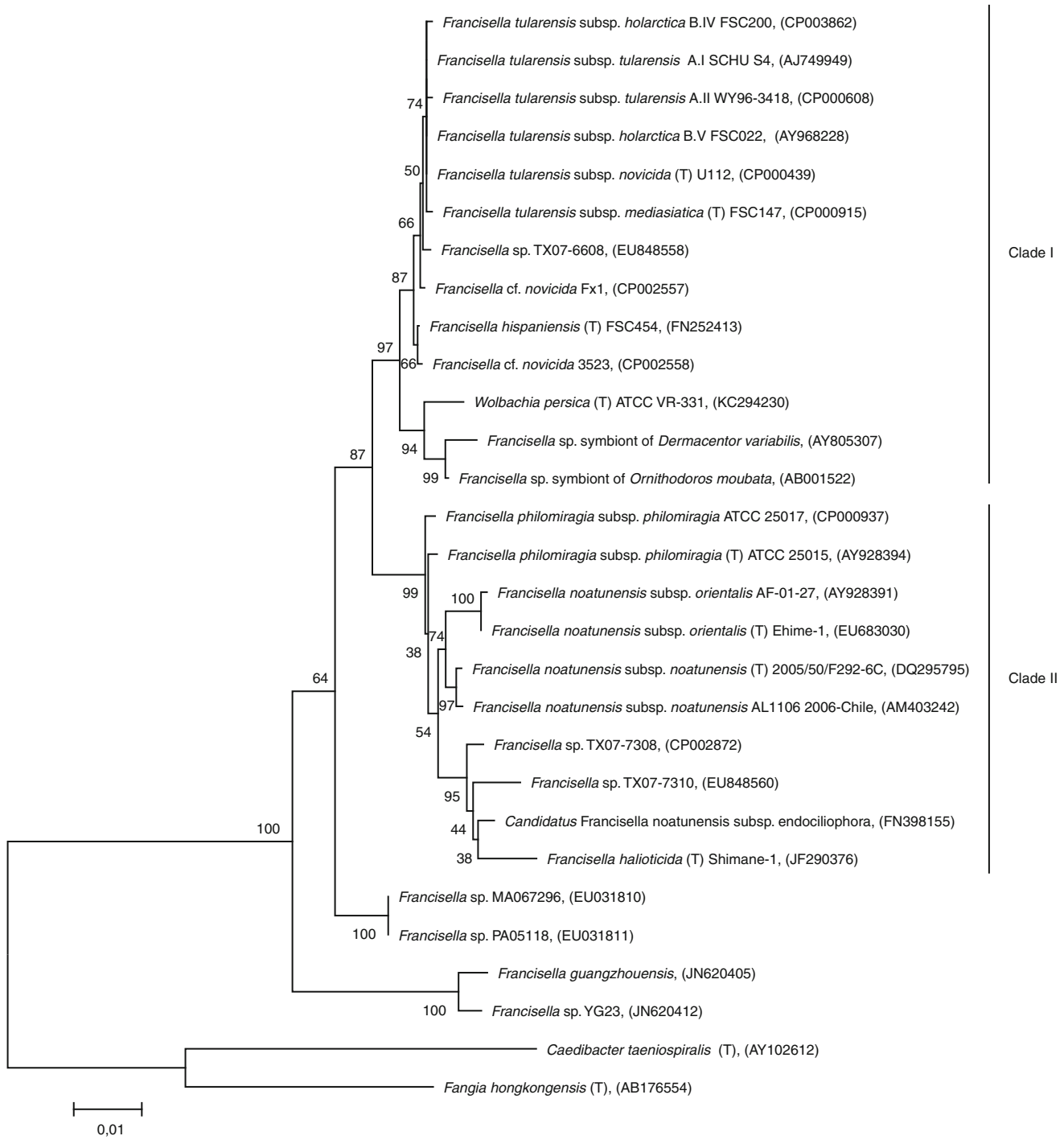

Fig. 14.2

Phylogeny of *Francisella* taxa based on 16S rRNA gene sequences (neighbor-joining method; Jukes-Cantor distances; gaps and missing data were eliminated). Bootstrap support values were obtained using 1,000 pseudo-replicates and are shown next to the branches. Multiple alignment was performed using Muscle (Edgar 2004) and evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011)

complete genome sequences representing all subspecies of *F. tularensis*, *F. philomiragia*, and several environmental *Francisella* species have now been published (Table 14.2). In addition, a large number of *Francisella* draft genome sequences are available in sequence databases worldwide.

Francisella genomes comprise single chromosomes of approximately 2 Mbp, of relatively low GC content (Table 14.2). Pairwise genomic analyses of average nucleotide identities (ANI) reveal levels of genomic relatedness of ≈85 % between members of *Francisella* clades I and II which is

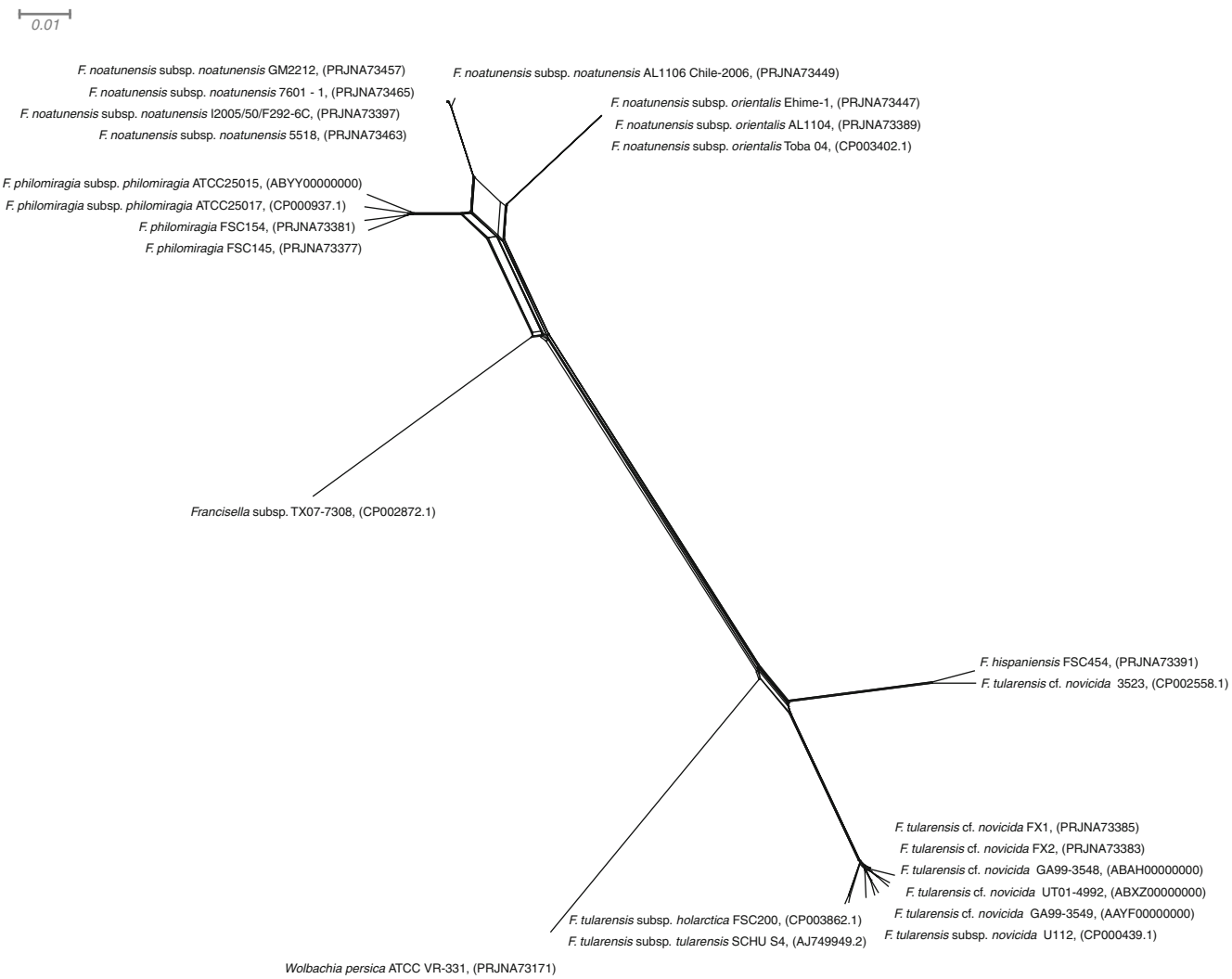

Fig. 14.3

Phylogenetic network based on a whole-genome alignment of 535,591 nucleotide positions for 25 *Francisella* isolates. Multiple alignment was performed using progressiveMauve (Darling et al. 2010). Nucleotide positions within 30 bp of indels were removed. The phylogenetic network was calculated by SplitsTree (Huson and Bryant 2006) using NeighborNet model and P-distances. GenBank accession numbers (where available) or GenBank bioproject numbers for sequence data are provided in parentheses

comparable with those identified between *Salmonella* spp. and *Escherichia coli* (Konstantinidis and Tiedje 2005). Members of *F. tularensis* demonstrate very high intraspecies ANI (≥98 %), particularly if only the virulent subspp., i.e., *tularensis*, *mediasiatica*, and *holarctica*, are considered (≥99.3 %). This is also true for intra-subspecies comparisons within *F. noatunensis* subsp. *noatunensis* (99.95 %) and subsp. *orientalis* (99.98 %). In clade II, ANI values between *F. noatunensis* subspp. *noatunensis*, *orientalis*, and *F. philomiragia* are in the range 93–94 %. As an ANI value of 95 % has been proposed for species demarcation (Richter and Rosselló-Móra 2009), it is notable that ANI for the two *F. noatunensis* subspp. fall below this limit. Of extrachromosomal elements, no evidence of recent prophage insertions has been identified and plasmids appear rare within the genus. Two plasmids (pF242 and pF243) have been isolated from *F. philomiragia* strains ATCC 25016 and ATCC 2517, respectively (Le Pihive et al. 2009), while plasmid pFNL10 has been isolated from a *F. tularensis* subsp. *novicida*-like strain GA99-3549 (aka F6168) (Pavlov et al. 1996; Pomerantsev et al. 2001). Plasmids pFNL10 and pF243 (3,990 bp and 5,072 bp, respectively) are theta-replicating and exhibit extensive sequence homology, while plasmid pF242 (3,936 bp) is unrelated and thought to replicate by the rolling circle mechanism. No plasmids have been described from the pathogenic taxa *F. noatunensis* and *F. tularensis* subspp. *tularensis*, *mediasiatica*, and *holarctica*.

Through comparison of gene content, it has become apparent that substantial differences in the repertoire of gene functions exist among the different members of the *Francisella* (Sjödin et al. 2012; Siddaramappa et al. 2012) and that despite the continued limited understanding of functional diversity within the genus, genomic patterns have been identified which suggest that different *Francisella* have followed different evolutionary trajectories. For example, comparison of the environmental isolate

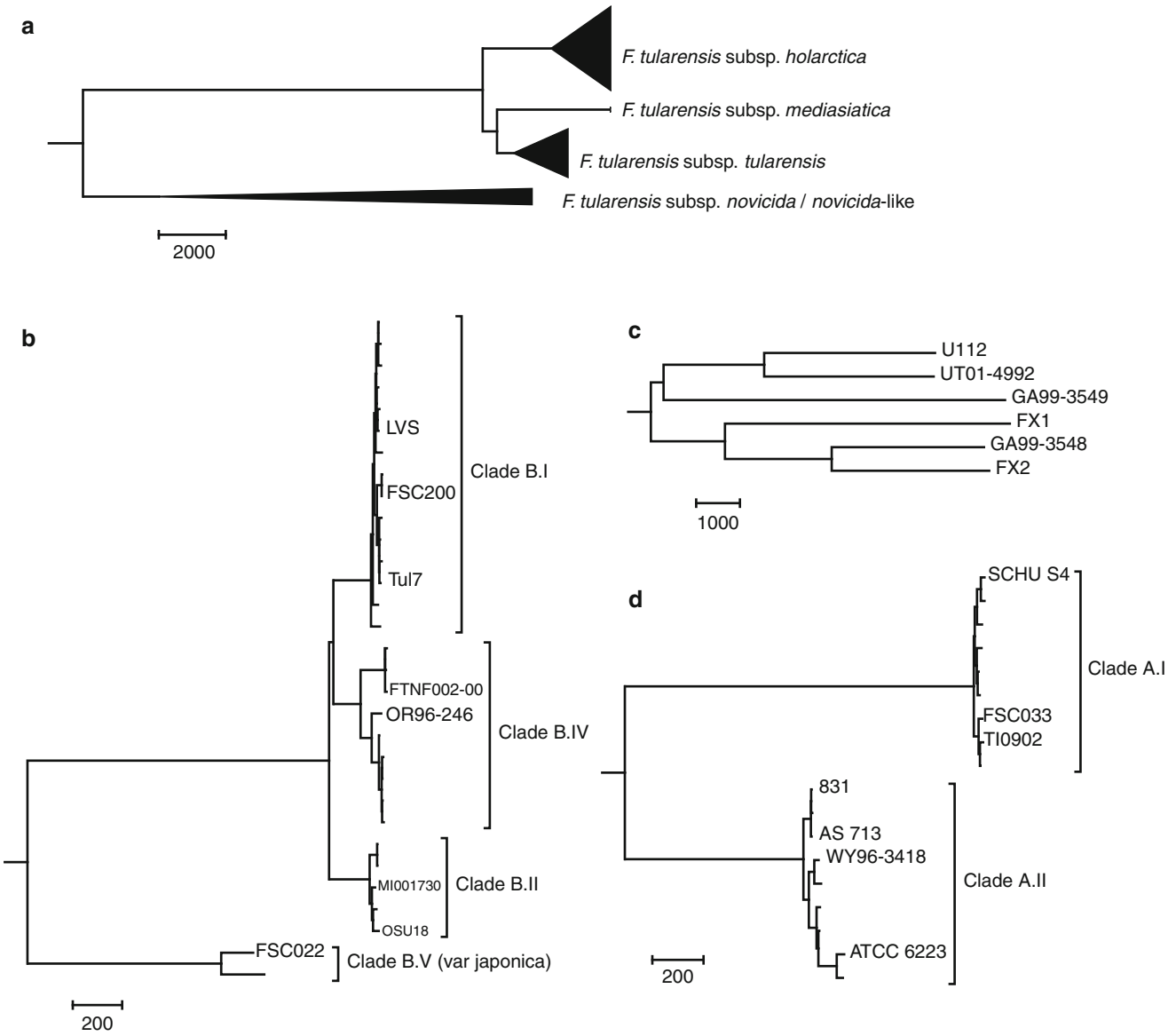

Fig. 14.4

(a–d) Phylogeny of *Francisella tularensis* taxa based on whole-genome alignment of 1,215,018 positions using the neighbor-joining method (number of differences, distances, gaps and missing data, and nucleotide positions within 30 bp of indels were eliminated) for 58 *F. tularensis* isolates. Multiple alignment was performed using progressiveMauve (Darling et al. 2010), and evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011). Genomic sequences for taxa in the analysis include both publically available data and unpublished data. (a) Overview of phylogenetic relationships for *F. tularensis*. (b) Overview of phylogenetic relationships for *F. tularensis* subsp. holarctica. (c) Overview of phylogenetic relationships for *F. tularensis* subsp. *novicida*/*novicida*-like isolates. (d) Overview of phylogenetic relationships for *F. tularensis* subsp. *tularensis*. Accession numbers/bioproject numbers for publically available genomic data are as follows. The genome sequence for *F. tularensis* cf. novicida 3523 was used as out-group for the analysis. *F. tularensis* subsp. *novicida*/*novicida*-like isolates: U112 (CP000439.1), UT01-4992 (ABXZ00000000), GA99-3549 (AAYF00000000), FX1 (CP002557.1), GA99-3548 (ABAH00000000); FX2 (PRJNA73383), and 3523 (CP002558.1). *F. tularensis* subsp. *tularensis* isolates: SCHU S4 (AJ749949.2), FSC033 (AAYE00000000), TI902 (CP003049.1), 831 (AMPV00000000), AS 713 (AMPU00000000), WY96-3418 (CP000608.1), and ATCC 6223 (PRJNA30629). *F. tularensis* subsp. *mediasiatica* isolates: FSC147 (). *F. tularensis* subsp. *holarctica* isolates: LVS (AM233362.1), FSC200 (), Tul7 (CP003862.1), FTN002-00 (CP000803.1), OR96-246 (PRJNA30669), MI001730 (PRJNA30635), OSU18 (CP000437.1), and FSC022 (AAYD00000000)

TX07-7308, *F. philomiragia* ATCC 25017, and *F. tularensis* subsp. *novicida* U112 genomes, 200–250 protein-coding genes were found to be specific to each organism (Siddaramappa et al. 2012). In the same study, the differential presence of genes involved in thiamine biosynthesis, lactose, and glucuronate metabolism was further noted. A systematic interpretation of the diversification of gene function and potential implications for ecology within the genus has, however, yet to be published.

Table 14.2

Genomic characteristics of completely sequenced *Francisella* isolates. Unless stated otherwise, statistics are obtained from PGAT (Brittnacher et al. 2011). Data for other noncoding RNA obtained NCBI Blastn v. 2.2.25+ search using 1e-10 cutoff (for small RNAs FtrABC) (Postic et al. 2010, 2012) and using Rfam v. 11 (Burge et al. 2012) searched with Infernal (Nawrocki et al. 2009), 1e-10 cutoff. Copy numbers for the *Francisella* pathogenicity island (FPI) have been obtained by blastn searches and inspection of genome annotations. Two completely sequenced genomes are excluded from the comparison as they represent derivative strains: the FSC198 (Chaudhuri et al. 2007), derived from strain SCHU S4, and the TIGB03 (Modise et al. 2012), derived from strain TI0902

Species	Subspecies	Strain	Genome size	Coding genes	Pseudo %	Coding %	GC %	rRNA	tRNA	Other ncRNA[a]	IS elements	FPI	Acc.	References
F. tularensis	*tularensis*	SCHU S4	1892819	1465	277	72.98	32.26	10	38	9	89	2	AJ749949.2	Larsson et al. (2005)
	tularensis	TI09-02	1892744	1460	319	72.96	32.26	10	38	9	85[d]	2	CP003049.1	Modise et al. (2012)
	tularensis	NE06-1598	1892681	1459	324	73.08	32.26	10	38	9	81[d]	2	CP001633.1	Nalbantoglu et al. (2010)
	tularensis	WY96-3418	1898476[b]	1509[c]	186[b]	75.9[c]	32.27[b]	10[b]	38[b]	9	84[d]	2	CP000608.1	Beckstrom-Sternberg et al. 2007
	mediasiatica	FSC147	1893886	1416	328	71.08	32.25	10	38	9	83	2	CP000915.1	Larsson et al. (2009)
	holarctica	F92	1886888	1386	421	68.28	32.17	10	38	9	109[d]	2	CP003932.1	Unpublished
	holarctica	LVS	1895994	1438	324	70.72	32.15	10	38	9	101	2	AM233362.1	Unpublished
	holarctica	FSC200	1790358	1440	321	70.84	32.15	10	38	9	107	2	CP003862.1	Svensson et al. (2012)
	holarctica	OSU18	1895727	1424	336	70.01	32.16	10	38	9	108	2	CP000437.1	Petrosino et al. (2006)
	holarctica	FTNF002-00 (FTA)	1890909	1434	325	70.68	32.16	10	38	9	109	2	CP000803.1	Barabote et al. (2009)
	novicida	U112	1910031	1726	2	89.16	32.48	10	38	12	26	1	CP000439.1	Rohmer et al. (2007)
	cf. *novicida*	Fx1	1913619	1691	63	87.58	32.54	10	38	12	19	1	CP002557.1	Siddaramappa et al. (2011)
	cf. *novicida*	3523	1945310	1436	77	86.36	32.32	10	38	9	5[e]	1	CP002558.1	Siddaramappa et al. (2011)
Francisella sp.		TX07-7308	2035931	1795	122	85.24	32.87	10	39	8	12[f]	1	CP002872.1	Siddaramappa et al. (2012)
F. philomiragia	*philomiragia*	ATCC 25017	2045775	1971	196	82.89	32.57	10	39	8	78[f]	1	CP000937.1, CP000938.1	Zeytun et al. (2012)
F. noatunensis	*orientalis*	Toba 04	1847202	1391	496	67.16	32.22	4	35	8	0[f]	1	CP003402.1	Sridhar et al. (2012)

[a]Noncoding RNAs include riboswitches but exclude CRISPR motifs. For small RNAs FtrA and B, present variants but judged to be truncated in relation to reference sequences are not listed

[b]Data obtained from Beckstrom-Sternberg et al. (2007)

[c]Number of coding genes and coding sequence percentage recalculated after removal insertion sequences in Beckstrom-Sternberg et al. (2007)

[d]Number of IS elements estimated using NCBI Blastn v. 2.2.25+ and sequences for IS*Ftu*1-6 as reference

[e]Number of IS elements obtained using NCBI Blastn v. 2.2.25+ and sequences for IS*Ftu*1-6 as reference in combination with reference publication

[f]Number of IS elements obtained from reference publications

Of the presence/effects of horizontal gene transfer (HGT), one locus having a lower G+C content than the rest of the genome, and therefore suspected as a HGT candidate, was identified in *F. tularensis* subsp. *novicida* (Nano et al. 2004) and proposed to represent a pathogenicity island. Recent genomic studies have identified the *Francisella* pathogenicity island (FPI) as a ubiquitous feature within members of the genus, suggesting that the (putative) horizontal transfer of this genomic region must have occurred in a distant *Francisella* ancestor (Larsson et al. 2009). While the FPI is considered critical for intracellular virulence (Nano et al. 2004) and is duplicated in the more virulent *F. tularensis* subspp. (Larsson et al. 2005), its presence in nonpathogenic environmental strains creates some doubt as to whether it does in fact represent a true pathogenicity island. The presence of other putative genomic islands has been suggested in *F. tularensis* subsp. *novicida* U112, cf. *novicida* 3523, cf. *novicida* Fx1, *F. philomiragia* ATCC 25017, and *Francisella* sp. TX07-7308, although the supporting evidence has not been described in detail (Siddaramappa et al. 2011, 2012).

A diversity of transposable elements has been detected in the various lineages of *Francisella*, with the exception of the fish pathogen *F. noatunensis* subsp. *orientalis* (Sjödin et al. 2012; Sridhar et al. 2012). In *F. tularensis* subspp. variable numbers of at least six types of IS elements, or remnants thereof, have been identified (ISFtu1-6)(Larsson et al. 2005; Rohmer et al. 2007). Four of these have also been found in *F. philomiragia* complemented by six additional types not found in *F. tularensis* (Zeytun et al. 2012). Genomic analyses of *F. tularensis* subspp. *tularensis*, *mediasiatica*, and *holarctica* have, in contrast to the situation in subsp. *novicida*, revealed considerable expansion of two types of IS elements, ISFtu1 and ISFtu2 (Rohmer et al. 2007; Larsson et al. 2009; Champion et al. 2009) (*Table 14.2*). All genome-sequenced non-*novicida F. tularensis* are also typified by an abundance of disrupted genes (Larsson et al. 2005, 2009; Rohmer et al. 2007; Champion et al. 2009), with more than 20 % of open reading frames in *F. tularensis* subspp. *tularensis*, *mediasiatica*, and *holarctica*, predicted to represent pseudogenes or gene fragments (*Table 14.2*). IS expansion has been related to both genome reduction and the emergence of pathogenicity in several host-restricted pathogens (Yang et al. 2005; Moore et al. 2004; Moran and Plague 2004; Parkhill et al. 2003). Interestingly, patterns of gene decay similar to that observed in *F. tularensis* have also recently been observed in the emerging fish pathogenic *F. noatunensis* subspecies which could indicate parallel evolutionary and ecological paths for these organisms (Sridhar et al. 2012). A remarkable contrast in IS element content has, meanwhile, been identified between the two *F. noatunensis* subspecies. With at best only a remnant of an IS element identified in subsp. *orientalis*, an IS element expansion trend comparable with that of *F. tularensis* has been identified in subsp. *noatunensis* (Sjödin et al. 2012; Sridhar et al. 2012).

IS elements have impacted significantly on the genome structure of virulent *F. tularensis* subspp. via IS element-mediated genomic excisions (Broekhuijsen et al. 2003; Rohmer et al. 2007) and rearrangements (Petrosino et al. 2006; Rohmer et al. 2007; Beckstrom-Sternberg et al. 2007; Larsson et al. 2009; Champion et al. 2009; Dempsey et al. 2006; Nalbantoglu et al. 2010). Petrosino et al. (2006) identified significant rearrangements in subsp. *tularensis* SCHU S4 and subsp. *holarctica* OSU18 in which 49 of 51 syntenic breakpoints arose from homologous recombination around IS*Ftu1* or IS*Ftu2* sequences. Whether these extensive structural changes confer fitness benefits is not understood. In support of an adaptive role for IS elements, it is thought that such elements were involved in duplication of the *Francisella* pathogenicity island (FPI). Thus IS elements may have contributed to adaptation of the more virulent *Francisella* subspecies from their nonhuman pathogenic relatives. Other functions associated with *Francisella* IS elements include global regulation of gene transcription in response to environmental cues (Carlson et al. 2007).

The extent and impact of recombination has been found to vary considerably among different *Francisella* lineages. While genomic analyses of the different lineages indicate generally moderate levels of recombination, these processes appear to have significantly impacted the evolution of environmental *Francisella* taxa (Sjödin et al. 2012; Larsson et al. 2009; Nübel et al. 2006). Network-based phylogenetic analysis (*Fig. 14.3*), which supports divisions without presupposing a tree-like structure, identifies pronounced reticulate relationships due to conflicting phylogenetic signals for *F. philomiragia, F. noatunensis* subspp. *noatunensis* and *orientalis*, and the environmental *Francisella* TX07-77308. This suggests the considerable impact of homologous recombination during their evolution. Similar patterns are found (not shown), although the evolutionary distances are shorter, within *F. tularensis* subsp. *novicida* and *novicida*-like isolates. In contrast, evidence of recombination events has not been identified in *F. tularensis* subspp. *tularensis*, *mediasiatica*, and *holarctica*, or within *F. noatunensis* subsp. *noatunensis* (Svensson et al. 2005; Johansson et al. 2004a; Sjödin et al. 2012; Nübel et al. 2006; Larsson et al. 2009), indicating that these taxa evolved in clonal isolation, possibly as a result of an intracellular lifestyle. It should be noted that as the evolutionary distances within these lineages are small, rare homologous recombination events cannot be entirely discounted.

Phenotypic Features

The *Francisella* consist of small (0.7–1.7 μm), nonmotile, Gram-negative (staining faintly), heterotrophic, aerobic coccobacilli, which are weakly catalase positive, most (but not all) produce H_2S. Members of the genus grow with entire, slightly convex green/pale bluish white or gray, semitranslucent, mucoid colonies. Reversible phenotypic diversity in colony morphology (Eigelsbach et al. 1951) between smooth (blue) and rough (gray), due to LPS phase/antigenic variation, has been described in *F. tularensis*. Such changes in colony morphology have been associated with changes in virulence and immunogenicity (Gunn and Ernst 2007). Incubation time and temperature is dependent on species and strain, but they are relatively slowly growing. The genus can be divided into more and less metabolically competent members with the latter generally requiring enhanced levels of cysteine

Table 14.3

Phenotypic characteristics allowing differentiation of species within the genus *Francisella* (1 *F. tularensis* subsp. *tularensis*, 2 *F. tularensis* subsp. *holarctica*, 3 *F. tularensis* subsp. *mediasiatica*, 4 *F. tularensis* subsp. *novicida*, 5 *F. hispaniensis*, 6 *F. philomiragia*, 7 *F. noatunensis* subsp. *noatunensis*, 8 *F. noatunensis* subsp. *orientalis*, 9 *F. halioticida*)

Characteristic	1[a]	2[a]	3[a]	4[a]	5[b]	6[a, c, d, e]	7[c, d, e]	8[c, e, f]	9[c]
Cell size (μm)	<0.5	<0.5	<0.5	<1.5	<1.5	<1.5	<1.5	<1.5	<1
Capsule	+	+	+	+	n.d.	n.d.	n.d.	n.d.	–
Gram stain negative	w	w	w	w	+	w	w	+	+
Growth on MacConkey agar	–	–	–	v	–	v	w	n.d.	n.d.
Indole	–	–	–	–	–	+	w	w	–
Oxidase	–	–	–	–	+	+	–	–	–
Gelatin hydrolase	–	–	–	–	–	+	–	–	+
Optimal growth (°C)	37	37	37	37	37	25/37	22	25	20
Growth at 37 °C	+	+	+	+	+	(+)	–	–	–
Beta-lactamase production	+	+	–	+	Pen/clavulanic acid resistant	+	+	+	Penicillin resistant
Cysteine requirement for growth	+	+	+	–	Enhanced	–	+	+	n.d.
Halophilic	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+
Growth 6 % NaCl	–	–	–	v	n.d.	+	–	n.d.	+

w weak, *v* variable, *n.d.* not done
Information taken from:
[a]Sjöstedt (2005)
[b]Huber et al. (2010)
[c]Brevik et al. (2011a)
[d]Mikalsen et al. (2007)
[e]Mikalsen et al. (2009)
[f]Ottem et al. (2009)

(or cystine) in culture media. A relationship between metabolic competency and ecology would appear to exist with the pathogenic members of the genus generally displaying reduced metabolic competence and thereby relatively unreactive under phenotypical testing. Individual strain variation appears to exist in regard to cysteine dependency, with strains of *F. tularensis* subsp. *holarctica* and *tularensis* capable of growth on blood agar without added cysteine not unknown.

Identification

Differential identification of individual species/subspecies within the genus is poorly described. Basic morphological, cultural, and phenotypical differences between the various species are summarized in *Table 14.3*. Studies describing newer additions to the genus have utilized a wide and diverse range of phenotyping tools with comparisons limited to closer relationships. Kits used include the BIOLOG-GN2 system, the API rapid Id 32A, 32E, and ZYM. In the absence of a unifying determinative system, comprehensive phenotypical profiling of the genus is incomplete. Huber et al. (2010) presented what is probably the most comprehensive strain comparison in recent years (*Table 14.4*, Huber et al. 2010). *Table 14.5* modified from Ottem et al. (2009) shows differential characteristics between the two *F. noatunensis* subspecies, both of which were not included in the Huber article.

Lipid Profiles

Although all members of the genus have not been tested, those that have typically display polar lipid profiles dominated by diphosphatidylglycerol and phosphatidylethanolamine, with moderate amounts of phosphatidylcholine and minor amounts of phosphatidylglycerol (Huber, et al. 2010). While differences in the relative proportion of each fatty acid are reported for particular taxa (Jantzen et al. 1979; Huber et al. 2010), the overall constituent lipids appear stably represented within members tested and include long-chain saturated and mono-saturated C18–C24 (Jantzen et al. 1979; Hollis et al. 1989), saturated even-chain fatty acids (C10:0, C14:0, C16:0), and long-chain hydroxyl acids (C16:0-3OH and C18:0-3OH). This unusual fatty acid profile is thought to be unique to the *Francisella* (Huber et al. 2010).

Surface Components and Characteristics

Genes coding for type IV pili are present in *F. tularensis* isolates although not all type IV-associated genes appear to be functional in all subspecies and strains, reviewed by Salomonsson et al. (2011). Release of outer membrane vesicles (OMVs), spherical membrane-bound structures released from the bacterial surface, has been described for several members of the *Francisella* including *F. tularensis* subsp. *novicida* and *F. philomiragia*

Table 14.4

Differential metabolic characteristics within the genus *Francisella*. From Huber et al. (2010). (1 *F. hispaniensis* FhSp1T/cFSC454/DSM22475^T, 2 *F. novicida* ATCC 15482^T, 3 *F. philomiragia* ATCC 25015^T, 4 *F. piscicida* (syn. *F. noatunensis* subsp. *noatunensis*) DSM 18777^T, 5 *F. tularensis* subsp. *tularensis* ATTC 6223^T, 6 *F. tularensis* subsp. *mediasiatica* FSC147/GIEM 543^T, 7 *F. tularensis* subsp. *holarctica* FSC257/GIEM503^T. Tests performed with the BIOLOG GN2 microplate system)

Characteristic	1	2	3	4	5	6	7
Catalase	(+)	(+)	+	+	+	+	(+)
Oxidase	+	–	+	–	–	–	–
Oxidation of							
Dextrin	+	–	–	–	–	–	–
Glycogen	+	–	–	–	–	–	–
N-acetyl-D-glucosamine	+	+	+	+	+	–	+
Cellobiose	+	+	–	–	–	–	–
D-galactose	+	+	–	–	–	+	–
Maltose	–	–	+	–	–	–	–
Sucrose	+	+	–	–	–	–	–
Trehalose	+	–	+	–	–	–	–
Succinic acid monomethyl ester	+	+	+	+	–	+	+
Acetic acid	+	–	–	–	–	–	–
α-hydroxybutyric acid	+	–	+	–	–	–	–
β-hydroxybutyric acid	+	+	+	–	–	–	–
α-ketobutyric acid	+	+	+	–	+	+	+
α-ketoglutaric acid	+	–	–	–	–	–	–
DL-lactic acid	+	+	+	–	–	–	+
Succinic acid	+	+	–	–	–	–	–
Bromosuccinic acid	+	–	–	–	–	–	–
Succinamic acid	–	–	+	–	–	–	–
L-alaninamide	+	+	+	+	–	–	–
D-alanine	+	+	+	–	–	–	–
L-alanine	+	+	+	+	–	+	+
L-alanyl glycine	+	+	+	+	–	–	–
L-asparagine	+	+	+	+	–	–	+
L-aspartic acid	+	+	+	–	–	–	+
Glycyl-L-aspartic acid	+	–	–	–	–	–	–
Glycyl-L-glutamic acid	+	+	+	–	–	–	–
Hydroxy-L-proline	+	–	–	–	–	–	–
L-ornithine	+	+	–	–	–	–	–
L-pyroglutamic acid	+	+	+	–	–	–	–
L-threonine	+	+	+	–	–	–	+
γ-aminobutyric acid	+	–	–	–	–	–	–
Inosine	+	+	+	–	–	–	–
Uridine	+	+	+	–	–	–	+
Thymidine	+	+	+	–	–	–	+
Glycerol	+	+	+	–	+	+	–
DL-α-glycerol phosphate	+	+	+	–	+	+	+
A-D-glucose 6-phosphate	+	–	+	–	–	–	–
D-glucose 6-phosphate	+	–	+	–	–	–	–

Table 14.5
Differential biochemical and phenotypic characteristics at 22 °C (1 *F. noatunensis* subsp. *orientalis* DSM21254^T, 2 *F. noatunensis* subsp. *noatunensis* 2005/50/F292-6C^T and *F. philomiragia* 3 1951, 4 CCUG 12603, 5 CCUG 13404, 6 CCUG 19701, and 7 ATCC 25015^T (Modified from Ottem et al. 2009))

Characteristic	1	2	3	4	5	6	7
α-Glucosidase	–	–	–	–	–	–	+
Mannose and raffinose fermentation	–	–	+	+	+	+	+
Arylamidases, leucyl glycine, leucine, pyroglutamic acid, glutamyl glutamic acid, glycine, valine, cystine, serine	–	–	+	+	+	+	+
Phenylalanine arylamidase	+	–	+	+	+	+	+
Tyrosine arylamidase	–	+	+	+	+	+	+
Histidine arylamidase	–	+	+	+	+	+	+
D-maltose, D-trehalose, colistin, coumarate, *O*-nitrophenyl *N*-acetyl-BD-glucosaminide	–	–	+	+	+	+	+
P-nitrophenyl-BD-galactopyranoside	–	–	–	+	–	–	+
Indoxyl phosphate	+	–	+	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	–	+	+	+	+	+
β-Galactosidase	–	–	–	+	–	–	+
N-Acetyl-β-glucosaminidase, oxidase, H_2S slant, triple iron sugar, gelatin hydrolysis	–	–	+	+	+	+	+

(Pierson et al. 2011). OMVs contain periplasmic and cytosolic components. An extracellular capsule has been demonstrated (Hood 1977; Gunn and Ernst 2007) and described (Apicella et al. 2010) in *F. tularensis*. Although capsules are suspected in other species (Soto et al. 2010), these have not yet been confirmed.

The *Francisella* lipopolysaccharide (LPS) molecule appears to differ structurally and biologically from other Gram-negative bacteria. All examined species have identical core-lipid A backbones, i.e., 4-β-Man-4-α-Man-5-α-Kdo-2-6-β-GlcN-6-GlcN (Kay et al. 2006). The major species of lipid A-associated fatty acids are 16 and 18 carbons in length, but minor species with varying fatty acid composition (including C14:0) also exist. The lipooligosaccharide residues (LOS, O-antigen) of *F. tularensis* types A and B appear identical (Gunn and Ernst 2007). Several studies have linked LPS variation to virulence (Pechous et al. 2009). Longer polymers of O-antigen (as well as other high molecular weight carbohydrates) are produced in *F. tularensis* in response to host adaption which impede recognition of the cell by antibody, complement, and toll-like receptors (Zarrella et al. 2011).

Metabolism

Like most heterotrophs, *Francisella* are capable of subsisting on a variety of carbon and nitrogen substrates for extra- and intracellular growth. The bacteria utilizes (in vitro) different carbohydrate sources for energy and grows better on hexoses (glucose and fructose) than on pentoses (xylose and ribose) (Raghunathan et al. 2010). Only minor differences have been observed within the genus regarding the ability to utilize glucose as sole carbon source. Optimum growth on artificial media may, however, require amino acid supplementations (Nagle et al. 1960; Traub et al. 1955), with the different species/subspecies showing obvious requirement differences. Although the relationship between nutritional requirement and metabolic activity is not well defined in *Francisella*, the recent increase in genome sequence analysis has provided some insight into their metabolic processes. Genes coding for diverse essential enzymes in critical metabolic pathways have been identified in various subspecies/strains. The genome of *F. tularensis* subsp. *tularensis* Schu S4 contains approximately 350 genes predicted to be involved in 155 small molecule metabolic pathways, including the major carbon and nitrogen metabolic pathways (Larsson et al. 2005). Pathways for utilization of galactose, glucose, mannose, fructose, glycerol, glutamate, *N*-Acetylglucosamine, glutamine, proline, asparagine, pyrimidine, and purine have been identified (Karlsson et al. 2000; Enstrom et al. 2012). In addition, many metabolic genes for fatty acid, glycerol, phospholipid, thiamine, and various vitamin/cofactor biosynthesis are present (Karlsson et al. 2000; Siddaramappa et al. 2012). The genome sequences predict complete glycolytic pathways, suggesting that glycolysis may be a major route for sugar catabolism in *Francisella* (Rohmer et al. 2007; Enstrom et al. 2012). Nevertheless, many biosynthetic pathways remain incomplete. Apparently incomplete central metabolic pathways include the pentose phosphate pathway (Raghunathan et al. 2010). In the fish pathogenic subspecies (*F. noatunensis* subsp. *orientalis* Toba04), 999 enzymes were predicted to be involved in small molecule metabolic pathways, of which 329 were proposed to be incomplete (Sridhar et al. 2012). The pathways for asparagine and histidine are completely missing in this species. In *F. tularensis* types A and type B, amino acid and nucleotide metabolic pathways are mostly disrupted. Several pathways including those for sulfate assimilation, threonine, valine, isoleucine, methionine, arginine, histidine, lysine, and tyrosine biosynthesis appear incomplete or missing, consistent with the auxotrophic nature of this bacterium for most of these substrates. Unlike the parasitic *Francisella* pathogens, the metabolically competent species (*F. philomiragia* subsp. *philomiragia* and *F. tularensis novicida*) possess major functional biosynthetic pathways including those for sulfate assimilation and asparagine biosynthesis and may potentially share the least number of pathways in common with other members of the genus (Neary et al. 2007). The large number of disrupted genes in the less metabolically competent members is consistent with the genome decay often found in intracellular pathogenic bacteria, which suggest that their metabolism may be tailored to exploit the intracellular niche. The in vivo metabolic requirements of the human and fish pathogenic species/subspecies may differ somewhat. The pathway for asparagine biosynthesis is absent in *F. noatunensis* subsp. *orientalis*, but present in *F. tularensis*, which probably reflects evolutionary differences related to host. It is thought that since the fish pathogen does not have the pathway for asparagine biosynthesis and asparagine is required for growth, the bacterium may adjust

its intracellular metabolism to exploit the nutrient supply of the host cell. Changes in metabolism upon entry of *Francisella* into the host cell have been reported. Several genes involved in carbon metabolism are shown to be upregulated during growth of *F. tularensis* in macrophages. In particular, genes for amino acid biosynthesis, sugar, and fatty acid metabolism are all upregulated. Emerging evidence suggests intra-macrophage metabolic changes for *Francisella* during infection. *Francisella* tend to preferentially utilize amino acids for energy and fatty acids as gluconeogenic substrates rather than relying on carbohydrate sources like glucose and fructose during infection. Glutathione provides a source of cysteine during intracellular multiplication/growth of *F. tularensis* subspecies *holarctica* strain LVS in macrophages (Alkhuder et al. 2009). The need for multiple amino acids for active intracellular growth is highlighted by the expression of several genes involved in specific amino acid biosynthetic pathways (Wehrly et al. 2009).

Isolation, Enrichment, and Maintenance Procedures

While some members of the genus do not require cysteine (or cystine) supplementation, all grow better in its presence. *F. tularensis* subspecies (with the exception of subsp. *novicida*) generally require cysteine supplementation, although isolates growing without such supplementation are, however, not unknown (Owen et al. 1964; Bernard et al. 1994).

Isolation and culture of members of the genus *Francisella* can be challenging (Petersen et al. 2004), due to their generally fastidious nature, slow growth, and that they are readily inhibited by other contaminating bacteria (Petersen et al. 2004; Colquhoun and Duodu 2011). Recovery of field specimens of *F. tularensis* is improved by on-site inoculation of media and antibiotic supplementation of media for culture from contaminated specimens (Petersen et al. 2004).

Media recommended for primary isolation of *Francisella* spp. from clinical samples are generally based on chocolatized blood agar containing elevated levels of cysteine (or cystine) and glucose (Birkbeck and Bordevik 2007; Hsieh et al. 2006; Huber et al. 2010; Jensen et al. 1969; Kamaishi et al. 2005; Kamaishi et al. 2010; Mikalsen et al. 2009; Olsen et al. 2006; Petersen et al. 2004; Soto et al. 2009a). Secondary laboratory culture may be achieved on simpler media including cation-adjusted Mueller-Hinton broth (CAMBH) supplemented with IsoVitaleX (Baker et al. 1985), tryptic soy broth (TSBC) supplemented with cysteine (Tresselt and Ward 1964), or brain heart infusion broth supplemented with cysteine, iron, β-NAD, and glucose (McGann et al. 2009).

Selective Media

Isolation of *Francisella* is commonly inhibited by growth of contaminating bacteria. Use of selective agar incorporating various antibiotics may, however, allow cultivation from a contaminated sample.

Selective agar is particularly relevant in diagnostic investigations and when attempting culture from environmental samples. Several agars selective for *Francisella* species have been published, including cysteine heart agars containing colistin, amphotericin, lincomycin, trimethoprim, and ampicillin for selective culture of *Francisella tularensis* (Petersen et al. 2004) or polymyxin B, amphotericin B, cycloheximide, cefepime, and vancomycin (Petersen et al. 2009a). Selective agars containing polymyxin B with and without ampicillin were also used successfully for isolation of *Francisella noatunensis* subsp. *orientalis* by Soto et al. (2009a).

Culture Temperature for Primary Isolation

Isolates belonging to the *F. tularensis* lineage are best cultivated at 37 °C (Sjöstedt 2005). Most isolates of *F. philomiragia* grow well at 37 °C although some strains may have lower temperature optima (Sjöstedt 2005). For isolation of environmental isolates and pathogens of poikilotherms, lower incubation temperatures should be used, with 22–25 °C probably resulting in growth of all known recognized species (Soto et al. 2009a; Mikalsen et al. 2007; Kamaishi et al. 2005).

Ecology

As mentioned previously, an increasing diversity of *Francisella* species and *Francisella*-like bacteria in the environment is recognized, for many of which we have no or little ecological knowledge (Broman et al. 2011; Barns et al. 2005; Duodu et al. 2012a; Berrada and Telford 2010). However, that in excess of 300 species of warm- and cold-blooded hosts (mammals, fish, insects, arthropods, freshwater protozoans) are reported susceptible to infection by members of this genus (Mörner and Addison 2001; Colquhoun and Duodu 2011) indicates a remarkable degree of adaptability within these organisms. The environmental reservoirs of both pathogenic and nonpathogenic strains of *Francisella* are not, however, well defined.

Genome sequence analyses of pathogenic and environmental strains support previous studies proposing the existence of varying ecological lifestyles among members of the *Francisella*. Pathogenic species exemplified by *F. tularensis* subspp. *tularensis* and *holarctica* and *F. noatunensis* subsp. *noatunensis* are characterized by a clonal population structure under weak purifying selection. Consequent loss of biosynthetic pathways and almost no exchange of genetic material between strains suggest a host-dependent (natural) life cycle for these taxa (Larsson et al. 2005, 2009; Sjödin et al. 2012). However, the (relatively) greater number of coding genes among *F. tularensis* subsp. *tularensis* compared to *F. tularensis* subsp. *holarctica* might suggest a more diverse ecological niche panorama for this subspecies. The more environmental members, exemplified by *F. tularensis* subsp. *novicida* show a higher degree of metabolic competence, higher levels of recombination, and dN/dS ratios consistent with a free-living environmental niche (Larsson et al. 2009;

Sjödin et al. 2012; Siddaramappa et al. 2011, 2012). *Francisella* appear to have evolved in and from the marine environment although *F. tularensis* subspp. *tularensis* and *holarctica* have not been directly associated with seawater. The relatively common detection of *F. tularensis* subsp. *novicida* and *F. philomiragia*-related species/strains from diverse saline, brackish, and seawater environments points toward an original marine niche (Duodu et al. 2012a; Petersen et al. 2009a; Barns et al. 2005). Berrada and Telford (2011) found enhanced survival of *F. tularensis* subsp. *tularensis* in brackish water and suggested that the salt influenced (marine) environment may promote bacterial survival. The same study also identified similarities in environmental stability between *F. tularensis* subspecies *tularensis* and *F. tularensis* subspecies *holarctica*. Brackish water and sediments often contain relatively high concentrations of sulfur residues which aid survival of *F. tularensis* (Parker et al. 1951). The fish pathogenic *F. noatunensis* subspecies *noatunensis* and *orientalis* appear to be the only *F. philomiragia*-related isolates to have been reported from freshwater environments. However, both fish pathogenic species also show reduced survival in freshwater compared to seawater (Duodu and Colquhoun 2010; Soto and Revan 2012), supporting the hypothesis that the *F. philomiragia* clade in particular is ecologically adapted to a marine environment.

While the presence of *Francisella and Francisella*-like organisms has been documented in many arthropod species, the ecological status of such associations, whether pathogenic or endosymbiont in nature, is not always clear. *Francisella*-harboring arthropod species include fleas, lice, midges, bedbugs, ticks, mosquitoes, and flies, only some of which host reasonably stable bacterial populations (Petersen et al. 2009b). Long-term survival of *F. tularensis* does not occur in, e.g., tabanid flies and transmission by the deer fly is purely mechanical. While *F. tularensis* infection has a high fitness cost for infected ticks and transovarial transmission is doubtful (Jellison 1974; Mani et al. 2012; Hopla 1974; Hopla and Hopla 1994), infected tick mortality is low (Mani et al. 2012; Reese et al. 2011). It is therefore probable that the bacteria can circulate in a tick-animal cycle for several years (Gyuranecz et al. 2011; Gyuranecz et al. 2012a). The stable prevalence of *F. tularensis* subsp. *tularensis* in questing *D. variabilis* on Martha's Vineyard suggests that such infections provide a significant persistence mechanism for the bacterium (Goethert et al. 2004; Goethert and Telford 2009). Uninfected ticks live longer than infected ticks, but mortalities appear to be associated with genotype of the infecting bacteria rather than bacterial load (Goethert and Telford 2011) suggesting better host adaptation in certain bacterial genotypes. Evidence exists for the existence of different ecological niches within *F. tularensis* subsp. *tularensis* strains, with genotypes AI and AII occupying different ecological niches associated with different arthropod vectors, while AIa and AIb do not (Nakazawa et al. 2010).

Mosquitoes of several species have been shown to carry *F. tularensis*, but experimental infections indicate that the bacteria are not capable of multiplication in these organisms (Petrisheva 1965) and that infection probably has a negative effect on mosquito fitness (Mahajan et al. 2011). Recent identification of *F. tularensis* subsp. *holarctica* DNA in laboratory-reared, field-collected mosquito larvae implies, however, a transmission cycle originating in aquatic habitats (Lundström et al. 2011). Modeling of predicted mosquito abundance and clinical tularemia cases were also positively correlated (Rydén et al. 2012). Mosquitoes are not, however, considered to play a role in transmission or maintenance of *F. tularensis* subspecies *tularensis* (Petersen et al. 2009b). Of the less pathogenic, metabolically competent *Francisella*, infection and transmission of *F. tularensis* subsp. *novicida* was readily achieved in the tick *Dermacentor andersoni* (Reif et al. 2011), but this bacterium was not transstadially maintained in mosquitoes (Triebenbach et al. 2010) and insect antimicrobial peptides have also been shown to inhibit growth of this species (Vonkavaara et al. 2012). *F. tularensis* subsp. *novicida* has been detected in brackish and saltwater sources but has not been identified in association with arthropod vectors or other organisms (Larson et al. 1955; Petersen et al. 2009b; Berrada and Telford 2010; Brett et al. 2012).

F. tularensis subsp. *holarctica* may persist for up to 3 years in the environment (Broman et al. 2011; Thelaus et al. 2009). Genetic subpopulations persist in close but segregated natural foci with little genetic variation (Svensson et al. 2009a; Rydén et al. 2012; Karlsson et al. 2012). Experimental evidence indicates that pathogenic *Francisella* spp. remain culturable from water for variable periods after which the cells may enter a viable but non-culturable (VBNC) physiological stage (Forsman et al. 2000; Thelaus et al. 2009; Duodu and Colquhoun 2010). VBNC cells, although capable of persisting in the environment for extended periods (Forsman et al. 2000; Thelaus et al. 2009), do not appear capable of causing disease (Forsman et al. 2000; Duodu and Colquhoun 2010).

Intracellular survival of *Francisella* within protozoa (Berdal et al. 1996; Abd et al. 2003; Thelaus et al. 2009; El-Etr et al. 2009; Santic et al. 2011) has been reported. *F. tularensis* subsp. *tularensis* strains survive and replicate in the amoeba *Acanthamoeba castellanii* causing rapid trophozoite encystment following infection. The induction of rapid encystment is essential for survival (El-Etr et al. 2009), and the bacterium can survive in *A. castellanii* cysts for at least 3 weeks postinfection. The ability to survive and resuscitate from amoebal cysts may explain long-term persistence of *F. tularensis* subsp. *tularensis* strains in water. Coexistence within endosymbiotic marine ciliates has recently been demonstrated for a closely related strain of *F. noatunensis* (Schrallhammer et al. 2011). Both fish pathogenic *F. noatunensis* subspecies are highly infectious, but their existence in natural aquatic environments outside their fish hosts is not well documented. Studies have shown that *F. philomiragia* is more resistant than *F. tularensis* subsp. *holarctica* to grazing by protists and readily forms an association with other unicellular organisms (e.g., *Tetrahymena pyriformis, Acanthamoeba castellanii*) in their natural habitat (Thelaus et al. 2009; Abd et al. 2003). The ability of *F. philomiragia* to form biofilms and interact with aquatic protists may therefore contribute to its greater survival and persistence in open water environments. Lack of biofilm formation in *F. tularensis* subsp. *holarctica* (Zogaj et al. 2012), a trait normally associated with persistence

within the environment, further supports an intracellular niche for this bacterium.

Little is known of the potential habitat, host, and/or vector associations of *F. tularensis* subsp. *mediasiatica*, uncommonly isolated from ticks and rodents in Central Asia, *F. hispaniensis* (Huber et al. 2010) isolated from human clinical specimens in Spain, and "*F. novicida*" strains likely to represent *F. hispaniensis* isolated in Australia (Whipp 2003; Siddaramappa et al. 2011) and Thailand (Leelaporn et al. 2008; Sjödin et al. 2012) or an isolate initially described as *F. cantonensis* (Qu et al. 2009), later *F. guangzhouensis* (Qu et al. 2013), originating from an air-conditioning system, probably as part of a biofilm. Nothing is known of the ecology of the many *F. philomiragia*-like strains identified via non-culture-based techniques. More recently, *F. halioticida* was isolated in Japan from diseased cultured giant abalone (*Haliotis gigantea*) for which the bacterium is highly virulent and infectious (Kamaishi et al. 2010). Besides requiring additional salt in culture media, little is known of the ecology of this bacterium.

F. tularensis subsp. *novicida* has been detected in brackish and saltwater sources, but no association with arthropod vectors or animals has been demonstrated in nature (Larson et al. 1955; Petersen et al. 2009a; Berrada and Telford 2010; Brett et al. 2012). Thus, little is known of reservoirs and or niches for *F. novicida.*

Epidemiology of Tularemia and Francisellosis

Tularemia caused by *F. tularensis* occurs over almost the entire Northern Hemisphere, but with great variation in geographic and temporal occurrence. Outbreaks may be associated with exposure to arthropods, infected animals, food, water, fomites, or aerosol-borne bacteria. Historically, tularemia has constituted a major health problem in the Old World, especially in times of social disruption or war (Pollitzer 1967; Reintjes et al. 2002).

On the American continent, tularemia has been reported from the USA, Mexico, and Canada. In the USA cases have been reported from all states except Hawaii. Persistent foci exist in the southern central USA, Pacific Northwest, and parts of Massachusetts, including Martha's Vineyard. In the period 2001–2010, 1208 cases were reported to Centers for Disease Control and Prevention (CDC 2011). The incidence of tularemia in the USA has decreased since the 1930s, possibly as a result of changes in society with less exposure to vectors and wild animal hosts.

In Europe, tularemia occurs in all countries except Iceland and the British Isles, with 1,004 cases reported between 2006 and 2010 (ECDC Annual Epidemiological report 2013 http://www.ecdc.europa.eu/en/publications/Publications/). The Scandinavian countries represent important foci of infection with annual averages of 305 and 298 cases, respectively, in Sweden and Finland, while a single outbreak reported in Norway in 2011 involved 180 cases (Larssen et al. 2011). Continental Europe (Tärnvik et al. 2004) has a history of only sporadic cases although the disease appears to have recently reemerged with natural foci in Germany and Austria (Splettstoesser et al. 2009; Kaysser et al. 2008; Lang and Kleines 2012).

Tularemia caused by *F. tularensis holarctica* also occurs in Central Asia, China, Mongolia, Korea, Japan, and Iran (Zhang et al. 2006, 2008; Ebright et al. 2003; Arata et al. 1973). Major differences in important mechanisms of transmission are apparent in different endemic areas. While mosquitoes (Eliasson et al. 2002) appear to be responsible for most cases in Sweden and Finland, contamination of drinking water by infected rodents was considered the dominating route of infection in the geographically close Norwegian outbreak of 2011 (Larssen et al. 2011). In southeast Europe water-borne tularemia dominates (Leblebicioglu et al. 2008; Kantardjiev et al. 2006; Simşek et al. 2012). In the United States, two main mechanisms of infection dominate, i.e., via vectors such as ticks and deerflies or via direct contact with infected mammals. Respiratory tularemia, although sporadic, has been reported in both the USA and Europe in association with animal carcasses and exposure to aerosols of soil, dust, or hay (Matyas et al. 2007; Cerny 2001; Dahlstrand et al. 1971). Tick-borne infection also has been described in Japan (Ohara et al. 1991). In Europe and Asia, while many animal hosts, primarily Rodentia (reviewed by Paul Keim et al. 2007), are associated with tularemia caused by *F. tularensis* subsp. *holarctica*, lagomorphs appear to be particularly important in Central Europe and northern latitudes (Morner et al. 1988). Numerous species of bird can be naturally infected with *F. tularensis* (Friend 2006), and transmission via migratory birds may occur either directly following death and contamination of water, through bacterial shedding in excrement (Cabelli et al. 1964), through direct contact (Padeshki et al. 2010), or by transport of infected ticks (Mörner and Krogh 1984). Recently, *F. tularensis* subsp. *holarctica* was detected in migratory shorebirds in Portugal by PCR on sampled blood, indicating a history of recent exposure (de Carvalho I et al. 2012). Bird migrations are unlikely to be a significant mode of transmission, however, since tularemia does not occur along migration routes to the Southern Hemisphere (Tärnvik et al. 2004).

Tularemia in Europe is almost exclusively caused by *F. tularensis* subsp. *holarctica*, while in North America *F. tularensis* subsp. *tularensis* (or type A) is the most common etiological agent (80–90 % of cases), despite the copresence of *F. tularensis* subsp. *holarctica* in this region (Jellison et al. 1961; McChesney and Narain 1983). *F. tularensis* subsp. *tularensis* (type A) is exclusively found in North America. The few isolations of subsp. *tularensis* in Europe (Guryčová 1998) have all been related to escape of laboratory strain Schu S4 originating from the USA (Chaudhuri et al. 2007; Sjödin et al. 2010).

F. tularensis type A can be genetically subdivided into AIa, AIb, AIIa, and AIIb, with subpopulation AIb associated with highest virulence and associated mortality (Farlow et al. 2005; Johansson et al. 2004a; Staples et al. 2006; Kugeler et al. 2009; Molins et al. 2010). Subpopulations AI and AII form distinct groups, with AII predominantly occurring in the west and AI in the east of the United States (Farlow et al. 2005) with AIb strains dominating along the Atlantic seaboard (Kugeler et al. 2009; Vogler et al. 2009). Also in Alaska, AII might be geographically separated from other *F. tularensis* genetic types (Hansen et al. 2011)

Of the four major *F. tularensis* type B clades, type BI dominates in Europe between Scandinavia and the Black Sea (Svensson et al. 2009a; Vogler et al. 2009; Chanturia et al. 2011; Miklós Gyuranecz et al. 2012b; Karlsson et al. 2012). BI strains are also intrinsic to Japan (Fujita et al. 2008). The other dominating European subtype is a subclade of BIV which can be found over a large area of Western and Central Europe (Dempsey et al. 2007; Pilo et al. 2009; Vogler et al. 2011; Miklós Gyuranecz et al. 2012; Gehringer et al. 2012). The BII clade, dominant in the USA, is also found in Europe, while clade BV mainly represents biovar *japonica* strains (Vogler et al. 2009; Karlsson et al. 2012). *F. tularensis* subsp. *holarctica* biovar japonica, originally thought confined to the Japanese islands, has recently been isolated in Australia, China and Turkey, (Jackson et al. 2012; Wang et al. 2011; Kiliç et al. 2013).

Biovar I Ery^S (see section ❯ Antibiotic Resistance) is present in Western Europe, North America, Eastern Siberia, and the Far East, while biovar II Ery^R is present in the European part of Russia as well as Northern and Eastern Europe (Kudelina and Olsufiev 1980; Petersen and Molins 2010; Kreizinger et al. 2013; Georgi et al. 2012; Yesilyurt et al. 2011). A mixture of both Ery biotypes has been reported in Sweden, Norway, Bulgaria, Russia, Kazakhstan (Kudelina and Olsufiev 1980; Petersen and Molins 2010; Kunitsa et al. 2012; Müller et al. 2013). Isolation of both Ery biovars from local rodent populations and water samples in Russia (Kudelina and Olsufiev 1980) indicates biovar coexistence within epidemiological foci. Strains representing much of the currently known genetic diversity of *F. tularensis* subsp. *holarctica* (Svensson et al. 2009a; Karlsson et al. 2012) have also been isolated in Sweden, which might suggest historical spread of disease from Scandinavia to other parts of Eurosiberia.

Although less is known of the epidemiology of francisellosis in fish, the disease has undoubtedly emerged as a global problem in aquaculture. Both fish pathogens result in similar pathological changes, but while *F. noatunensis* subsp. *noatunensis* affects relatively cold-water fish species, e.g., Atlantic cod and Atlantic salmon in seawater and freshwater, respectively (Birkbeck and Bordevik 2007; Olsen et al. 2006; Nylund et al. 2006), *F. noatunensis* subsp. *orientalis* affects a number of fish species (Colquhoun and Duodu 2011), primarily tilapia (Mauel et al. 2007; Soto et al. 2009a; Chern and Chao 1994; Hsieh et al. 2006; Mauel et al. 2005; Jeffery et al. 2010) in warmer waters. Francisellosis caused by *F. noatunensis* subsp. *noatunensis* has been identified in the eastern northern Atlantic and North Sea areas (van Banning 1987; Zerihun et al. 2011) and also in freshwater in Chile (Birkbeck and Bordevik 2007; Bohle et al. 2009). Francisellosis caused by *F. noatunensis* subsp. *orientalis* has been identified in many Asian countries, many states within the USA, Central America, and the UK (see Colquhoun and Duodu 2011; Birkbeck et al. 2011 for reviews). The natural geographical distributions of these two bacteria are not known to overlap, but following importation of tilapia to the UK and associated outbreaks of francisellosis (*F. noatunensis* subsp. *orientalis*) (Jeffery et al. 2010), as well as previous outbreaks of francisellosis in cod (*F. noatunensis* subsp. *noatunensis*) in English coastal waters (Zerihun et al. 2011), their ranges may now cross. An MLVA study separated *F. noatunensis* subsp. *noatunensis* from farmed and wild Norwegian cod into at least nine clades (Brevik et al. 2011b). Whole-genome SNP analysis (Larsson et al. unpublished results) was also capable of separating *F. noatunensis* subsp. *noatunensis* isolates, both within and between strains isolated from geographically disparate areas. Although the number of subsp. *orientalis* strains examined is low, there are indications from whole-genome sequence analysis that both subspecies appear to be highly clonal in nature (Sjödin et al. 2012; Brevik et al. 2011b; Sridhar et al. 2012). Given the strong association between the distribution and probable clonal nature of *F. noatunensis* subsp. *orientalis* and tilapia spp., it is tempting to speculate that the bacterium has been spread around the world with transport of these fish. Retrospective identification of *F. noatunensis* subsp. *noatunensis* in wild caught Atlantic cod during the 1980s (Zerihun et al. 2011) and 2000s (Alfjorden et al. 2006; Ottem et al. 2008) indicates that wild Atlantic cod represents the reservoir of this bacterium.

Pathogenicity

Two species in the genus *Francisella* are particularly notable for their pathogenicity: *F. tularensis*, causing tularemia in mammals, including humans, and *F. noatunensis* causing francisellosis in fish. Although the virulence mechanisms of these *Francisella* species remain, in general, poorly understood, several studies over the past decade have significantly enhanced our understanding of *F. tularensis* pathogenesis, while less is known of the relatively newly discovered fish pathogens *F. noatunensis* subsp. *noatunensis* and subsp. *orientalis* and the molluscan pathogen *F. halioticida*.

F. tularensis has been shown to infect a variety of cell types including dendritic cells, neutrophils, hepatocytes, epithelial, endothelial cells, and erythrocytes (Buddingh and Womack 1941; Shepard 1959; Anthony et al. 1991; Conlan and North 1992; McCaffrey and Allen 2006; Craven et al. 2008; Horzempa et al. 2011). However, the macrophage represents the main cell type in which *F. tularensis* multiplies within the host organism (Anthony et al. 1991; Fortier et al. 1995). The recently discovered ability to invade human erythrocytes may only represent a persistency mechanism, since replication in erythrocytes has not been demonstrated. *F. tularensis* enters macrophages via phagocytosis and resides initially within a phagosome. Maturation of the *Francisella*-containing phagosome is arrested at the late phagosomal stage where after the bacterium induces degradation of the phagosomal membrane and rapidly escapes into the cytosol to replicate to high numbers (Santic et al. 2005; Golovliov et al. 2003). When present in the cytosol, *F. tularensis* may direct apoptosis and pyroptosis of host cells and may also reenter the endocytic compartments via an autophagy-mediated process (Checroun et al. 2006; Wehrly et al. 2009). Invasion of cells, interference with phagosomal trafficking, and multiplication within the host cell cytosol represent fundamental parts of the *F. tularensis* life cycle. Few classical virulence factors are

encoded in *F. tularensis* (Larsson et al. 2005). *F. tularensis* has evolved other mechanisms which allow survival and reproduction within the host environment. A principle survival strategy is avoidance of recognition by the immune system, which entails active manipulation of the host response. Almost the entire genome is required for full virulence (Maier et al. 2007; Su et al. 2007; Alkhuder et al. 2009; Kraemer et al. 2009; Schulert et al. 2009; Weiss et al. 2007), with many genes coding for metabolic and nutritional functions essential for virulence. Hypothetical proteins appear important for virulence but the functional basis for this remains poorly understood. A number of genes and structures in *F. tularensis* have, however, been linked to specific virulence functions.

F. tularensis type A and type B strains display an electron-dense structure on their surfaces (Sandström et al. 1988; Sjöstedt 2005; Sorokin et al. 1996), believed to represent a capsule. This structure disappears under hypertonic conditions (Hood 1977), and repeated passage in a defined medium may enhance its expression (Cherwonogrodzky et al. 1994). To date, two components of the *F. tularensis* capsule have been described, a high molecular weight glycoprotein (Bandara et al. 2011; Zarrella et al. 2011) and a tetrasaccharide subunit polymer, structurally similar to the *F. tularensis* lipopolysaccharide O-antigen (Apicella et al. 2010). Many, but not all genes of the *wbt* locus, essential for expression of the LPS O-antigen are required for synthesis of the O-antigen capsule (Apicella et al. 2010), demonstrating the close relationship between the O-antigen and O-antigen capsule. Curiously, the *F. tularensis* genome also harbors an operon, *capBCA*, with homology to genes that are required for the production of a poly-D-glutamic acid capsule in *Bacillus* species (Michell et al. 2010). Although mutations in this operon have led to an attenuated phenotype in mice (Michell et al. 2010; Su et al. 2011), poly-D-glutamic acid has not been detected in *Francisella*, and its role in capsule production in *F. tularensis* is unclear.

The lipopolysaccharide (LPS) of *F. tularensis* is structurally and biologically unusual, exhibiting very low endotoxicity and stimulation of inflammatory pathways (Hajjar et al. 2006; Vinogradov and Perry 2004). In particular, structural features of the lipid A moiety of *F. tularensis* have been found to contribute to virulence. The lipid A component in *F. tularensis* is hypoacetylated, lacks a phosphate group at the 4' position usually present in lipid A of other bacteria (Vinogradov et al. 2002; Wang et al. 2007), and is modified by the addition of two carbohydrate moieties. Unique to *Francisella* species, 70 % of the total lipid A in the outer membrane has been found to exist in a "free" form, lacking core and O-antigen polysaccharides, and is further deacetylated in comparison to the complete LPS (Wang et al. 2006; Zhao and Raetz 2010). Several proteins responsible for lipid A modifications have been linked to virulence (Kanistanon et al. 2008; Wang et al. 2007; Llewellyn et al. 2012). Similarly, mutations in the O-antigen gene cluster (*wbt* locus), resulting in the loss of O-antigen, have been found to greatly increase serum sensitivity in both *F. tularensis* type A and type B, hinder intracellular replication, and attenuate the pathogen in animal models (Li et al. 2007; Raynaud et al. 2007; Lai et al. 2010). Due to the common repertoire of genes required for synthesis of both O-antigen and O-antigen capsule, however, the relative contribution to virulence by these structures is unclear.

Type IV pili (Tfp) are dynamic filamentous bacterial surface structures known to contribute to several physiological roles in bacteria, including adhesion to host cell surfaces and protein secretion. Analysis of genome sequences from *F. tularensis* subspp. *tularensis*, *holarctica*, and *novicida* has revealed the presence of Tfp homologs. In support of a functional Tfp apparatus in *F. tularensis*, Tfp-like structures have been observed on the bacterial surface (Gil et al. 2004) and disruption of predicted Tfp genes has abolished the presence of the Tfp-like structures (Chakraborty et al. 2008). Such mutants have also resulted in an attenuated virulence phenotype in mice models (Chakraborty et al. 2008; Salomonsson et al. 2011). Six major pilin subunits exist in *F. tularensis* genomes, PilE1 (or PilA)-PilE6, but PilA is the only subunit shown to be required for full virulence in both type A and type B strains (Forslund et al. 2006; Forslund et al. 2010). The complement of putative pili genes also differs among the various subspecies. While *F. tularensis* subspecies *tularensis* and *novicida* harbor a complete set, several predicted Tfp determinants are missing or are present as pseudogenes in subspecies *holarctica* (Chakraborty et al. 2008), notably the Tfp component responsible for retraction (PilT) (Forslund et al. 2010). In *F. tularensis* subsp. *novicida*, mutational inactivation of predicted Tfp genes and ancillary factors abolished the secretion of multiple proteins, suggesting that the Tfp may also have secretory activity (Hager et al. 2006).

The *Francisella* pathogenicity island (FPI) is a genomic region of approximately 30 kb coding 16–19 genes, most of which have been shown to be required for intracellular survival, phagosomal escape, and virulence (Nano et al. 2004; Twine et al. 2005; Santic et al. 2007; Vonkavaara et al. 2008; Ludu et al. 2008; Bröms et al. 2009; Ahlund et al. 2010). Two predicted operons are encoded within the FPI, the intracellular growth locus (igl) and pathogenicity determining protein locus (*pdp*). It is thought that several genes in the FPI encode components of a secretion system, similar to the type VI system recently discovered in other organisms (Bingle et al. 2008; Bröms et al. 2010). However, the effector molecules of the *Francisella* T6SS are yet to be discovered. The FPI was initially designated a pathogenicity island (Nano et al. 2004) due to the presence of virulence genes, a lower G+C content, and because it is duplicated and flanked by repeat sequences in the pathogenic subspecies *tularensis* and *holarctica* while present as a single copy in the less virulent *F. tularensis* subsp. *novicida* (Larsson et al. 2005). All *Francisella* genomes analyzed to date have, however, been found to contain the FPI, including subsp. *mediasiatica* (Champion et al. 2009), and more distantly related isolates including *F. noatunensis* (Sridhar et al. 2012), *F. philomiragia* (Zeytun et al. 2012), and several *novicida*- and *philomiragia*-like environmental isolates (Siddaramappa et al. 2012). This suggests that the FPI represents a ubiquitous feature deeply rooted within the *Francisella* and that its presence cannot be directly linked to pathogenicity. While the *Francisella* pathogenicity island (FPI) is present and conserved in both fish pathogenic subspecies (Soto et al. 2009b)

in common with *F. philomiragia*, the pathogenicity determining protein locus genes *pdpC* and *pdpE*, generally present in the tularemia clade, are absent (Larsson unpublished results). The ecological significance of the presence/absence of these genes is, however, not fully understood.

Transcription factors are critical for virulence in most bacterial pathogens. During recent years several transcription factors important for *F. tularensis* virulence have been identified, including MglA, SspA, PmrA, FevR, MigR, and Hfq. Importantly, *F. tularensis* contains few regulators with only one recognized alternative sigma factor and no classically arranged two-component system. All transcription factors known to regulate virulence genes are encoded within the FPI, further demonstrating the importance of this locus. MglA, the first transcriptional virulence regulator identified in *F. tularensis*, encoded in the bi-cistronic macrophage growth locus operon (*mglAB*) was subsequently found to regulate expression of more than 100 genes within and external to the FPI (Lauriano et al. 2004). Deletion of genes within the *mglAB* operon results in defective intracellular replication (Baron and Nano 1998), possibly due to an inability to regulate expression of the *iglABCD* genes of the FPI (Brotcke et al. 2006). In *F. tularensis* LVS, MglA and a second regulator with close homology to MglA, SspA, have been found to influence gene expression through an association with RNA polymerase (Costante-Hamm et al. 2007). The response regulator, PmrA also regulates expression of several genes within the FPI (Mohapatra et al. 2007). Disruption of PmrA in *F. tularensis* leads to susceptibility to antimicrobial peptide killing, lack of phagosomal escape in macrophages, and attenuation in the mouse model. The FevR transcriptional regulator is required for replication of *F. novicida* and *F. tularensis* LVS in murine and human macrophages (Brotcke and Monack 2008), in human epithelial cell lines (Buchan et al. 2009), and in mice (Brotcke and Monack 2008). *F. tularensis* LVS FevR mutants remain trapped within the phagosome and are compromised in their ability to block NADPH oxidase activity following infection of neutrophils (Buchan et al. 2009). FevR is also positively regulated by the response regulator PmrA (Mohapatra et al. 2007). The transcriptional regulator MigR lacks close homologs, and *F. tularensis* LVS *migR* mutants are attenuated for growth in human monocyte-derived macrophages. In addition to its ability to regulate the *igl* locus in LVS, MigR is also a positive regulator of transcription factor FevR (Buchan et al. 2009). The only known transcriptional repressor is an RNA-binding protein Hfq, which is shown to regulate expression of several genes in *Francisella*, including 10 FPI genes (Meibom et al. 2009; Chambers and Bender 2011). While Hfq deletion mutants have not indicated a major role for the protein in intracellular multiplication in murine macrophages, it does appear important for *Francisella* virulence in vivo (Meibom et al. 2009).

Francisella require iron for growth and virulence. Genes in the *fsl* operon (aka *fig* operon in subsp. *novicida*) are thought to encode components for production, release, and capture of a polycarboxylate siderophore responsible for ferric ($Fe3^+$) iron uptake (Sullivan et al. 2006). In contrast to most Gram-negative bacteria, the internalization pathway for siderophore-mediated iron uptake appears to occur independently of TonB, as no TonB homolog has been detected in *F. tularensis* (Crosa et al. 2009; Larsson et al. 2005). Interestingly, while the protein FslE has been identified as the likely ferric siderophore receptor, FupA, a paralog to FslE located outside the *fsl* operon, was recently shown to facilitate uptake of ferrous ($Fe2^+$) ions. Both FslE and FupA are required for full virulence (Ramakrishnan et al. 2012). Besides the *fsl* locus and *fopA*, another possible iron transporter in *F. tularensis* is the Feo system. The genes *feoA* and *feoB* have been identified in *F. tularensis* but not in a single operon (Sullivan 2006). Disruption of feoB has attenuated growth in a murine model of respiratory tularemia (Kraemer et al. 2009), but has otherwise not been studied in detail.

Acid phosphatases in *F. tularensis* (designated AcpA, B, C, and Hap) have attracted considerable interest and have in subsp. *novicida* been shown to directly participate in dephosphorylation of the host cell Phox components of NADPH oxidase, thus reducing production of reactive oxygen species (Mohapatra et al. 2008, 2010). However, as disruption of acid phosphatase production in *F. tularensis* subsp. *tularensis* SCHU S4 did not result in lower virulence, their importance for pathogenicity is uncertain (Child et al. 2010; McCaffrey et al. 2010).

Gene loss as a result of host specialization and adaptation in the human pathogenic *F. tularensis* lineages may also be linked to increased virulence. Decreased secretion of PepO conferred higher virulence in *F. novicida* mutants (Hager et al. 2006), possibly owing to the predicted vasoconstrictor activity of this protein, by enabling increased dissemination of the bacterium during infection. Analyses of the genomes of subspecies *holarctica* and *tularensis* have revealed a lack of expression of *PepO*, suggesting that their higher virulence may in part be attributed to this effect. Similarly, the human pathogenic subspecies also lost genes necessary for synthesis and degradation of cyclic di-GMP (cdGMP), which in subsp. *novicida* was found to promote biofilm formation and reduce virulence (Zogaj et al. 2012).

Clinical Relevance

Infection by *Francisella tularensis* causes tularemia, which is a serious and potentially life-threatening disease in humans. Tularemia is characterized by flu-like symptoms with enlarged and tender lymph nodes regional to the site of infection. Onset is abrupt, usually within 3–5 days, but can be as rapid as 1 day or as prolonged as 21 days postexposure (Penn 2010). Infections can initiate through the skin, mucosal membranes, lungs, and gastrointestinal tract, to produce different tularemia manifestations that principally depend on the site of infection (ulceroglandular, glandular, oropharyngeal, oculoglandular, and pneumonic forms). If other forms of tularemia are left untreated, pneumonic tularemia may follow as a result of hematogenous spread of the bacteria to the lungs, and the disease may also progress to septic shock.

The severity of the disease depends on strain/subspecies of infecting bacteria and the clinical presentation, where pneumonic tularemia is the most serious form. Strains of *F. tularensis* subsp. *tularensis* (type A) are typically more aggressive than those belonging to subsp. *holarctica* (type B). The clinical significance of the two remaining *F. tularensis* subspecies is limited. *F. tularensis* subsp. *mediasiatica* is not reported in the English language literature as causing human disease, and *F. tularensis subsp. novicida* has been found to be infectious predominantly in immuno-compromised humans. Human infection with *F. tularensis* subsp. *novicida* is exceedingly rare, with only six cases published in the English literature (Brett et al. 2012).

The overall case-fatality rate of human infections by *F. tularensis* type A is currently less than 2 % but was significantly higher during the pre-antibiotic era (Dennis et al. 2001). Overall mortality levels of 5–15 %, with 30–60 % for severe forms, are reported for untreated tularemia (Dennis et al. 2001). Recent epidemiological data has, however, identified substantial virulence differences also within *F. tularensis* type A, where isolates belonging to subclade AIb demonstrated a significantly higher case-fatality rate than members of subclades AIa and AII (Molins et al. 2010). The case-fatality rate for infections by *F. tularensis* subsp. *holarctica* is negligible.

A distinctive characteristic of *F. tularensis* is its high infectivity, being one of the most infectious human pathogens known. In previous studies on human volunteers, conducted during the 1950s and 1960s, it was determined that the dose of this pathogen needed to cause an infection is as low as 10 organisms. Using previous data, it has been further estimated that, although susceptibility varies, approximately 30 % of individuals who inhale a single *F. tularensis* bacterium will develop tularemia (Jones et al. 2005). Although *F. tularensis* can be cultured from pharyngeal washings and sputum specimens from patients with respiratory tularemia, suggesting a potential for aerosol spread, secondary (person-to-person) transmission of tularemia is not known to occur. However, due to its exceptional infectiousness, tularemia remains one of the most commonly reported laboratory-associated bacterial infections (Pike 1976; Shapiro and Schwartz 2002). For this reason precautionary measures must be practiced when handling cultures of this pathogen.

F. philomiragia, although originally described as a pathogen of semiaquatic rodents (Jensen et al. 1969), is rarely isolated as such. Human infections with *F. philomiragia*, although frequently fatal, are rare and have in the main either been associated with an immuno-compromised condition or near-drowning incidents (Hollis et al. 1989; Wenger et al. 1989). While *F. hispaniensis* (Huber et al. 2010) and *F. novicida* strains likely to represent *F. hispaniensis* (Whipp 2003; Siddaramappa et al. 2011; Leelaporn et al. 2008) have been identified from several clinical human cases worldwide, this bacterium does not appear to pose a significant threat to human or animal health.

Francisella noatunensis subspp. *noatunensis* and *orientalis* have been described as the causative agents of francisellosis in fish. This disease often presents as systemic, chronic, granulomatous infections with varying associated mortality levels. Since the first outbreaks caused by subspecies *noatunensis* were observed in farmed Atlantic cod in 2004/2005 (Nylund et al. 2006; Olsen et al. 2006), the disease has increased in prevalence and has now been diagnosed in clinically infected cod in numerous aquaculture facilities spanning most of the mid- and southern Norwegian coastline, as well as isolated cases in Denmark (Ottem et al. 2007a, 2008) and Ireland.

Initial reports of francisellosis caused by *F. noatunensis* subsp. *orientalis* date back to the early 1990s, when outbreaks of a systemic granulomatous condition in cultured tilapia *Oreochromis* sp., defined initially as Rickettsia-like organism (RLO) infections, were discovered in Taiwan (Chern and Chao 1994; Chen et al. 1994). Since then, clinical cases have been reported from several warmwater aquaculture species including hybrid striped bass *Morone chrysops* × *M. saxatilis*, three-line grunt *Parapristipoma trilineatum*, and ornamental cichlid species in the USA (Hawaii, California, Florida, Texas, and midwestern states), Costa Rica, Haiti, Jamaica, the UK, and Japan (Birkbeck et al. 2011; Colquhoun and Duodu 2011; Soto et al. 2011a). Mortalities of up to 40 % have been described (Olsen et al. 2006) in cod, while mortality rates as high as 95 % have been reported in tilapia (Chen et al. 1994). Little is known of the mortalities associated with the subsp. *noatunensis* strain causing sporadic disease in Atlantic salmon juveniles cultured in freshwater in Chile (Birkbeck and Bordevik 2007). Environmental conditions, in particular temperature, appear to play a significant role in the rate of mortality (Kamaishi et al. 2010; Ostland et al. 2006; Soto and Revan 2012). Infectivity of subsp. *noatunensis* in cod is high (Mikalsen et al. 2009) and as few as 23 cfu subsp. *orientalis* can cause significant mortality in tilapia fingerlings (Soto et al. 2009b). However, mortalities associated with natural infections in other fish species may not be as high (Bohle et al. 2009; Ostland et al. 2006). More recently, a species highly pathogenic for abalone (Kamaishi et al. 2010), *F. halioticida*, has been described (Brevik et al. 2011a). Inoculation by intramuscular injection of 32 cfu per individual abalone resulted in 100 % mortality after 16 days at 15 °C (Kamaishi et al. 2010). Presently, little is understood of the impact of wild fish infection on cultured fish and vice versa. Unlike the human pathogens, the fish pathogenic *Francisella* species pose no risk of zoonotic infection in humans.

Antibiotic Resistance

While the antibiotic susceptibilities of the major human pathogens, i.e., *F. tularensis* subspp. *tularensis* and *holarctica*, are relatively well described (Ikaheimo et al. 2000; Baker et al. 1985; Kreizinger et al. 2013) and treatment regimes for clinical disease established (Johansson et al. 2000a; Dennis et al. 2001), relatively little is known regarding intrinsic and/or acquired antibacterial resistance among the fish and mollusc pathogenic species/subspecies and even less regarding the more "environmental" strains/species.

F. tularensis is susceptible to many common antibiotics, including streptomycin, gentamicin, doxycycline, chloramphenicol, and

quinolones (WHO 2007, http://www.cdc.gov/tularemia/resources/whotularemiamanual.pdf). The pathogen is not known to rapidly develop resistance (WHO 2007), and the lack of contact with other bacteria, demonstrated by the very low influence of genetic recombination, probably restricts uptake of mobile resistance determinants. The absence of human-to-human transmission also means that this is not an important driver of resistance evolution. High intrinsic expression of beta-lactamase, a trait also common to *F. tularensis novicida*, *F. philomiragia*, and *F. noatunensis* (Hollis et al. 1989; Mikalsen et al. 2007) and most probably in penicillin resistant *F. noatunensis* subsp. *orientalis* and *F. halioticida* (Soto et al. 2012b; Brevik et al. 2011a), awards these bacteria a high degree of resistance to ampicillin and most other penicillin derivatives.

Susceptibility to erythromycin is variable within the genus and within some species. *F. tularensis* type B strains, prevalent in Europe but not in North America and *F. tularensis subsp. holarctica* are heterogeneous with respect to erythromycin sensitivity. The variable sensitivity to erythromycin provided a basis for separation of these strains into biovars I (susceptible) and II (resistant) (Olsufiev and Meshcheryakova 1982; Sandström et al. 1992). Both *F. noatunensis* subspp. are reported to be resistant to erythromycin (Isachsen et al. 2012; Soto et al. 2012b), while *F. halioticida* and *F. hispaniensis* are sensitive (Brevik et al. 2011a; Huber et al. 2010). Resistance to erythromycin is reported to be variable within *F. philomiragia* and *F. tularensis* subsp. *novicida* (Hollis et al. 1989). The molecular mechanisms of resistance to erythromycin have not been functionally established, but mutations identified in domain V of the 23S rRNA in *F. tularensis* type B, biovar II, could provide a likely explanation (Biswas et al. 2008).

F. philomiragia and *F. tularensis subsp. novicida* were further deemed susceptible to nalidixic acid, norfloxacin and ciprofloxacin, streptomycin, gentamicin, tobramycin, amikacin, tetracycline, chloramphenicol, moxalactam, cefotaxime, and cefoxitin. *F. hispaniensis* is reportedly resistant to meropenem, amoxicillin plus clavulanic acid, ampicillin, and aztreonam and susceptible to chloramphenicol, tetracycline, doxycycline, gentamicin, tobramycin, streptomycin, nalidixic acid, ciprofloxacin, levofloxacin, and rifampicin (Huber et al. 2010).

Isachsen et al. (2012) recently demonstrated the susceptibility of several subsp. *noatunensis* isolated from Norwegian Atlantic cod to florfenicol, oxolinic acid, flumequine, and rifampin. The same isolates were resistant to oxytetracycline, trimethoprim/sulfadiazine (Tribrissen), ciprofloxacin, and streptomycin sulfate. Similarly, Bohle et al. (2009) reported comparable levels of sensitivity for flumequine and oxolinic acid in Chilean isolates from Atlantic salmon. *F. noatunensis* subsp. *orientalis* appears susceptible to gentamicin, neomycin, oxytetracycline, tetracycline, florfenicol, streptomycin, novobiocin, amikacin, ciprofloxacin, and other fluoroquinolones, while resistant to amoxicillin, ampicillin, piperacillin, oxacillin, cefuroxime, vancomycin and potentiated sulfonamides (Soto et al. 2012b). Differences apparent between (and within) the fish pathogenic subspecies include susceptibilities to oxytetracycline and ciprofloxacin (Soto et al. 2012b; Isachsen et al. 2012; Bohle et al. 2009; Ottem et al. 2007b) with all isolates of subsp. *orientalis* tested being susceptible to these agents and subsp. *noatunensis* isolates varying in susceptibility (Ottem et al. 2007b; Isachsen et al. 2012). *F. halioticida* is reported to be sensitive to ceftazidime, ciprofloxacin, gentamicin, and tetracycline and resistant to ampicillin, cefuroxime, and penicillin (Brevik et al. 2011a).

F. tularensis as a Biological Weapon

Trials on human volunteers exposed to aerosols of *F. tularensis* type A (10–50 cells of highly virulent SchuS4 strain) resulted in systematic infection in 16 of 20 volunteers within 4–7 days of exposure (Saslaw et al. 1961). This high attack rate by the respiratory route combined with low infective dose, potential for human disease and death, and relative ease of production and dissemination has led to classification of *F. tularensis* as a category A bioterrorism agent by the Centers for Disease Control and Prevention (CDC) (Rotz et al. 2002).

Research into use of *F. tularensis* as a biological weapon, initiated by Japanese research units in the period 1932–1945 (Dennis et al. 2001), was further developed by both the USA and USSR. These studies were related to aerosol dispersion technology and aerosol survival from wet and dry states at different temperatures and relative humidity (Cox 1971; Ehrlich and Miller 1973), as well as introduction of antibiotic resistance (SIPRI, 1973). *F. tularensis*, possibly antibiotic resistant, was one of the principal agents designated by the USSR for tactical or operational battlefield use (Davis 1999). A World Health Organization report (WHO 1970) estimated that aerosol dispersal of 50 kg virulent *F. tularensis* over a metropolitan area with five million inhabitants would result in 250,000 incapacitating casualties, including 19,000 deaths.

Development of disease following a suspected deliberate attack with *F. tularensis* has been described (Hodges and Penn 2010), and recent research emphasis has shifted toward defense against bioterrorism rather than attack (Dennis et al. 2001). The potential impact of this organism as a bioterrorist weapon was estimated to include 82,500 cases involving 6,188 deaths for every 100,000 people exposed (Kaufmann et al. 1997). Recently, a model involving daytime release of *F. tularensis* in central London predicted 2.4 million people exposed, 130,000 infected, and 24,000 deaths (Egan et al. 2011).

For at least tularemia endemic areas, detailed information on endemic strains would greatly assist in differentiating between naturally occurring cases and intentional releases. Reference data based on whole-genome sequencing of outbreak strains would provide a powerful tool in understanding natural population structures and may be crucial in microbial forensics in the event of bioterrorism.

Treatment and Prevention

The bactericidal aminoglycosides have a long history of proven clinical efficacy in treatment of tularemia (Enderlin et al. 1994).

Streptomycin is the primary choice, although gentamicin is an acceptable substitute for seriously ill patients (Penn 2010). Ototoxic and nephrotoxic side effects combined with the absence of oral formulations do, however, limit the use of aminoglycosides in clinical practice. For uncomplicated forms of tularemia, the bacteriostat doxycycline is generally preferred, despite the relatively high risk of relapse, as it may be administered orally. Recent microbiological and clinical trials have shown that the quinolone ciprofloxacin probably constitutes an effective first-line therapy for tularemia, particularly for outpatients or in outbreak situations with limited treatment options (Tärnvik and Chu 2007; Ulu Klç et al. 2011, 2012). Quinolones offer several advantages; they can be administered orally, are bactericidal, have high bioavailability, achieve high intracellular concentrations, and have lower toxicity compared to aminoglycosides. However, while levels of experience relating to quinolone treatment of tularemia are excellent in Europe, such experiences are lacking in North America, particularly in relation to treatment of serious cases (Tärnvik and Chu 2007; Ulu-Kilic et al. 2012). Antibiotic susceptibility analysis supports the hypothesis that quinolone treatment is likely to constitute an effective therapy for severe *F. tularensis* subsp. *tularensis*-related tularemia (Johansson et al. 2002). Early initiation of treatment is, however, critical for successful doxycycline or ciprofloxacin therapy (Enderlin et al. 1994; Ulu-Kilic et al. 2012). While a degree of success relating to treatment of francisellosis in farmed fish has been described, this has been exclusively related to tetracycline (Mauel et al. 2005; Ostland et al. 2006) and oxytetracycline (Chern and Chao 1994) treatment of *F. noatunensis* subsp. *orientalis* infections in warmwater fish. Attempted field treatments of francisellosis caused by *F. noatunensis* subsp. *noatunensis* in Atlantic cod, i.e., cold-water fish, have not been successful. Due to the intracellular nature of the infection, high prevalence of infected fish, high transmissibility, and low infective dose combined with high morbidity and lack of appetite in severely infected fish, there is reason to believe that antibiotic therapy is unlikely to provide a lasting effect in infected populations.

Currently no effective vaccines are approved or commercially available for treatment of tularemia in humans or francisellosis in fish. However, a vaccine based on *F. tularensis* subsp. *holarctica* was developed in the former USSR and used with apparent success (Sjöstedt 2007). Subsequent development of this vaccine by US researchers resulted in the live vaccine strain (LVS) shown to be effective in inducing protection against tularemia in both animal models and humans (Tigertt 1962; Sandström 1994; Conlan 2011). However, LVS is suboptimal against respiratory compared with systemic routes of infection, and due to the uncharacterized nature of the vaccine strain and difficulties in standardization, it is not licensed for general use. In the USA the LVS vaccine is available to at-risk personnel via the Special Immunization Program administrated by the Department of Defense (Conlan 2011). Vaccination using variants of the vaccine developed in the former USSR is still used in parts of Russia. A vaccine based on a ΔiglC mutant has recently been described for francisellosis in tilapia (Soto et al. 2011b).

Detection/Diagnosis/Typing

Until recently, clinical diagnosis of *Francisella* infection has primarily relied on classical microbiological and histopathological methods. Various immuno-based assays including microagglutination, ELISA, direct immunofluorescence, and immunohistochemistry methods have been used for detection of *F. tularensis* in clinical specimens (Mörner et al. 1988; Petersen et al. 2004), with higher detection specificity obtained by direct or indirect fluorescent antibody staining or immunohistochemical methods. Histological examination of formalin-fixed paraffin-embedded tissues (FFPE) is one of the most commonly used diagnostic procedures in fish francisellosis investigation (Colquhoun and Duodu 2011). Although serology can confirm infection retrospectively, cross-reactions occur among different *Francisella* strains and subspecies as well as with other unrelated organisms including *Brucella*, *Salmonella*, *Yersinia*, and *Legionella* (Francis and Evans 1926; Ohara et al. 1974; Birkbeck and Bordevik 2007; Ottem et al. 2007b). Serology may be less suitable in acute treatment decision making for humans, as antibodies are not produced until 10–14 days postexposure.

Culturing remains the "gold standard" for confirmatory identification of both human and fish pathogenic strains, although there are several challenges associated with this approach. *Francisella* are known to be highly fastidious in their requirements for growth and easily inhibited by concomitant flora. The bacteria can take several days to grow on laboratory media, and cultivation of *F. tularensis* is often avoided, since it poses a considerable risk for laboratory-acquired infection.

The polymerase chain reaction (PCR) has emerged as a rapid and reliable diagnostic tool for direct detection of *Francisella* DNA in clinical specimens (Johansson et al. 2000b, 2004b). Conventional PCR assays targeting one or a few genes, e.g., *IpnA*, *fopA*, *groEL*, and 16S rDNA, have been used to establish the absence or presence of *Francisella* in clinical samples (Forsman et al. 1994; Fulop et al. 1996; Long et al. 1993; Sjöstedt et al. 1997; Caipang et al. 2010). Real-time quantitative PCR (qPCR) offers higher sensitivity and specificity (Fujita et al. 2006; Kugeler et al. 2006; Molins et al. 2009; Ottem et al. 2008; Soto et al. 2010; Tomaso et al. 2007; Versage et al. 2003). Existing real-time qPCR protocols are able to distinguish between different *Francisella* subspecies. qPCR assays separating the highly virulent subsp. *tularensis* from the less virulent subsp. *holarctica* have been developed, as well as a single and multiplex qPCRs that discriminates all four *F. tularensis* subspecies and even subpopulations (World Health Organization 2007; Molins-Schneekloth et al. 2008; Gunnell et al. 2012). More recently, qPCR protocols based on unique genes identified by whole-genome sequence comparisons have been developed for specific detection of all pathogenic *F. tularensis* and *F. noatunensis* subspecies (Mitchell et al. 2010; Duodu et al. 2012b). In situ hybridization technology using either digoxigenin (DIG) probes directed at the 16S rRNA molecule or fluorescence probes based on the 23S rRNA molecule has also been developed for rapid identification and differentiation of various *Francisella* infections (Hsieh et al. 2007; Splettstoesser et al. 2010).

An array of genotypic methods has have been applied for typing of *Francisella* using DNA-based technology. Methods including ribosomal gene typing, multilocus sequence typing (MLST), restriction and amplified fragment length polymorphism (AFLP) typing, PFGE, deletion-based typing schemes, multiple-locus variable-number tandem repeat analysis (MLVA), single-nucleotide polymorphisms (canSNPs) (Svensson et al. 2009b), and recently whole-genome sequencing (reviewed by Johansson and Petersen 2010) have all been used in the characterization of *Francisella*, contributing significantly to surveillance and epidemiological investigations of these organisms. These methods vary with respect to level of resolution, depending on whether gene-order arrangement or nucleotide sequence difference is analyzed (for review, see Keim et al. 2007). The pre-genome sequencing methods could only provide resolution to the subspecies level (De la Puente-Redondo et al. 2000; Johansson et al. 2000c, 2004a; Thomas et al. 2003). By genotyping polymorphic sequences with higher mutational rates such as variable number of tandem repeats (VNTR) identified from whole-genome sequencing, resolution to the strain level can be achieved (Johansson et al. 2004a; Larsson et al. 2007; Vogler et al. 2009). Clearly, whole-genome sequence analyses represent the most powerful methods for reconstruction of accurate phylogenies as well as distinguishing individual closely related strains. Nevertheless, application of these different typing methods during recent years has highlighted difficulties connected with the limited genetic diversity between the pathogenic and closely related nonpathogenic *Francisella* spp. For clinical samples this is normally not a problem, but during environmental surveillance false-positive detections are frequent (Shea and Lister 2012; Kman and Bachmann 2012). This is because current methods for DNA detection of *Francisella* were developed with limited knowledge of the natural genetic diversity. In a recent study, published DNA markers and their corresponding PCR primers were evaluated against 42 genomes representing the currently known diversity within the genus *Francisella*. The results highlight that PCR assays for *F. tularensis* detection are complicated by low specificity, resulting in a high probability of false positives if applied on environmental samples (Ahlinder et al. 2012). Application of deep sequencing technologies may ultimately allow specific detection of *Francisella* (Kuroda et al. 2012).

Concluding Remarks

The genus *Francisella* represents a closely related and increasingly recognized population of bacterial taxa of diverse ecological niche. While several taxa are serious human/animal pathogens, the ecological roles played by the majority of members of the genus remain unknown. The molecular adaptations behind the extraordinary ability of the pathogenic species and strains to cause disease remain elusive. Future research will hopefully answer these questions.

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15 The Family *Granulosicoccaceae*

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Abstract
Granulosicoccaceae is a family belonging to the order *Chromatiales* within the class *Gammaproteobacteria*. There are two species validly ascribed to this family, both of which belong to the genus *Granulosicoccus*: the type species *G. antarcticus* and *G. coccoides*. The branching within *Chromatiales* based on the analysis of the 16S rRNA gene sequences of *G. antarcticus* and *G. coccoides*, as well as their chemotaxonomic profiles, is indicative that these two species do form a generic lineage distinct from the other members of *Chromatiales* known to date. Both species were originally isolated from marine environments and test positive for oxidase, catalase, gelatinase, and utilization of various sugars and amino acids.

This contribution is a modified and updated version of the original species descriptions (Lee et al. J Microbiol Biotechnol 17:1483–1490, 2007; Kurilenko et al. Int J Syst Evol Microbial 60:972–976, 2010).

Taxonomy, Historical and Current

Short Description of the Family

Granulosicoccaceae Lee, Lee, Choi, Kim and Cho 2007 1483^{VP}

Gra.nu.lo.si.co.cca'ce.ae. N.L. masc. n. *Granulosicoccus* type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. *Granulosicoccaceae* the family of the genus *Granulosicoccus*.

The family *Granulosicoccaceae* encompasses obligately chemoheterotrophic Gram-negative bacteria within the order *Chromatiales* in the class *Gammaproteobacteria*. The delineation of the family was primarily determined by the phylogenetic position of 16S rRNA gene sequences and the phenotypic properties of the genus *Granulosicoccus* (Lee et al. 2007).

The family *Granulosicoccaceae* belongs to the order *Chromatiales*, within the class *Gammaproteobacteria*. The type and only genus of the family is *Granulosicoccus*. Currently there are only two species that have been classified as belonging to *Granulosicoccus*: the type species *G. antarcticus* (Lee et al. 2007) and *G. coccoides* (Kurilenko et al. 2010). Cells are Gram-negative cocci, which are typically 1–1.2 μm in diameter. Motile by tuft flagella. Strictly aerobic, chemoorganoheterotrophs, capable of utilizing various carbohydrates. Major fatty acids include 11-octadecenoic acid ($C_{18:1}$ $\omega 7c$), 9-hexadecenoic acid ($C_{16:1}$ $\omega 7c$), and hexadecanoic acid ($C_{16:0}$). Ubiquinone Q-8 is the major isoprenoid quinone. The signature 16S rRNA gene sequence positions are 16:573 (C-G), 574:583 (T-T), 586:607 (T-G), 609:611 (G-T), 613:615 (A-C), 617:637 (T-G), 640:672 (A-C), 674:700 (G-C), 702:850 (G-G), 852:992 (A-T), 994:1002 (G-T), 1004:1009 (A-T), 1011:1013 (A-T), 1015:1029 (G-C), 1031:1123 (A-C), 1125:1132 (C-G), and 1134:1491 (G-A). The G+C content of DNA ranges from 58.0 to 60.2 mol%. Isolated from marine environments.

Phylogenetic Position

The branching of the phylogenetic tree in ➊ *Fig. 15.1* does not show clear distinction between *Granulosicoccaceae* and *Thioalkalispiraceae*; however, based on phenotypic data and 16S rRNA gene sequence analysis, the two species of *Granulosicoccus* clearly form a distinct phylogenetic lineage. It is expected that this region of the tree of life will undergo significant additions and perhaps reorganization in the future as more isolates of the *Chromatiales* are identified.

List of type strains used for dendrogram construction: *Chromatium okenii* DSM 169^T, *Thiocystis violacea* DSMZ 207^T, *Thiodictyon bacillosum* DSM 234^T, *Thiocapsa roseopersicina* 1711^T, *Allochromatium vinosum* DSM 180 ^{T}, *Thiorhodococcus minor* CE2203^T, *Thiococcus pfennigii* 4250^T, *Thioflavicoccus mobilis* 8320^T, *Halochromatium roseum* JA134^T, *Thioprofundum lithotrophicum* 106^T, *Thioprofundum hispidum* gps61^T, *Thiohalophilus thiocyanatoxydans* HRhD 2^T, *Coxiella burnetii* ATCC VR-615^T, *Granulosicoccus antarcticus* IMCC3135^T, *Granulosicoccus coccoides* Z271^T, *Thioalkalispira microaerophila*

E. Rosenberg et al. (eds.), *The Prokaryotes – Gammaproteobacteria*, DOI 10.1007/978-3-642-38922-1_247,

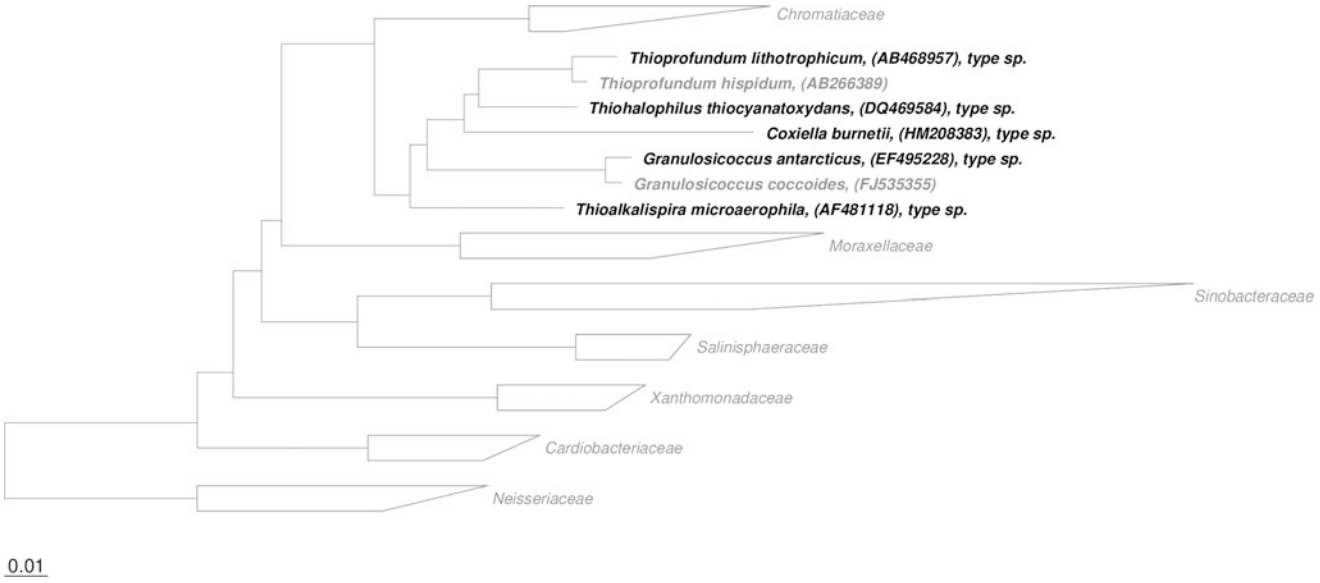

Fig. 15.1

Phylogenetic reconstruction of the family *Granulosicoccaceae* based on 16S rRNA gene sequences and created using the maximum likelihood algorithm RAxML (Stamatakis 2006). The sequence dataset and alignment were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010); (http://www.arb-silva.de/projects/living-tree). Representative sequences from closely related taxa were used as out-groups. In addition, a 40 % maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. Scale bar indicates estimated sequence divergence

ALEN 1^T, *Psychrobacter frigidicola* DSM 12411^T, *Psychrobacter okhotskensis* MD17^T, *Psychrobacter proteolyticus* 116^T, *Psychrobacter salsus* DD48^T, *Psychrobacter fulvigenes* KMM 3954^T, *Moraxella bovoculi* 237^T, *Moraxella lacunata* ATCC 17967^T, *Moraxella nonliquefaciens* CCUG 348^T, *Acinetobacter brisouii* 5YN5-8^T, *Acinetobacter schindleri* LUH5832^T, *Acinetobacter venetianus* ATCC 31012^T, *Alkanindiges illinoisensis* MVAB Hex1^T, *Solimonas variicoloris* MN 28^T, *Solimonas soli* DCY12^T, *Solimonas flava* CW-KD 4^T, *Solimonas aquatica* NAA16^T, *Panacagrimonas perspica* Gsoil 142^T, *Hydrocarboniphaga effusa* AP103^T, *Hydrocarboniphaga daqingensis* B2-9^T, *Nevskia ramosa* Soe1^T, *Nevskia soli* GR15-1^T, *Nevskia terrae* KIS13-15^T, *Alkanibacter difficilis* MN 154.3^T, *Steroidobacter denitrificans* FS^T, *Salinisphaera dokdonensis* CL-ES53^T, *Salinisphaera shabanensis* E1L3A^T, *Salinisphaera hydrothermalis* EPR70^T, *Ignatzschineria indica* FFA1^T, *Ignatzschineria ureiclastica* FFA3^T, *Ignatzschineria larvae* L1/68^T, *Wohlfahrtiimonas chitiniclastica* S5^T, *Cardiobacterium hominis* ATCC 15826^T, *Cardiobacterium valvarum* MDA3079^T, *Suttonella indologenes* ATCC 25869^T, *Suttonella ornithocola* B6/99/2^T, *Conchiformibius steedae* IAM 14972^T, *Alysiella filiformis* ATCC 15532^T, *Neisseria elongata* subsp. *glycolytica* ATCC 29315^T, *Neisseria gonorrhoeae* NCTC 83785^T, *Aquitalea magnusonii* TRO-001DR8^T, and *Chitinibacter tainanensis* BCRC 17254^T.

Molecular Analyses

Being a small family currently containing only two validly described species, the molecular analyses performed on *Granulosicoccaceae* have been limited. DNA-DNA hybridization has been performed to determine the overall genomic similarity between *G. antarcticus* and *G. coccoides*. These two species were shown to share 35 % of their genomic DNA (Kurilenko et al. 2010).

Phenotypic Analyses

Cells belonging to the genus of *Granulosicoccus* are Gram-negative cocci, which are typically 1–1.2 μm in diameter but can range in diameter between 0.8 μm and 2.2 μm. When grown on marine agar, colonies are generally between 0.3 mm and 1.5 mm in diameter, circular, regular, convex, opaque, and pale yellowish in color. Specific phenotypic traits can be found below in *Table 15.1*.

Isolation, Enrichment, and Maintenance Procedures

The original strain of *Granulosicoccaceae*, *Granulosicoccus antarcticus*, was first isolated from seawater collected on the coast of the Weaver Peninsula, King George Island, Antarctica (62°14′S, 58°47′E). A 100 μL aliquot was spread on oligotrophic plates consisting of R2A agar (Difco) diluted by a factor of 10 in aged seawater. Plates were incubated for 1 month at 20 °C, before individual colonies were selected and purified on marine agar (Difco), incubating for 1 week at 20 °C.

Table 15.1

Chemotaxonomic and morphological traits of members of the *Granulosicoccus* genus. Bacterial cells of both species are Gram negative, motile, nonpigmented, obligate aerobes, chemoorganoheterotrophs, accumulate PHB, and catalase and oxidase positive; ubiquinone 8 (Q-8) is a major respiratory quinone

Phenotypic trait	*Granulosicoccus antarcticus*	*Granulosicoccus coccoides*
Cellular morphology	Spherical	Spherical
Size	0.8–2.2 μm i.d.	~1 μm
Temperature requirements	3–25 °C, optimum 20 °C	5–28 °C, optimum 25 °C
NaCl requirements	0.5–5 %, optimum 2 %	0.5–5 %, optimum 2.5 %
Optimum pH	7.0	7.5
Budding	–	+
G + C %	58.0	60.2
Gelatinase	+	w
Major fatty acids	$C_{16:1}$ $\omega 7c$ and/or iso-$C_{15:0}$ 2-OH (38.9 %), $C_{18:1}$ $\omega 7c$ (20.4 %), $C_{16:0}$ (13.2 %), and $C_{10:0}$ 3-OH (9.5 %)	$C_{18:1}$ $\omega 7c$ (43.7 %), $C_{16:1}$ $\omega 7c$ (31.1 %), and $C_{16:0}$ (16.8 %)
Enzymatic profile[a]	Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, α-chymotrypsin	Esterase (C4), esterase lipase (C8), lipase (C14), acid phosphatase, naphthol-AS-BI-phosphohydrolase, *N*-acetyl-b-glucosaminidase
Carbon substrates[b]	L-Proline, L-threonine, putrescine, 2-aminoethanol, and D,L-α-glycerol phosphate	α-Cyclodextrin, β-cyclodextrin, dextrin, glycogen, inulin, mannan, L-arabinose, D-arabitol, cellobiose, D-mannitol, D-ribose, sucrose, D-xylose, acetic acid, inosine, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate

w weak reaction

[a]Enzymatic profile as determined using API ZYM strips (bioMérieux)

[b]In addition to the abovementioned substrates, both species are also able to utilize TWEEN 40, TWEEN 80, D-fructose, L-fucose, D-galactose, maltose, D-mannose, D-sorbitol, D-galacturonic acid, β-hydroxybutyric acid, α-ketoglutaric acid, malonic acid, succinamic acid, glucuronamide, glycyl-L-aspartic acid, glycyl-L-glutamic acid, and L-serine

G. coccoides was first isolated from the leaves of the seagrass *Zostera marina* collected from Troitza Bay of the Gulf of Peter the Great. Leaves were washed with sterile seawater, and 100 μL aliquots of the resulting suspension were cultivated by incubating at 28 °C for 1 week on plates containing 0.2 % (w/v) Bacto peptone (Oxoid), 0.2 % (w/v) casein hydrolysate (Merck), 0.2 % (w/v) Bacto yeast extract (Oxoid), 0.1 % (w/v) glucose, 0.02 % (w/v) KH_2PO_4, 0.005 % (w/v) $MgSO_4$, $7H_2O$, 1.5 % (w/v) Bacto agar (Oxoid), 50 % (v/v) natural seawater, and 50 % (v/v) distilled water at pH 7.8. Individual colonies were then selected and cultured on marine agar (BD) at 28 °C.

Ecology

Both species of *Granulosicoccaceae* were originally isolated from marine environments. *G. antarcticus* was collected growing planktonically in Antarctic seawater on the coast of the Weaver Peninsula, King George Island, Antarctica (62°14′S, 58°47′E). *G. coccoides* was first recovered from the surface of the leaves of the seagrass *Zostera marina*, which was collected from Troitza Bay of the Gulf of Peter the Great.

Pathogenicity, Clinical Relevance

As species of *Granulosicoccaceae* are marine bacteria there has been little work conducted investigating their pathogenicity. However, it is known that *G. antarcticus* is sensitive to the following antibiotics: chloramphenicol, erythromycin, gentamicin, kanamycin, rifampicin, streptomycin, and tetracycline.

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16 The Families *Hahellaceae* and *Litoricolaceae*

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Abstract
The families *Hahellaceae* and *Litoricolaceae* belong to the order *Oceanospirillales*, within the class *Gammaproteobacteria*. *Litoricolaceae* is a monogeneric family, containing only the type genus *Litoricola* (spp. *L. lipolytica* and *L. marina*), while a total of five genera have been assigned to the family *Hahellaceae*: the type genus *Hahella* (*H. chejuensis*, *H. ganghwensis* and *H. antarctica*), *Zooshikella* (*Z. ganghwensis*), *Halospina* (*H. denitrificans*), *Endozoicomonas* (*E. elysicola* and *E. montiporae*) and *Kistimonas* (*K. asteriae*). All known species of these genera are Gram-negative rod-shaped cells, with an absolute NaCl requirement for growth. Growth occurs aerobically; however, some species are facultative anaerobes.

Taxonomy: Historical and Current

Short Description of the Families

Hahellaceae Garrity, Bell and Lilburn 2005

Hahellaceae (Ha.hel.la'ce.ae. M.L. fem. n. Hahella type genus of the family; -aceae ending to denote family; M.L. fem. pl. n. Hahellaceae the Hahella family (Garrity et al. 2005).

The family *Hahellaceae* was circumscribed in 2005 on the basis of phylogenetic analysis of 16 s rDNA sequences, and is within the order *Oceanospirillales*. The family contains the genera *Hahella* (type genus) (Lee et al. 2001), *Zooshikella* (Yi et al. 2003), *Halospina* (Sorokin et al. 2006), *Endozoicomonas* (Kurahashi and Yokota 2007), and *Kistimonas* (Choi et al. 2010b). All described species are either obligately aerobic or facultatively anaerobic chemoorganotrophs.

Litoricolaceae Kim, Choo and Cho 2007

Litoricolaceae (Li.to.ri.co.la'ce.ae. N.L. fem. n. *Litoricola* type genus of the family; *-aceae* ending to denote a family; N.L. fem. pl. n. *Litoricolaceae* the family of the genus *Litoricola*) (Kim et al. 2007b).

The family *Litoricolaceae* is within the order *Oceanospirillales* and encompasses Gram-negative bacteria retrieved from marine environments. Currently, the type genus *Litoricola* (Kim et al. 2007b) is the only validly described genus in the family. The delineation of the family is primarily determined from the phylogenetic position of the 16S rRNA gene sequence. The detailed description is the same as given below for the genus *Litoricola*. Both of the only two species described to date are facultatively anaerobic chemoheterotrophs.

E. Rosenberg et al. (eds.), *The Prokaryotes – Gammaproteobacteria*, DOI 10.1007/978-3-642-38922-1_287,

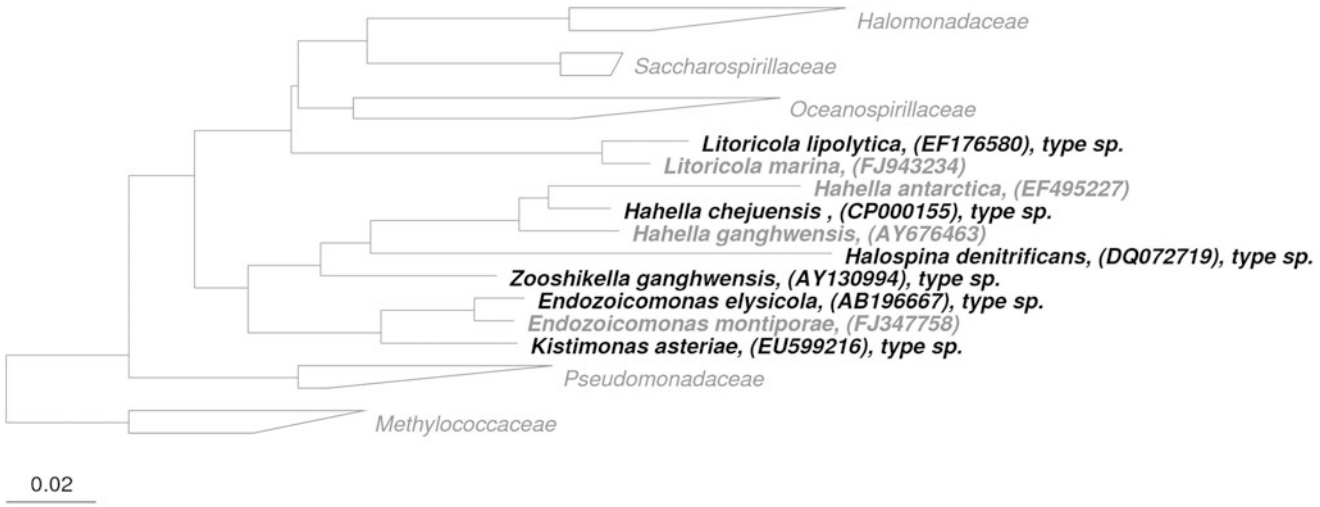

Fig. 16.1

Phylogenetic reconstruction of the families *Hahellaceae* and *Litoricolceae* based on 16S rRNA and created using the maximum likelihood algorithm RAxML (Stamatakis 2006). The sequence dataset and alignments were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). Representative sequences from closely related taxa were used as outgroups. In addition, a 40 % maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. Scale bar indicates estimated sequence divergence

Phylogenetic Position

Phylogenetic analysis via generation of dendograms using 16S gene sequences and maximum likelihood algorithms suggests that the nearest related families to *Hahellaceae* and *Litoricolaceae* are *Oceanospirillaceae*m, *Saccharospirillaceae*, *and Halomonadaceae*. The five families form a stable clade, and within this clade, a subclade is formed that excludes *Hahellaceae* (*Fig. 16.1*).

Molecular Analyses

Few studies have conducted DNA-DNA hybridization (DDH) analysis on *Hahellaceae* and *Litoricolaceae* families; however, the overall genome relatedness determined by DDH has been reported to be 14.5 % between *Endozoicomonas elysicola* and *E. montiporae* (Yang et al. 2010), and 28.1 % between *Litoricola lipolytica* and *L. marina* (Choi et al. 2010a). In addition to genome relatedness, the genome of *Hahella chejuensis* has been sequenced, and there are a few works that report on characterization of protein structures of the same species. The structures of three enzymes involved in the production of prodigiosin have been characterized (Hyo et al. 2008; Kang et al. 2010; Chawrai et al. 2012), as has the structure of a βγ-crystallin known as Hahellin (Srivastava et al. 2008; Srivastava et al. 2010; Srivastava and Chary 2011; Ramanujam et al. 2013).

Genome Analyses

The genome of *Hahella chejuensis*, the type species of the type genus of *Hahellaceae*, is the only one from either family that has been sequenced to date (Jeong et al. 2005). *H. chejuensis* has a single circular genome, 7.2 Mb in size. The G+C content is 54.8 mol%, which is in good agreement with that originally found using the thermal denaturation method (55 %) (Lee et al. 2001). The genome contains 69 genomic islands which are likely the result of horizontal gene transfer. Over 6,700 genes are predicted, include many encoding for enzymes involved in heterotrophic respiration, regulation, and transport. *H. chejuensis* also contains a putative carbon monoxide dehydrogenase, one hydrogenase and genes for iron utilization, which suggests it may be capable of facultative lithoheterotrophic growth. Other genes (many of which are contained in the genomic islands) include those related to production of exopolysaccharides, homologues of hemolysin and RTX toxin, two type three secretion systems and genes for biosynthesis of an algicidal compound, prodigiosin.

Phenotypic Analyses

Hahellaceae

Hahella Lee, Chun, Moon, Ko, Lee, Lee and Bae 2001, emend Baik et al. 2005

Cells belonging to the genus *Hahella* are Gram-negative rods, which are capable of reaching up to 9.0 μm in length. When grown on marine agar, colonies are generally between 0.5 and 1.0 mm in diameter, circular, and cream colored. *H. chejuensis* produces the red pigment prodigiosin, which is known to possess algicidal properties (Lee et al. 2001; Jeong et al. 2005; Kim et al. 2007a) and has anticancer potential

(Montaner and Pérez-Tomás 2001; Pérez-Tomás et al. 2003). *H. chejuensis* also produces exopolysaccharides with emulsifying properties.

Zooshikella Yi, Chang, Oh, Bae and Chun 2003

Cells of *Zooshikella* are Gram-negative, slightly curved rods, which range in size from 0.7 to 0.9 μm × 1.5–2.5 μm. When grown on marine agar for 36 h, the colonies are approximately 1 mm in diameter, circular, convex, have entire edges, glistening, opaque, and viscid. *Z. ganghwensis* cells produce a substantial amount of red pigment that appears to be similar to prodigiosin.

Halospina Sorokin, Tourova, Galinski, Belloch and Tindall 2006

Halospina cells are Gram-negative rods, 0.7–1.0 μm × 3.0–7.0 μm in size. Only one species has yet been described within this genus, and its major distinguishing phenotypic features are its denitrifying ability and high halophilicity. *H. denitrificans* cells can withstand 5 M concentrations of NaCl.

Endozoicomonas Kurahashi and Yokota 2007

Cells belonging to the genus *Endozoicomonas* are rod-shaped, Gram-negative, and range from 0.4 to 0.7 μm × 1.0–3.0 μm in size. When grown on marine agar, colonies are circular, convex, beige, and have entire edges. Species of *Endozoicomonas* have thus far been isolated exclusively from marine macroorganisms.

Kistimonas Choi, Kwon, Sohn and Yang 2010

Kistimonas cells are Gram-negative rods, 0.3–0.5 μm × 1.0–1.6 μm in size. They form light yellow colonies on nutrient agar, marine agar, TSA medium, and A1+C medium. *Kistimonas* cells have an unusual fatty acid profile; the three major fatty acids are not common among bacteria: anteiso-$C_{15:0}$, iso-$C_{15:0}$, and iso-$C_{14:0}$.

Litoricolaceae

Litoricola Kim, Choo and Cho 2007

Cells belonging to *Litoricola* are Gram-negative, short rods, ranging between 0.6 and 1.8 μm in length. Colonies on marine agar are very small, reaching only 0.5 mm diameter after 3 week incubation. Colonies are smooth, convex, circular, opaque, and cream colored. More detail on the phenotypic traits of both *Litoricolaceae* and *Hahellaceae* can be found listed in *Tables 16.1* and *16.2*.

Isolation, Enrichment, and Maintenance Procedures

Hahella

Hahella species were originally isolated from seawater and getbol samples collected in Korea and Antarctica. Samples were diluted with saline or sterile seawater, and streaked on plates of either marine agar or ZoBell's medium (5-g peptone, 1-g yeast extract, 0.01-g $FePO_4$, 15-g agar, 250-ml distilled water, 750-ml aged seawater). Selected colonies were then streaked on marine agar.

Zooshikella

Zooshikella ganghwensis was first isolated from a getbol sample collected from Ganghwa Island in Korea (37° 35′ 31.9″ N, 126° 27′ 24.5″ E). The sample was diluted in sterilized seawater, streaked on marine agar, and incubated at 25 °C for 3 weeks. Selected colonies were then streaked and maintained on marine agar.

Halospina

Halospina denitrificans was originally isolated from a hypersaline lake in the Kulunda Steppe, Russia. Culture enrichment was performed anaerobically in the following medium: 240 g L^{-1} NaCl, 2.5 g L^{-1} K_2HPO_4, 0.5 g L^{-1} $(NH_4)_2SO_4$, 10 mM sodium acetate, 0.1-g yeast extract, 2 mM $MgCl_2.6H_2O$, 1 mL L^{-1} trace metal solution, 5 mM KNO_3, and 50 mM N_2O. Pure cultures were isolated by serial dilution and streaking on solid medium with the same composition, with the exception that the NaCl concentration was 2 M.

Endozoicomonas

Both species of *Endozoicomonas* were originally isolated from marine organisms. *E. elysicola* was isolated from the sea slug *Elysia ornata*, and *E. monitporae* from *Montipora aequituberculata* coral. The macroorganisms were homogenized and diluted in sterile seawater, before spreading on marine agar. After incubating at 25 °C for 5–7 days, single colonies were selected and streaked on the same medium.

Kistimonas

The original type strain of the type species of *Kistimonas* was isolated from the skin of the starfish *Asterias amurensis*. The starfish were collected 10 m below the surface near the port of Im-Won on the Korean East Sea coast. The skin was homogenized and diluted with sterile seawater, before spreading on A1+C medium (10.0-g starch, 4.0-g peptone, 2.0-g yeast extract, 1.0-g $CaCO_3$, and 18.0-g agar, per liter of filtered seawater).

Table 16.1
Phenotypic properties of the genera belonging to *Hahellaceae* and *Litoricolaceae*

	Hahellaceae					*Litoricolaceae*
	Hahella	*Zooshikella*	*Halospina*	*Endozoicomonas*	*Kistimonas*	*Litoricola*
Shape	Rod-shaped	Slightly curved rod	Rod-shaped	Rod-shaped	Short rods	Short rods
Size (μm)	0.4–0.7 × 0.9–9.0	0.7–0.9 × 1.5–2.5	0.7–1.0 × 3.0–7.0	0.4–0.7 × 1.0–3.0	0.3–0.5 × 1.0–1.6	0.3–0.7 × 0.6–1.8
Gram stain	–	–	–	–	–	–
Motility	v	+	–	+	+	–
Pigment	Red/Brown	Red with metallic green	ND	Beige	Light yellow	–
Respiration	Aerobic/Facultative anaerobic	Aerobic	Facultatively anaerobic	Aerobic	Aerobic	Anaerobic/ Facultatively anaerobic
Temp. (°C)	15–45	15–45	NR	25–30	15–30	15–37
NaCl	0.5–10	1.0–7.0	11.7–29.2	2–3	0.1–11.0	1.0–10.0
pH	4.0–12.0	5.0–8.0	6.7–8.5	6.0–10.0	5.0–10.0	5.0–12.0
G+C (mol %)	44–56.4	40–42	60.1	50.0–50.4	47.6	57.8–59.6
Nitrate reduction	v	w	+	+	–	–
Catalase	ND	+	ND	+	ND	+
Oxidase	+	+	ND	+	ND	ND
Urease	–	ND	ND	ND	ND	ND
Indole	–	–	ND	–	ND	ND
Major quinone	Q-9	Q-9	Q-9	Q-9	Q-9	ND
Major fatty acids	$C_{16:0}$	$C_{16:1}\omega6c/C_{16:1}\omega7c$/ iso-$C_{15:0}$ 2-OH	$C_{16:0}$	$C_{16:1}\omega6c$/ $C_{16:1}\omega7c$	Anteiso-$C_{15:0}$	$C_{16:1}\omega6c/C_{16:1}\omega7c$/ iso-$C_{15:0}$ 2-OH
	$C_{16:1}\omega6c/C_{16:1}\omega7c$/ iso-$C_{15:0}$ 2-OH	$C_{16:0}$		$C_{16:0}$	Iso-$C_{15:0}$	$C_{18:1}\omega7c/C_{18:1}\omega6c$
	$C_{18:0}$	$C_{18:1}\omega7c/C_{18:1}\omega6c$		$C_{18:1}\omega7c$/ $C_{18:1}\omega6c$	Iso-$C_{14:0}$	$C_{16:0}$

All species are chemoheterotrophs; + positive, – negative, *w* weakly positive, *v* variable; *ND* not determined

Plates were incubated at 25 °C for 2 weeks, before selected colonies were streaked and purified on the same medium.

Litoricola

Both species of *Litoricola* isolated to date were originally recovered from seawater samples. *L. lipolytica* was recovered from a sample collected near Goseong, Korea, in the East Sea (38° 20′ N 128° 33′E), while *L. marina* was recovered from a sample collected near Yeongjong Do, Korea, in the Yellow Sea (37° 27′ 44″ N 126° 29′ 59″ E). Both samples were taken from within 10 m of the surface. Both samples were spread on an oligotrophic medium consisting of R2A agar diluted by a factor of 10 with aged seawater. After 2–4 week incubation at 20 °C, selected colonies were streaked and purified on marine agar.

Pathogenicity: Clinical Relevance

Little information of clinical relevance has been published on any members of either *Hahellaceae* or *Litoricolaceae*. However, antibiotic sensitivity has been reported for selected strains (*Table 16.2*).

Application

Hahella chejuensis has been reported to produce an exopolysaccharide (EPS-R) with useful emulsifying properties (Ko et al. 2000; Yim et al. 2004), and prodigiosin, a red pigment that has algicidal properties (Jeong et al. 2005; Kim et al. 2007a). This compound has the potential for use in controlling algal outbreaks, especially in ocean waters, and it has also been reported that prodigiosin and related compounds possess

Table 16.2
Antibiotic sensitivity/resistance of selected species of *Hahellaceae* and *Litoricolaceae*

	Endozoicomonas montiporae	*Hahella antarctica*	*Litoricola lipolytica*
Ampicillin	S	R	R
Chloramphenicol	S	R	S
Erythromycin	ND	R	S
Gentamycin	S	S	S
Kanamycin	S	S	S
Nalidixic acid	S	ND	ND
Novobiocin	S	ND	ND
Penicillin	S	S	R
Rifampicin	S	S	S
Streptomycin	S	S	S
Sulfamethoxazole	S	ND	ND
Tetracycline	S	S	S
Trimethoprim	S	ND	ND
Vancomycin	NR	R	S

S sensitive, *R* resistant, *ND* not determined

anticancer activity (Montaner and Pérez-Tomás 2001; Pérez-Tomás et al. 2003). A second strain purportedly belonging to the genus *Zooshikella* has also been reported to produce prodigiosin and cycloprodigiosin (Lee et al. 2011). This strain, however, as of yet, is not validly described.

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17 The Family *Halomonadaceae*

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Abstract
The family *Halomonadaceae*, within the class *Gammaproteobacteria*, consists mostly of marine and moderately halophilic microorganisms that are phenotypically rather diverse. As of January 2014, this family contains ten genera and 106 validly published species names and, therefore, constitutes the largest group of halophilic bacteria. In this chapter, the historical and current taxonomy have been reviewed along with molecular and phenotypic analyses. In addition, isolation and preservation procedures were considered, as well as the ecological habitats where the members of the family *Halomonadaceae* can be found and their clinical relevance as human pathogens. The increasing interest of this group of microorganisms due to its biotechnological and environmental applications has also been addressed.

Introduction

The family *Halomonadaceae*, within the class *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 genera and species descriptions within the family *Halomonadaceae* have been reported, and the increasing number of species led some authors to review its phylogeny (Arahal et al. 2002c; de la Haba et al. 2010a, 2012) 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 more than 10 years ago (Vreeland and Ventosa 2003) and can be taken as a sign of the increasing interest in this group of organisms.

Species of 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

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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.

Taxonomy, Historical and Current

Short Description of the Family

Ha.lo.mo.na.da'ce.ae. M.L. fem. n. *Halomonas*, type genus of the family; suff. *-aceae*, ending to denote a family; M.L. fem. pl. n. *Halomonadaceae*, the *Halomonas* family. The description of the family is identical to that given by Franzmann et al. (1988) and emended by Dobson and Franzmann (1996), Ntougias et al. (2007) and Ben Ali Gam et al. (2007).

The family *Halomonadaceae* belongs, together with the *Alcanivoraceae*, the *Hahellaceae*, the *Litoricolaceae*, the *Oceanospirillaceae*, the *Oleiphilaceae* and the "*Saccharospirillaceae*" to the order *Oceanospirillales*, within the class *Gammaproteobacteria* (Garrity et al. 2005a) 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), Ntougias et al. (2007) and Ben Ali Gam et al. (2007). At the start of 2014, this family included ten recognized genera: *Halomonas* (type genus), *Aidingimonas*, *Carnimonas*, *Chromohalobacter*, *Cobetia*, *Halotalea*, *Kushneria*, *Modicisalibacter*, *Salinicola*, and *Zymobacter* (Parte 2014). *Table 17.1* 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. 1980), *Chromohalobacter* (Ventosa et al. 1989) or *Cobetia* (Arahal et al. 2002a): *Pseudomonas beijerinckii* (basonym of *Chromohalobacter beijerinckii*), "*Chromobacterium marismortui*" (now *Chromohalobacter marismortui*), "*Arthrobacter marinus*" (earlier synonym of *Cobetia marina*), "*Achromobacter aquamarinus*" (*Halomonas aquamarina*), *Flavobacterium halmophilum* (basonym of *Halomonas halmophila*) and "*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* (Baumann et al. 1972). About one decade later, Baumann et al. (1983) proposed the creation of the 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. Additionally, it was concluded that *Alcaligenes aquamarinus* (currently *Halomonas aquamarina*) and *Halovibrio variabilis* (*Halomonas variabilis*) were members of the family *Halomonadaceae* and could perhaps be accomodated within existing genera of the family.

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. 2002c). The same year, the genus *Cobetia* was created by Arahal et al. (2002a) to accommodate the species *Halomonas marina*.

More recently, other five additional genera belonging to this family have been described: *Halotalea* (Ntougias et al. 2007), *Modicisalibacter* (Ben Ali Gam et al. 2007), *Kushneria* (Sánchez-Porro et al. 2009), *Aidingimonas* (Wang et al. 2009), and *Salinicola* (Anan'ina et al. 2007). Besides, the genera *Halomonas*, *Chromohalobacter*, and *Cobetia* were expanded to include new species since new descriptions were carried out. Moreover, several reclassifications have taken place among genera of the family *Halomonadaceae*: the species *Halomonas canadensis* and *H. israelensis* (Arahal et al. 2001a) were transferred to the genus *Chromohalobacter*; the species *H. avicenniae*, *H. indalinina*, and *H. marisflavi* to the genus *Kushneria* (Sánchez-Porro et al. 2009); and the species *H. salaria* and *Chromohalobacter salarius* to the

Table 17.1

Validly published genera and species names of the family *Halomonadaceae* (as to 31 January 2014). Basonyms/synonyms of microorganisms that have been transferred to other genera are not included. For genera/species whose descriptions have been emended only the most recent reference is included

Genus and species name	Reference	Type strain designation(s)
Aidingimonas		
A. halophila	Wang et al. (2009)	YIM 90637 = CCTCC AB 207002 = KCTC 12885
Carnimonas		
Car. nigrificans[a]	Garriga et al. (1998)	CTCBS1 = ATCC BAA-78 = CECT 4437 = CIP 105703
Chromohalobacter		
Chr. beijerinckii	Peçonek et al. (2006)	ATCC 19372 = CCUG 49679 = CIP 106957 = DSM 7218 = JCM 13305 = JCM 21422 = LMG 2148 = NBRC 103041 = NCCB 35008 = NCIMB 9041 = NRRL B-3153
Chr. canadensis	Arahal et al. (2001a)	ATCC 43984 = CECT 5385 = CCM 4919 = CIP 105571 = DSM 6769 = LMG 19547 = NCIMB 13767 = NRCC 41227
Chr. israelensis	Arahal et al. (2001a)	Ba1 = ATCC 43985 = CECT 5287 = CCM 4920 = CIP 106853 = DSM 6768 = LMG 19546 = NCIMB 13766
Chr. japonicus	Sánchez-Porro et al. (2007)	43 = CCM 7416 = CECT 7219
Chr. marismortui[a]	Ventosa et al. (1989)	CCM 3518 = ATCC 17056 = DSM 6770 = JCM 21220 = LMG 3935 = NBRC 103155
Chr. nigrandesensis	Prado et al. (2006)	LTS-4 N = CECT 5315 = DSM 14323
Chr. salexigens	Arahal et al. (2001b)	ATCC BAA-138 = CECT 5384 = CCM 4921 = CIP 106854 = DSM 3043 = NCIMB 13768 = 1H11
Chr. sarecensis	Quillaguamán et al. (2004a)	LV4 = ATCC BAA-761 = CCUG 47987 = DSM 15547
Cobetia		
Cob. amphilecti	Romanenko et al. (2013)	CCUG 49560 = KMM 1561 = NRIC 0815
Cob. crustatorum	Kim et al. (2010b)	JO1 = JCM 15644 = KCTC 22486
Cob. litoralis	Romanenko et al. (2013)	CCUG 49563 = KMM 3880 = NRIC 0814
Cob. marina[a]	Arahal et al. (2002a)	219 = ATCC 25374 = CCUG 49558 = CCUG 49558 = CECT 4278 = CIP 104765 = DSM 4741 = LMG 2217 = NBRC 102605 = NCIMB 1877
Cob. pacifica	Romanenko et al. (2013)	CCUG 49562 = KMM 3879 = NRIC 0813
Halomonas[b]		
H. alimentaria	Yoon et al. (2002)	YKJ-16 = DSM 15356 = KCCM 41042 = JCM 10888
H. alkaliantarctica	Poli et al. (2007)	CRSS = ATCC BAA-848 = DSM 15686
H. alkaliphila	Romano et al. (2006)	18bAG = ATCC BAA-953 = DSM 16354
H. almeriensis	Martínez-Checa et al. (2005)	M8 = CECT 7050 = LMG 22904
H. andesensis	Guzmán et al. (2010)	LC6 = CCUG 54844 = DSM 19434 = LMG 24243
H. anticariensis	Martínez-Cánovas et al. (2004a)	FP35 = CECT 5854 = LMG 22089
H. aquamarina	Dobson and Franzmann (1996)	ZoBell and Upham 558 = ATCC 14400 = CCUG 16157 = CIP 105454 = DSM 30161 = IAM 12550 = LMG 2853 = NCIMB 557
H. arcis	Xu et al. (2007)	AJ282 = CGMCC 1.6494 = JCM 14607 = LMG 23978
H. axialensis	Kaye et al. (2004)	Althf1 = ATCC BAA-802 = CECT 5812 = DSM 15723
H. beimenensis	Wang et al. (2012)	NTU-107 = BCRC 17999 = JCM 16084 = KCTC 22876
H. boliviensis	Quillaguamán et al. (2004b)	LC1 = ATCC BAA-759 = DSM 15516
H. campaniensis	Romano et al. (2005)	5AG = ATCC BAA-966 = DSM 15293
H. campisalis	Mormile et al. (1999)	4A = ATCC 700597 = CIP 106639
H. caseinilytica	Wu et al. (2008b)	AJ261 = CGMCC 1.6773 = JCM 14802
H. cerina	González-Domenech et al. (2008b)	SP4 = CECT 7282 = LMG 24145
H. cibimaris	Jeong et al. (2013)	10-C-3 = JCM 16914 = KACC 14932
H. cupida	Dobson and Franzmann (1996)	79 = ATCC 27124 = CCUG 16075 = CIP 103199 = DSM 4740 = JCM 20632 = LMG 3448 = NBRC 102219
H. daqiaonensis	Qu et al. (2011)	YCSA28 = CGMCC 1.9150 = NCCB 100305 = MCCC 1B00920
H. daqingensis	Wu et al. (2008a)	DQD2-30 = CGMCC 1.6443 = LMG 23896
H. denitrificans	Kim et al. (2007)	M29 = DSM 18045 = KCTC 12665

Table 17.1 (continued)

Genus and species name	Reference	Type strain designation(s)
H. desiderata	Berendes et al. (1996)	FB2 = CIP 105505 = DSM 9502 = LMG 19548
H. elongata[a]	Vreeland et al. (1980)	1H9 = ATCC 33173 = CIP 104264 = DSM 2581 = NBRC 15536 = JCM 21044 = LMG 9076
H. eurihalina	Mellado et al. (1995b)	F9-6 = ATCC 49336 = CIP 106091 = DSM 5720
H. flava	Chen et al. (2011)	YIM 94343 = CCTCC AB 2010382 = KCTC 23356
H. fontilapidosi	González-Domenech et al. (2009)	5CR = CECT 7341 = LMG 24455
H. gomseomensis	Kim et al. (2007)	M12 = DSM 18042 = KCTC 12662
H. gudaonensis	Wang et al. (2007b)	SL014B-69 = CGMCC 1.6133 = LMG 23610
H. halmophila	Dobson et al. (1990)	ACAM 71 = ATCC 19717 = CIP 105455 = DSM 5349 = NBRC 15537 = JCM 21222 = LMG 4023 = NCIMB 1971
H. halocynthiae	Romanenko et al. (2002)	KMM 1376 = DSM 14573 = CIP 107736
H. halodenitrificans	Dobson and Franzmann (1996)	ATCC 13511 = CIP 105456 = DSM 735 = CCM 286 = CECT 5012 = IAM 13950 = KCTC 5069 = NBRC 14912
H. halophila	Dobson and Franzmann (1996)	F5-7 = ATCC 49969 = CCM 3662 = CIP 103512 = DSM 4770 = JCM 20791 = LMG 6456 = NBRC 102604
H. hamiltonii	Kim et al. (2010a)	W1025 = DSM 21196 = KCTC 22154
H. hydrothermalis	Kaye et al. (2004)	Slthf2 = ATCC BAA-800 = CECT 5814 = DSM 15725
H. ilicicola	Arenas et al. (2009)	SP8 = CCM 7522 = CECT 7331 = DSM 19980
H. janggokensis	Kim et al. (2007)	M24 = KCTC 12663 = DSM 18043
H. jeotgali	Kim et al. (2011)	Hwa = JCM 15645 = KCTC 22487
H. johnsoniae	Kim et al. (2010a)	T68687 = DSM 21197 = KCTC 22157
H. kenyensis	Boltyanskaya et al. (2007)	AIR-2 = DSM 17331 = VKM B-2354
H. koreensis	Lim et al. (2004)	SS20 = JCM 12237 = KCTC 12127
H. korlensis	Li et al. (2008)	XK1 = CGMCC 1.6981 = DSM 19633
H. kribbensis	Jeon et al. (2007)	BH843 = DSM 17892 = KCTC 12584
H. lutea	Wang et al. (2008a)	YIM 91125 = CCTCC AB 206093 = KCTC 12847
H. magadiensis	Duckworth et al. (2000)	21 MI = CIP 106823 = CIP 106874 = DSM 15367 = NCIMB 13595
H. maura	Bouchotroch et al. (2001)	S-31 = ATCC 700995 = CECT 5298 = DSM 13445
H. meridiana	James et al. (1990)	ACAM 246 = ATCC 49692 = CIP 104043 = DSM 5425 = NBRC 15608 = UQM 3352
H. mongoliensis	Boltyanskaya et al. (2007)	Z-7009 = DSM 17332 = VKM B-2353
H. muralis	Heyrman et al. (2002)	LMG 20969 = CIP 108825 = DSM 14789
H. nanhaiensis	Long et al. (2013)	YIM M 13059 = JCM 18142 = CCTCC AB 2012911
H. neptunia	Kaye et al. (2004)	Eplume1 = ATCC BAA-805 = CECT 5815 = DSM 15720
H. nitroreducens	González-Domenech et al. (2008a)	11S = CECT 7281 = LMG 24185
H. olivaria	Amouric et al. (2014)	TYRC17 = DSM 19074 = CCUG 53850B
H. organivorans	García et al. (2004)	G-16.1 = CCM 7142 = CECT 5995
H. pacifica	Dobson and Franzmann (1996)	62 = ATCC 27122 = CIP 103200 = DSM 4742 = JCM 20633 = LMG 3446 = NBRC 102220
H. pantelleriensis	Romano et al. (1996)	AAP = ATCC 700273 = CIP 105506 = DSM 9661 = LMG 19550
H. qijiaojingensis	Chen et al. (2011)	YIM 93003 = CCTCC AB 208133 = KCTC 22228
H. ramblicola	Luque et al. (2012)	RS-16 = CECT 7896 = LMG 26647
H. rifensis	Amjres et al. (2011)	HK31 = CECT 7698 = LMG 25695
H. sabkhae	Kharroub et al. (2008)	5-3 = CECT 7246 = DSM 19122 = LMG 24084
H. saccharevitans	Xu et al. (2007)	AJ275 = CGMCC 1.6493 = JCM 14606 = LMG 23976
H. salifodinae	Wang et al. (2008b)	BC7 = CGMCC 1.6774 = JCM 14803
H. salina	Dobson and Franzmann (1996)	F8-11 = ATCC 49509 = CIP 106092 = DSM 5928 = JCM 21221
H. shengliensis	Wang et al. (2007a)	SL014B-85 = CGMCC 1.6444 = LMG 23897
H. sinaiensis	Romano et al. (2007)	ALO Sharm = ATCC BAA-1308 = DSM 18067
H. smyrnensis	Poli et al. (2013)	AAD6 = DSM 21644 = JCM 15723
H. stenophila	Llamas et al. (2011)	N12 = CECT 7744 = LMG 25812

Table 17.1 (continued)

Genus and species name	Reference	Type strain designation(s)
H. stevensii	Kim et al. (2010a)	S18214 = DSM 21198 = KCTC 22148
H. subglaciescola	Franzmann et al. (1987)	ACAM 12 = ATCC 43668 = CIP 104042 = DSM 4683 = NBRC 14766 = JCM 21045 = LMG 8824 = UQM 2926
H. subterranea	Xu et al. (2007)	ZG16 = CGMCC 1.6495 = JCM 14608 = LMG 23977
H. sulfidaeris	Kaye et al. (2004)	Esulfide1 = ATCC BAA-803 = CECT 5817 = DSM 15722
H. taeanensis	Lee et al. (2005)	BH539 = DSM 16463 = KCTC 12284
H. titanicae	Sánchez-Porro et al. (2010)	BH1 = ATCC BAA-1257 = CECT 7585 = JCM 16411 = LMG 25388
H. variabilis	Dobson and Franzmann (1996)	isolate III = ATCC 49240 = CIP 105504 = DSM 3051 = IAM 14440 = JCM 21223 = NBRC 102410
H. ventosae	Martínez-Cánovas et al. (2004b)	Al12 = CECT 5797 = DSM 15911
H. venusta	Dobson and Franzmann (1996)	86 = ATCC 27125 = CCUG 16063 = CIP 103201 = DSM 4743 = JCM 20634 = LMG 3445 = NBRC 102221
H. vilamensis	Menes et al. (2011)	SV325 = DSM 21020 = LMG 24332
H. xianhensis	Zhao et al. (2012)	A-1 = CGMCC 1.6848 = JCM 14849
H. xinjiangensis	Guan et al. (2010)	TRM 0175 = CCTCC AB 208329 = KCTC 22608
H. zhanjiangensis	Chen et al. (2009)	JSM 078169 = CCTCC AB 208031 = DSM 21076 = KCTC 22279
H. zincidurans	Xu et al. (2013)	B6 = CGMCC 1.12450 = JCM 18472
Halotalea		
Halot. alkalilenta[a]	Ntougias et al. (2007)	AW-7 = CECT 7134 = DSM 17697
Kushneria		
K. aurantia[a]	Sánchez-Porro et al. (2009)	A10 = CCM 7415 = CECT 7220
K. avicenniae	Sánchez-Porro et al. (2009)	MW2a = CCM 7396 = CECT 7193 = CIP 109711
K. indalinina	Sánchez-Porro et al. (2009)	CG2.1 = CECT 5902 = CIP 109528 = DSM 14324 = LMG 23625
K. marisflavi	Sánchez-Porro et al. (2009)	SW32 = CIP 107103 = DSM 15357 = JCM 10873 = KCCM 80003
K. sinocarnis	Zou and Wang (2010)	Z35 = CCTCC AB 209027 = DSM 23229 = NRRL B-59197
Modicisalibacter		
M. tunisiensis[a]	Ben Ali Gam et al. (2007)	LIT2 = CCUG 52917 = CIP 109206
Salinicola		
S. halophilus	de la Haba et al. (2010b)	CG4.1 = CECT 5903 = LMG 23626
S. peritrichatus	Huo et al. (2013)	DY22 = CGMCC 1.12381 = JCM 18795
S. salarius	de la Haba et al. (2010b)	M27 = DSM 18044 = KCTC 12664
S. socius[a]	Anan'ina et al. (2007)	SMB35 = DSM 19940 = VKM B-2397
Zymobacter		
Z. palmae[a]	Okamoto et al. (1993)	T109 = ATCC 51623 = DSM 10491 = IAM 14233 = JCM 21091 = NBRC 102412

Abbreviations of culture collections are: *ACAM* Australian Collection of Antarctic Microorganisms, *ATCC* American Type Culture Collection, *BCRC* Bioresource Collection and Research Center, *CCM* Czech Collection of Microorganisms, *CCTCC* China Center for Type Culture Collection, *CCUG* Culture Collection University of Göteborg, *CECT* Colección Española de Cultivos Tipo, *CIP* Collection de L'Institut Pasteur, *CGMCC* China General Microbiological Culture Collection Center, *DSM* Deutsche Sammlung von Mikroorganismen und Zellkulturen, *IAM* Institute of Applied Microbiology, *JCM* Japan Collection of Microorganisms, *KACC* Korean Agricultural Culture Collection, *KCCM* Korean Culture Center of Microorganisms, *KCTC* Korean Collection for Type Cultures, *KMM* Collection of. Marine Microorganisms, *LMG* Belgian Co-ordinated Collections of Microorganisms, *MCCC* Marine Culture Collection of China, *NBRC*, NITE Biological Resource Center, *NCCB* Netherlands Culture Collection of Bacteria, *NCIMB* The National Collection of Industrial, Marine and Food Bacteria, *NRCC* National Research Council of Canada, *NRIC*, *NODAI* Research Institute Culture Collection, *NRRL* Agricultural Research Service Culture Collection, *UQM* Australian Collection of Microorganisms, *VKM* All-Russian Collection of Microorganisms

[a]Type species of the genus

[b]Type genus of the family

genus *Salinicola* (de la Haba et al. 2010b). In 2013, Romanenko et al. (2013) classified *H. halodurans* as a later heterotypic synonym of *Cobetia marina*, invalidating the former species name.

As of January 2014 there are 106 validly published species names within the family *Halomonadaceae*, 79 belonging to the genus *Halomonas*, eight to *Chromohalobacter*, five to *Cobetia*, five to *Kushneria*, four to *Salinicola*, and the genera *Aidingimonas*, *Carnimonas*, *Halotalea*, *Modicisalibacter*, and *Zymobacter* with a single species each one. The list of articles in press of the International Journal of Systematic and Evolutionary Microbiology (http://ijs.sgmjournals.org/content/early/recent)

includes the description of a new *Halomonas* species for which the name *H. huangheensis* has been proposed.

A valuable effort to address the taxonomy of the group from an entirely phenotypic point of view was the study of Mata et al. (2002). In that article, they presented 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.

In 2007, the International Committee on Systematics of Prokaryotes–Subcommittee on the Taxonomy of the *Halomonadaceae*, following Recommendation 30b of the Bacteriological Code (1990 Revision) (Lapage et al. 1992), published the minimal standards for describing new taxa within this family (Arahal et al. 2007). This paper evaluates many different approaches to ensure that a rich polyphasic characterization is given and must be considered as guidelines for authors to prepare descriptions of novel taxa. Although a list of traits that are required and recommended is provided, the manuscript does not attempt to limit the characterization of new isolates to the features that are indicated in the text. Moreover, current or yet to be described species may show new and interesting characteristics not listed in the paper of Arahal et al. (2007) and such features may prove to be of taxonomic importance. More recently, the Subcommittee on the Taxonomy of *Halomonadaceae*, in an open meeting held in Storrs, Connecticut, USA (June 2013), agreed that the current minimal standards document are still adequate in combination with Notes on the characterization of prokaryote strains for taxonomic purposes (Tindall et al. 2010), but the following additions were suggested: (i) It was stressed that it is highly desirable to base the description of new species on more than one strain. (ii) A good database of sequences of suitable genes for multilocus sequence analysis is now available (de la Haba et al. 2012), and inclusion of multilocus sequence analysis in the minimal standards is highly desirable. (iii) Fatty acid analysis should be added as required rather than recommended in the recommended standards for describing new taxa of the family *Halomonadaceae* (Oren and Ventosa 2013).

Phylogenetic Structure of the Family and Its Genera

Seven 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 *Deleya aquamarina* (now *Halomonas aquamarina*), *Deleya halophila* (*Halomonas halophila*), *Deleya marina* (*Cobetia marina*), *Halomonas elongata*, *Halomonas meridiana*, *Halovibrio variabilis* (*Halomonas variabilis*) and *Halomonas subglaciescola* and analyzed them together with the sequences of *Halomonas halmophila* and other species belonging to the *Gammaproteobacteria*. They showed that the level of 16S rRNA sequence similarity among members of the family *Halomonadaceae* is 100–92.6 % 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. 2002c; Ntougias et al. 2007; Ben Ali Gam et al. 2007), as new members have been described. Currently, the 16S rRNA sequence signature characteristics of the family *Halomonadaceae* are defined by the following positions: 484 (A or G), 486 (C or U), 640 (A or G), 660 (A), 668 (A), 669 (A), 737 (U), 738 (U), 745 (U), 776 (U), 1124 (U or G), 1297 (U), 1298 (C), 1423 (A), 1424 (C or U), 1439 (U or C), 1462 (A or G) and 1464 (C or U). However, fullsequence 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 corresponding to *Chromohalobacter marismortui* (four strains), *Volcaniella eurihalina* (now *Halomonas eurihalina*), *Deleya salina* (*Halomonas 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 for the genus *Halomonas* since its heterogeneity was (and still is) too large for a single genus.

Further studies by Dobson and Franzmann (1996) determined another seven 16S rRNA sequences corresponding to the type strains of *Halomonas subglaciescola*, *Deleya cupida* (currently *Halomonas cupida*), *Deleya pacifica* (*Halomonas pacifica*), *Deleya salina* (*Halomonas salina*), *Deleya venusta* (*Halomonas venusta*), *Halomonas halodurans* (*Cobetia marina*), and *Halomonas 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*.

Arahal et al. (2002c) evaluated the phylogenetic status of the family *Halomonadaceae* using 16S and 23S rRNA gene sequences. In addition to the new sequences determined in their study, 18 for the 23S rRNA and 7 for the 16S rRNA, 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-based 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 halodurans* (now *Cobetia marina*), 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 did 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. 2002c). With respect to the genus *Chromohalobacter*, the four species described in those days within this genus formed a group closely related to *Halomonas*. The average rRNA sequence similarity of species of *Chromohalobacter* was 98.6 % (for the 23S rRNA) and 98.5 % (for the 16S rRNA). Within this group fell the sequence of *Pseudomonas beijerinckii*, which was later reclassified as *Chromohalobacter beijerinckii* (Peçonek et al. 2006). When the *Chromohalobacter* sequences were compared to those of other halomonads, values below 95 % (generally accepted as a good borderline for genus separation) were 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 the new genus *Cobetia* (Arahal et al. 2002a). Finally, in this study, the sequences of *Zymobacter palmae* and *Carnimonas nigrificans* showed a deeper branching in the tree. Their 16S rRNA sequence similarity was 93.5 % and even lower values were obtained when comparing any of the two with the other members of the family (Arahal et al. 2002c).

More recently, de la Haba et al. (2010a) updated the comparative analysis based on 23S and 16S rRNA gene sequences of Arahal et al. (2002c) including the 49 novel species that had been described since 2002. A total of 28 new complete 23S rRNA sequences were obtained in this study. Additionally, following the recommended minimal standards for the description of new members of the family *Halomonadaceae*, seven already-sequenced 16S rRNA genes of type strains were resequenced to resolve undetermined positions and to reach the established quality standards. In that sense, some suggestions were included in the paper about the recommended sequences to be used for future comparative phylogenetic analysis. In general, there was excellent agreement between the phylogenies based on both rRNA genes, but the 23S rRNA gene showed higher resolution in the differentiation of species of the family *Halomonadaceae* due to the slower evolutionary rate for the 16S rRNA gene. As previously reported by Arahal et al. (2002c), the genus *Halomonas* resulted to be not monophyletic and comprised two clearly separated phylogenetic groups that now contained larger numbers of species. Group 1, representing *Halomonas sensu stricto*, was formed by *Halomonas elongata* (the type species of the genus), *H. eurihalina*, *H. caseinilytica*, *H. halmophila*, *H. sabkhae*, *H. almeriensis*, *H. halophila*, *H. salina*, *H. organivorans*, *H. koreensis*, *H. maura* and *H. nitroreducens*. The mean 16S rRNA gene sequence similarity for this group was 97.8 %, whereas a lower value was obtained with the 23S rRNA gene sequences (97.0 %). Group 2, included the 16 species *Halomonas aquamarina*, *H. meridiana*, *H. axialensis*, *H. magadiensis*, *H. hydrothermalis*, *H. alkaliphila*, *H. venusta*, *H. boliviensis*, *H. neptunia*, *H. variabilis*, *H. sulfidaeris*, *H. subterranea*, *H. janggokensis*, *H. gomseomensis*, *H. arcis* and *H. subglaciescola*. This group displays mean similarities of 97.4 % and 97.5 % for the 16S and 23S rRNA gene sequences, respectively. Similarity values between groups 1 and 2 were low enough as to suggest that they could constitute two different genera, however, neither chemotaxonomic nor more general phenotypic studies have permitted their separation. The other 27 species at that time assigned to the genus *Halomonas* did not appear to be included clearly in either of these phylogenetic groups. One of these species, *H. salaria*, formed a separate cluster with the species *Chromohalobacter salarius* and *Salinicola socius*, which was confirmed by further studies proposing the transference of the first two species to the genus *Salinicola* (de la Haba et al. 2010b). An important finding in the paper of de la Haba et al. (2010a) is the fact that the type species of *Halomonas halodurans* and *Cobetia marina* shared 100 % sequence similarity (16S and 23S rRNA) and, according to their data, there was not sufficient evidence to determine whether they were members of the same or different species. A recent publication has demonstrated that, actually, they belong to the same genospecies (Romanenko et al. 2013). Concerning the genus *Chromohalobacter*, all the species described until then clustered together (the mean 16S and 23S rRNA gene sequence similarity of this group was 98.0 % and 97.8 %, respectively) with the only exception being *Chromohalobacter salarius*, as discussed previously (de la Haba et al. 2010a). Finally, the phylogenetic distinctness of the remaining genera at that time included in the family *Halomonadaceae* (*Carnimonas*, *Cobetia*, *Halotalea*, *Kushneria*, *Modicisalibacter*, *Salinicola*, and *Zymobacter*) was confirmed in the mentioned paper, being stable in the trees produced from all methods of analysis.

In the meanwhile, 26 new species have been proposed within the genus *Halomonas*, some of them belonging to the group 1 (*H. beimenensis*, *H. sinaiensis*, *H. smyrnensis*, and *H. stenophila*), others to the group 2 (*H. alkaliantarctica*, *H. andesensis*, *H. cibimaris*, *H. hamiltonii*, *H. jeotgali*, *H. johnsoniae*, *H. nanhaiensis*, *H. olivaria*, *H. stevensii*, *H. titanicae*, *H. vilamensis*, and *H. zhanjiangensis*) and others that cannot be assigned to any of these two groups (*H. daqiaonensis*, *H. flava*, *H. fontilapidosi*, *H. ilicicola*, *H. qijiaojingensis*, *H. ramblicola*, *H. rifensis*,

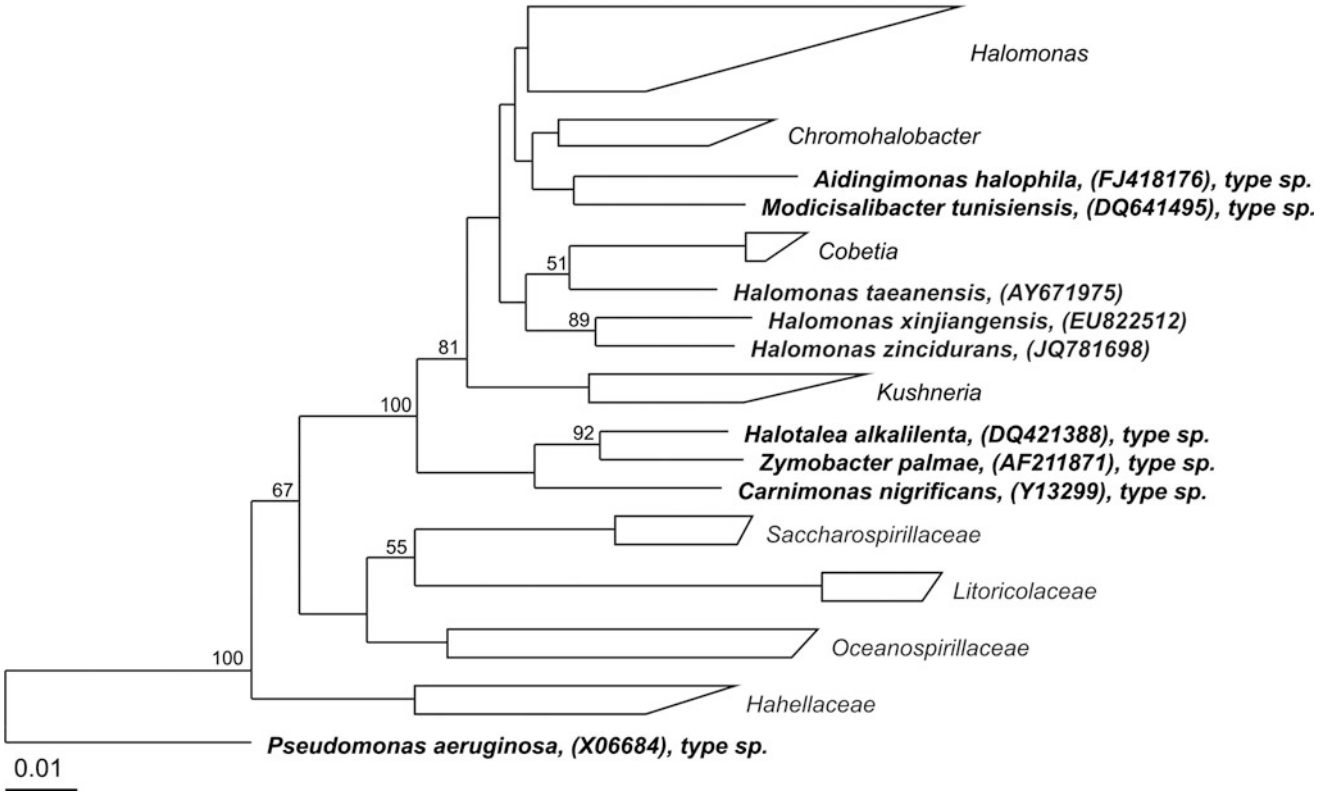

Fig. 17.1

Phylogenetic reconstruction of the family *Halomonadaceae* based on 16S rRNA and created using the neighbour-joining algorithm with the Jukes-Cantor correction. The sequence datasets and alignments were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). 1,000 resampling boostrap values over 50 % are shown. Scale bar indicates estimated sequence divergence

H. xianhensis, *H. xinjiangensis*, and *H. zincidurans*) (Fig. 17.1). Besides, four novel species of the genus *Cobetia*, and one of the genera *Kushneria* and *Salinicola*, respectively, have been described, all of them forming a monophyletic branch with the other relatives of their respective genera (Fig. 17.1). Additionally, the new genus *Aidingimonas*, not included in the study of de la Haba et al. (2010a), clustered together with the genus *Modicisalibacter* (Fig. 17.1), but they share ≤95 % 16S rRNA similarity values among them and with respect to the other genera of the family *Halomonadaceae*.

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 become better defined once more in-between sequences become available.

The most recent phylogenetic study conducted within the family *Halomonadaceae* was carried out by de la Haba et al. (2012). A multilocus sequence analysis (MLSA) of 52 representative species was performed for the first time in a moderately halophilic bacterial group with the purpose of investigating in detail the phylogenetic relationships of species from the family *Halomonadaceae* and helping clarify the current classification of this complex and dynamic family. A total of six loci were selected for the analysis, the 16S and 23S rRNA and the following four protein-encoding genes (housekeeping genes): *atpA* (F1-ATP synthase, α subunit), *gyrB* (DNA gyrase, B subunit), *rpoD* (RNA polymerase, β subunit) and *secA* (protein translocase, SecA subunit). Different nucleotide substitution models and tree-constructing algorithms were compared. The average pairwise sequence similarity values for 16S rRNA, 23S rRNA, *atpA*, *gyrB*, *rpoD* and *secA* were 94.0 %, 93.1 %, 86.8 %, 79.7 %, 79.6 % and 76.6 %, respectively, indicating that the *secA* gene had the highest theoretical discriminatory power, although some halomonads *secA* gene sequences were identical, suggesting gene flow. In any case, the six genes studied were not always sufficient to correctly assign a new strain to the genus *Halomonas* since there was a large overlap between the intrageneric and intergeneric sequence similiarities, that is, halomonads sequences could be more similar to those from species belonging to other genera of the family than to those from species of the genus *Halomonas*. This overlap was mainly due to the enormous variability within the genus *Halomonas*, suggesting that the genus should be divided into two or more genera. Besides, the overlap problem also lies with the huge sequence similarity of the pair *Halomonas halodurans-Cobetia marina*, whose taxonomic status has been recently revised by

Romanenko et al. (2013) and concluded that they are member of the same species. With respect to the phylogenetic trees, the different methods produced variable results, with those generated from the maximum-likelihood and neighbour-joining algorithms being more similar than those obtained by maximum-parsimony methods. Except *atpA* gene, the other five genes studied showed a consistent evolutionary history (with some exceptions probably due to lateral gene transfer events, and other intrinsic and extrinsic factors, such as the size of the dataset and the taxa included); therefore *atpA* gene may not be useful as an individual gene phylogenetic marker within the family *Halomonadaceae*. The *gyrB*-based tree was the only that formed monophyletic branches for the different genera of the family, including the genus *Halomonas*. Although there were some exceptions, in general, the two groups defined within this genus by Arahal et al. (2002c) and de la Haba et al. (2010a) (group 1 or *Halomonas sensu stricto* and group 2) could be clearly distinguished in the phylogenetic trees for each gene. Concatenation of the six loci enhanced the phylogenetic reconstruction and optimized the taxonomic resolution by adding more informative data and minimizing the weight of recombination events. Trees resulting from the six-gene concatenation demonstrated a monophyletic and well-supported separation of the different genera, including the genus *Halomonas*. The only exceptions were the pairs *Halomonas halodurans-Cobetia marina* (mentioned above, with concatenated sequence similarity of 99.7 %) and *Halomonas muralis–M. tunisiensis* (with concatenated sequence similarity of 90.8 %). With regard to the intrageneric groups, the six-gene concatenations resolved *Halomonas* groups 1 and 2 monophyletically, demonstrating to be a very good tool for the delineation of taxonomic relationships on a broad scale, including intrageneric and intergeneric relationships, at the level of the family *Halomonadaceae*. In order to simplify the MLSA approach within this family, de la Haba et al. (2012) attempted to reduce the number of genes to be analyzed but retaining the resolution obtained with the six concatenated gene sequences. With this idea the general use of the individual and concatenated 16S rRNA, *gyrB* and *rpoD* genes is suggested for future taxonomic studies using MLSA within the family *Halomonadaceae*. This proposal has been recently endorsed and recommended within the minimal standards by the ICSP-Subcommittee on the Taxonomy of the *Halomonadaceaae* (Oren and Ventosa 2013). Finally, the paper of de la Haba et al. (2012) analyze the phylogeny of this family within the domain *Bacteria* by comparing the sequences obtained in this study with those of 445 bacterial species with sequenced genomes available from the GenBank/EMBL/DDBJ databases. The results showed that the family *Halomonadaceae* constituted a robust and monophyletic branch within the domain *Bacteria* for three of the six analysed genes, 16S rRNA, *gyrB* and *rpoD*. Besides, according to *Figs. 17.1*, *17.2*, and *17.3* the family is related to families *Saccharospirillaceae*, *Litoricolaceae*, *Oceanospirillaceae*, and *Hahellaceae*.

Molecular Analyses

DNA-DNA Hybridization Studies

DNA–DNA hybridization data between type strains of species contained in the family *Halomonadaceae* are widely available. Actually, the vast majority of descriptions include results of DNA-DNA hybridization (DDH). As indicated in the recomended minimal standards for this family, DDH studies remains essential when novel species are described (Arahal et al. 2007), and only in those proposals based on a single isolate that possess less than 97 % 16S rRNA gene sequence similarity with its closest relative can DDH data be considered redundant.

With respect to DDH between strains belonging to the same species within the family *Halomonadaceae* data are very limited, mainly due to the fact that the majority of the species have been described based on a single isolate.

Multilocus Sequence Analysis (MLSA)

With the objective to overcome the limitations attached to DNA-DNA hybridization studies (time-consuming, expensive, lack of uniformity and reproducibility problems, etc.) and to 16S rRNA gene sequence based taxonomy (high levels of conservation, microheterogeneity among the different copies, lateral gene transfer, etc.), additional rRNA and protein-encoding genes (called housekeeping genes) have been suggested as phylogenetic markers (Stackebrandt et al. 2002; Zeigler 2003; Arahal et al. 2007; Tindall et al. 2010) to perform multilocus sequence analysis (MLSA).

Although some authors (Arahal et al. 2002c; Lee et al. 2005; de la Haba et al. 2010a; Okamoto et al. 2004; González-Domenech et al. 2010) had previously used some housekeeping genes (23S rRNA, *gyrB*, *ectBC*, *narH*, *nirS*, and *nosZ*) for studying the family *Halomonadaceae*, the first MLSA study was conducted by de la Haba et al. (2012) based on six phylogenetic markers: 16S rRNA, 23S rRNA, *atpA*, *gyrB*, *rpoD*, and *secA* genes. However, the correlation of DDH values against the MLSA data could not be determined and, therefore, this approach cannot be taken yet as an alternative to DDH for species circumscription.

DNA Fingerprinting Methods

Determination of inter- and intraspecies relatedness by rapid DNA typing methods (AFLP, RAPD, rep-PCR, BOX-PCR, PFGE, ribotyping of ribosomal ribonucleic operons, ARDRA) has not been widely applied to the *Halomonadaceae* except for the studies by Mellado et al. (1998) and Llamas et al. (2002), who applied PFGE, Garriga et al. (1998), who used RAPD, Heyrman et al. (2002), who reported rep-PCR, and Li et al. (2008) who carried out BOX-PCR genomic fingerprinting.

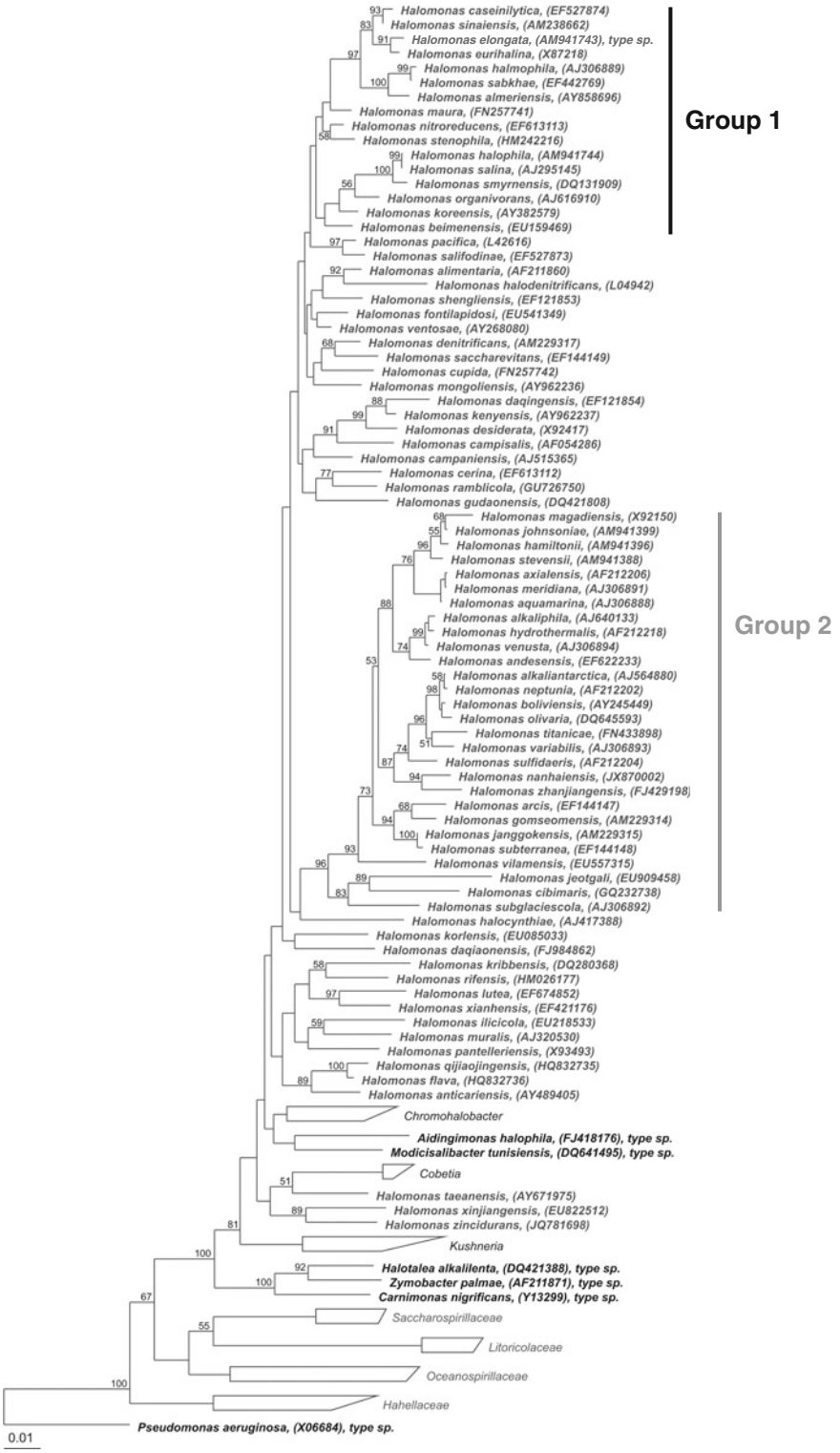

Fig. 17.2

Phylogenetic distribution of the genus *Halomonas* based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes-Cantor correction. The sequence datasets and alignments were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). 1,000 resampling boostrap values over 50 % are shown. Scale bar indicates estimated sequence divergence

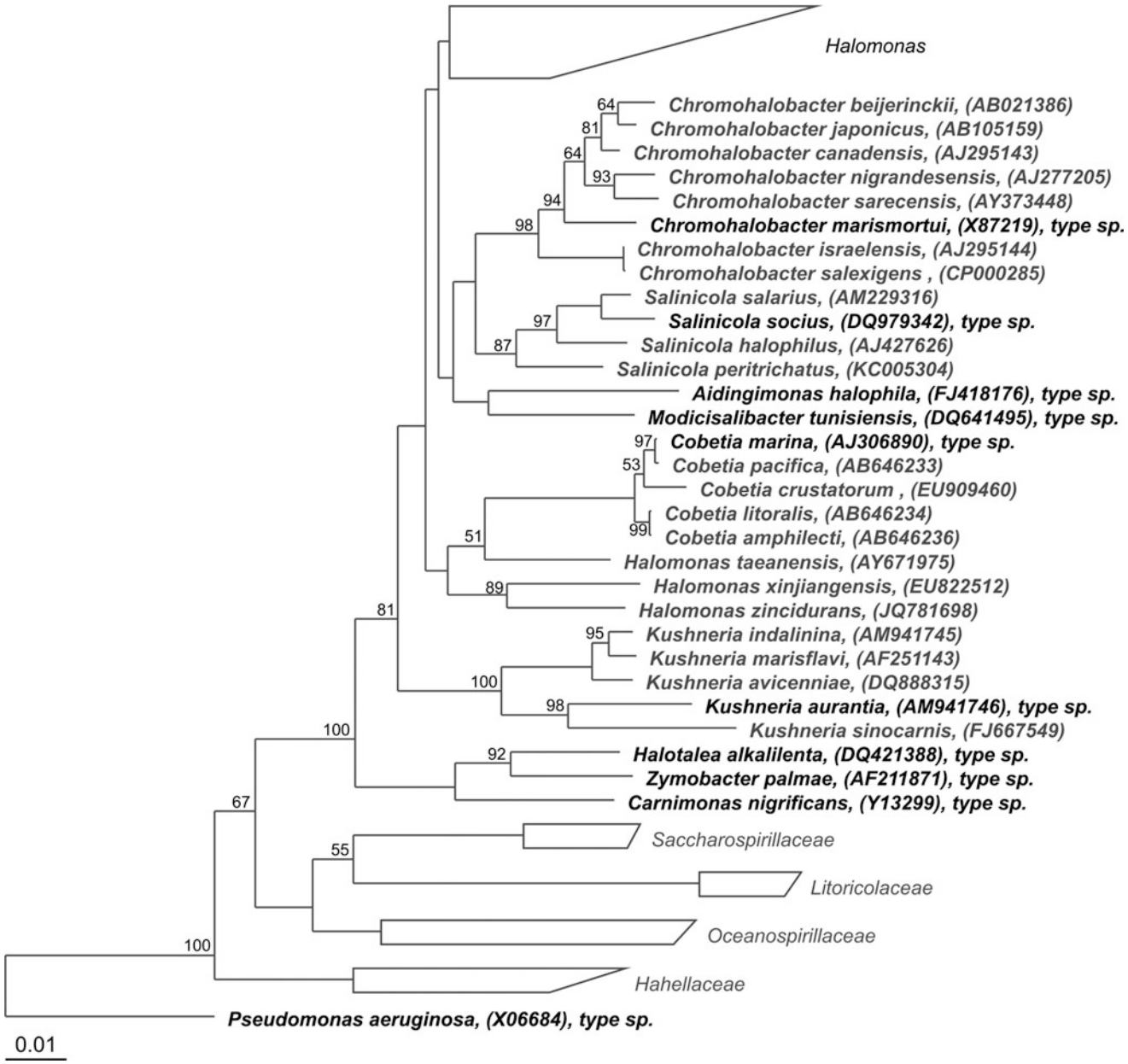

Fig. 17.3

Phylogenetic distribution of genera within the family *Halomonadaceae* other than *Halomonas* based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes-Cantor correction. The sequence datasets and alignments were usedaccording to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). 1,000 resampling boostrap values over 50 % are shown. Scale bar indicates estimated sequence divergence

Genes Sequenced and Characterized

Several genes from different members of the family *Halomonadaceae* have been sequenced and characterized, mainly from *Halomonas elongata* (Göller et al. 1998; Grammann et al. 2002; Kraegeloh et al. 2005; Schwibbert et al. 2011) and *Chromohalobacter salexigens* (Cánovas et al. 1998, 2000; Copeland et al. 2011), but also from *Cobetia marina* (Kraiwattanapong et al. 1999), *Halomonas eurihalina* (Llamas et al. 2003), *Halomonas halodenitrificans* (Sakurai and Sakurai 1998; Sakurai et al. 2006), *Halomonas maura* (Llamas et al. 2006), *Halomonas meridiana* (Coronado et al. 2000b), *Halomonas organivorans* (Moreno et al. 2011), *Halomonas salina* (Sripo et al. 2002), and *Zymobacter palmae* (Raj et al. 2002).

Genome Sizes and Plasmid

Before the genomic era, estimations of the genome size of 11 *Halomonas* and *Chromohalobacter* strains were carried out by using pulsed-field gel electrophoresis (Mellado et al. 1998; Llamas et al. 2002; Quesada et al. 2004). Additionally, the presence of plasmids (and megaplasmids) in strains of *Halomonas*, *Chromohalobacter* and *Cobetia* has been investigated (Fernández-Castillo et al. 1992; Vargas et al. 1995; Mellado et al. 1995a; Llamas et al. 1997; Argandoña et al. 2003).

Genome Comparison

As of January 2014, complete or draft genome sequences were available for type strains of the following species: *Carnimonas nigrificans* (JAGO00000000), *Chromohalobacter salexigens* (CP000285), *Halomonas anticariensis* (ASTJ00000000, AUAB00000000), *Halomonas boliviensis* (AGQZ00000000), *Halomonas elongata* (FN869568), *Halomonas halocynthiae* (AUDZ00000000), *Halomonas jeotgali* (AMQY00000000), *Halomonas lutea* (ARKK00000000), *Halomonas smyrnensis* (AJKS00000000), *Halomonas stevensii* (AJTS00000000), *Halomonas titanicae* (AOPO00000000), *Halomonas zhanjiangensis* (ARIT00000000), and *Kushneria aurantia* (ARNK00000000). Although they have not been sequenced yet, the type species of *Halomonas halodenitrificans*, *Halotalea alkalilenta*, and *Zymobacter palmae* are part of the Genomic Encyclopedia of Type Strains, Phase I: the 1,000 microbial genomes (KMG) project.

Additional sequenced strains are: *Halomonas* sp. 23_GOM-1509m (JADJ01000000), *Halomonas* sp. A3H3 (CBRE000000000), *Halomonas* sp. BJGMM-B45 (AVBC00000000), *Halomonas* sp. GFAJ-1 (AHBC00000000), *Halomonas* sp. HAL1 (AGIB00000000), *Halomonas* sp. HTNK1 (Gi05412), *Halomonas* sp. KM-1 (BAEU00000000), and *Halomonas* sp. TD01 (AFQW00000000).

A summary of the genome characteristics of the sequenced species of the family *Halomonadaceae* is presented in *Table 17.2*.

Phenotypic Analyses

Halomonadaceae Franzmann et al. (1989), emend. Dobson and Franzmann (1996), Ntougias et al. (2007), and Ben Ali Gam et al. (2007)

Ha.lo.mo.na.da'ce.ae. M.L. fem. n. *Halomonas*, type genus of the family; suff. *-aceae*, ending to denote a family; M.L. fem. pl. n. *Halomonadaceae*, the *Halomonas* family.

The members of the family *Halomonadaceae* cannot be defined by a reasonably large number of common-to-all features. This 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. Cells are Gram-negative, straight or curved, rod-shaped. They are either slight or moderate halophiles or halotolerant, except species of the genus *Zymobacter* (Okamoto et al. 1993), growing in the presence of high concentrations of sugars. Aerobic or facultatively anaerobic, chemoorganotrophs (Dobson and Franzmann 1996; Garrity et al. 2005b). Mata et al. (2002) reviewed in depth the phenotypic features of the genus *Halomonas*, and confirmed the enormous diversity among the species of this genus. In addition, 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*.

Genotypic diversity within the *Halomonadaceae* is also huge, as indicated for the genomic DNA G+C content, which ranges from 52.0 to 74.3 mol% (Martínez-Cánovas et al. 2004b; Arahal and Ventosa 2006).

From the chemotaxonomic point of view, the fatty acid profile is available for most species described within this family whereas the analysis of respiratory lipoquinones or polar lipids has been addressed for only some of them. There are a set of features shared for the vast majority of the species belonging to the *Halomonadaceae*. The major respiratory lipoquinone is ubiquinone 9 (Q9), although ubiquinone 8 (Q8) and ubiquinone 10 (Q10) are also present in several species. With regards to polar lipid composition all the members possess phosphatidylethanolamine and phosphatidylglycerol (Franzmann and Tindall 1990), except for the species *Aidingimonas halophila*, which contains diphosphatidylglycerol instead of phosphatidylglycerol (Wang et al. 2009). The fatty acid profile notably varies depending on the growing media and conditions, but in general, the main fatty acids are $C_{16:0}$, $C_{18:1}\omega7c$, $C_{16:1}\omega7c$, $C_{19:0}$ cyclo ω8c, $C_{17:0}$ cyclo, and $C_{12:0}$ 3-OH.

Table 17.3 shows the phenotypic and chemotaxonomic features, as well as the DNA G+C content comparison among the genera of the family *Halomonadaceae*. The type genus of the family is *Halomonas* (Vreeland et al. 1980).

Halomonas Vreeland et al. (1980), emend. Dobson and Franzmann (1996)

Ha.lo.mo'nas. Gr. n. *hals*, halos salt of the sea; Gr. n. *monas* a unit, monad; M.L. fem. n. *Halomonas* salt(-tolerant) monad.

Gram-negative, straight or curved, rod-shaped cells, generally 0.6–0.8 × 1.6–1.9 μm, except the species *H. halodenitrificans* that presents coccoid cells. Some species may produce poly-β-hydroxyalcanoates and/or exopolysaccharides. Endospores are not formed. Motile by means of peritrichous, lateral or polar flagella or nonmotile. Colonies are white to yellow, turning light brown with age. Slight to moderate halophiles, that are able to grow in NaCl concentrations ranging from 0.1 % to 32.5 % (w/v). Possess a mainly respiratory type of metabolism with oxygen as the terminal electron acceptor, but some species are also capable of anaerobic growth in the

Table 17.2
Genome characteristics of some members of the family *Halomonadaceae*

Strain	Accession number	Genome size (Mb)	Predicted ORFs	G+C content (mol %)	References
Carnimonas nigrificans ATCC BAA-78^T	JAGO01000000	2.7	2,500	56.0	Unpublished
Chromohalobacter salexigens 1H11^T	CP000285	3.7	3,412	63.9	Copeland et al. (2011)
Halomonas anticariensis FP35^T *Halomonas anticariensis* DSM 16096^T	ASTJ00000000 AUAB00000000	5.1 5.0	4,652 4,807	58.5 58.5	Tahrioui et al. (2013a) Unpublished
Halomonas boliviensis LC1^T	AGQZ00000000	4.2	3,915	54.7	Guzmán et al. (2012)
Halomonas elongata DSM 2581^T	FN869568	4.1	3,556	63.6	Schwibbert et al. (2011)
Halomonas halocynthiae DSM 14573^T	AUDZ00000000	2.9	2,772	53.8	Unpublished
Halomonas jeotgali Hwa^T	AMQY00000000	2.8	2,636	62.9	Unpublished
Halomonas lutea DSM 23508^T	ARKK00000000	4.5	4,368	59.1	Unpublished
Halomonas smyrnensis AAD6^T	AJKS00000000	3.6	3,326	67.9	Sogutcu et al. (2012)
Halomonas sp. 23_GOM-1509m	JADJ01000000	5.4	5,025	54.6	Unpublished
Halomonas sp. A3H3	CBRE000000000	5.6	5,279	55.6	Unpublished
Halomonas sp. BJGMM-B45	AVBC00000000	4.8	4,209	58.5	Unpublished
Halomonas sp. GFAJ-1	AHBC00000000	3.6	3,347	53.9	Phung et al. (2012)
Halomonas sp. HAL1	AGIB00000000	4.4	4,212	54.1	Lin et al. (2012)
Halomonas sp. HTNK1	Gi05412	4.4	4,221	53.0	Unpublished
Halomonas sp. KM-1	BAEU00000000	5.0	4,685	64.1	Kawata et al. (2012)
Halomonas sp. TD01	AFQW00000000	4.1	3,889	52.6	Cai et al. (2011)
Halomonas stevensii S18214^T	AJTS00000000	3.7	3,523	60.2	Kim et al. (2012)
Halomonas titanicae BH1^T	AOPO00000000	5.3	3,314	54.6	Sánchez-Porro et al. (2013)
Halomonas zhanjiangensis DSM 21076^T	ARIT00000000	4.1	3,739	54.5	Unpublished
Kushneria aurantia DSM 21353^T	ARNK00000000	3.8	3,598	62.8	Unpublished

presence of nitrate. Some species have been reported to grow under anaerobic conditions in the absence of nitrate if supplied with glucose (but not other carbohydrates or amino acids). Some species reduce nitrate to nitrite; nitrogen gas is not formed. Catalase positive and most of them are also oxidase positive. Chemoorganotrophic. Carbohydrates, organic acids, polyols, and amino acids, can be used as sole carbon and energy sources or as sole carbon, nitrogen and energy sources (Vreeland et al. 1980; Dobson and Franzmann 1996; Vreeland 2005).

The major respiratory lipoquinone is ubiquinone 9 (Vreeland 2005), with the exception of *H. alkaliphila* that mainly possess ubiquinone 8 and ubiquinone 6 (Romano et al. 2006). The major fatty acids are $C_{16:1}\omega7c$, $C_{17:0}$ cyclo, $C_{16:0}$, $C_{18:1}\omega7c$, and $C_{19:0}$ cyclo $\omega8c$ (Vreeland 2005). The predominant polar lipids are phosphatidylethanolamine and phosphatidylglycerol.

DNA G+C content ranges between 52.0 and 74.3 mol% (Martínez-Cánovas et al. 2004b; Arahal and Ventosa 2006), demonstrating the enormous heterogeneity within this genus.

Halomonas is the type genus of the family *Halomonadaceae*. The species *Halomonas elongata* is the type species of the genus.

Aidingimonas Wang et al. (2009)

Ai.ding.i.mo'nas. N.L. n. *Aiding* a lake located in Xinjiang province of north-west China; L. fem. n. *monas*, *monad* a unit, a monad; N.L. fem. n. *Aidingimonas* a monad from Aiding Lake.

Cells are Gram-negative, facultatively anaerobic, non-endospore-forming, short rods. Non-motile, without flagella. Moderately halophilic. Positive for catalase activity. Negative for oxidase activity and nitrate reduction. Ubiquinone 9 is present. Major fatty acids are $C_{19:0}$ cyclo $\omega8c$ and $C_{16:0}$.

Table 17.3

Phenotypic and chemotaxonomic characteristics and DNA G+C content of genera of the family *Halomonadaceae*

Characteristic	*Aidingi-monas*	*Carnimonas*	*Chromo-halobacter*	*Cobetia*	*Halomonas*	*Halotalea*	*Kushneria*	*Modici-salibacter*	*Salinicola*	*Zymobacter*
Oxidase	–	+	D	–	D	+	–	–	D	–
Motility	–	–	+	D	D	+	D	+	+	+
Acid production from:										
L-arabinose	+	–	ND	–	D	–	D	ND	ND	ND
D-fructose	+	+	ND	+	D	+	+	ND	+	ND
D-glucose	+	+	+	+	D	+	+	ND	+	ND
Lactose	+	ND	+	–	D	–	D	ND	+	ND
Maltose	+	+	+	D	D	+	D	ND	+	ND
D-mannitol	–	+	ND	D	D	–	D	ND	–	ND
D-mannose	ND	+	+	+	D	+	D	ND	+	ND
D-melezitose	–	–	ND	–	D	ND	–	ND	–	ND
Sucrose	–	+	D	D	D	–	D	ND	–	ND
Hydrolysis of:										
DNA	ND	–	–	D	D	–	–	ND	D	ND
Starch	–	+	–	–	D	–	–	ND	D	–
Casein	–	–	D	D	D	–	–	ND	D	ND
Aesculin	–	+	–	D	D	ND	D	–	–	ND
Indole production	–	–	D	–	–	–	–	–	–	–
Methyl red	–	ND	D	–	D	–	D	ND	+	+
Voges-Proskauer	V	–	–	–	D	–	–	ND	–	+
Citrate utilization	+	+	D	–	D	+	+	–	+	–
Arginine dihydrolase	+	–	ND	–	D	–	–	–	ND	–
Lysine decarboxylase	–	ND	D	–	–	–	–	ND	–	–
Ornithine decarboxylase	–	ND	D	–	D	–	D	ND	–	–
H_2S production	–	ND	D	–	D	–	–	–	D	ND
Nitrate reduction	–	–	D	–	D	–	D	+	D	–
Phosphatase	+	ND	D	+	D	ND	+	+	+	ND
Urease	+	–	D	D	D	–	–	–	D	ND
Lecithinase	ND	–	ND	V	D	ND	ND	ND	ND	ND
Phenylalanine deaminase	–	–	–	–	D	–	D	ND	–	–

ONPG	−	+	ND	+	D	ND	+	−	−	−
O/F (D-glucose)	ND	ND	ND	O	D	ND	O	ND	F	F
Growth on:										
MacConkey agar	ND	+	ND	+	+	ND	D	ND	ND	ND
Cetrimide agar	ND	+	ND	+	D	ND	ND	ND	ND	ND
Utilization of:										
Starch	ND	ND	+	−	D	ND	−	ND	D	ND
L-arabinose	+	−	ND	−	D	ND	+	ND	ND	ND
Aesculin	ND	ND	+	D	D	ND	−	ND	D	ND
D-fructose	+	ND	D	+	D	+	+	+	D	ND
D-glucose	+	ND	+	+	D	+	+	+	D	ND
Lactose	+	ND	+	−	D	−	D	ND	D	ND
Maltose	+	+	D	+	D	+	+	−	D	ND
D-mannose	+	+	+	D	D	ND	+	−	D	ND
Ribose	+	ND	D	D	D	ND	+	−	D	ND
Sucrose	+	ND	+	D	D	+	D	ND	D	ND
D-xylose	ND	ND	+	−	D	+	D	ND	D	ND
Glycerol	+	ND	+	+	D	+	+	ND	+	ND
myo-inositol	+	ND	D	D	D	ND	−	ND	D	ND
D-mannitol	+	−	+	D	D	+	D	ND	+	ND
Sorbitol	+	ND	D	−	D	+	D	ND	+	ND
Gluconate	ND	+	+	D	D	ND	+	ND	ND	ND
Propionate	ND	ND	D	+	D	ND	−	ND	+	ND
Succinate	+	ND	D	+	D	+	+	ND	−	ND
Polar lipids	DPG, PE	DPG, PG, PE	DPG, PG, PE	PE, PG	PE, PG	ND	PG, DPG, PE	ND	PG, PE	ND
Respiratory quinones	Q9	Q9	Q9, Q8	Q9, Q8	Q9, Q8	Q9	Q9, Q8, Q10	ND	Q9, Q10, Q8	Q9
Fatty acids	$C_{19:0}$ cyclo, $C_{16:0}$	$C_{16:0}$, $C_{16:1}$, $C_{18:1}$, $C_{19:0}$ cyclo	$C_{16:0}$, $C_{19:0}$ cyclo, $C_{18:1}$, $C_{17:0}$ cyclo, $C_{12:0}$ 3-OH	$C_{16:1}$, $C_{16:0}$, $C_{12:0}$ 3-OH $C_{18:1}$, $C_{17:0}$ cyclo,	$C_{16:1}$, $C_{17:0}$ cyclo, $C_{16:0}$, $C_{18:1}$, $C_{19:0}$ cyclo	$C_{18:1}$, $C_{16:0}$, $C_{19:0}$ cyclo, $C_{12:0}$ 3-OH, $C_{16:1}$/iso-$C_{15:0}$ 2-OH	$C_{16:0}$, $C_{18:1}$, $C_{19:0}$ cyclo, $C_{12:0}$ 3-OH, $C_{17:0}$ cyclo	$C_{16:0}$, $C_{18:1}$, $C_{16:1}$, $C_{19:0}$ cyclo, $C_{17:0}$	$C_{16:1}$, $C_{16:0}$, $C_{18:1}$, $C_{19:0}$ cyclo, $C_{12:0}$ 3-OH	$C_{16:0}$, $C_{19:0}$ cyclo, $C_{18:1}$, $C_{12:0}$ 3-OH
DNA G+C content (mol%)	57.2–57.5	56.0	56.1–66.0	61.4–64.2	52.0–74.3	64.4	59.0–61.7	53.7	58.8–63.6	55.4–56.2

Data from original descriptions and from Franzmann and Tindall (1990) and Arahal and Ventosa (2006). + positive, − negative, *V* variable among different studies, *D* different results for species belonging to the same genus, *ONPG* ortho-nitrophenyl-β-D-galactopyranoside, *O* oxidative, *F* fermentative, *ND* no data available, *DPG* diphosphatidylglycerol, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerol

The polar lipid pattern consists of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides, two unknown phospholipids, two unknown phosphoglycolipids and one unknown glycolipid. The DNA G+C content is 57.2–57.5 mol% (Wang et al. 2009).

The only species within this genus is *Aidingimonas halophila*, which was isolated from a salt lake in Xinjiang province, northwest China. Cell size ranges between 0.1–0.3 × 0.7–1.5 μm. It forms colourless to yellow brown colonies, flat and opaque with slightly irregular edges. Growth occurs at 10–45 °C, at pH 5.0–10.0 and in 1–25 % (w/v) NaCl, with optimal growth at 37 °C, pH 7.0–8.0 and 5–10 % NaCl. Does not contain poly-β-hydroxybutyrate granules or produce exopolysaccharide. Growth occurs under anoxic conditions in the presence of nitrate ion as electron acceptor. The Voges–Proskauer test is variable. Indole and H_2S are not produced. Milk peptonization and coagulation and the methyl red test are negative. Gelatin, aesculin, casein, starch, and Tweens 40, 60 and 80 are not hydrolysed, but positive for hydrolysis of Tween 20 and urea. ONPG, phenylalanine deaminase and lysine and ornithine decarboxylase tests are negative, but positive for arginine dihydrolase. Citrate and other substrates can be utilized as a sole carbon or nitrogen and energy sources. Acid is produced from different carbohydrates and organic acids. The type strain is YIM 90637^T, with a DNA G+C content of 57.5 mol% (Wang et al. 2009).

Carnimonas Garriga et al. (1998)

Car.ni'mo.nas. L. gen. n. carnis of meat; Gr. n. monas a unit, monad. *Carnimonas* a monad of meat.

Straight or slightly curved rods, 0.5–0.6 × 1.0–1.7 μm, occurring singly or in pairs. Gram-negative. Does not form endospores. Nonmotile. Oxidase and catalase positive. Aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Slightly or moderate halophile. No growth occurs in the presence of more than 8 % (w/v) NaCl. Optimum temperature for growth is 28–30 °C. No growth occurs at 5 °C or 37 °C. Chemoorganotrophic. Acid, but no gas, is produced from D-glucose, D-xylose, melibiose, maltose and sucrose. β-galactosidase (ONPG) activity occurs. Forms dark spots on the surface of raw, cured meat products. The main respiratory quinone is ubiquinone-9. Main components in the polar lipid composition are diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylethanolamine. Major fatty acids are $C_{16:0}$, $C_{16:1}$, $C_{18:1}$, and $C_{19:0}$ cyclo. The G+C content of the DNA is 56.0 mol% (Garriga et al. 1998; 2005).

The type and the single species within this genus is *Carnimonas nigrificans*. In the species description a total of nine strains, CTCBS1^T to CTCBS9 were reported to be isolated from cured meat products. Colonies are non-pigmented, white, convex, shiny and circular. Aesculin and starch are hydrolysed. Gelatin, casein and DNA are not hydrolysed. Voges-Proskauer negative. Arginine dihydrolase, urease, lecithinase and phenylalanine deaminase negative. Indole is not produced. Nitrate is not reduced (Garriga et al. 1998; 2005).

Chromohalobacter Ventosa et al. (1989) emend. Arahal et al. (2001a)

Chro.mo.ha'lo.bac'ter. Gr. n. *chroma* color; Gr. n. *halos* the sea, salt; M.L. n. *bacter* rod; M.L. masc. n.*Chromohalobacter* colored salt rod.

Gram-negative, straight or sometimes slightly curved, rods (0.4–1.2 × 0.8–6.1 μm). Motile by polar or peritrichous flagella. Cells occur singly, in pairs, and in short chains. Colonies are cream to brown-yellow pigmented, with the exception of *C. nigrandesensis* that shows black pigmentation. Endospores are not formed. Moderately halophilic. Salt is required for growth. The optimum salt concentration for growth is between 8 % and 10 %. May grow at salt concentrations up to 30 %. The broader ranges of temperature and pH observed for growth are 0–45 °C (optimal 30–37 °C) and pH 5.0–10.0 (optimal pH 7.5), respectively. Aerobic. Chemoorganotrophic. Catalase positive. Oxidase negative, with the exception of *C. beijerinckii* (Peçonek et al. 2006) and *C. sarecensis* (Quillaguamán et al. 2004a). Some strains reduce nitrates, but H_2S and urease are not produced, with the exception of *C. nigrandesensis* (Prado et al. 2006) and *C. salexigens* (Arahal et al. 2001b). Phenylalanine deaminase test is negative. Starch, Tween 80, aesculin, DNA, and tyrosine are not hydrolyzed. The species *C. japonicus* is the only able to hydrolyse gelatin (Sánchez-Porro et al. 2007). Acid is produced aerobically from D-glucose and other carbohydrates. Carbohydrates, amino acids, and some polyols can serve as sole carbon or nitrogen sources (Arahal et al. 2001a; Ventosa 2005).

Predominant fatty acids are $C_{16:0}$, $C_{19:0}$ cyclo $\omega 8c$, and $C_{18:1}$ $\omega 7c$; $C_{17:0}$ cyclo, and $C_{12:0}$ 3-OH are present in smaller amounts. Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and two unknown phospholipids are major to moderate compounds in the polar lipid profile. The quinone system consists of the major compound Q-9 (>95 %) and small amounts of Q-8 (Peçonek et al. 2006; Sánchez-Porro et al. 2007). The DNA G+C base composition ranges from 56.1 to 66.0 mol%.

The type species of the genus is *Chromohalobacter marismortui*, previously named as "*Chromobacterium marismortui*", which was originally isolated from the Dead Sea (Ventosa et al. 1989).

Cobetia Arahal et al. (2002b) emend. Romanenko et al. (2013)

Co.be'ti.a. N.L. fem. n. *Cobetia* named after A. B. Cobet, who originally described the type species as "*Arthrobacter marinus*".

Gram-negative, straight, rod-shaped cells that are 1.6–4.0 × 0.8–1.2 μm, and occur singly and in pairs. Strains are non-motile or motile by means of a single polar flagellum and/or two to

seven lateral flagella. Some strains can produce fimbria-like structures and capsules. Colonies are round, bright, smooth and cream pigmented. Poly-β-hydroxyalkanoate is accumulated. Oxidase negative. Aerobic; unable to grow anaerobically in the presence of nitrate or arginine. Sodium ions are not essential for growth. Most strains can grow without addition of NaCl to the medium, but optimal growth occurs in the presence of 5–6 % (w/v) salts and they can considered as slightly halophiles. Good growth is also obtained up to 20 % (w/v) but not at higher salinities. Hydrolyses tyrosine, but not gelatine, starch or Tween 80. Hydrolysis of casein, DNA and aesculin is strain-dependent (negative reaction for most strains). Negative for chitin hydrolysis and Simmons' citrate test. ONPG positive. Phosphatase positive. Negative for phenylalanine deaminase, methyl red, indole production, and nitrate reduction. H_2S is not produced. Acid is produced from glucose and other carbohydrates (Arahal et al. 2002a; Romanenko et al. 2013).

Polar lipids include phosphatidylethanolamine, phosphatidylglycerol, unknown phospholipids, unknown lipids, an unknown aminolipid and phosphatidic acid. The major fatty acids are $C_{16:1}$ $\omega7c$, $C_{16:0}$, $C_{12:0}$ 3-OH, $C_{18:1}$ $\omega7c$ and $C_{17:0}$ cyclo. The DNA G+C content varies between 61.4 and to 64.2 mol% (Kim et al. 2010b; Romanenko et al. 2013).

The species *Cobetia marina* is the type of the genus and strain DSM 4741^T is the type strain of this species. Strain DSM 5160, formerly the type strain of the species *Halomonas halodurans*, has been proposed as member of *C. marina* based on phylogenetic analysis and DNA-DNA hybridization and chemotaxonomic characteristics (Romanenko et al. 2013).

Halotalea Ntougias et al. (2007)

Ha.lo.ta.le'a. Gr. n. *hals halos* salt; L. fem. n. *talea* a staff, rod; N.L. fem. n. *Halotalea* rod-shaped cells living in saline conditions.

Cells are Gram-negative, rods, motile by peritrichous flagella and forming small, non-pigmented pale yellow colonies. Endospores are not formed. Strictly aerobic. Halotolerant and alkalitolerant. Sugar-tolerant. Oxidase- and catalase-positive. Chemo-organotrophic. Ubiquinone-9 is present in the respiratory chain. The major fatty acids are $C_{18:1}$ $\omega7c$, $C_{16:0}$, $C_{19:0}$ cyclo $\omega8c$, $C_{12:0}$ 3-OH and $C_{16:1}$ $\omega7c$/iso-$C_{15:0}$ 2-OH. The DNA G+C content is 64.4 mol% (Ntougias et al. 2007).

The type species is *Halotalea alkalilenta*, which tolerates up to 15 % (w/v) NaCl, with an optimum salt concentration of 0–3 % (w/v) NaCl. Tolerates up to 45 % and 60 % w/v (+)-D-glucose and maltose, respectively. Grows at pH 5–11, with optimum at pH 7. The temperature range for growth is 5–45 °C, with an optimum temperature of 32–37 °C. Negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase, nitrate reduction, H_2S production from L-cysteine, indole production, methyl red and Voges-Proskauer. Acid is produced from (+)-D-glucose and other carbohydrates. Hydrolyses Tween 20, but does not hydrolyse casein, DNA, gelatin, starch, Tween 80 or urea. The type strain is AW-7^T, isolated from olive mill waste (alkaline alpeorujo) obtained from the premises of the Toplou Monastery in the region of Sitia, Crete, Greece (Ntougias et al. 2007).

Kushneria Sánchez-Porro et al. (2009)

Kush.ne'ri.a. N.L. fem. n. *Kushneria* from the name Kushner, honouring Dr Donn J. Kushner, a Canadian microbiologist who carried out pioneering studies on halophilic micro-organisms.

Cells are Gram-negative, motile rods (0.5–2.0 × 1.7–5.0 μm). Endospores are not formed. Strictly aerobic. Colonies are yellow-orange to cream. Moderately halophilic; Na^+ is required for growth. The optimal NaCl range supporting the growth is 0.5–12 % (w/v). Mesophilic (able to grow from 4 to 42° C, optimum at 25–37° C). Growth occurs at pH 4.5–10.0 (optimally at pH 7.0–8.0). Chemo-organotrophic. Catalase-positive and oxidase-negative. Aesculin and gelatin are hydrolysed, with the exception of *Kushneria sinocarnis* that does not possess gelatinase activity (Zou and Wang 2010). Casein, starch, Tween 80, DNA, and tyrosine are not hydrolysed. Indole and H_2S production, and Voges-Proskauer test are negative. Phosphatase is produced, but urease, arginine dihydrolase, and lysine decarboxylase are not. Except *K. indalinina* (Cabrera et al. 2007) and *K. sinocarnis* (Zou and Wang 2010), the other species do not reduce nitrate to nitrite. The major respiratory quinone is Q9. Major fatty acids are $C_{16:0}$, $C_{18:1}$ $\omega7c$, $C_{19:0}$ cyclo $\omega8c$, $C_{12:0}$ 3-OH, and $C_{17:0}$ cyclo. Polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and unidentified phospholipids and glycolipids. The DNA G+C content is 59.0–61.7 mol% (Yoon et al. 2001; Cabrera et al. 2007; Soto-Ramírez et al. 2007; Sánchez-Porro et al. 2009; Zou and Wang 2010).

The type species of the genus is *Kushneria aurantia*, with the type strain A10^T isolated from the leaf surface of *Avicennia germinans* (black mangrove).

Modicisalibacter Ben Ali Gam et al. (2007)

Mo'di.ci.sa'li.bac'ter. L. adj. *modicus* moderate, limited; L. n. *sal, salis* salt; N.L. masc. n. *bacter* a rod; N.L. masc. n. *Modicisalibacter* a moderately halophilic rod.

Cells are Gram-negative, non-endospore-forming, motile rods. Moderately halophilic. Strictly aerobic and require Na^+ for growth. Mesophilic, growing well at 15–45 °C, oxidase-negative and reduce nitrate. Predominant fatty acids are $C_{16:0}$, $C_{18:1}$ $\omega7c$, $C_{16:1}$ $\omega7c$, $C_{19:0}$ cyclo $\omega8c$ and $C_{17:0}$. Contents of $C_{19:0}$ cyclo $\omega8c$ and $C_{17:0}$ differ significantly from those of other members of the *Halomonadaceae*. The DNA G+C content is 53.7 mol% (Ben Ali Gam et al. 2007).

The genus contains a single species, namely *Modicisalibacter tunisiensis*, whose cells are approximately 1.0–4.0 μm long × 0.6–1.0 μm wide. Colonies on marine agar are circular, smooth, convex and 2–3 mm in diameter after 48 h of incubation at

37 °C. Cells grow at 4–45 °C, with optimum at 37 °C. The pH range for growth is 5–10, with an optimum at pH 7.2. Growth occurs in the range 0.1–25 % (w/v) NaCl and optimally at 10 % (w/v) NaCl. Catalase reaction is positive. ONPG hydrolysis is negative. Citrate is not utilized. Urease and arginine dihydrolase are not produced. Gelatin, alginate and aesculin are not hydrolysed. H_2S and indole are not produced. D-glucose, D-fructose, tryptone, peptone and Casamino acids are utilized. The type strain is LIT2^T, which was isolated from a sample of oilfield-water injection collected in the Sidi Litayem area near Sfax, Tunisia (Ben Ali Gam et al. 2007).

Salinicola Anan'ina et al. (2008)

Sa.li.ni.co'.la. L. fem. pl. n. *salinae* salterns, salt-works; N.L. suff. *-cola*, derived from *incola*, inhabitant; N.L. masc. n. *Salinicola*, inhabitant of salterns.

Cells are Gram-negative, non-endospore-forming rods (0.5–1.3 × 1.0–3.2 μm). Motile by means of a single lateral/polar flagellum or by peritrichous flagella (Huo et al. 2013). Colonies are circular, smooth, convex and creamy yellow-coloured. Aerobic and chemoorganotrophic. Moderately halophilic that grows in the range of 0–30 % (w/v) NaCl with the optimum of 0.5–20 % (w/v) NaCl, at pH 4.5–10.0 (optimum pH 5.0–8.0). Mesophilic, growing in the range 4–45 °C (optimal growth temperature is 25–37 °C). Exopolysaccharides are not produced, but the species *Salinicola salarius* and *S. socius* produce poly-β-hydroxyalkanoate (de la Haba et al. 2010b). Respiration on fumarate, nitrate and nitrite is negative. Shows positive reaction in the oxidation/fermentation of D-glucose. Catalase reaction is positive, oxidase reaction is negative, except for *S. salarius* (Kim et al. 2007). Tween 20 is hydrolysed. Tyrosine and aesculin are not hydrolysed. β-Galactosidase is not produced. Phosphatase activity is present. For all species, except *S. peritrichatus*, methyl red test is positive and Voges-Proskauer is negative (de la Haba et al. 2010b; Huo et al. 2013). Indole is not produced. Lysine- and ornithine-decarboxylases and phenylalanine deaminase are negative. Gluconate is not oxidized. Selenite is not reduced. The predominant respiratory lipoquinone is ubiquinone with nine isoprene units (Q9). The major fatty acids are $C_{16:1}$ $\omega7c$, $C_{16:0}$, $C_{18:1}$ $\omega7c$, $C_{19:0}$ cyclo $\omega8c$, and $C_{12:0}$ 3-OH. The DNA G+C content ranges between 58.8 and 63.6 mol% (Anan'ina et al. 2007; Kim et al. 2007; Aguilera et al. 2007; de la Haba et al. 2010b; Huo et al. 2013).

The type species of the genus is *Salinicola socius*, isolated from the microbial community obtained from the soil of salt mines (Berezniki, Perm region of Russia), which grows on naphthalene as a sole carbon and energy source. The other three species comprising the genera are *S. halophilus* (formerly *Chromohalobacter salarius*), *S. peritrichatus*, and *S. salarius* (basonym of *Halomonas salaria*). The species name *S. zeshunii* (Cao et al. 2013) has been effectively, but not validly published.

Zymobacter Okamoto et al. (1995)

Zy.mo.bac'ter. Gr. n. *zyme* leaven, ferment; M.L. n. *bacter* masc. equivalent of Gr. neut. n. *bakterion* rod; M. L. masc. n. *Zymobacter* the fermenting rod.

Non-endospore forming rod-shaped cells with rounded ends, 1.3–2.4 × 0.7–0.9 μm; usually single. Motile by as many as 20 peritrichous flagella that are non-sheathed. Gram-negative. Facultatively anaerobic. Chemoorganotrophic. Grow on and ferment 1 mol of glucose or hexose moiety of maltose to produce approximately 2 mol each of ethanol and CO_2, with a trace amount of acids. Ferments hexoses, α-linked di- and trisaccharides and sugar alcohols. Growth initiates at pH values of 4.7–8.1. Catalase-positive and oxidase-negative. The major cellular fatty acids are $C_{16:0}$, $C_{19:0}$ cyclo, $C_{18:1}$ $\omega9c$, and $C_{12:0}$ 3-OH. Quinone system is ubiquinone-9. The DNA G+C base composition ranges from 55.4 to 56.2 mol% (Okamoto et al. 1993, 2005).

Currently, the genus include a single species, *Zymobacter palmae*, with the type strain T109^T isolated from palm sap. Colonies are round, entire, smooth, opaque, and milky white. Colonies of similar size develop under aerobic and anaerobic growth conditions. Growth is better in static cultures than in shaken cultures. Requires nicotinic acid for growth. Growth does not occur in the absence of a sugar or sugar alcohol. Growth occurs at 15–37 °C (optimum, 30 °C) and pH 4.7–8.1 (optimum, pH 6.0). The organisms are neither halophilic nor halotolerant, but they are considerably tolerant to ethanol and produce ethanol mainly from maltose (5.8 % ethanol after 6 days of fermentation), but also from glucose, fructose, sucrose, melibiose, raffinose, sorbitol, mannitol, and –depending on the strain– mannose and galactose. Methyl red, Voges-Proskauer and α-glucosidase are positive. The following tests are negative: indole production, utilization of citrate, nitrate reduction, chromogenicity, gelatin liquefaction, hydrolysis of starch, phenylalanine deaminase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and β-galactosidase (Okamoto et al. 1993, 2005).

Isolation and Maintenance Procedures

Isolation

Halomonads may be isolated following standard microbiological techniques on complex 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 17.4*), 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 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^{2+} to prevent the growth of extremely halophilic archaea (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 *Aidingimonas*, *Chromohalobacter*, some *Halomonas* (such as *H. alkaliantarctica*, *H. alkaliphila*, *H. almeriensis*, *H. anticariensis*, *H. aquamarina*, *H. beimenensis*, *H. campaniensis*, *H. cerina*, *H. cibimaris*, *H. cupida*, *H. daqiaonensis*, *H. daqingensis*, *H. denitrificans*, *H. eurihalina*, *H. fontilapidosi*, *H. gomseomensis*, *H. gudaonensis*, *H. halmophila*, *H. halodenitrificans*, *H. halophila*, *H. ilicicola*, *H. janggokensis*, *H. jeotgali*, *H. korlensis*, *H. lutea*, *H. maura*, *H. olivaria*, *H. organivorans*, *H. pantelleriensis*, *H. sabkhae*, *H. shengliensis*, *H. sinaiensis*, *H. smyrnensis*, *H. stenophila*, *H. subglaciescola*, *H. taeanensis*, *H. variabilis*, *H. ventosae*, *H. vilamensis*, and *H. xinjiangensis*), and the species *Kushneria aurantia*, *K. indalinina*, *K. sinocarnis*, *Modicisalibacter tunisiensis*, *Salinicola halophilus*, and *S. salarius* 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 at 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 0.5–5 %.

Other species (*Cobetia amphilecti*, *Cob. crustatorum*, *Cob. litoralis*, *Cob. marina*, *Cob. pacifica*, *Halomonas alimentaria*, *H. boliviensis*, *H. campisalis*, *H. elongata*, *H. flava*, *H. hamiltonii*, *H. hydrothermalis*, *H. koreensis*, *H. kribbensis*, *H. mongoliensis*, *H. muralis*, *H. nitroreducens*, *H. qijiaojingensis*, *H. ramblicola*, *H. rifensis*, *H. saccharevitans*, *H. salina*, *H. stevensii*, *H. xianhensis*, *H. zincidurans*, *K. avicenniae*, *K. marisflavi*, and *Salinicola socius*) are also moderately halophilic, but their optimum salinity for growth is lower (around 5 %). Most of these species show no requirement of salts or require only as little as 0.5–1 %.

The slightly halophilic species (*Halomonas andesensis*, *H. arcis*, *H. axialensis*, *H. caseinilytica*, *H. desiderata*, *H. halocynthiae*, *H. johnsoniae*, *H. kenyensis*, *H. magadiensis*, *H. meridiana*, *H. nanhaiensis*, *H. neptunia*, *H. pacifica*, *H. salifodinae*, *H. subterranea*, *H. sulfidaeris*, *H. titanicae*, *H. venusta*, *H. zhanjiangensis*, *Halotalea alkalilenta* and *S. peritrichatus*) grow optimally at around 3 % NaCl. However, these species are halotolerant of up to 15–20 % NaCl (25 % in the case of *H. neptunia* and *H. titanicae* and 10 % in the case of *H. kenyensis*).

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 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.

On the contrary, *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 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, α-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. alkaliantarctica*, *H. alkaliphila*, *H. campaniensis*, *H. campisalis*, *H. daqingensis*, *H. desiderata*, *H. kenyensis*, *H. korlensis*, *H. magadiensis*, *H. mongoliensis*, and *H. pantelleriensis* that grow optimally at pH 9.0 or 9.5 or *Cobetia crustatorum*, *Salinicola peritrichatus*, and *Zymobacter palmae*, with optimal growth at pH 5 or 6 (Table 17.4).

Finally, although all halomonads are mesophilic, some differences in their behavior towards temperature can be used for selective isolation. *Aidingimonas halophila*, *Chromohalobacter japonicus*, *C. marismortui*, *C. salexigens*, *Cobetia amphilecti*, *C. litoralis*, *C. marina*, *C. pacifica*, *Halomonas alkaliphila*, *H. beimenensis*, *H. campaniensis*, *H. desiderata*, *H. elongata*, *H. flava*, *H. fontilapidosi*, *H. halophila*, *H. ilicicola*, *H. kenyensis*, *H. lutea*, *H. magadiensis*, *H. meridiana*, *H. mongoliensis*, *H. organivorans*, *H. qijiaojingensis*, *H. ramblicola*, *H. rifensis*, *H. sabkhae*, *H. smyrnensis*, *H. titanicae*, *H. xinjiangensis*, *Halotalea alkalilenta*, *Kushneria aurantia*, *K. sinocarnis*, *Modicisalibacter tunisiensis*, *Salinicola peritrichatus*, and *S. socius* grew well at 37 °C while the rest grew only suboptimally (or not at all). Temperatures around 30 °C fit all known species so far. In the lower limit most species show no apparent growth below 4–15 °C (20 °C in the case of *H. anticariensis*, *H. flava*, *H. magadiensis*, *H. qijiaojingensis*, *Kushneria aurantia*, and *Z. palmae*; 25 °C in the case of *H. ilicicola*, *H. rifensis*, and *H. sinaiensis*; 30 °C in the case of *H. sabkhae*), but *Chromohalobacter sarecensis*, *H. boliviensis*, *H. subglaciescola* are capable of growing at 0 °C, and *H. axialensis*, *H. neptunia*, *H. sulfidaeris* at −1 °C. With respect to the upper limit, the species *H. alkaliphila*, *H. beimenensis*, *H. campisalis*, *H. daqingensis*, *H. denitrificans*, *H. mongoliensis*, *H. olivaria*, *H. sabkhae*, *H. sinaiensis*, *H. ventosae*, and *H. xinjiangensis* are able to grow up to 50 °C, although the most thermotolerant species is *H. kenyensis*, growing up to 55 °C (Table 17.4).

Maintenance and 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

Table 17.4
Physiological features of member of the family *Halomonadaceae*

Species	Salt range (%, w/v)											Temperature (°C)							pH							
	0	0.5	1	2	3	3.5	5	10	15	20	25	4–5	10	15	20	35	37	45	5	6	7	8	9	10	11	12
A. halophila	–	–	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	–	–
Car. nigrificans	+	+	+	+	+	+	+	+	–	–	–	–	ND	ND	ND	ND[a]	–	–	ND	ND	ND	ND	ND	ND	ND	ND
Chr. beijerinckii	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+[b]	+	–	+	+	+	+	–	–	–	–
Chr. canadensis	–	–	–	–	+	+	+	+	+	+	+	–	–	+	+	+[b]	+	+	+	+	+	+	+	–	–	–
Chr. israelensis	–	–	–	–	–	+	+	+	+	+	–	–	–	+	+	+[b]	+	+	+	+	+	+	+	–	–	–
Chr. japonicus	–	–	–	–	–	–	+	+	+	+	+	–	–	+	+	+	+	–	–	+	+	+	+	–	–	–
Chr. marismortui	–	–	–	+	+	+	+	+	+	+	+[c]	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
Chr. nigrandesensis	–	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+[b]	+	+	+	+	+	+	+	+	–	–
Chr. salexigens	–	–	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–
Chr. sarecensis	+	+	+	+	+	+	+	+	+	+	+	+[d]	+	+	+	+	+	+	+	+	+	+	+	+	–	–
Cob. amphilecti	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	–[e]	+	+	+	+	+	+	–	–
Cob. crustatorum	–	–	–	–	–	+	+	+	–	–	–	+	+	+	+[f]	–[g]	–	–	+	+	+	+	+	+	–	–
Cob. litoralis	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	–[e]	+	+	+	+	+	+	–	–
Cob. marina	–	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	+	+	+	+	+	+	–	–
Cob. pacifica	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	–[e]	+	+	+	+	+	+	+	–
H. alimentaria	–	ND	+	+	+	+	+	+	+	+	–	+	+	+	+	+[b]	+	+	+	+	+	ND	ND	ND	ND	ND
H. alkaliantarctica	–	–	–	+	+	+	+	+	+	+	–	–	+	+	+	+[b]	+	–	–	–	+	+	+	+	–	–
H. alkaliphila	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+[h]	–	–	–	+	+	+	–	–
H. almeriensis	–	–	–	–	–	–	+	+	+	+	+	–	–	+	+	+	+	–	–	+	+	+	+	+	–	–
H. andesensis	–	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	–	+	+	+	+	+	+	–
H. anticariensis	–	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	–	+	+	+	+	–	–	–
H. aquamarina	–	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+	–	+	+	+	+	+	+	–	–
H. arcis	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+[b]	+	+	–	+	+	+	+	+	–	–
H. axialensis	–	+	+	+	+	+	+	+	+	+	–	+[i]	+	+	+	+[b]	–	–	+	+	+	+	+	+	+	+
H. beimenensis	+	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+	+[h]	–	+	+	+	+	–	–	–
H. boliviensis	+	+	+	+	+	+	+	+	+	+	+	+[d]	+	+	+[f]	+[b]	+	+	–	+	+	+	+	+	+	ND
H. campaniensis	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+	+	–	–	–	+	+	+	+	–	–
H. campisalis	–	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+[b]	+	+[h]	–	–	–	+	+	+	+	–
H. caseinilytica	–	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+[b]	+	+	+	+	+	+	+	–	–	–
H. cerina	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+[b]	+	+	+	+	+	+	+	+	–	–
H. cibimaris	–	–	–	–	+	+	+	+	+	–	–	–	–	+	+[f]	+[b]	–	–	–	+	+	+	+	–	–	–
H. cupida	+	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+	–	+	+	+	+	+	+	–	–

H. daqiaonensis	–	–	–	+	+	+	+	+[j]	+	–	–	+	+	+	+	+[b]	+	–[e]	–	+	+	+	+	–	–	–
H. daqingensis	–	–	+	+	+	+	+	+	+	–	–	–	+	+	+	+[b]	+	+[h]	–	–	–	+	+	+	–	–
H. denitrificans	–	–	–	+	+	+	+	+	+	+	–	+	+	+	+[f]	+	+	+[h]	–	–	+	+	+	+	–	–
H. desiderata	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+	–	–	+	+	+	+	+	–
H. elongata	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+[b]	+	+	+	+	+	+	+	+	–	–
H. eurihalina	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
H. flava	–	+	+	+	+	+	+	+	+	+	–[k]	–	–	–	+	+	+	–[e]	–	+	+	+	+	–	–	–
H. fontilapidosi	–	–	–	–	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–
H. gomseomensis	–	–	+	+	+	+	+	+	+	+	–	+	+	+	+[f]	+[b]	+	+	–	+	+	+	+	+	–	–
H. gudaonensis	–	–	+	+	+	+	+	+	+	+	–	–	+	+	+	+[b]	+	–	–	–	–	+	+	–	–	–
H. halmophila	–	–	–	–	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+	+	+	–	–
H. halocynthiae	–	+	+	+	+	+	+	+	+	–	–	–	+	+	+[f]	+	–	–	+	+	+	+	+	+	+	–
H. halodenitrificans	–	–	–	–	+	+	+	+	+	+	–	+	+	+	+[f]	+[b]	+	–	+	+	+	+	+	+	–	–
H. halophila	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+[b]	+	+	+	+	+	+	+	+	–	–
H. hamiltonii	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	–	+	+	+	+	+	–	–
H. hydrothermalis	–	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+[b]	+	–	+	+	+	+	+	+	+	+
H. ilicicola	–	–	–	+	+	+	+	+	+	–	–	–	–	–	–[l]	+	+	–	–	+	+	+	+	–	–	–
H. janggokensis	–	–	+	+	+	+	+	+	+	+	–	+	+	+	+[f]	+[b]	+	+	–	+	+	+	+	+	–	–
H. jeotgali	–	–	–	–	–	–	+	+	+	+	+	–	+	+	+[f]	–[g]	–	–	+	+	+	+	+	+	–	–
H. johnsoniae	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	–	–	+	+	+	+	–	–
H. kenyensis	–	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+	+	+[m]	–	–	–	+	+	+	–	–
H. koreensis	–	–	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+	–	+	+	+	+	+	–	–
H. korlensis	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+[b]	+	–	–	+	+	+	+	+	–	–
H. kribbensis	–	–	+	+	+	+	+	+	–	–	–	–	+	+	+	+	+	–	–	+	+	+	+	–	–	–
H. lutea	–	–	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
H. magadiensis	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	–
H. maura	–	–	+	+	+	+	+	+	+	+	–	–	+	+	+	+[b]	+	+	+	+	+	+	+	+	–	–
H. meridiana	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+[b]	+	+	+	+	+	+	+	+	–	–
H. mongoliensis	–	–	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+[h]	–	–	–	+	+	+	–	–
H. muralis	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+[f]	+	–	–	–	+	+	+	+	+	–	–
H. nanhaiensis	–	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+[b]	+	–[e]	–	+	+	+	+	+	–	–
H. neptunia	–	+	+	+	+	+	+	+	+	+	+	+[i]	+	+	+	+[b]	–	–	+	+	+	+	+	+	+	+
H. nitroreducens	–	–	–	–	+	+	+	+	+	+	–	+	+	+	+	+[b]	+	+	+	+	+	+	+	+	–	–
H. olivaria	+	+	+	+	+	+	+	+[j]	+	+	+	+	+	+	+	+	+	+[h]	+	+	+	+	+	+	+	–
H. organivorans	–	–	–	+	+	+	+	+	+	+	+[c]	–	–	+	+	+	+	+	–	+	+	+	+	+	–	–
H. pacifica	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
H. pantelleriensis	–	–	+	+	+	+	+	+	+	–	–	–	+	+	+	+	+	+	–	+	+	+	+	+	+	–

Table 17.4 (continued)

Species	Salt range (%, w/v)											Temperature (°C)							pH							
	0	0.5	1	2	3	3.5	5	10	15	20	25	4–5	10	15	20	35	37	45	5	6	7	8	9	10	11	12
H. qijiaojingensis	–	+	+	+	+	+	+	+	+	+	–[k]	–	–	–	+	+	+	–[e]	–	+	+	+	+	–	–	–
H. ramblicola	–	–	+	+	+	+	+	+[j]	+	+	+[c]	+	+	+	+	+[b]	+	–[e]	+	+	+	+	+	+	–	–
H. rifensis	–	+	+	+	+	+	+	+[j]	+	+	–	–	–	–	–[l]	+[b]	+	+	+	+	+	+	+	+	–	–
H. sabkhae	–	–	–	–	–	–	+	+	+	+	+	–	–	–	–	+[g]	+	+[h]	–	+	+	+	+	–	–	–
H. saccharevitans	–	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+[b]	+	+	–	+	+	+	+	+	–	–
H. salifodinae	–	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+[b]	+	+	–	+	+	+	+	–	–	–
H. salina	–	–	–	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
H. shengliensis	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+[b]	+	–	–	–	–	+	+	–	–	–
H. sinaiensis	+	+	+	+	+	+	+	+	+	+	+[c]	–	–	–	–[l]	+	+	+[h]	–	+	+	+	+	–	–	–
H. smyrnensis	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	–[e]	–	+	+	+	+	–	–	–
H. stenophila	–	–	–	–	+	+	+	+	+	–	–	–	–	+	+	+[b]	+	–	–	+	+	+	–	–	–	–
H. stevensii	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	–	–	+	+	+	+	+	–
H. subglaciescola	–	+	+	+	+	+	+	+	+	+	–	+[d]	+	+	+[f]	+	+	+	+	+	+	+	+	+	–	–
H. subterranea	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+[b]	+	+	–	+	+	+	+	+	–	–
H. sulfidaeris	–	+	+	+	+	+	+	+	+	+	–	+[i]	+	+	+	+	–	–	+	+	+	+	+	+	–	–
H. taeanensis	–	–	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	–	–	+	+	+	+	–	–
H. titanicae	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+[b]	+	–	–	+	+	+	+	–	–	–
H. variabilis	–	–	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	–	–	+	+	+	+	–	–	–
H. ventosae	–	–	–	–	+	+	+	+	+	ND	ND	–	–	+	+	+	+	+[h]	–	+	+	+	+	+	–	–
H. venusta	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
H. vilamensis	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+[b]	+	–[e]	+	+	+	+	+	+	–	–
H. xianhensis	–	+	+	+	+	+	+	+[j]	+	+	+	–	+	+	+	+[b]	+	–[e]	–	+	+	+	+	–	–	–
H. xinjiangensis	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+	+[h]	–	+	+	+	+	–	–	–

H. zhanjiangensis	−	−	+	+	**+**	**+**	**+**	+	+	+	−	+	+	+	**+**[f]	**+**[b]	+	−	−	+	**+**	**+**	+	+	−	−
H. zincidurans	−	+	+	+	+	+	**+**	+	+	−	−	+	+	+	+	**+**	+	−	+	+	**+**	+	+	−	−	−
Halot. alkalilenta	**+**	**+**	**+**	**+**	**+**	+	+	+	+	−	−	+	+	+	+	**+**[b]	**+**	+	+	+	**+**	+	+	+	+	−
K. aurantia	−	−	−	−	−	−	+	**+**	+	−	−	−	−	−	+	+	**+**	−	−	+	**+**	**+**	−	−	−	−
K. avicenniae	+	+	+	+	+	+	**+**	+	+	+	+	−	−	+	+	**+**	+	−	+	+	**+**	**+**	+	−	−	−
K. indalinina	−	−	ND	ND	+	+	+	**+**	+	+	+	−	−	+	+	**+**	+	−	+	+	**+**	+	+	−	−	−
K. marisflavi	−	**+**	**+**	**+**	**+**	**+**	**+**	**+**	+	+	+	+	+	+	**+**[f]	**+**[b]	+	−	+	+	**+**	**+**	+	+	−	−
K. sinocarnis	−	−	−	−	−	−	+	**+**	**+**	+	+[c]	+	+	+	+	+	**+**	−	+	+	**+**	+	−	−	−	−
M. tunisiensis	−	−	+	+	+	+	+	**+**	+	+	+	−	−	+	+	+	**+**	+	+	+	**+**	+	+	+	−	−
S. halophilus	−	−	−	−	+	+	+	**+**	+	+	+	−	−	+	+	**+**	+	+	+	+	**+**	+	+	−	−	−
S. peritrichatus	+	**+**	**+**	**+**	+	+	+	+	+	+	−	+	+	+	+	**+**	**+**	−[e]	**+**	**+**	**+**	+	+	−	−	−
S. salarius	+	+	+	+	+	+	+	**+**	**+**	**+**	+	−	+	+	**+**[f]	**+**[b]	+	+	+	+	**+**	**+**	+	+	−	−
S. socius	−	+	+	+	**+**	**+**	**+**	**+**	+	+	+[c]	−	−	+	+	**+**[b]	**+**	−	+	**+**	**+**	**+**	+	−	−	−
Z. palmae	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	−	−	−	+[n]	**+**[b]	+	−	+[o]	**+**	+	+	+	+	−	−

Data from the original description of the species and from Mata et al. (2002)

A Aidingimonas, Car *Carnimonas*, Chr *Chromohalobacter*, Cob *Cobetia*, H *Halomonas*, Halot *Halotalea*, K *Kushneria*, M *Modicisalibacter*, S *Salinicola*, Z *Zymobacter*

+ growth, − not growth, *ND* no data available. Optimal values are show with + symbol in bold

[a]Available information very limited: species was described as unable to grow at 5° C and 37° C and it grows optimally at 28–30 °C

[b]Optimal temperature for growth 30 °C

[c]Also grows with 30 % (w/v) of salts

[d]Also grows at 0 °C

[e]Grows at 40–42 °C

[f]Optimal temperature for growth 25 °C

[g]Grows at 30 °C

[h]Also grows at 50 °C

[i]Also grows at −1 °C

[j]Optimal growth at 7–8 % (w/v) of salts

[k]Grows at 23 % (w/v) of salts

[l]Grows at 25 °C

[m]Also grows at 55 °C

[n]Grows at 21 °C

[o]Minimal pH for growth 3.0

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 medium (Ventosa et al. 1982)	
Yeast extract	10 g
Proteose peptone	5 g
Glucose	1 g
NaCl	81 g
$MgCl_2 \cdot 6H_2O$	7 g
$MgSO_4 \cdot 7H_2O$	9.6 g
$CaCl_2 \cdot 2H_2O$	0.36 g
KCl	2 g
$NaHCO_3$	0.06 g
NaBr	0.026 g
Distilled water q.s.	1 L

Adjust pH to 7.2 with 1 M KOH or NaOH. Add agar (20 g liter^{-1}) 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 2002 Mata et al. employed MH medium at 7.5 % salt content to maintain 104 *Halomonas* strains (including 21 type strains). For the vast majority of the strains the pH was adjusted to 7.2–7.5, except for two of the strains, *H. magadiensis* NCIMB 13595^T and *H. campisalis* ATCC 700597^T, for which the pH was adjusted to 9. All the *Halomonas* species described from 2002 up to now can be mantained in the same medium at pH 7.2–7.5, with the exception of *H. alkaliantarctica*, *H. alkaliphila*, *H. campaniensis*, *H. daqingensis*, *H. desiderata*, *H. kenyensis*, *H. korlensis*, *H. mongoliensis*, and *H. pantelleriensis* that grow optimally at pH 9.0 or 9.5.

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, 2013; Kim et al. 2010b). 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 non-halophilic species) (Garriga et al. 1998). With regards to the species *Zymobacter palmae* (non-halophile) the MY medium is recommended (Okamoto et al. 1993), which consisted of 1 % Bacto yeast extract (Difco), 2 % maltose, 0.2 % KH_2PO_4 and 0.5 % NaCl, pH 6.0. The species *Cobetia crustatorum* and *Salinicola peritrichatus* are also slightly acidophiles (with optimal growth at pH 5 or 6), but they can be mantained in MH medium at 7.5 % salts.

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.

Ecology

Habitat

According to the original description of the family *Halomonadaceae* (Franzmann et al. 1988), its members typically occurr 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 17.5*). Indeed the only exceptions to the above definition are *Carnimonas nigrificans*, *Chromohalobacter beijerinckii*, *Chr. canadensis*, *Chr. japonicus*, *Cobetia crustatorum*, *Halomonas alimentaria*, *H. cibimaris*, *H. daqingensis*, *H. desiderata*, *H. halodenitrificans*, *H. hamiltonii*, *H. jeotgali*, *H. johnsoniae*, *H. muralis*, *H. olivaria*, *H. stevensii*, *Halotalea alkalilenta*, *Kushneria sinocarnis*, *Modicisalibacter tunisiensis*, and *Zymobacter palmae*. Of these 20 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. Additionally, three species have been isolated from blood patients and from dialysis machines of a renal care centre.

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 microorganims, 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.

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.

Table 17.5

Habitats of members of the family *Halomonadaceae* from which the original strains were isolated

Species	Isolation place
Aidingimonas	
A. halophila	Salt lake located in in Xinjiang province (China)
Carnimonas	
Car. nigrificans	Raw cured-meat products (Spain)
Chromohalobacter	
Chr. beijerinckii	Salted beans and herrings
Chr. canadensis	Contaminant on medium containing 25 % NaCl
Chr. israelensis	Dead Sea
Chr. japonicus	Japanese salty food
Chr. marismortui	Dead Sea and solar salterns
Chr. nigrandesensis	Hypersaline sediment of Lake Tebenquiche (Chile)
Chr. salexigens	Solar salterns
Chr. sarecensis	Saline soil around a hypersaline lake (Bolivia)
Cobetia	
Cob. amphilecti	Internal tissue of the sponge *Amphilectus digitatus*
Cob. crustatorum	Traditional fermented seafood (Korea)
Cob. marina	Marine
Cob. litoralis	Sediment collected from the shore of the Sea of Japan, Russia
Cob. pacifica	Sediment collected from the shore of the Sea of Japan, Russia
Halomonas	
H. alimentaria	Jeotgal (traditional Korean fermented seafood)
H. alkaliantarctica	Saline lake Cape Russell in Antarctica
H. alkaliphila	Salt pool in Campania (Italy)
H. almeriensis	Solar saltern (Spain)
H. andesensis	Saline lake Laguna Colorada in Bolivia
H. anticariensis	Saline soil (Spain)
H. aquamarina	Marine
H. arcis	Hypersaline environments (China)
H. axialensis	Deep-sea hydrothermal-vent environments
H. beimenensis	Abandoned saltern
H. boliviensis	Soil around a hypersaline lake (Bolivia)
H. campaniensis	Mineral pool (Italy)
H. campisalis	Soil below a crystalline salt surface
H. caseinilytica	Saline lake on the Qinghai-Tibet Plateau (China)
H. cerina	Saline soils (Spain)
H. cibimaris	Traditional Korean fermented seafood
H. cupida	Marine
H. daqiaonensis	Littoral saltern
H. daqingensis	Oilfield soil
H. denitrificans	Saline water (Korea)
H. desiderata	Municipal sewage
H. elongata	Solar salterns
H. eurihalina	Hypersaline habitats (soils, salt ponds) and seawater
H. flava	Salt lake
H. fontilapidosi	Saline soil at Fuente de Piedra (Spain)
H. gomseomensis	Saline water (Korea)

Table 17.5 (continued)

Species	Isolation place
H. gudaonensis	Saline soil contaminated by crude oil (China)
H. halmophila	Dead Sea
H. halocynthiae	Gill tissues of the ascidian *Halocynthia aurantium*
H. halodenitrificans	Meat-curing brines
H. halophila	Saline soils
H. hamiltonii	Renal care centre
H. hydrothermalis	Deep-sea hydrothermal-vent environments
H. ilicicola	Solar saltern (Spain)
H. janggokensis	Saline water (Korea)
H. jeotgali	Traditional fermented seafood
H. johnsoniae	Renal care centre
H. kenyensis	Soda lake (Kenya)
H. koreensis	Solar saltern (Korea)
H. korlensis	Saline and alkaline soil
H. kribbensis	Solar saltern (Korea)
H. lutea	Salt lake
H. magadiensis	Littoral sediments of haloalkaline East African lakes
H. maura	Solar saltern (Morocco)
H. meridiana	Antarctic saline lakes
H. mongoliensis	Soda lake (Mongolia)
H. muralis	Biofilm covering a wall and a mural (Austria)
H. nanhaiensis	Sediment sample from the South China Sea
H. neptunia	Deep-sea hydrothermal-vent environments
H. nitroreducens	Solar saltern in Cahuil (Chile)
H. olivaria	Olive-processing effluents
H. organivorans	Saline soils (Spain)
H. pacifica	Marine
H. pantelleriensis	Hard sand from Pantelleria island (Italy)
H. qijiaojingensis	Salt lake
H. ramblicola	Hypersaline rambla (Spain)
H. rifensis	Solar saltern
H. sabkhae	Algerian sabkha
H. saccharevitans	Hypersaline environments (China)
H. salifodinae	Salt mine (China)
H. salina	Hypersaline soils, salt ponds, salt lakes, seawater
H. shengliensis	Crude-oil-contaminated saline soil (China)
H. sinaiensis	Salt lake (Egypt)
H. smyrnensis	Saltern area (Turkey)
H. stenophila	Saline soil
H. stevensii	Renal care centre
H. subglaciescola	Antarctic saline lake (Organic Lake)
H. subterranea	Hypersaline environments (China)
H. sulfidaeris	Deep-sea hydrothermal-vent environments
H. taeanensis	Solar saltern (Korea)
H. titanicae	Rusticles from the RMS Titanic
H. variabilis	North arm of the Great Salt Lake (USA)
H. ventosae	Saline soils (Spain)

Table 17.5 (continued)

Species	Isolation place
H. venusta	Marine
H. vilamensis	High-altitude Andean lakes
H. xianhensis	Saline soil contaminated by crude oil
H. xinjiangensis	Salt lake
H. zhanjiangensis	Sea urchin
H. zincidurans	Deep-sea environment
Halotalea	
Halot. alkalilenta	Alkaline olive mill wastes
Kushneria	
K. aurantia	Salty leaves of *Avicennia germinans* (Puerto Rico)
K. avicenniae	Salty leaves of *Avicennia germinans* (Puerto Rico)
K. indalinina	Solar saltern (Spain)
K. marisflavi	Marine
K. sinocarnis	Chinese traditional cured meat
Modicisalibacter	
M. tunisiensis	Oilfield-water injection sample
Salinicola	
S. halophilus	Solar saltern (Spain)
S. peritrichatus	Deep-sea sediment
S. salarius	Saline water (Korea)
S. socius	Salt mines (Russia)
Zymobacter	
Z. palmae	Palm sap in Okinawa Prefecture (Japan)

A recent study based on the metagenomic analysis of two hypersaline saltern ponds (19 % and 37 % NaCl) from Santa Pola (Spain) has demonstrated that the genera *Halomonas* and *Chromohalobacter*, which are commonly obtained in pure culture from similar salinity samples, are almost not represented in those ponds according to the metagenomic reads obtained (Ghai et al. 2011). This means that members of *Halomonadaceae* are less abundant in hypersaline environment than it was previously thought when using culture-dependent techniques.

The diversity and distribution of *Halomonas* populations in the hypersaline habitat Rambla Salada, situated in south-east Spain, have been studied using different molecular techniques (Oueriaghli et al. 2014). Denaturing gradient gel electrophoresis (DGGE) using specific primers for the 16S rRNA gene of *Halomonas* followed by a multivariate analysis of the results indicated that richness and evenness of the *Halomonas* populations were mainly influenced by the season, being the summer (the season with the highest salinity) the one with the highest value of diversity. Furthermore, canonical correspondence analysis (CCA) demonstrated that both salinity and pH significantly affected the structure of the *Halomonas* community. *Halomonas almeriensis* and two denitrifiers, *H. ilicicola* and *H. ventosae* were the predominant species. CARD-FISH showed that the percentage of *Halomonas* cells with respect to the total number of microorganisms in that habitat ranged from 4.4 % to 5.7 %. Finally, no significant differences between the types of samples studied, from either watery sediments or soil samples, were found (Oueriaghli et al. 2014). Classical cultivation methods have been also employed very recently to analyze the diversity of the halophilic bacterial community from Rambla Salada, being *Halomonas* the most abundant genus, representing 41.2 % of the 364 isolated strains (Luque, R., Béjar, V., Quesada, E., and Llamas, I., unpublished).

Pathogenicity, Clinical Relevance

Members of the *Halomonadaceae* were thought to be 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.

In 2007, Berger et al. (2007) reported an outbreak of "*Halomonas phocaeensis*" bacteraemia in a neonatal intensive care unit in Tunisia, attributed to contamination from a water bath used to warm fresh frozen plasma.

In a study performed seeking bacterial DNA signatures in unexplained deaths and critical illnesses, polymerase chain reaction of 16S RNA gene from culture-negative materials identified

an unspeciated *Halomonas* organism in one patient's blood (Nikkari et al. 2002). The phylogenetic analysis of the sequence data determined that this organism was part of the *H. variabilis–H. boliviensis–H. neptunia* cluster, but in an apparently distinct position (Stevens et al. 2009). Additionally, Stevens et al. (2009) isolated a total of 14 strains recognized as human pathogens causing infection and contamination in a dyalisis center. Exhaustive taxonomic characterization of these isolates led to the description of three new *Halomonas* species, *H. stevensii*, *H. hamiltonii* and *H. johnsoniae* (Kim et al. 2010a).

More recently, a patient developed a bacteremia caused by *Halomonas johnsoniae* (previously reported only as dialysis unit environmental contaminants) (Stevens et al. 2013). The medical community is alerted to the pathogenic potential of the genus in humans, but also in algae and animals (Kim et al. 2013).

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 (Ventosa et al. 1998; Margesin and Schinner 2001; Mellado and Ventosa 2003; Quillaguamán et al. 2010; Oren 2010). Some of the most promising applications of members of *Halomonadaceae* include the production of compatible solutes and polyhydroxyalkanoates 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) and therefore could be useful for industrial and clinical purposes. 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). Very recently, an *ectD* (ectoine hydroxylase) deficient *H. elongata* mutant has been proved to produce ectoine from a variety of sugars derived from lignocellulosic biomass and thus has tremendous potential as a host for producing useful compounds from biomass resources (Tanimura et al. 2013). Concerning other halomonads, a process comprising two-step fed-batch cultivation has been investigated for the production of ectoine and hydroxyectoine using *Halomonas boliviensis* DSM 15516^T (Guzmán et al. 2009; Van-Thuoc et al. 2010a, b). The first cultivation was performed under optimal conditions for cell growth and resulted in a high cell mass concentration. During the second cultivation at higher salt concentration, accumulation of ectoines increased while cell mass decreased. Maximum productivity of total ectoines reached was 10 g l^{-1} d^{-1} at 18.5 % NaCl, which is among the highest reported so far. The accumulated ectoines were released by subjecting the cells to hypoosmotic shock and the cells were further recycled for the production process (Van-Thuoc et al. 2010a). A similar method in two stages has been employed for efficient ectoine production using *Halomonas salina* DSM 5928^T (Zhang et al. 2009; Lang et al. 2011). An ectoine absorption defective *H. salina* DSM 5928^T mutant (lacking the ectoine-specific transporter TeaABC), which compromised the negative feedback regulation of ectoine synthesis, was constructed to improve the efficiency of ectoine production up to 9.93 g l^{-1} d^{-1} (Xu and Zhang 2012). Apart from *Halomonas* species, only the species *Chromohalobacter salexigens* has been optimized for ectoine and hydroxyectoine production (Fallet et al. 2010; Rodríguez-Moya et al. 2013). Ectoine and its derivative hydroxyectoine are used in the cosmetic industry because of their moisturizing properties. The potential use of the *Chromohalobacter salexigens* and *Halomonas elongata ect* 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.

Poly-β-hydroxyalkanoate (PHA) is a polymer accumulated by many prokaryotes, that can be used for the production of biodegradable plastics ("biological polyesters") with properties resembling that of polypropylene. Several bacterial species of the family *Halomonadaceae* accumulate PHAs: *Cobetia amphilecti*, *Cob. crustatorum*, *Cob. litoralis*, *Cob. marina*, *Cob. pacifica*, *Chromohalobacter salexigens*, *Chr. sarecensis*, *Halomonas almeriensis*, *H. andesensis*, *H. anticariensis*, *H. alkaliphila*, *H. aquamarina*, *H. boliviensis*, *H. campaniensis*, *H. campisalis*, *H. caseinilytica*, *H. cerina*, *H. cibimaris*, *H. cupida*, *H. daqiaonensis*, *H. daqingensis*, *H. desiderata*, *H. elongata*, *H. eurihalina*, *H. fontilapidosi*, *H. halmophila*, *H. halodenitrificans*, *H. halophila*, *H. hamiltonii*, *H. jeotgali*, *H. johnsoniae*, *H. magadiensis*, *H. maura*, *H. meridiana*, *H. nitroreducens*, *H. olivaria*, *H. pacifica*, *H. pantelleriensis*, *H. ramblicola*, *H. rifensis*, *H. salina*, *H. sinaiensis*, *H. stevensii*, *H. subglaciescola*, *H. variabilis*, *H. ventosae*, *H. venusta*, *H. zhanjiangensis*, *Kushneria marisflavi*, *Salinicola salarius*, and *S. socius*. From those, the strain *Halomonas boliviensis* LC1^T reached PHA yields and volumetric productivities close to the highest reported so far, accumulating the compound to up to 88 % of its dry weight (Quillaguamán et al. 2006, 2007). Furthermore, *H. boliviensis* and other *Halomonas* species are able to co-produce PHA and osmolytes, i.e., ectoines and hydroxyectoine, in one process (Quillaguamán et al. 2010).

Strains from several species of *Halomonas* are of interest as producers of exopolysaccharides (EPS). These include *H. alkaliantarctica*, *H. alkaliphila*, *H. almeriensis*, *H. anticariensis*, *H. caseinilytica*, *H. cerina*, *H. daqiaonensis*, *H. daqingensis*, *H. eurihalina*, *H. fontilapidosi*, *H. halophila*,

H. maura, *H. nitroreducens*, *H. olivaria*, *H. ramblicola*, *H. rifensis*, *H. sabkhae*, *H. salina*, *H. sinaiensis*, *H. smyrnensis*, *H. stenophila*, and *H. ventosae*. EPS production has also been described in the species *Cobetia crustatorum*. The nutritional and environmental factors influencing the production of EPS have been thoroughly investigated (Quesada et al. 1993; Béjar et al. 1996; Béjar et al. 1998; Bouchotroch et al. 1999; Bouchotroch et al. 2000; Martinez-Checa et al. 2002; 2007; Arias et al. 2003; Mata et al. 2006; Llamas et al. 2012). 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 polymers 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, biodetoxification of heavy-metal polluted environments, crude oil emulsification, viscosity enhancement in foods, protection against oxidative stress, among others (Quesada et al. 2004; Raveendran et al. 2013). Recently, it has been demonstrated that the EPS produced by *H. stenophila* strain B100 selectively induces apoptosis in human T leukaemia cells (Ruiz-Ruiz et al. 2011), suggesting that the search for new antineoplastic drugs should include the screening of other bacterial EPSs, particularly those isolated from halophiles. The properties of some of these EPSs such as mauran (from *H. maura*), H28 and V2-7 (from *H. eurihalina*) and those from strains Al12^T and Al16 (*H. ventosae*), from strains FP35^T and FP36 (*H. anticariensis*) and from strain M8^T (*H. almeriensis*) have been studied in detail highlighting in each case their biotechnological potentials (Pérez-Fernandez et al. 2000; Martinez-Checa et al. 2002, 2007; Arias et al. 2003; Mata et al. 2006; Llamas et al. 2012). Besides, genes involved in the biosynthesis of the exopolysaccharide mauran produced by *H. maura* have been identified, which form part of a gene cluster *epsABCDJ* (Arco et al. 2005). Furthermore, production of EPS in *H. anticariensis* was found to be influenced by a two-component regulatory system, GacS/GacA (Tahrioui et al. 2013b).

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 (Mellado et al. 2004). Sanchez-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. Another study with the aims to isolate and characterize the cultivable community of hydrolase producers inhabiting heavy-metal-contaminated soils in extreme conditions from the Atacama Desert showed that only 1 out of 25 isolates was closely related to the genus *Halomonas*, in particular to *H. organivorans*, possessing protease, lipase, amylase, DNase, and pullulanase activities (Moreno et al. 2012). Concerning the characterization of extracellular enzymes, an α-amylase produced by *Halomonas meridiana* has been studied at the biochemical and molecular level (Coronado et al. 2000a, b). 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 α-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 α-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). More recently, a novel endoglucanase (Cel8H) from *Halomonas* sp. strain S66-4 has been biochemically characterized, cloned, expressed in *E. coli* and purified. The purified recombinant enzyme had an optimal activity of 4.9 U/mg at pH 5 and 45° C toward the substrate carboxymethylcellulose. It exhibited extraordinary properties which differed from endoglucanases reported previously at the point of high salt tolerance above 5 M, simultaneously with high stability at pH 4–12 and 40–60° C (Huang et al. 2010). Moreover, an α-glucosidase (HaG) with a estimated molecular mass of 58 kDa was isolated from *Halomonas* sp. strain H11 (Ojima et al. 2012a). HaG showed high hydrolytic activities toward maltose, sucrose, and p-nitrophenyl-α-D-glucoside but to almost no other disaccharides or malto-oligosaccharides higher than trisaccharides. HaG showed optimum activity to maltose at 30 °C and pH 6.5. Monovalent cations, such as K^+, Rb^+, Cs^+, and NH_4^+ increased the enzymatic activity to 2- to 9-fold of the original activity. This enzyme was tested to be useful for efficient synthesis of α-D-glucosylglycerol and α-glucosylated 6-gingerol (Ojima et al. 2012a, b).

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, *p*-aminosalicylate,

Table 17.6

Organic compounds and chemicals that can be degraded or transformed by *Halomonadaceae* strains under hypersaline conditions

Compound	Organism	NaCl (%, w/v)	References
Benzoate	*Cobetia marina*	3–20	Rosenberg (1983)
Aminomethane sulfonate	*Chromohalobacter marismortui* VH1	5–15	Ternan and McMullan (2002)
Organophosphonates	*Chromohalobacter marismortui* VH1	5–15	Hayes et al. (2000)
Phenol	*Halomonas campisalis* A4^T	0–15[a]	Alva and Peyton (2003)
p-aminosalicylate	*Halomonas organivorans* G-16.1^T	10	García et al. (2004)
Benzoate	*Halomonas organivorans* G-16.1^T	10	García et al. (2004); Moreno et al. (2011)
Cinnamate	*Halomonas organivorans* G-16.1^T	10	García et al. (2004)
p-coumarate	*Halomonas organivorans* G-16.1^T	10	García et al. (2004)
Ferulate	*Halomonas organivorans* G-16.1^T	10	García et al. (2004)
p-hydroxybenzoate	*Halomonas organivorans* G-16.1^T	10	García et al. (2004)
Phenol	*Halomonas organivorans* G-16.1^T	10	García et al. (2004); Moreno et al. (2011); Bonfá et al. (2013)
Phenylacetate	*Halomonas organivorans* G-16.1^T	10	García et al. (2004)
Phenylpropionate	*Halomonas organivorans* G-16.1^T	10	García et al. (2004)
Salicylate	*Halomonas organivorans* G-16.1^T	10	García et al. (2004)
Crude-oil	*Halomonas organivorans* PG-31	2.8	Hassanshahian et al. (2012)
Phenol	*Halomonas venusta*	8	Muñoz et al. (2001)
Phenol	*Halomonas* sp.	1–14	Hinteregger and Streichsbier (1997)
3-chlorobenzoate	*Halomonas* sp. EF43	1[c]	Kleinsteuber et al. (2001)
2,4-dichlorophenoxyacetate	*Halomonas* sp. EF43	1[c]	Kleinsteuber et al. (2001)
2,4-dichlorophenoxyacetate	*Halomonas* sp. I18	3.5–5.8[b]	Maltseva et al. (1996)
Formaldehyde	*Halomonas* sp. MA-C	0–20	Azachi et al. (1995)
Selenate	*Halomonas* sp. MPD-51 (and three more isolates)	0–32.5	De Souza et al. (2001)
Uranium compounds	*Halomonas* sp. WIPP1A	20	Francis et al. (2000)
Phenol	*Modicisalibacter tunisiensis* HU	10	Bonfá et al. (2013)

[a]At pH 8–11
[b]At pH 7.5–9.8
[c]At pH 10

organophosphonates, benzoate, 3-chlorobenzoate, cinnamate, *p*-coumarate, crude-oil, 2,4-dichlorophenoxyacetate, ferulate, formaldehyde, *p*-hydroxybenzoate, phenol, phenylacetate, phenylpropionate, salicylate, selenate and uranium compounds) are shown in *Table 17.6*. 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). Kleinsteuber 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. In 2004, García et al. (2004) described a novel species, *Halomonas organivorans*, which was 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. Later, 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 (García et al. 2005). Recently, *cat* and *ben* genes, involved in phenol and benzoate degradation, respectively, from *H. organivorans*, have been cloned, characterized and analyzed (Moreno et al. 2011), providing an ideal model system to investigate the potential use of this group of extremophiles in the decontamination of saline environments. Other recent publications also show the ability of *H. organivorans* to degradate contaminants, such as crude-oil (Hassanshahian et al. 2012) and phenol (Bonfá et al. 2013). 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. *Cobetia crustatorum*, *Halomonas alimentaria*, *H. cibimaris*, and *H. jeotgali* were isolated from the traditional Korean fermented seafood (Kim et al. 2010b; Yoon et al. 2002; Jeong et al. 2013; Kim et al. 2011). 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 *Km* for pyruvate of all four known bacterial PDCs.

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18 The Family *Idiomarinaceae*

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Abstract
Idiomarinaceae, a family within the order *Alteromonadales* of the class *Gammaproteobacteria*. This family embraces the genera *Aliidiomarina* and *Idiomarina*. Initially *Idiomarina* was the only genus of this family; recently another genus, *Aliidiomarina*, has been included. *Aliidiomarina* encompasses one species *Aliidiomarina taiwanensis*, while the genus *Idiomarina* comprises at this time twenty species. The members of this family are Gram-negative, mesophilic, and, with the exception of *Idiomarina xiamenensis*, require NaCl for growth. Members of this family have been isolated from saline habitats with a wide range of salinities, such as coastal and oceanic waters, solar salterns, submarine hydrothermal fluids, and inland hypersaline wetlands.

Taxonomy, Historical and Current

Short Description of the Family

The family *Idiomarinaceae* was proposed by Ivanova et al. (2004) based on the species *Idiomarina abyssalis* (Ivanova et al. 2000), *I. zobellii* (Ivanova et al. 2000), *I. baltica* (Brettar et al. 2003), and *I. loihiensis* (Donachie et al. 2003). The species of the genus *Idiomarina* are slightly to moderately halophilic, and the organisms have specific signature nucleotide positions. However, Jean et al. (2006) emended the family *Idiomarinaceae* and proposed the genus *Pseudidiomarina*, for the species *P. taiwanensis*. Later, the genus *Idiomarina* was split into two genera, to include several species in the genus *Pseudidiomarina* (Jean et al. 2009). The genus *Pseudidiomarina* was proposed to include *Idiomarina*-like organisms without motility or flagella, unable to grow at pHs below 6, without DNAse activity and the substitution of one nucleotide in the 16S rRNA gene, that were considered important to distinguish the species of both genera. The species of the genera *Idiomarina* and *Pseudidiomarina* as defined by Jean et al. (2006) form two rRNA groups with specific signature nucleotides that can be used as a distinctive feature (Taborda et al. 2009). However, as more species were described, it became apparent that there were no distinguishing phenotypic characteristics that could be used to differentiate the two genera; therefore, the organisms classified in the genus *Pseudidiomarina* have been reclassified in the genus *Idiomarina* (Taborda et al. 2009, 2010a, b).

More recently, a new species of a novel genus of the family *Idiomarinaceae* named *Aliidiomarina taiwanensis* was proposed by Huang et al. (2012), but again it is difficult, if not impossible to distinguish this new organism from those of the genus *Idiomarina*.

Idiomarinaceae Ivanova et al. 2004; Emend Jean et al. 2006

Idiomarinaceae (I.di.o.ma.ri.na' ce.ae. N.L. fem. n. *Idiomarina*, type genus of the family; suff. *-aceae*, ending to denote a family; N.L. fem. pl. n. *Idiomarinaceae*, the *Idiomarina* family).

The members of the family *Idiomarinaceae* of the order *Alteromonadales* stain Gram-negative and forms rod-shaped cells. Do not form endospores. Strictly aerobic, one species grows under anaerobic conditions and chemoorganotrophic, non-fermentative. Ubiquinone-8 (U-8) is the major respiratory lipoquinone. Iso-branched (saturated and monounsaturated) fatty acids, straight-chain (saturated and monounsaturated) fatty acids, and iso- or straight-chain 3-OH fatty acids are present. The 16S rRNA sequences have the following signature nucleotide positions: 143 (C or A or U), 662 (A), 682 (A or U), 830 (U), and 856 (A) (Huang et al. 2012; Ivanova et al. 2004; Jean et al. 2006; Taborda et al. 2009; Wang et al. 2011; Zhang et al. 2012). All organisms of this family, with the exception of *Idiomarina xiamenensis* (Wang et al. 2011), require NaCl for growth and have been isolated from saline environments. The type genus of the family is *Idiomarina* (Ivanova et al. 2000). *Aliidiomarina* is an additional genus of the family (Huang et al. 2012).

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Phylogenetic Structure of the Family and Its Genera

These species of the genus *Idiomarina* appear to form four 16S RNA groups (Huang et al. 2012; Jean et al. 2006; Taborda et al. 2009; Wang et al. 2011; Zhang et al. 2012); 16S rRNA group 1 corresponds to the species *I. abyssalis* (Ivanova et al. 2000), *I. baltica* (Brettar et al. 2003), *I. fontislapidosi* (Martínez-Cánovas et al. 2004b), *I. loihiensis* (Donachie et al. 2003), *I. ramblicola* (Martínez-Cánovas et al. 2004b), *I. seosinensis* (Choi and Cho 2005), and *I. zobellii* (Ivanova et al. 2000). 16S RNA group 2 corresponds to the species that include *I. insulisalsae* (Taborda et al. 2009), *I. aquimaris* (Chen et al. 2012), and those species formerly classified in the genus *Pseudidiomarina*, namely, *I. tainanensis* (Jean et al. 2009), *I. maritima* (Wu et al. 2009), *I. marina* (Jean et al. 2009), *I. donghaiensis* (Wu et al. 2009), *I. taiwanensis* (Jean et al. 2006), *I. sediminum* (Hu and Li 2007), *I. homiensis* (Kwon et al. 2006), *I. salinarum* (Yoon et al. 2007), and *I. aestuarii* (Park et al. 2010). One species, *I. xiamenensis*, forms 16S RNA group 3 by itself, while the species *I. maris* represents 16S RNA group 4 (Wang et al. 2011; Zhang et al. 2012). The 16S RNA groups are supported by signature nucleotides (Jean et al. 2006; Taborda et al. 2009; Zhang et al. 2012; Wang et al. 2011). Despite the 16S RNA distinctiveness, no phenotypic (including chemotaxonomic) characteristics support a classification other than that all species belong to the genus *Idiomarina*. Recently, Huang et al. (2012) classified a new species closely related to the genus *Idiomarina*, as *Aliidiomarina taiwanen*sis. This organism is, based on 16S rRNA gene sequence analysis, most closely related to *I. maris* (Zhang et al. 2012), but the proposal of a new genus was based on very minor differential phenotypic characteristics. The authors based their proposal on the sum of two major fatty acids, iso-$C_{17:0}$ and summed feature 9 (most likely iso-$C_{17:1}\omega9c$), which reached 52.4 % of the total fatty acids, in contrast to the sum of these two fatty acids in the species of the genus *Idiomarina* that reach between 17.8 and 36.7 % of the total fatty acids. The two genera could also be distinguished because *A. taiwanensis* possessed iso-$C_{15:0}$ 3-OH (2.2 % of the total), while the species of *Idiomarina* did not. Had the authors used the sum, for example, of iso-$C_{15:0}$ and iso-$C_{17:0}$ the distinctive characteristic for proposing the genus *Aliidiomarina* would be insignificant. The publication of the description of *Idiomarina maris* (Zhang et al. 2012) was almost simultaneous with the proposal of *Aliidiomarina taiw*anensis. Therefore, the characteristics of the two organisms could not be compared. Our 16S rRNA gene analysis using the neighbor-joining indicates that both species are very closely related (94 % similarity) (*Fig. 18.1*). However, the sum of the iso-$C_{17:0}$ and iso-$C_{17:1}\omega9c$ fatty acids is 31.8 %, which is within the range of the other species considered by Huang et al. (2012) to belong to the genus *Idiomarina*. Moreover, the species *I. maris* also possess iso-$C_{15:0}$ 3-OH (6.2 % of the total).

The 16S rRNA signature nucleotides at positions 143 (A or C or U), 662 (A), 682 (A or U), 830 (U), and 856 (A) are considered to be indicative of the family *Idiomarinaceae* (Huang et al. 2012; Ivanova et al. 2004; Jean et al. 2006; Taborda et al. 2009; Wang et al. 2011; Zhang et al. 2012). *A. taiwanensis* also has these signature nucleotides, but possesses other nucleotides considered specific for the new genus (Huang et al. 2012). However, each 16S rRNA group of the other four groups that make up the species of the genus *Idiomarina* possesses specific signature nucleotides. The question remains whether *A. taiwanensis* should be considered a separate genus or is a member of the genus *Idiomarina*, particularly when taking into account the phenotypic characteristics of *I. maris*, which also forms a separate 16S RNA group.

Phenotypic Analyses

The main features of members of the family *Idiomarinaceae* are listed in *Table 18.1*. The main features of members of the genus *Idiomarina* are listed in *Table 18.2* and of *Aliidiomarina taiwanensis* in *Table 18.3*.

Idiomarina Ivanova et al. 2000; Emend. Taborda et al. 2009; Emend. Taborda et al. 2010a, b

Idiomarina [I.di.o.ma.ri' na. Gr. adj. *idios*, pertaining to oneself, private, personal; L. fem. adj. *marina*, of the sea, marine; N.L. fem. n. *Idiomarina*, pertaining to the peculiar, true marine nature of microorganisms from the ocean (seawater)].

Idiomarina forms rod-shaped cells and most strains are motile by means of one polar flagellum. Catalase (*I. sediminum* appears to be catalase negative) and oxidase positive. The organisms are mesophilic. The species of the genus have a salt range between about 0 % and 25 %; optimum growth in salt-containing medium is variable and ranges from about 1 % to 10 %. The optimum pH is around neutral. Phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylglycerol are the major polar lipids. The fatty acids are primarily iso-$C_{15:0}$, iso-$C_{17:0}$, and iso-$C_{17:1}\omega9c$. The assimilation of single carbon and energy has not been generally examined or given negative results for the carbon sources examined (except *I. abyssalis* and *I. zobellii* which appear to use D-arginine and L-tyrosine; *I. aquimaris* utilizes mannose and maltose on the API 20 NE) because the organisms grow very poorly on minimal media supplemented with single carbon sources. Acid is generally not produced from carbohydrates using the API 50CH or classical methods. Therefore, carbon metabolism is based on the results of the Biolog GN2 with variable results for the same organism. The DNA G+C content varies between 45 and 56.4 mol%. The species of this genus have been isolated from saline environments. The type species is *Idiomarina abyssalis*. The type strain is KMM 227^T (=ATCC BAA-312^T) (Ivanova et al. 2000).

At present this genus comprises twenty species, namely, *I. abyssalis* (Ivanova et al. 2000), *I. aestuarii* (Park et al. 2010; Wang et al. 2011), *I. aquimaris* (Chen et al. 2012), *I. baltica* (Brettar et al. 2003), *I. donghaiensis* (Wu et al. 2009;

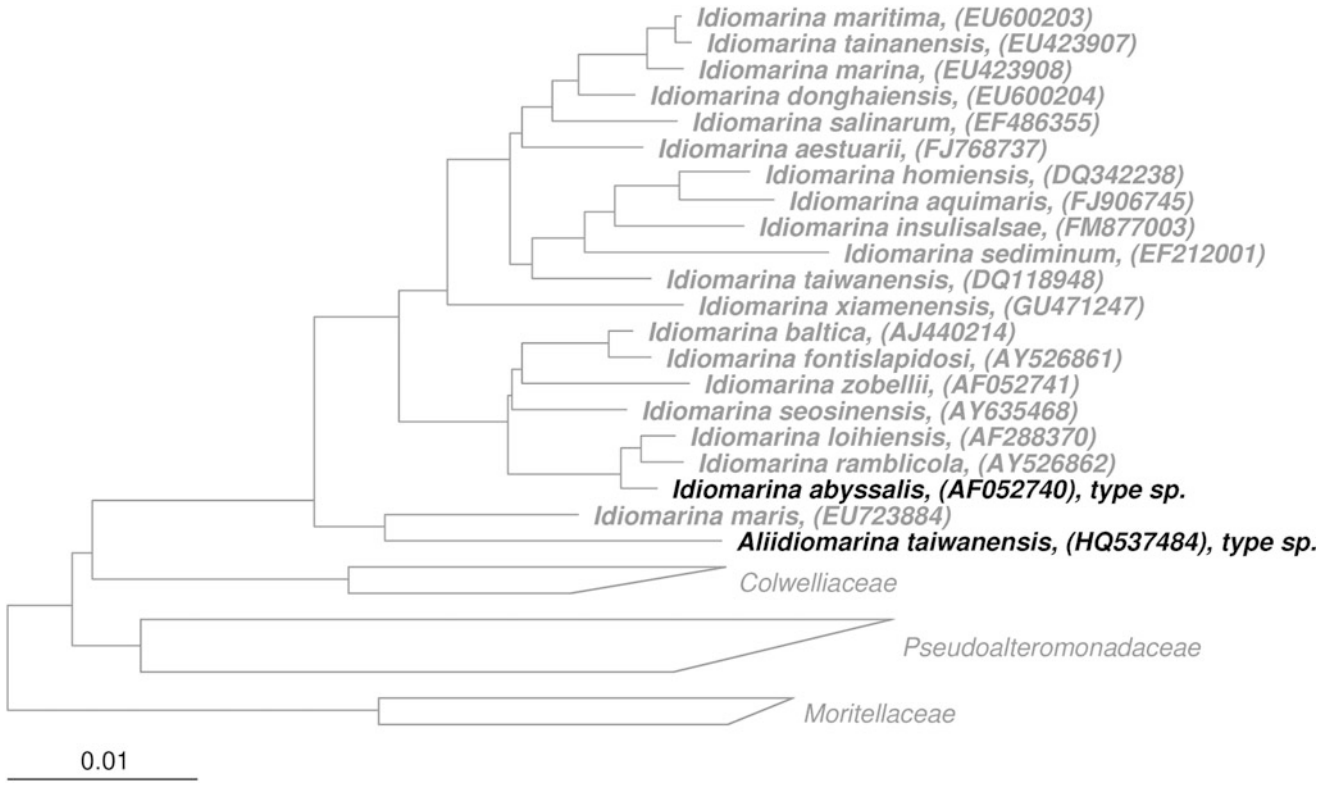

Fig. 18.1

Phylogenetic reconstruction of the family *Idiomarinaceae* based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes-Cantor correction. The sequence dataset and alignment were used according to the All-Species Living Tree Project (LTP) database (Yarza et al., 2010; http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high quality type strain sequences proportionally distributed among the different bacterial and archaeal phyla. In addition, a 40% maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. Scale bar indicates estimated sequence divergence

Taborda et al. 2009), *I. fontislapidosi* (Martínez-Cánovas et al. 2004b), *I. homiensis* (Kwon et al. 2006), *I. insulisalsae* (Taborda et al. 2009), *I. loihiensis* (Donachie et al. 2003), *I. marina* (Jean et al. 2009; Taborda et al. 2009), *I. maris* (Zhang et al. 2012), *I. maritima* (Wu et al. 2009; Taborda et al. 2009), *I. ramblicola* (Martínez-Cánovas et al. 2004b), *I. salinarum* (Yoon et al. 2007), *I. sediminum* (Hu and Li 2007; Taborda et al. 2009), *I. seosinensis* (Choi and Cho 2005), *I. tainanensis* (Jean et al. 2009; Taborda et al. 2009), *I. taiwanensis* (Jean et al. 2006; Taborda et al. 2009), *I. xiamenensis* (Wang et al. 2011), and *I. zobellii* (Ivanova et al. 2000).

Aliidiomarina

Aliidiomarina (A.li.i.di.o.ma.ri' na. L. pronoun. *alius*, other, another; N.L. fem. n. *Idiomarina*, a name of a bacterial genus; N.L. fem. n. *Aliidiomarina*, the other *Idiomarina*).

Aliidiomarina forms straight or slightly curved rods that are motile by means of a single polar flagellum. Catalase and oxidase positive. Mesophilic and require NaCl for growth. Phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylglycerol are the major polar lipids. The major fatty acids are iso-$C_{15:0}$, iso-$C_{17:0}$, and iso-$C_{17:1}\omega 9c$. The DNA G+C content of the type strain is 51.5 mol %. The species of this genus have been isolated from saline environments. The carbon and energy sources of the sole species of the genus is not known, since the attempt to grow the organism in a minimal medium supplemented with several individual carbohydrates proved to be negative for all those examined; the organism produces acid from several carbohydrates with the API 50CH and metabolizes several organic compounds on the Biolog GN2. The type species is *Aliidiomarina taiwanensis*. The type strain AITI^T (=JCM 16052^T = BCRC 80035^T) was isolated from shallow coastal seawater of Bitou Harbour, New Taipei City, Taiwan (Huang et al. 2012).

Isolation, Enrichment, and Maintenance Procedures

The species of the genera *Idiomarina* and *Aliidiomarina* have all been isolated from saline environments, many by different

Table 18.1
Phenotypic and chemotaxonomic characteristics of genera of *Idiomarinaceae*

	Idiomarina[a,b,c,d,e]	*Aliidiomarina*[f]
Morphology	Rods	Rods
Gram-stain	Negative	Negative
Pigmentation	Nonpigmented or slightly yellowish colored	Light brown
Motility	Variable	+
Metabolism	Aerobic	Aerobic
Nitrate reduction	Variable	+
Presence of		
Oxidase	+	+
Catalase	Variable	+
Temperature for growth (°C)		
Range	4–46	4–45
Optimum	20–40	30–40
pH for growth		
Range	5.0–11	7.0–9.0
Optimum	7.0–9.0	8.0
Requires NaCl for growth	+ (negative for *I. xiamenensis*)	+
NaCl concentration for growth (%)		
Range	0.1–25 (*I. xiamenensis* grows without additional NaCl)	0.5–10
Optimum	1–10	1.5–5.0
Major fatty acids	Iso-$C_{15:0}$, iso-$C_{17:0}$, iso-$C_{17:1}\omega 9c$	Iso-$C_{15:0}$, iso-$C_{17:0}$, iso-$C_{17:1}\omega 9c$
Major polar lipids[g]	DPG, PG, PE	DPG, PG, PE
Major respiratory lipoquinone[h]	U-8	U-8
G+C content (mol %)	45.0–56.4	51.5

[a]Chen et al. (2012); [b]Ivanova et al. (2000); [c]Taborda et al. (2009); [d]Wang et al. (2011); [e]Zhang et al. (2012); [f]Huang et al. (2012); [g]*DGP* diphosphatidylglycerol, *PG* phosphatidylglycerol, *PE* phosphatidylethanolamine. [h]*U* ubiquinone
Symbols: +, positive; −, negative

methods and with different media, making it difficult to produce a general method to isolate new strains of these organisms. It is, therefore, necessary to describe briefly the method used for the isolation of each organism.

Idiomarina abyssalis and *Idiomarina zobellii* were isolated from water samples collected from a depth of 4,000 ± 5,000 m (salinity, 34 ‰; temperature, 2 °C) in the northwestern area of the Pacific Ocean (Ivanova et al. 2000). Isolation of the strains was achieved at atmospheric pressure by plating 0.1 mL seawater on oligotrophic agar prepared with full-strength seawater, amended with 0.3 % (w/v) Bacto peptone and 1.5 % (w/v) Bacto agar at 25 °C. The strains were subsequently purified on medium B, containing 0.2 % (w/v) Bacto peptone, 0.2 % (w/v) casein hydrolysate (Merck), 0.2 % (w/v) Bacto yeast extract, 0.1 % (w/v) glucose, 0.002 % (w/v) KH_2PO_4, 0.005 % (w/v) $MgSO_4 \cdot 7H_2O$, 50 % (v/v) natural seawater, and 50 % deionized water. The strains are maintained and routinely grown on medium B.

Idiomarina baltica was isolated during a cruise of the RV *Poseidon* from the oxic part of the water column (30 m, 6 °C, 8 ‰ NaCl) of a basin in the Central Baltic Sea (Brettar et al. 2003). Medium for isolation was ZoBell agar (5 g Bacto peptone, 1 g yeast extract, 15 g Bacto agar, 250 mL aged seawater, 750 mL deionized water, pH 7.2) (Oppenheimer and Zobell 1952) at 20 °C. *Idiomarina baltica* grows well on ZoBell agar, in marine broth (MB, Difco), or on marine agar (Difco).

Idiomarina loihiensis was recovered from hydrothermal fluids (163 °C) venting into seawater (4 °C) at a depth of 1,296 m during a cruise of the RV *Ka'imikai-o-Kanaloa* on the Lō'ihi Seamount, Hawai'i (Donachie et al. 2003). The water sample was centrifuged and spread plates were prepared with 200 µl of the pellet on marine agar (MA, Difco) and incubated at 30 °C. Single colony was transferred to MA for purification. *Idiomarina loihiensis* is routinely cultured on MA or marine broth (MB, Difco) at 30 °C.

Idiomarina fontislapidosi and *Idiomarina ramblicola* were found during two different samplings made in 1998 (Martínez-Cánovas et al. 2004a). *Idiomarina fontislapidosi* was isolated from a sample of soil taken from the temporally emerged banks of a hypersaline pool in the

Table 18.2

Comparison of selected characteristics of members of the genera of *Idiomarina*.

	I. abyssalis[a] KMM 227^T	*I. aestuarii*[b,c] KYW314^T	*I. aquimaris*[d] SW15^T	*I. baltica*[e] OS145^T	*I. donghaiensis*[f,g] 908033^T	*I. fontislapidosi*[h] F23^T
All of the organisms are oxidase, catalase, and DNAse positive. All of the organisms are urease negative. None of the strains hydrolyze starch						
Morphology	Rods	Rods	Rods	Slightly curved rods	Slightly curved rods	Slightly curved rods
Cell size (μm)	0.7–0.9 × 1.0–1.8	0.3 × 0.7–1.4	0.7–0.9 × 1.2–2.0	0.4–0.7 × 0.7–1.6	0.4–0.6 × 1.0–1.4	0.75 × 3.0–4.0
Pigmentation	Light yellowish	Nonpigmented	Light yellow	Nonpigmented to slightly yellowish	Nonpigmented	Cream
Colony morphology	Uniformly round and opaque	Circular, convex, smooth, opaque with entire margins	Circular and convex with entire edges	Circular and smooth	Circular and smooth	Round, convex, and mucoid
Motility	+ (single polar flagellum)	–	+ (single polar flagellum)	+ (single polar flagellum)	+ (peritrichous flagella)	+ (single polar flagellum)
Temperature for growth (°C)						
Range	4–30	10–37	10–45	8–46	15–45	4–45
Optimum	20–22[a] 20–25[d]	30	20–30	30–40	37	32
pH for growth						
Range	5.5–9.5[a] 6.0–9.0[d]	5.0–10.0	6.0–11.0	nd	6.5–10.0	5–10
Optimum	7.5–8.0[a] 7.0–8.0[d]	7.0	7.0–8.0	nd	8.0–9.0	7.0–8.0
NaCl concentration for growth (%)						
Range	0.6–15[a] 0.5–15[d]	1–10	0.5–15	0.8–10	0.5–10	0.5–25
Optimum	3–5	3	3–4	3–6	3	3–5
Nitrate reduction	+	–	+	–	–	–
Selenite reduction	nd	nd	nd	nd	–	+
Production of						
Poly-β-hydroxybutyrate	–	nd	–	nd	nd	–
Exopolysaccharide	–	nd	nd	–	nd	+
Indole	–	–	–	–	–	–
H_2S	nd	–	–	+	+	+
Citrate utilization	nd	–	–	nd	nd	nd
Methyl red	nd	nd	nd	–	–	–
Voges-Proskauer	nd	+	–	nd	nd	–
Presence of						
Lecithinase	nd	nd	nd	nd	+	–
Chitinase	–	nd	–	–	nd	nd
β-*galactosidase*	–	–	–	–	–	+
Arginine dihydrolase	–	–	–	–	–	nd
Lysine decarboxylase	nd	–	–	nd	–	nd
Ornithine decarboxylase	nd	–	–	nd	–	nd

Table 18.2 (continued)

	I. abyssalis[a] KMM 227^T	*I. aestuarii*[b,c] KYW314^T	*I. aquimaris*[d] SW15^T	*I. baltica*[e] OS145^T	*I. donghaiensis*[f,g] 908033^T	*I. fontislapidosi*[h] F23^T
Tryptophan deaminase	nd	−	+	nd	nd	nd
Phenylalanine deaminase	nd	nd	nd	nd	nd	−
Enzymes (Api Zym)						
Alkaline phosphatase	+	+	+	+	+	nd
Esterase (C 4)	+	−	+	+	+	nd
Esterase lipase (C 8)	+	−	+	+	+	nd
Lipase (C14)	−	−	+	−	+	−
Leucine arylamidase	+	−	+	+	+	nd
Valine arylamidase	−[o],+[d]	−	+	+	+	+
Cystine arylamidase	−[o],+[d]	−	+	+	+	−
Trypsin	−	−	+	−	+	−
α-chymotrypsin	−[o],+[d]	+	+	+	+	+
Acid phosphatase	+	−	+	+	+	nd
Naphthol-AS-BI-phosphohydrolase	+	−	+	+	+	+
α-galactosidase	−[o],+[d]	−	−	−	−	nd
β-galactosidase	−	−	−	−	−	nd
β-glucuronidase	−	−	−	−	−	nd
α-glucosidase	−	−	−	−	−	nd
β-glucosidase	−	−	−	+[e,g],−[o]	−	w
N-acetyl-β-glucosaminidase	−	−	−	−	−	nd
α-mannosidase	−	−	−	−	−	nd
α-fucosidase	−	−	−	−	−	nd
Hydrolysis of						
Gelatin	+[a,d,m],−[i,o]	−[b],+[c]	+	+	+	+
Starch	−	−	−	−	−	−
Esculin	−	+	+	+[e,o],−[g]	−	+
Casein	+	+	−	+	+	+
Agar	−	nd	nd	nd	nd	nd
Tyrosine	+	+	nd	nd	+	−
Alginate	nd	nd	+	nd	nd	nd
Tween 20	+	+	+	nd	+	+
Tween 40	+	nd	−	nd	nd	nd
Tween 80	−[i,m],+[a,d]	+	+	+	+	+
CM-cellulose	nd	−	−	nd	nd	nd
Xylan	nd	−	nd	nd	nd	nd
Egg yolk	nd	−	nd	nd	nd	nd
Biolog GP2 MicroPlate						
α-cyclodextrin	+[a],−[d]	−	+	−	nd	−
Dextrin	+[a],−[d]	−	−	−	nd	−
Glycogen	+[a],−[d,o]	−	−	−[e,o],+[g]	nd	+
Tween 40	−[a],+[d,o]	−	+	+	nd	−

Table 18.2 (continued)

	I. abyssalis[a] KMM 227^T	*I. aestuarii*[b,c] KYW314^T	*I. aquimaris*[d] SW15^T	*I. baltica*[e] OS145^T	*I. donghaiensis*[f,g] 908033^T	*I. fontislapidosi*[h] F23^T
Tween 80	−[a],+[d,o]	+	+	+	nd	−
n-acetyl-D-glucosamine	−	−	−	−	nd	nd
Adonitol	−	−	−	−	nd	nd
L-arabinose	−	−	−	+[e],−[g,o]	nd	nd
i-erythritol	−	+	−	−	nd	nd
D-fructose	−	−	+	−	nd	nd
L-fucose	−	−	+	−	nd	nd
Galactose	−	+	+	−	nd	nd
Gentiobiose	−	+	+	−	nd	−
α-D-glucose	−	+	+	−	nd	nd
m-inositol	−	+	−	−	nd	nd
α-D-lactose	−	+	−	−	nd	nd
Maltose	−	−	−	−	nd	nd
D-mannitol	−	−	−	−	nd	nd
D-mannose	−	+	−	−	nd	−
D-melibiose	−	−	−	−	nd	nd
β-methyl-D-glucoside	−	+	−	−	nd	nd
D-psicose	−	−	−	−[e,o],+[g]	nd	−
L-rhamnose	−	+	−	−	nd	nd
D-sorbitol	−	+	−	−	nd	nd
Sucrose	−	−	+	−	nd	nd
Trehalose	−	−	+	−	nd	nd
Turanose	−	−	+	−	nd	nd
Xylitol	−	−	+	−	nd	nd
Pyruvic acid methyl ester	+	−	−	−[e],+[g],w[o]	nd	+
Succinic acid monomethyl ester	+[a],−[d,o]	−	−	−	nd	−
Acetic acid	+[a,o],−[d]	+	+	+[e,o],−[g]	nd	+
cis-aconitic acid	−	−	−	−	nd	nd
Citric acid	−	+	−	−	nd	−
Formic acid	−	−	−	−	nd	−
D-galactonic acid lactone	−	−	−	−	nd	nd
D-galacturonic acid	−	−	−	−	nd	nd
D-gluconic acid	−	−	+	−	nd	nd
D-glucosaminic acid	−	−	−	−	nd	−
D-glucuronic acid	−	−	−	−	nd	nd
α-hydroxybutyric acid	−	−	−	−	nd	−
β-hydroxybutyric acid	−[a,o],+[d]	−	−	−[e,g],w[o]	nd	+
γ-hydroxybutyric acid	−	+	−	−	nd	−

Table 18.2 (continued)

	I. abyssalis[a] KMM 227^T	*I. aestuarii*[b,c] KYW314^T	*I. aquimaris*[d] SW15^T	*I. baltica*[e] OS145^T	*I. donghaiensis*[f,g] 908033^T	*I. fontislapidosi*[h] F23^T
Itaconic acid	−	−	−	−	nd	nd
α-ketobutyric acid	+	−	+	+	nd	nd
α-ketoglutaric acid	$-^{a}$,$+^{d}$	−	+	−	nd	+
α-ketovaleric acid	+	−	−	$+^{e,o}$,$-^{g}$	nd	+
DL-lactic acid	$-^{a,o}$,$+^{d}$	−	−	−	nd	nd
Malonic acid	−	−	−	−	nd	nd
Propionic acid	+	+	−	$-^{e}$,$+^{g,o}$	nd	nd
D-saccharic	−	−	−	−	nd	−
Succinic acid	$+^{a,d}$,w^{o}	−	−	$-^{e,o}$,$+^{g}$	nd	+
Succinamic acid	$-^{a,o}$,$+^{d}$	−	−	−	nd	nd
L-alaninamide	+	+	+	$-^{e,g}$,$+^{o}$	nd	+
D-alanine	−	−	+	−	nd	+
L-alanine	+	−	+	$-^{e}$,$+^{g,o}$	nd	+
L-alanyl-glycine	+	−	−	$-^{e,g}$,$+^{o}$	nd	+
L-asparagine	$-^{a,o}$,$+^{d}$	−	+	$-^{e,g}$,$+^{o}$	nd	+
L-aspartic acid	$-^{a,o}$,$+^{d}$	−	+	−	nd	−
L-glutamic acid	$-^{a,o}$,$+^{d}$	−	+	$-^{e}$,$+^{g,o}$	nd	+
Glycyl L-aspartic acid	$-^{a,o}$,$+^{d}$	−	+	$-^{e}$,$+^{g,o}$	nd	+
Glycyl L-glutamic acid	+	−	+	$-^{e}$,$+^{g,o}$	nd	+
L-histidine	−	−	−	−	nd	nd
Hydroxy-L-proline	−	−	+	−	nd	nd
L-leucine	$-^{a,o}$,$+^{d}$	−	−	−	nd	+
L-ornithine	−	−	−	$-^{e,o}$,$+^{g}$	nd	+
L-phenylalanine	−	−	+	−	nd	nd
L-proline	$+^{a,d}$,$-^{o}$	−	+	$-^{e}$,$+^{g,o}$	nd	+
L-pyroglutamic acid	$-^{a,o}$,$+^{d}$	+	+	−	nd	−
D-serine	−	−	−	−	nd	−
L-serine	$-^{a}$,$+^{d,o}$	−	−	$-^{e}$,$+^{g,o}$	nd	+
L-threonine	−	−	+	$-^{e,g}$,$+^{o}$	nd	+
DL-carnitine	−	−	−	−	nd	−
γ-aminobutyric	−	−	−	−	nd	nd
Uronic acid	−	−	−	−	nd	nd
Inosine	−	−	+	−	nd	nd
Uridine	−	−	+	−	nd	−
Thymidine	−	+	−	−	nd	nd
Putrescine	$-^{a,o}$,$+^{d}$	−	+	−	nd	nd
2-aminoethanol	$-^{a,o}$,$+^{d}$	−	+	−	nd	nd
2,3-butanediol	−	−	+	−	nd	−
Glycerol	$+^{a}$,$-^{d,o}$	−	+	−	nd	−
DL-α-glycerol phosphate	−	−	+	−	nd	nd
α-D-Glucose 1-phosphate	−	−	+	−	nd	nd
Glucose 6-phosphate	$+^{a}$,$-^{d,o}$	−	−	−	nd	−

Table 18.2 (continued)

	I. abyssalis[a] KMM 227^T	*I. aestuarii*[b,c] KYW314^T	*I. aquimaris*[d] SW15^T	*I. baltica*[e] OS145^T	*I. donghaiensis*[f,g] 908033^T	*I. fontislapidosi*[h] F23^T
Acid production from						
D-glucose	–	–	nd	+	–	–[h],+[g]
D-fructose	–	nd	nd	nd	–	–
D-arabinose	–	nd	nd	–	nd	–
D-ribose	–	nd	nd	–	–	–
D-xylose	–	nd	nd	nd	–	–
L-xylose	–	nd	nd	nd	nd	–
D-maltose	–	–	nd	nd	–	–[h],+[g]
L-sorbose	–	nd	nd	nd	–	–
Gentiobiose	–	nd	nd	+	nd	+
D-turanose	–	nd	nd	nd	nd	–
D-lyxose	–	nd	nd	nd	nd	–
D-tagatose	+	nd	nd	+	nd	–
5-Ketogluconate	+	nd	nd	+	nd	+
Sensitivity to						
Erythromycin	+	+	nd	nd	+	+
Streptomycin	+	–	+	nd	nd	+
Gentamicin	+	–	+	nd	nd	nd
Kanamycin	–	–	+	nd	–	+
Ampicillin	–	+	+	nd	+	+
Benzylpenicillin	–	nd	nd	nd	nd	nd
Lincomycin	–	nd	nd	nd	nd	nd
Tetracycline	–	–	–	nd	+	nd
Oxacillin	–	nd	nd	nd	nd	nd
Oleandomycin	–	nd	nd	nd	nd	nd
Vancomycin	–	+	nd	nd	nd	nd
O/129	–	nd	nd	nd	nd	nd
Amoxicillin	nd	nd	nd	nd	+	+
Carbenicillin	nd	nd	nd	nd	+	+
Cefotaxime	nd	nd	nd	nd	+	+
Cefoxitin	nd	nd	nd	nd	+	+
Chloramphenicol	nd	+	+	nd	+	+
Nalidixic acid	nd	+	+	nd	nd	+
Nitrofurantoin	nd	nd	nd	nd	+	+
Polymyxin B	nd	+	nd	nd	+	+
Rifampicin	nd	nd	+	nd	+	+
Sulfamide	nd	nd	nd	nd	nd	–
Trimethoprim/ sulfamethoxazole	nd	nd	–	nd	nd	+
Tobramycin	nd	nd	nd	nd	–	+
Novobiocin	nd	nd	+	nd	+	nd
Penicillin G	nd	–[b],+[c]	–	nd	+	nd
Minocycline	nd	nd	nd	nd	+	nd
Bacitracin	nd	nd	nd	nd	–	nd
Neomycin	nd	nd	nd	nd	–	nd

Table 18.2 (continued)

	I. abyssalis[a] KMM 227^T	*I. aestuarii*[b,c] KYW314^T	*I. aquimaris*[d] SW15^T	*I. baltica*[e] OS145^T	*I. donghaiensis*[f,g] 908033^T	*I. fontislapidosi*[h] F23^T
Nystatin	nd	nd	nd	nd	–	nd
Amikacin	nd	–	nd	nd	nd	nd
G+C content (mol%)	50.4	56.4	51.1	49.7	45.5	46.0

	I. homiensis[i] PO-M2^T	*I. insulisalsae*[g] CVS-6^T	*I. loihiensis*[j] L2-TR^T	*I. marina*[g,k] PIM1^T	*I. maris*[l] CF12-14^T	*I. maritima*[f,g] 908087^T	*I. ramblicola*[h] R22^T
All of the organisms are oxidase, catalase, and DNAse positive. All of the organisms are urease negative. All of the strains hydrolyze gelatin. None of the strains hydrolyze starch. In Api ZYM tests all organisms are negative for β-glucosidase							
Morphology	Straight or slightly curved rods	Rods	Straight to slightly curved rods	Straight rods	Straight to slightly curved rods	Slightly curved rods	Slightly curved rods
Cell size (μm)	0.4–0.6 × 0.7–2.0	0.8 × 1.6–2.4	0.35–0.45 × 0.7–1.8	0.5–0.9 × 2.5–3.0	0.3–0.6 × 0.8–2.4	0.4–0.6 × 1.4–2.0	0.75 × 2.0–3.0
Pigmentation	Light yellowish	Nonpigmented	Beige to yellow	Off-white	White to yellow	Nonpigmented	Cream
Colony morphology	Round and convex	nd	Circular, low convex to raised, smooth, shiny, and entire	Circular, convex, and nonluminescent, with entire edges	Circular and slightly convex	Circular and smooth	Round, convex, and mucoid
Motility	+ (single polar flagellum)	+ (single polar flagellum)	+ (single polar or subpolar flagellum)	–	+ (single polar flagellum)	+ (peritrichous flagella)	+ (single polar flagellum)
Temperature for growth (°C)							
Range	4–45	10–45[g] 10–50[d]	4–46	10–42[k] 10–40[d]	4–42	10–45	15–40
Optimum	25–30	37–40[g] 35–40[d]	30	30–35	30–35	37	32
pH for growth							
Range	6.0–9.0	5.5–10.0[g] 6.0–9.0[d]	nd	6.0–10.0	6.0–11.5	6.5–10.0	5–10
Optimum	7.0–8.0	7.5[g] 7.0–8.0[d]	nd	7.0–8.0	8.0–9.5	8.0–9.0	7.0–8.0
NaCl concentration for growth (%)							
Range	1–15	1–12[g] 1–15[d]	0.5–20	0.5–15	0.1–15	0.5–15	0.5–15
Optimum	3–5[i] 2–5[b]	5	7.5–10.0	2–5	2–3	3	3–5
Nitrate reduction	+	–	+[j],–[g,i,o]	–	+	–	–
Selenite reduction	nd	nd	nd	nd	nd	+	+
Production of							
Poly-β-hydroxybutyrate	nd	nd	nd	–	–	nd	–
Exopolysaccharide	nd	nd	–	nd	nd	nd	+
Indole	–	nd	–	nd	–	–	–
H_2S	+	nd	nd	–	–	–	+

Table 18.2 (continued)

	I. homiensis[i] PO-M2^T	*I. insulisalsae*[g] CVS-6^T	*I. loihiensis*[j] L2-TR^T	*I. marina*[g,k] PIM1^T	*I. maris*[l] CF12-14^T	*I. maritima*[f,g] 908087^T	*I. ramblicola*[h] R22^T
Methyl red	nd	nd	nd	nd	nd	–	–
Voges-Proskauer	–	nd	nd	nd	–	nd	–
Presence of							
Lecithinase	–	nd	nd	nd	nd	–	–
Chitinase	–	nd	nd	nd	–	nd	nd
β-galactosidase	–	nd	–	nd	nd	–	nd
Arginine dihydrolase	–	nd	–	–	–	–	nd
Lysine decarboxylase	nd	nd	nd	–	–	–	nd
Ornithine decarboxylase	nd	nd	nd	–	–	–	nd
Tryptophan deaminase	nd	nd	nd	nd	–	nd	nd
Phenylalanine deaminase	–	nd	nd	nd	nd	nd	–
Enzymes (Api Zym)							
Alkaline phosphatase	+	+	+	+	+	+	nd
Esterase (C 4)	+	+	+	+	+	+	nd
Esterase lipase (C 8)	+	+	+	+	+	+	nd
Lipase (C14)	–	–	–	+	–	+	–
Leucine arylamidase	+	+	+	+	+	+	nd
Valine arylamidase	+[d,i],–[b]	–	–	+	–	+	–
Cystine arylamidase	+	–	–	+	–	+	–
Trypsin	+	+	–[j],+[g]	+	+	+	–
α-chymotrypsin	+	+	–	+	+	+	+
Acid phosphatase	+	+	+	+	+	+	nd
Naphthol-AS-BI-phosphohydrolase	+	+	+	+	+	+	+
α-galactosidase	–	–	–	–[k],+[d]	–	–	nd
β-galactosidase	–	–	–	–	–	–	nd
β-glucuronidase	–	–	–	–	–	–	nd
α-glucosidase	–	–	–	–	–	–	nd
N-acetyl-β-glucosaminidase	–	–	–	–	–	–	nd
α-mannosidase	–	–	–	–	–	–	nd
α-fucosidase	–	–	–	–	–	–	nd
Hydrolysis of							
Esculin	+	–	–	–[k],+[d]	–	–	+
Casein	–	–	+	–	+	+	+
Agar	nd	nd	nd	–	nd	nd	nd
Tyrosine	+	nd	nd	nd	nd	–	–
Alginate	–	nd	nd	–	nd	nd	nd
Tween 20	+	+	nd	+	nd	+	+

Table 18.2 (continued)

	I. homiensis[i] PO-M2^T	*I. insulisalsae*[g] CVS-6^T	*I. loihiensis*[j] L2-TR^T	*I. marina*[g,k] PIM1^T	*I. maris*[l] CF12-14^T	*I. maritima*[f,g] 908087^T	*I. ramblicola*[h] R22^T
Tween 40	–	+	nd	+	+	nd	nd
Tween 60	nd	+	nd	nd	nd	nd	nd
Tween 80	+	–	+	–	–	+	+
CM-cellulose	–	nd	nd	nd	–	nd	nd
Egg yolk	–	nd	nd	nd	nd	nd	nd
Arbutin	nd	+	nd	nd	nd	nd	nd
Hippurate	nd	+	nd	nd	nd	nd	nd
Biolog GP2 MicroPlate							
α-cyclodextrin	–[i],+[d]	–	–	–[k],+[d]	–	nd	–
Dextrin	–	–	+[j],–[g,o]	–	–	nd	–
Glycogen	–[i],+[d]	–	+	–[k],+[d]	–	nd	–
Tween 40	–[i],+[d]	–	+[j,o],–[g]	–[k],+[d]	–	nd	–
Tween 80	–[i],+[d]	–	+[j,o],–[g]	–[k],+[d]	–	nd	–
N-acetyl-D-galactosamine	–	–	–	–	–	nd	nd
Adonitol	–	–	–	–	–	nd	nd
L-arabinose	–	–	–	–	–	nd	nd
i-erythritol	–	–	–	–[k],+[d]	–	nd	nd
D-fructose	–	–	–	–	–	nd	nd
L-fucose	–	–	–	–	–	nd	nd
D-galactose	–	–	–	–	–	nd	nd
Gentiobiose	–	+	–	–	–	nd	–
α-D-glucose	–	–	–[g,j],+[o]	–	–	nd	nd
m-inositol	–	–	–	–	–	nd	nd
α-lactose	–	–	–	–	–	nd	nd
Maltose	–	–	+[j],–[g,o]	–	–	nd	nd
D-mannitol	–[i],+[d]	–	–	–	–	nd	nd
D-mannose	–	+	–	–	–	nd	–
Melibiose	–	–	–	–[k],+[d]	–	nd	nd
β-methyl-D-glucoside	–	–	–	–	–	nd	nd
D-psicose	–	–	–[j,o],+[g]	–	–	nd	–
L-rhamnose	–	–	–	–	–	nd	nd
D-sorbitol	–	–	–	–	–	nd	nd
Sucrose	–	–	–	–	–	nd	nd
Trehalose	–	–	–	–	–	nd	nd
Turanose	–[i],+[d]	–	–	–	–	nd	nd
Xylitol	–	–	–	–	–	nd	nd
Pyruvic acid methyl ester	–[i],+[d]	–	+	–[k],+[d]	–	nd	–
Succinic acid monomethyl ester	–	–	+[j],–[g,o]	–	–	nd	–
Acetic acid	–	–	+	–[k],+[d]	–	nd	+
cis-aconitic acid	–	–	+[j],–[o]	–	–	nd	nd
Citric acid	–	–	+[g,j],–[o]	–	–	nd	–
Formic acid	–	+	–	–	–	nd	–

Table 18.2 (continued)

	I. homiensis[i] PO-M2[T]	*I. insulisalsae*[g] CVS-6[T]	*I. loihiensis*[j] L2-TR[T]	*I. marina*[g,k] PIM1[T]	*I. maris*[l] CF12-14[T]	*I. maritima*[f,g] 908087[T]	*I. ramblicola*[h] R22[T]
D-galactonic acid lactone	−[i],+[d]	−	−	−[k],+[d]	−	nd	nd
D-galacturonic acid	−	−	−	−[k],+[d]	−	nd	nd
D-gluconic acid	−	−	+[j],−[g,o]	−	−	nd	nd
D-glucosaminic acid	−	−	−[j,o],+[g]	−	−	nd	−
D-glucuronic acid	−	−	−	−[k],+[d]	−	nd	nd
α-hydroxybutyric acid	−	+	−	−	−	nd	−
β-hydroxybutyric acid	−	−	+[g,j],w[o]	−	−	nd	−
γ-hydroxybutyric acid	−	−	+	−	−	nd	−
Itaconic acid	−	−	−	−[k],+[d]	−	nd	nd
α-ketobutyric acid	−[i],+[d]	+	+	−[k],+[d]	−	nd	nd
α-ketoglutaric acid	−[i],+[d]	−	+	−[k],+[d]	−	nd	−
α-ketovaleric acid	−[i],+[d]	−	−[j],+[g,o]	−[k],+[d]	−	nd	−
DL-lactic acid	−[i],+[d]	−	+[j],−[g,o]	−	−	nd	nd
Malonic acid	−	−	+[j],−[g,o]	−	−	nd	nd
Propionic acid	−	+	+[g,j],w[o]	−	−	nd	nd
D-saccharic acid	−	−	−[j,o],+[g]	−	−	nd	−
Succinic acid	−	−	−[j],w[o],+[g]	−[k],+[d]	−	nd	−
Succinamic acid	−	−	−	−	−	nd	nd
L-alaninamide	−[i],+[d]	−	−[j],+[g,o]	−[k],+[d]	−	nd	+
D-alanine	−[i],+[d]	+	+[j],−[g,o]	−	−	nd	−
L-alanine	−[i],+[d]	+	+	−[k],+[d]	−	nd	+
L-alanyl-glycine	−[i],+[d]	+	−[j],+[g,o]	−[k],+[d]	−	nd	+
L-asparagine	−[i],+[d]	−	+	−[k],+[d]	−	nd	+
L-aspartic acid	−[i],+[d]	−	−	−[k],+[d]	−	nd	−
L-glutamic acid	−[i],+[d]	−	+	−[k],+[d]	−	nd	+
Glycyl L-aspartic acid	−[i],+[d]	+	−[j],+[g,o]	−[k],+[d]	−	nd	+
Glycyl L-glutamic acid	−[i],+[d]	+	−[g,j],+[o]	−[k],+[d]	−	nd	+
L-histidine	−[i],+[d]	−	−	−	−	nd	nd
Hydroxy-L-proline	−[i],+[d]	−	−	−	−	nd	nd
L-leucine	−	−	−[j,o],+[g]	−	+	nd	−
L-ornithine	−[i],+[d]	+	−[j,o],+[g]	−	−	nd	−
L-phenylalanine	−[i],+[d]	−	−	−	−	−	nd
L-proline	−[i],+[d]	+	+	−[k],+[d]	−	nd	+
L-pyroglutamic acid	−[i],+[d]	−	−[j,o],+[g]	−[k],+[d]	−	nd	−
D-serine	−[i],+[d]	−	+[j],−[g,o]	−	−	nd	−
L-serine	−[i],+[d]	+	−[j],+[g,o]	−	−	nd	+
L-threonine	−[i],+[d]	−	−[j],+[g,o]	−[k],+[d]	−	nd	+
D,L-carnitine	−[i],+[d]	+	−	−	−	nd	−
γ-aminobutyric acid	−[i],+[d]	−	−	−	−	nd	nd
Uronic acid	−	−	−	−[k],+[d]	−	nd	nd

Table 18.2 (continued)

	I. homiensis[i] PO-M2^T	*I. insulisalsae*[g] CVS-6^T	*I. loihiensis*[j] L2-TR^T	*I. marina*[g,k] PIM1^T	*I. maris*[l] CF12-14^T	*I. maritima*[f,g] 908087^T	*I. ramblicola*[h] R22^T
Inosine	–[i],+[d]	–	+[j],–[g,o]	–	–	nd	nd
Uridine	–	–	–	–	–	nd	–
Thymidine	–	–	–	–	–	nd	nd
Putrescine	–[i],+[d]	–	–	–	–	nd	nd
2-aminoethanol	–	–	–	–	–	nd	nd
2,3-butanediol	–	+	+[g,j],–[o]	–	–	nd	–
Glycerol	–	–	+[j],–[g,o]	–	–	nd	–
DL-α-glycerol phosphate	–	–	–	–[k],+[d]	–	nd	nd
α-D-Glucose 1-phosphate	–	–	–	–	–	nd	nd
Glucose 6-phosphate	–	–	–	–[k],+[d]	–	nd	–
Acid production from							
D-glucose	–	–	–	–	–	–	–[h],+[g]
D-fructose	–	+	–	–	nd	–	–
D-arabinose	–	w	–	–	–	nd	–
D-ribose	–	+	–[j],w[g]	–	nd	–	–
D-xylose	–	w	–	–	nd	nd	–
L-xylose	–	w	–	–	nd	nd	–
D-maltose	–	–	–	–	nd	–	–
L-sorbose	–	+	–[j],w[g]	–	nd	–	–
Gentiobiose	–	–	–	–	nd	nd	+
D-turanose	–	+	–	–	nd	nd	–
D-lyxose	–	+	–[j],w[g]	–	nd	nd	–
D-tagatose	–	+	–[j],+[g]	–	nd	nd	–
5-ketogluconate	–	+	–[j],+[g]	–	nd	nd	+
Sensitivity to							
Erythromycin	nd	nd	nd	–	nd	+	+
Streptomycin	nd	nd	nd	–	+	nd	–
Gentamicin	nd	nd	nd	–	+	nd	nd
Kanamycin	nd	nd	nd	–	+	–	–
Ampicillin	nd	nd	nd	+	+	+	+
Lincomycin	nd	nd	nd	–	–	nd	nd
Tetracycline	nd	nd	nd	+	–	+	nd
Oxacillin	nd	nd	nd	–	nd	nd	nd
Vancomycin	nd	nd	nd	+	–	nd	nd
Amoxicillin	nd	nd	nd	nd	nd	+	+
Carbenicillin	nd	nd	nd	–	+	+	+
Cefotaxime	nd	nd	nd	nd	nd	+	+
Cefoxitin	nd	nd	nd	nd	nd	+	+
Chloramphenicol	nd	nd	nd	+	+	+	+
Nalidixic acid	nd	nd	nd	+	+	nd	+
Nitrofurantoin	nd	nd	nd	nd	nd	+	+
Polymyxin B	nd	nd	nd	+	nd	+	+
Rifampicin	nd	nd	nd	nd	nd	+	+
Sulphamide	nd	nd	nd	nd	nd	nd	+

Table 18.2 (continued)

	I. homiensis[i] PO-M2^T	*I. insulisalsae*[g] CVS-6^T	*I. loihiensis*[j] L2-TR^T	*I. marina*[g,k] PIM1^T	*I. maris*[l] CF12-14^T	*I. maritima*[f,g] 908087^T	*I. ramblicola*[h] R22^T
Trimethoprim/ sulfamethoxazole	nd	nd	nd	nd	nd	nd	+
Tobramycin	nd	nd	nd	nd	nd	–	–
Cephalothin	nd	nd	nd	+	nd	nd	nd
Colistin	nd	nd	nd	+	nd	nd	nd
Clindamycin	nd	nd	nd	–	nd	nd	nd
Novobiocin	nd	nd	nd	–	nd	+	nd
Penicillin G	nd	nd	nd	+	nd	+	nd
Minocycline	nd	nd	nd	nd	–	+	nd
Bacitracin	nd	nd	nd	nd	nd	–	nd
Neomycin	nd	nd	nd	–	nd	–	nd
Nystatin	nd	nd	nd	nd	nd	–	nd
Cefazolin	nd	nd	nd	nd	+	nd	nd
Doxycycline hydrochloride	nd	nd	nd	nd	–	nd	nd
G+C content (mol%)	45.1	51.6	47.4	46.6	50.4	45.2	48.7

	I. salinarum[m] ISL-52^T	*I. sediminum*[g,n] c121^T	*I. seosinensis*[o] CL-SP19^T	*I. tainanensis*[g,k] PIN1^T	*I. taiwanensis*[g,p] PIT1^T	*I. xiamenensis*[c] 10-D-4^T	*I. zobelli*[a] KMM 231^T
All of the organisms are oxidase and catalase positive. None of the strains hydrolyze starch. In Api ZYM tests all organisms are positive for alkaline phosphatase, esterase (C 4), esterase lipase (C 8), and leucine arylamidase and negative for α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase							
Morphology	Straight or curved rods	Rods	Straight or slightly curved rods	Straight rods	Straight rods	Rods	Rods
Cell size (μm)	0.3–0.6 × 0.8–3.5	0.3 × 1.2–1.8	0.3–0.6 × 1.0–1.9	0.6–0.9 × 2.5–3.2	0.6–0.8 × 0.8–2.4	0.4–0.6 × 1.2–1.7	0.7–0.9 × 1.0–1.8
Pigmentation	Pale yellow	Nonpigmented	Slightly yellowish	Yellow	Nonpigmented	Grey	Light yellowish
Colony morphology	Circular to slightly irregular, raised, smooth, and glistening	Circular, smooth, and convex with entire edge	Entire circular, convex, opaque, and shiny	Circular, convex, and nonluminescent, with entire edges	Circular, translucent, and nonluminescent	Smooth with regular edges	Uniformly round and opaque
Motility	+	–	+ (single polar flagellum)	–	–	+ (single polar flagellum)	+ (single polar flagellum)
Temperature for growth (°C)							
Range	4–42[m] 4–40[d]	13–42[n] 15–40[d]	4–40	10–42	15–42[p] 15–40[d]	4–42	4–30
Optimum	30–37[m] 30–35[d]	30–40	30–35	30–35	30–35	25	20–22
pH for growth							
Range	6–8	6.3–10.5[n] 6.0–10.0[d]	6.0–10.0	6.0–10.0	7.0–9.0[p] 7.0–8.0[d]	6.0–10.0	5.5–9.5

Table 18.2 (continued)

	I. salinarum[m] ISL-52^T	*I. sediminum*[g,n] c121^T	*I. seosinensis*[o] CL-SP19^T	*I. tainanensis*[g,k] PIN1^T	*I. taiwanensis*[g,p] PIT1^T	*I. xiamenensis*[c] 10-D-4^T	*I. zobelli*[a] KMM 231^T
Optimum	7.0–8.0	8.0–9.0	nd	7.0–8.0	8.0	7.0–8.0	7.5–8.0
NaCl concentration for growth (%)							
Range	0.5–14	0.5–15	1–20	0.5–15	0.5–11	0–15	1–10
Optimum	2–3	1–8	5–10	2–5	1–4	1–3	3–6
Nitrate reduction	+	–	+	–	+	–	–
Production of							
Poly-β-hydroxybutyrate	nd	nd	nd	–	–	nd	–
Exopolysaccharide	nd	nd	nd	nd	nd	nd	–
Indole	–	–	–	–	–	–	–
H_2S	nd	–	nd	–	–	–	nd
Presence of							
DNAse	+	–[d,n],+[g]	+	–[k] + [g]	–[p],+[g]	nd	+
Urease	–	–	–	–	nd	+	–
Chitinase	nd	–	nd	nd	nd	nd	+
β-galactosidase	–	+	–	–	nd	–	–
Arginine dihydrolase	nd	–	–	–	–	+	–
Lysine decarboxylase	nd	nd	nd	–	–	nd	nd
Ornithine decarboxylase	nd	nd	nd	–	–	nd	nd
Enzymes (Api Zym)							
Lipase (C14)	–	+[g],–[d]	–	+	+	+	–
Valine arylamidase	–[m],+[b,d]	–[b],+[d]	–	+	+[d,p],–[b]	+	–
Cystine arylamidase	–	+	–	+	+	+	–
Trypsin	+[d,m],–[b]	–	–	+	+	+	–
α-chymotrypsin	+[d,m],–[b]	–	–	+	+	+	w
Acid phosphatase	–	+	+	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	–	+	–	+	+	+	w
Hydrolysis of							
Gelatin	–[b,d,m],+[c]	+	–	+	+	+	+[a,m],–[i,o]
Esculin	–[b,c,m],+[d]	+	+	–	+	+	–
Casein	–	–	nd	–	–	nd	nd
Agar	nd	–	nd	–	–	nd	–
Tyrosine	–[m],+[b]	+	nd	nd	+	nd	+
Alginate	nd	nd	nd	–	nd	nd	nd
Tween 20	+	+	nd	nd	–	nd	nd
Tween 40	+	+	nd	nd	+	nd	nd
Tween 80	+	+	+	–	+	–	+[a],–[i,m]
Egg yolk	–	–	nd	nd	–	nd	nd

Table 18.2 (continued)

	I. salinarum[m] ISL-52[T]	*I. sediminum*[g,n] c121[T]	*I. seosinensis*[o] CL-SP19[T]	*I. tainanensis*[g,k] PIN1[T]	*I. taiwanensis*[g,p] PIT1[T]	*I. xiamenensis*[c] 10-D-4[T]	*I. zobelli*[a] KMM 231[T]
Biolog GP2 MicroPlate							
α-cyclodextrin	+[d],−[g]	−	−	−	−	nd	−
Dextrin	−	+[d],−[g]	−	−	−	nd	−
Glycogen	−	+[d],−[g]	+	−	+	nd	−[a],w[o]
Tween 40	−[d],+[g]	+	+	−	−	nd	−[a],+[o]
Tween 80	−	+	+	−	−[p],+[d]	nd	−[a],+[o]
N-acetyl-D-galactosamine	−	−	−	−	−[p],+[d]	nd	−
Adonitol	−	−	−	−	−[p],+[d]	nd	−
L-arabinose	−	−	−	−	−	nd	−
i-erythritol	−	−	−	−	−	nd	−
D-fructose	−	−	−	−	−	nd	−
L-fucose	−	−	−	−	−	nd	−
D-galactose	−	−	−	−	−	nd	−
Gentiobiose	−	−	−	−	−	nd	−
α-D-glucose	−	−	−	−	−[p],+[d]	nd	−[a],+[o]
m-inositol	−	−	−	−	−[p],+[d]	nd	−
α-D-lactose	−	−	−	−	−[p],+[d]	nd	−
Maltose	−	−	−	−	−[p],+[d]	nd	−
D-mannitol	−	−	−	−	−[p],+[d]	nd	−
D-Mannose	−	−	−	−	−	nd	−
D-melibiose	+[d],−[g]	−	−	−	−	nd	−
β-methyl-D-glucoside	−	−	−	−	−	nd	−
L-rhamnose	−	−	−	−	−	nd	−
D-sorbitol	−	−	−	−	−	nd	−
Sucrose	−	−	−	−	−	nd	−
Trehalose	−	−	−	−	−	nd	−
Turanose	−	−	−	−	−	nd	−
Xylitol	−	−	−	−	−	nd	−
Pyruvic acid methyl ester	−	+	−	−	−	nd	−[a],w[o]
Succinic acid monomethyl ester	+[d],−[g]	−	w	−	−	−	−
Acetic acid	+[d],−[g]	−[d],+[g]	−	−	−	−	−
cis-aconitic acid	−	−	−	−	−	nd	−
Citric acid	+[d],−[g]	−	−	−	−	nd	−
Formic acid	−	−	−	−	−	nd	−
D-galactonic acid lactone	−	−	−	−	−	nd	−
D-galacturonic acid	−	−	−	−	−	nd	−
D-gluconic acid	−	−	−	−	−	nd	−
D-glucuronic acid	+[l],−[d,g]	−	−	−	−	nd	−

Table 18.2 (continued)

	I. salinarum[m] ISL-52[T]	*I. sediminum*[g,n] c121[T]	*I. seosinensis*[o] CL-SP19[T]	*I. tainanensis*[g,k] PIN1[T]	*I. taiwanensis*[g,p] PIT1[T]	*I. xiamenensis*[c] 10-D-4[T]	*I. zobelli*[a] KMM 231[T]
α-hydroxybutyric acid	–	–	–	–	–	nd	–
β-hydroxybutyric acid	–	–[d],+[g]	w	–	–	nd	–
γ-hydroxybutyric acid	–	–[d],+[g]	–	–	–	nd	–
α-ketobutyric acid	+[d],–[g]	+[d],–[g]	+	–	–[p],+[d]	nd	+
α-ketoglutaric acid	+[d],–[g]	–	–	–	–[p],+[d]	nd	–
α-ketovaleric acid	+[d],–[g]	+	+	–	–[p],+[d]	nd	+
DL-lactic acid	–	+[d],–[g]	–	–	–	nd	–
Malonic acid	–	–	–	–	–	nd	–
Propionic acid	+[d],–[g]	–	–	–	–	nd	–
Succinic acid	+[d],–[g]	–[d],+[g]	+	–	+[p],–[d]	nd	+
Succinamic acid	–	+[d],–[g]	–	–	–	nd	–
L-alaninamide	–[d],+[g]	–[d],+[g]	+	–	–[p],+[d]	nd	+
D-alanine	–[d],+[g]	–[d],+[g]	+	–	–[p],+[d]	nd	–
L-alanine	–[g,l],+[d]	+[d],–[g]	+[o],–[l]	–	+[p],–[d]	nd	+
L-alanyl-glycine	+	+	+	–	–	nd	+
L-asparagine	+	+	+	–	–	nd	–[a],+[o]
L-aspartic acid	–	+	+	–	–	nd	–[a],+[o]
L-glutamic acid	–[d],+[g]	+	+	–	+	nd	–[a],+[o]
Glycyl L-aspartic acid	+	+	–	–	–	nd	–[a],+[o]
Glycyl L-glutamic acid	+	+	+	–	+[p],–[d]	nd	+
L-histidine	–	–	–	–	–	nd	–
Hydroxy-L-proline	–	–	–	–	–	nd	–
L-leucine	+[l],–[d,g]	–	–[o],+[l]	–	–[p],+[d]	nd	–[a],w[o]
L-ornithine	–	+[d],–[g]	–	–	–[p],+[d]	nd	+[a],–[o]
L-phenylalanine	–	+	–	–	–[p],+[d]	nd	–
L-proline	–	+	+	–	+	nd	–
L-pyroglutamic acid	–	+[d],–[g]	–	–	–	nd	–
D-serine	–	+[d],–[g]	–	–	+[p],–[d]	nd	–
L-serine	–[d],+[g]	+	–	–	+[p],–[d]	nd	–[a],+[o]
L-threonine	–[d],+[g]	+	+	–	–	nd	–[a],+[o]
D,L-carnitine	–	–	–	–	–	nd	–
γ-aminobutyric acid	–	+[d],–[g]	–	–	–	nd	–
Urocanic acid	–	–	–	–	–	nd	–
Inosine	–	–	–	–	–	nd	–
Uridine	–	–	–	–	+[p],–[d]	nd	–

Table 18.2 (continued)

	I. salinarum[m] ISL-52[T]	*I. sediminum*[g,n] c121[T]	*I. seosinensis*[o] CL-SP19[T]	*I. tainanensis*[g,k] PIN1[T]	*I. taiwanensis*[g,p] PIT1[T]	*I. xiamenensis*[c] 10-D-4[T]	*I. zobelli*[a] KMM 231[T]
Putrescine	–	–	–	–	–	nd	–
2-aminoethanol	–	+[d],–[g]	–	–	–	nd	–
2,3-butanediol	–	–	–	–	–	nd	–
Glycerol	–	–	–	–	–	nd	–
DL-α-glycerol phosphate	–	–	–	–	–	nd	–
α-D-Glucose 1-phosphate	–	–	–	–	–	nd	–
Glucose 6-phosphate	+	–	–	–	–	nd	–
Acid production from					–		–
D-glucose	–	–	–	–	–[p],+[b]	nd	–
D-fructose	–	–	w	–	–	nd	nd
D-arabinose	–	–	–	–	–	nd	–
D-ribose	–	–	w	–	–	nd	–
D-xylose	–	–	–	–	–	nd	nd
L-xylose	–	–	–	–	–	nd	nd
D-maltose	–	+	–	–	–	nd	nd
L-sorbose	–	–	w	–	–	nd	nd
Gentiobiose	w	–	–	–	–	nd	nd
D-turanose	–	–	–	–	–	nd	nd
d-lyxose	w	–	w	–	–	nd	nd
D-tagatose	+	+	+	–	–	nd	nd
5-ketogluconate	+	+	+	–	–	nd	nd
Sensitivity to							
Erythromycin	nd	nd	nd	+	+	+	+
Streptomycin	+	nd	nd	–	–	–	–
Gentamicin	+[m],–[c]	nd	nd	+	+	–	–
Kanamycin	–	nd	nd	–	–	+	–
Ampicillin	–	nd	nd	+	+	+	–
Benzylpenicillin	nd	nd	nd	nd	nd	nd	–
Lincomycin	–	nd	nd	–	+	–	–
Tetracycline	–	nd	nd	–	+	–	–
Oxacillin	nd	nd	nd	–	–	–	–
Oleandomycin	–	nd	nd	nd	nd	nd	–
Vancomycin	+	nd	nd	–[k],+[c]	+	–	–
O/129	nd	nd	nd	nd	–	nd	–
Carbenicillin	nd	nd	nd	+	+	+	nd
Chloramphenicol	+	nd	nd	–	+	nd	nd
Nalidixic acid	nd	nd	nd	+	+	nd	nd
Polymyxin B	+	nd	nd	+	+	+	nd
Rifampicin	nd	nd	nd	nd	nd	+	nd
Cephalothin	–	nd	nd	–	+	nd	nd

Table 18.2 (continued)

	I. salinarum[m] ISL-52^T	*I. sediminum*[g,n] c121^T	*I. seosinensis*[o] CL-SP19^T	*I. tainanensis*[g,k] PIN1^T	*I. taiwanensis*[g,p] PIT1^T	*I. xiamenensis*[c] 10-D-4^T	*I. zobelli*[a] KMM 231^T
Colistin	nd	nd	nd	+	+	nd	nd
Clindamycin	nd	nd	nd	–	–	–	nd
Novobiocin	–	nd	nd	–	+	nd	nd
Penicillin G	–	nd	nd	–[k],+[c]	+	–	nd
Neomycin	nd	nd	nd	+	nd	–	nd
Cefazolin	nd	nd	nd	nd	nd	+	nd
Chloromycetin	nd	nd	nd	nd	nd	+	nd
Ciprofloxacin	nd	nd	nd	nd	nd	+	nd
Norfloxacin	–	nd	nd	+	nd	+	nd
Ofloxacin	–	nd	nd	+	nd	+	nd
Rocephin	nd	nd	nd	nd	nd	+	nd
Cefalexin	nd	nd	nd	nd	nd	–	nd
Cefobid	–	nd	nd	+	nd	–	nd
Cephradine	–	nd	nd	+	nd	–	nd
Co-trimoxazole	+	nd	nd	+	nd	–	nd
Furazolidone	nd	nd	nd	nd	nd	–	nd
Metronidazole	nd	nd	nd	nd	nd	–	nd
Minomycin	–	nd	nd	+	nd	–	nd
Piperacillin	nd	nd	nd	+	nd	–	nd
Vibramycin	nd	nd	nd	nd	nd	–	nd
G+C content (mol %)	53.9	50.0	45.0	46.9	49.3	50.4	48.0

For symbols, see *Table 18.1*. *nd* not determined, *w* weakly positive
[a]Ivanova et al. (2000); [b]Park et al. (2010); [c]Wang et al. (2011); [d]Chen et al. (2012); [e]Brettar et al. (2003); [f]Wu et al. (2009); [g]Taborda et al. (2009); [h]Martínez-Cánovas et al. (2004b); [i]Kwon et al. (2006); [j]Donachie et al. (2003); [k]Jean et al. (2009); [l]Zhang et al. (2012); [m]Yoon et al. (2007); [n]Hu and Li (2007); [o]Choi and Cho (2005); [p]Jean et al. (2006)

Fuente de Piedra (Málaga, Spain), an inland, hypersaline wetland (wetland wild-fowl reserve). *Idiomarina ramblicola* was isolated from a hypersaline water sample taken from Rambla Salada (Murcia, Spain), a hypersaline rambla (a steep-sided riverbed, normally dry but subject to flash flooding) (Martínez-Cánovas et al. 2004b). The isolation medium was MY (Moraine and Rogovin 1966) supplemented with 7.5 % (w/v) salts (Rodríguez-Valera et al. 1981); its composition is as follows (per liter of deionized water): 51.3 g NaCl, 9 g $MgCl_2{\cdot}6H_2O$, 13 g $MgSO_4{\cdot}7H_2O$, 0.2 g $CaCl_2{\cdot}2H_2O$, 1.3 g KCl, 0.05 g $NaCO_3H$, 0.15 g NaBr, traces $FeCl_3{\cdot}6H_2O$, 10 g glucose, 3 g yeast extract, 3 g malt extract, and 5 g proteose peptone (pH 7.0). Both strains are kept and routinely grown in MH medium (Quesada et al. 1983) at 32 °C; its composition is the following (per liter of deionized water): 51.3 g NaCl, 9.0 g $MgCl_2{\cdot}6H_2O$, 13 g $MgSO_4{\cdot}7H_2O$, 0.2 g $CaCl_2{\cdot}2H_2O$, 1.3 g KCl, 0.05 g $NaCO_3H$, 0.15 g NaBr, traces $FeCl_3{\cdot}6H2O$, 1 g glucose, 10 g yeast extract, and 5 g proteose peptone (pH 7.0).

Idiomarina seosinensis was isolated from hypersaline water from a solar saltern located in Seosan, Korea (Choi and Cho 2005). The sample was spread on a plate of marine agar 2216 (MA, Difco). The plate was incubated at 30 °C for 2 weeks. A single slightly yellowish colony was isolated from the plate and subsequently purified on MA at 30 °C. The strain is routinely cultured on MA at 30 °C.

Idiomarina homiensis was isolated from seashore sand collected in Homi Cape, near Pohang, Korea (Kwon et al. 2006). The strain was isolated by means of the dilution-plating technique on marine agar 2216 (MA, Difco). *Idiomarina homiensis* is routinely cultured on MA.

Idiomarina taiwanensis was recovered from seawater samples collected at the shallow coastal region of An-Ping Harbour, Taiwan (Jean et al. 2006). The sample was decimally diluted with sterile NaCl/Tris buffer (30 g NaCl and 0.24 g Tris in 1 L deionized water, pH 8.0). Aliquots (0.1 mL) of the dilutions were spread on polypeptone-yeast extract plate medium (PY contained the following: 3 g polypeptone, 1 g Bacto yeast extract, 25 g NaCl, 5 g $MgCl_2{\cdot}6H_2O$, 15 g Bacto agar, 1 L deionized water, pH 7.8) (Shieh et al. 2000). The plates were incubated at 25 °C for 7 days. Colonies were isolated and purified by streaking on PY plates. The isolate was maintained at 25 °C in PY stab medium (3 g polypeptone, 1 g Bacto yeast extract, 25 g NaCl, 5 g

Table 18.3
Phenotypic and chemotaxonomic properties of *Aliidiomarina taiwanensis*

Characteristic	*Aliidiomarina taiwanensis*[a] AIT1[T]
Morphology	Rods
Cell size (μm)	0.5–0.8 × 1.5–2.8
Pigmentation	Light brown
Colony morphology	Circular, convex, and nonluminescent with entire edges
Gram-stain	Negative
Motility	+ (single polar flagellum)
Temperature for growth (°C)	
Range	4–45
Optimum	30–40
pH for growth	
Range	7.0–9.0
Optimum	8.0
NaCl concentration for growth (%)	
Range	0.5–10
Optimum	1.5–5.0
Metabolism	Aerobic
Nitrate reduction	+
Production of	
Poly-β-hydroxybutyrate	–
Indole	–
H_2S	–
Presence of	
Oxidase	+
Catalase	+
DNAse	+
Urease	–
Arginine dihydrolase	–
Lysine decarboxylase	–
Ornithine decarboxylase	–
Enzymes (Api Zym)	
Alkaline phosphatase	–
Esterase (C 4)	–
Esterase lipase (C 8)	–
Lipase (C14)	–
Leucine arylamidase	–
Valine arylamidase	+
Cystine arylamidase	+
Trypsin	+
α-chymotrypsin	+
Acid phosphatase	+
Naphthol-AS-BI-phosphohydrolase	+
α-galactosidase	–
β-galactosidase	–
β-glucuronidase	–
α-glucosidase	–
β-glucosidase	–
N-acetyl-β-glucosaminidase	–
α-mannosidase	–
α-fucosidase	–
Hydrolysis of	
Gelatin	+
Esculin	–
Starch	–
Agar	–
Casein	–
Tween 80	–
Biolog GP2 MicroPlate	
Acetic acid	+
N-acetyl-D-galactosamine	+
N-acetyl-D-glucosamine	+
L-asparagine	+
L-aspartic acid	+
Bromosuccinic acid	+
Dextrin	+
D-fructose	+
L-fucose	+
α-D-glucose	+
D-glucose 6-phosphate	+
D-gluconic acid	+
D-glucosaminic acid	+
L-glutamic acid	+
Glycogen	+
Glycyl L-aspartic acid	+
L-histidine	+
p-hydroxyphenylacetic acid	+
Inosine	+
DL-lactic acid	+
Maltose	+
D-mannitol	+
D-mannose	+
D-psicose	+
Pyruvic acid methyl ester	+
L-serine	+
Sucrose	+
Trehalose	+

Table 18.3 (continued)

Characteristic	*Aliidiomarina taiwanensis*[a] AIT1^T
Thymidine	+
Turanose	+
Uridine	
Acid production from carbohydrates using API 50CH	
Glycerol	+
Erythritol	+
L-arabinose	+
D-ribose	+
D-galactose	+
D-glucose	+
D-fructose	+
D-mannose	+
D-mannitol	+
N-acetylglucosamine	+
Maltose	+
Sucrose	+
Trehalose	+
Starch	+
Glycogen	+
L-fucose	+
Potassium gluconate	+
Fermentation of carbohydrates in PYC stab media[b]	
D-arabinose	–
L-arabinose	–
Cellobiose	–
Dulcitol	–
D-fructose	–
D-galactose	–
D-glucose	–
Myo-inositol	–
Lactose	–
Maltose	–
D-mannitol	–
D-mannose	–
Melezitose	–
Melibiose	–
Raffinose	–
D-ribose	–
D-sorbitol	–
Sucrose	–
Trehalose	–
D-xylose	–
Sensitivity to	
Carbenicillin	+
Cephalothin	+
Chloramphenicol	+
Colistin	+
Erythromycin	+
Gentamicin	+
Nalidixic acid	+
Novobiocin	+
Penicillin G	+
Polymyxin B	+
Tetracycline	+
Ampicillin	+
Kanamycin	+
Neomycin	+
Streptomycin	+
Oxacillin	–
Vancomycin	–
G+C content (mol%)	51.5

[a]Huang et al. (2012)

[b]PYC stab media, polypeptone-yeast extract-carbohydrate stab media were prepared from two parts. The first contained the 3 g polypeptone, 1 g Bacto yeast extract, 25 g NaCl, 5 g $MgCl_2{\cdot}6H_2O$, 0.24 g Tris (Sigma), 0.03 g bromothymol blue, and 10 g Bacto agar dissolved in 900 mL deionized water, pH 7.8. The second contained 5 g of carbohydrate dissolved in 100 mL deionized water. The two parts were autoclaved separately and mixed at about 50 °C

For symbols, see Table 18.1

$MgCl_2{\cdot}6H_2O$, 3.5 g Bacto agar, 1 L deionized water, pH 7.8) (Shieh et al. 2000). *Idiomarina taiwanensis* is routinely grown in PY broth or on PY agar medium at 30 °C.

Idiomarina sediminum was isolated from a sediment sample collected from a coastal region of Luoyuan Bay in Fujian province, PR China (Hu and Li 2007). Aliquots of the diluted sediment sample were spread on plates of 2216E medium (5 g Bacto peptone, 1 g Bacto yeast extract, 0.1 g $FePO_4$, 1 L seawater, pH 7.6–7.8). The plates were incubated at 28 °C in the dark for 7 days under aerobic conditions. Colonies were isolated and purified by successive streaking on 2216E plates. *Idiomarina sediminum* is routinely grown on 2216E medium at 28 °C.

Idiomarina salinarum was isolated from a marine solar saltern of the Yellow Sea in Korea (Yoon et al. 2007). The strain was isolated by means of the dilution-plating technique at 30 °C on marine agar 2216 (MA, Difco) supplemented with 8 % (w/v) NaCl. *Idiomarina salinarum* is routinely cultured on MA at 37 °C.

Idiomarina donghaiensis and *Idiomarina maritima* were isolated from a seawater sample collected from the coastal of the East China Sea, China, at a depth of 70 m (temperature 16.7 °C; salinity 33.95 ‰) (Wu et al. 2009). 150 µl of the sample was plated on marine agar 2216 (MA, Difco) and incubated at 25 °C for 3 days under aerobic conditions. Colonies were isolated and purified by successive streaking. *Idiomarina donghaiensis* and *Idiomarina maritima* are

maintained and routinely cultured on halophilic medium (HM) containing 3 % NaCl (w/v) at 37 °C. HM contained the following: 30 g NaCl, 2 g KCl, 1 g $MgSO_4 \cdot 7H_2O$, 0.36 g $CaCl_2 \cdot 2H_2O$, 0.23 g NaBr, 0.06 g $NaHCO_3$, traces $FeCl_3$, 10 g yeast extract, 5 g peptone, 1 g glucose, and 1 L deionized water, pH 7.5 (Ventosa et al. 1982).

Idiomarina marina and *Idiomarina tainanensis* were recovered from a seawater sample collected from the shallow coastal region of An-Ping Harbour, Tainan, Taiwan, during a survey of the diversity of phenanthrene-degrading bacteria (Jean et al. 2009). Aliquots (1 mL) of the seawater samples were transferred to culture bottles containing 50 mL of mineral/phenanthrene medium (MP, liquid medium used for enrichment cultivation of phenanthrene-degrading bacteria, contained the following: 0.54 g NH_4Cl, 30 g NaCl, 3 g $MgCl_2 \cdot 6H_2O$, 2 g K_2SO_4, 0.2 g K_2HPO_4, 0.01 g $CaCl_2$, 0.006 g $FeCl_3 \cdot 6H_2O$, 0.005 g $Na_2MoO_4 \cdot 7H_2O$, 0.004 g $CuCl_2 \cdot 2H_2O$, 6 g Tris, 1 g phenanthrene, 1 L deionized water, pH 8.0). All culture bottles were incubated aerobically at 30 °C in the dark for 2–3 weeks. Cultures in bottles that developed visible turbidity were streaked on polypeptone-yeast (PY) plate medium (Shieh et al. 2000). Colonies that appeared on the plates were picked and purified by successive streaking on PY plates. PY stab cultures of the isolates were maintained at 25 °C under aerobic conditions. The isolates were not able to grow in MP medium as pure cultures, in spite of the fact that they were isolated from enrichment cultures with phenanthrene as sole carbon source. *Idiomarina marina* and *Idiomarina tainanensis* are routinely grown in PY broth or on PY agar medium at 30 °C.

Idiomarina insulisalsae was isolated from a soil sample retrieved from a solar saltern on the Island of Sal, Cape Verde (Taborda et al. 2009). A suspension was prepared by mixing 3 g of the soil sample with 10 mL of sterile deionized water. Enrichment cultures were established by spreading the mixture on R3A-V agar (1 g yeast extract, 1 g proteose peptone (Difco no. 3), 1 g casamino acids, 1 g glucose, 0.6 g K_2HPO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.05 g Na pyruvate, 15 g agar, 50 mL of a macronutrients solution 10x concentrated, 5 mL of a micronutrients solution 100x concentrated, 845 mL deionized water, pH 7.0, and 100 mL of a phosphate buffer at a concentration of 1 M autoclaved separately and mixed after cooling) containing 5 % (w/v) NaCL, followed by incubation at 37 °C. The 10x concentrated macronutrients solution contained per liter of deionized water: 1 g nitrilotriacetic acid, 0.6 g $CaSO_4 \cdot 2H_2O$, 1 g $MgSO_4 \cdot 7H_2O$, 0.8 g NaCl, 1.03 g KNO_3, 6.89 g $NaNO_3$, 1.11 g $NaHPO_4$. The 100x concentrated micronutrients solution contained per liter of deionized water: 0.22 g $MnSO_4 \cdot H_2O$, 0.05 g $ZnSO_4 \cdot 7H_2O$, 0.05 g H_3BO_3, 0.0025 g $CuSO_4 \cdot 5H_2O$, 0.0025 g $Na_2MoO_4 \cdot 2H_2O$, and 0.0046 g $CoCl_2 \cdot 6H_2O$ (Tiago et al. 2006). Cultures were purified by subculturing on the same medium. *Idiomarina insulisalsae* is routinely culture on marine agar (MA, Difco) or in marine broth (MB, Difco) at 37 °C.

Idiomarina aestuarii was isolated from seawater collected from the South Sea, Republic of Korea (Park et al. 2010). The strain was isolated using the standard dilution-plating technique on marine agar 2216 (MA, Difco) at 30 °C for 7 days. *Idiomarina aestuarii* is routinely cultured on MA at 30 °C.

Idiomarina xiamenensis was isolated from surface seawater around Xiamen Island, PR China (Wang et al. 2011). 150 mL of seawater was subsampled into sterilized bottles. To start the enrichment, the seawater was supplemented with 1 mL sterilized crude oil (as a carbon source) and nitrogen, phosphorus and iron sources with concentrations as in NH medium (Wang et al. 2010). Cultures were incubated at 25 °C with shaking (150 rpm) for 10 days. Serial dilutions of enrichments were streaked on HLB (HLB was modified from Luria-Bertani (LB) medium with the concentration of NaCl increased to 30 g L^{-1}) agar plates (Liu and Shao 2005) and incubated at 25 °C. Colonies were picked and restreaked on HLB plates to obtain pure cultures. *Idiomarina xiamenensis* is routinely cultured on MA (Difco).

Idiomarina maris was isolated from deep-sea sediment of the South China Sea at a water depth of 1,153 m (Zhang et al. 2012; Zhou et al. 2009). Sample was serially diluted with sterile artificial seawater. Aliquots of 100 µl were spread on screening plates with a medium composed of 0.2 % (w/v) yeast extract, 0.3 % (w/v) casein, 0.5 % (w/v) gelatin, 1.5 % (w/v) agar, and artificial seawater (pH 7.0). The plates were incubated at 15 °C to form detectable colonies. Colonies were selected and further purified by repeatedly streaking on the same medium. *Idiomarina maris* is routinely cultivated at 30 °C in TYS broth, containing 0.5 % (w/v) tryptone, 0.1 % (w/v) yeast extract, and artificial seawater, or on TYS agar [1.5 % (w/v) agar].

Idiomarina aquimaris was isolated from a sample of a reef-building coral (*Isopora palifera*) collected on the coast at Kenting, in Pingtung County, in southern Taiwan (Chen et al. 2012). Sample was completely ground and then plated on marine agar 2216 (MA, Difco), using a standard dilution-plating method. A single colony was isolated after incubation at 25 °C for 5 days. *Idiomarina aquimaris* is routinely cultured on marine agar (MA, Difco) or in marine broth (MB, Difco) at 30 °C.

Aliidiomarina taiwanensis was recovered from a seawater sample collected from the shallow coastal region of Bitou Harbour, New Taipei, on the north coast of Taiwan (Huang et al. 2012). The sample was diluted tenfold with sterile NaCl/Tris buffer (30 g NaCl and 0.24 g Tris base, 1 L deionized water, pH 8.0). Aliquots (0.1 mL) of dilutions were spread on polypeptone-yeast (PY) agar (Shieh et al. 2000) and incubated at 30 °C for 7 days. Colonies were isolated and purified by streaking on PY agar. The isolate was maintained at 25 °C in PY stab medium. *Aliidiomarina taiwanensis* is routinely grown in PY broth or on PY agar medium.

Members of this family do not require special procedures for maintenance and long-term storage. Generally strains can be maintained on marine agar (MA, Difco) medium at 4 °C for a few days and can be stored frozen at −70 °C in marine broth (MB, Difco) medium containing 15 % glycerol without loss of viability. Long-term preservation is by lyophilization.

Ecology

Habitat

All validly named members of the family *Idiomarinaceae* have been isolated from saline habitats, some with salinities higher than the surrounding seawater, clearly indicating that these organisms are part of saline/marine ecosystems. One species, namely, *I. insulisalsae* (Taborda et al. 2009), was isolated from a mixture of soil and salt associated with a seawater evaporation pond on the Island of Salt in the Cape Verde Archipelago and *I. salinarum* from a marine solar saltern in Korea (Yoon et al. 2007), but these organisms are unlikely to grow near the saturation level of the salt in these ponds, since the maximum salt range for growth occurs in medium with around 12 % NaCl. The species *I. fontislapidosi* and *I. ramblicola* (Martínez-Cánovas et al. 2004b) were isolated from inland hypersaline wetlands and a normally dry riverbed in Spain. The species *I. abyssalis* and *I. zobellii* were isolated from an abyssal site in the Pacific Ocean at a depth of over 4,000 m with a water temperature of 2 °C (Ivanova et al. 2000), while the *I. maris* was isolated from a deep-sea sample with 1,153 m (Zhang et al. 2012) and *I. loihiensis* from a deep-sea sample at 1,296 m (Donachie et al. 2003). On the other hand, the type strain of *I. aquimaris* was isolated from a sample taken from a coral (Chen et al. 2012). Other species of *Idiomarina* and *Aliidiomarina taiwanensis* have been isolated from coastal and oceanic waters.

Several undescribed strains of the genera *Idiomarina* and *Aliidiomarina* and many 16S rRNA clone sequences related to these organisms at a similarity level of 95 % or higher to known species have been retrieved from different types of marine or hypersaline environments. Some 16S rRNA sequences or strains, more closely related to *Aliidiomarina taiwanensis*, originate from soda lakes, namely, GQ891120, GQ202581, X92128, and EF554896, and from a cured skate (GQ891120). Environclones clones and isolates of the genus *Idiomarina* have been obtained from a large variety of saline environments; examples are abyssal water and sediments (AB526349, D87345, EU935267, JQ032271), euryhaline lakes (AB167017, AB166933, HQ190037, EU722645), surface marine water and sediments (JX391102, FJ497406, FJ746576, EU491881, EU440984, EF409425, EU600202, EF437160), petroleum-contaminated soils, oil pipelines (JF421177, DQ235567, FJ152962), salt terns (EU308446), activated sludge (EF648142), alkaline olive oil wastes (AY914068), and alkaline soil (FJ152962).

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19 The Family *Legionellaceae*

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Abstract
This chapter provides a brief description of *Legionella*, the only genus in the family of *Legionellaceae*.

The family *Legionellaceae* contains a single genus, *Legionella*, with over 50 species (*Fig. 19.1*). They are obligate aerobes, Gram-negative, non-spore-forming, rod-shaped cells, although they can take on a coccobacillary appearance in clinical samples. Most species are motile and possess up to three polar flagella. On agar surfaces, they can also grow as long filaments. The various *Legionella* species are widely distributed in aquatic environments and can survive temperatures of over 60 °C without loss of viability. The primary source of carbon and energy in all *Legionella* is the catabolism of amino acids. For laboratory growth, they require specific media containing cysteine and iron salts; however, in natural habitats, they frequently thrive in nutritionally poor aquatic reservoirs or grow intracellularly within protozoan hosts (Diederen 2008). Some *Legionella* can also utilize glucose via the Entner–Doudoroff pathway; this may be an important energy-generating mechanism during intracellular growth (Eylert et al. 2010; Harada et al. 2010). The distinct steps of invasion and intracellular growth of pathogenic *Legionella* in free-living amoeba resemble the life cycle of these organisms in macrophages and respiratory epithelial cells during human infections (Escoll et al. 2013). It is likely that the majority of *Legionella* in the environment are not free-living but in a parasitic relationship with protozoan or other eukaryotic hosts.

Although over half of the *Legionella* species can cause disease in humans, referred to as legionellosis, only a handful of these organisms account for most of the clinically important infections, particularly among immunocompromized individuals and the elderly. *Legionella pneumophila* and, less commonly, *Legionella longbeacheae* are responsible for Legionnaire's disease, a severe form of pneumonia. While the primary sources of *L. pneumophila* are various natural water reservoirs and man-made water systems (i.e., air-conditioning cooling systems), the majority of infections by *L. longbeachea* originated from potting soil containing animal manure. These two organisms, together with several other *Legionella* species (*Legionella feelei, Legionella anisa* and *Legionella micdadei*), can also cause a milder respiratory disease referred to as Pontiac fever. A more detailed description of taxonomy, ecology, and pathogenic characteristics is provided in the chapter "Legionnaires' Disease" in *The Prokaryotes—Human Microbiology*, Fourth Edition (Springer-Verlag Berlin Heidelberg, 2013).

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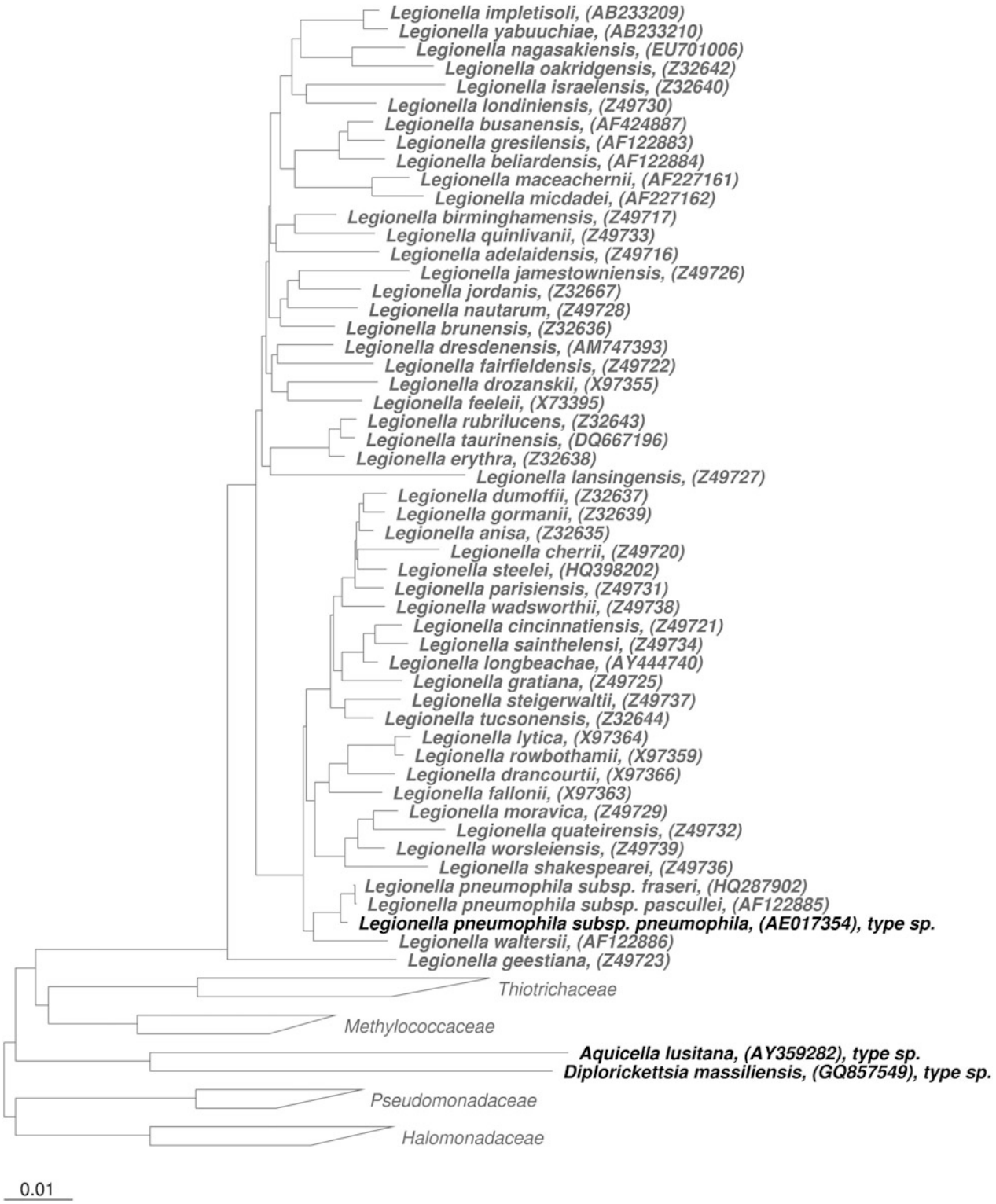

Fig. 19.1

Phylogenetic reconstruction of the family *Legionellaceae* based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes–Cantor correction. The sequence datasets and alignments were used according to ''The All-Species Living Tree'' Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high-quality-type strain sequences proportionally distributed among the different bacterial and archaeal phyla. In addition, a 40 % maximum-frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. The scale bar indicates the estimated sequence divergence

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20 The Family *Leucotrichaceae*

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Abstract
The family *Leucotrichaecae* contains filamentous, rosette-forming, aerobic or microaerophilic, neutrophilic, sulfide-oxidizing or heterotrophic bacteria that span the physiological range from obligate sulfur-based chemolithoautotrophy to obligately heterotrophic growth without any supplemental role for sulfur oxidation. In contrast to their cousins of the family *Beggiatoaceae*, the *Leucotrichaceae* filaments are non-motile and feature a very interesting dimorphic life cycle that involves differentiation into small motile cells, called gonidia, which attach to each other and to surfaces, creating a rosette-like cluster of elongating filaments. Their systematic position was debated for a long time; as some members of the *Beggiatoaceae*, the *Leucotrichaceae* were regarded as nonphotosynthetic versions to filamentous cyanobacteria. The *Leucotrichaecae* are now placed as a distinct lineage among the *Gammaproteobacteria* based on 5S, 16S, and 23S rRNA phylogenies. Currently, the *Leucotrichaceae* contain the two genera *Leucothrix* and *Thiothrix*, which differ mostly by the extent of their sulfur metabolism. While reduced sulfur sources provide auxiliary electron donors for the essentially heterotrophic genus *Leucothrix*, they sustain mixotrophic or lithotrophic growth of *Thiothrix*.

Taxonomy, Historical and Current

Early systematic considerations of non-motile, attached, rosette-forming filaments did not always attribute these bacteria in the same group of organisms. Characteristics shared with filamentous bacteria of other taxa placed the genus *Leucothrix* in close relationship with the *Oscillatoriaceae* (Oersted 1844), and the genus *Thiothrix* with the *Beggiatoaceae* (Rabenhorst 1865; Buchanan and Gibbons 1974). The establishment of the family *Leucotrichaceae* (Buchanan 1957) eventually provided a taxonomic home for the genera *Thiothrix* and *Leucothrix*, consolidating previously recognized similarities in morphology and physiology (Harold and Stanier 1955).

The genus *Thiothrix* was among the first to be studied by ribosomal RNA sequence analyses. Initially, 5S rRNA-based phylogeny studies placed *Thiothrix* into the *Gammaproto-bacteria*, separately from the filamentous sulfur oxidizer *Beggiatoa* (Stahl et al. 1987). *Thiothrix* was also placed into the *Gammaproteobacteria* in the first 16S rRNA sequencing survey of sulfur-oxidizing bacteria (Lane et al. 1992). The first 16S rRNA gene phylogeny that included the filamentous sulfur-oxidizing bacteria *Thiothrix nivea*, *Beggiatoa alba* and *Thiopoca ingrica* showed that *Thiothrix* on the one hand, and *Beggiatoa* and *Thioploca* on the other, form two separate lineages within the *Gammaproteobacteria* (Teske et al. 1995). Subsequently, a comprehensive 16S rRNA gene sequencing study showed that the genera *Thiothrix* and *Leucothrix* are sister taxa, and form a monophyletic lineage within the *Gammaproteobacteria*; the *Thiothrix*/*Leucothrix* lineage separates from the gammaproteobacterial lineage represented by *Beggiatoa* and *Thioploca* (Howarth et al. 1999). All later additions to the genera *Leucothrix* and *Thiothrix* fall consistently into the *Leucothrix* and *Thiothrix* branches of this lineage (*Fig. 20.1*). This phylogenetic framework is remarkably consistent with the validly published family *Leucotrichaceae* (Buchanan 1957; Brock 1974) that contains the genera *Leucothrix* and *Thiothrix*, and it therefore forms the basis for the family *Leucotrichaceae* as covered in this chapter. The *Leucotrichaceae* provide a clearly defined, monophyletic framework that can accommodate novel genera and species in the future.

Recently, the combined family name *Thiotrichaceae* was introduced to include the genera *Beggiatoa*, *Thioploca*, *Thiomargarita*, *Thiothrix*, *Leucothrix*, *Achromatium*, *Thiobacterium* and *Thiospira* (Garrity et al. 2005). However, the *Thiotrichaceae* comprise physiologically and phylogenetically divergent bacteria, including the type genera (*Beggiatoa*, *Leucothrix*, and *Achromatium*) of the validly published families *Beggiatoaceae* (Migula 1894; Leadbetter 1974; Strohl 1989; Salman et al. 2011), *Leucotrichaceae* (Buchanan 1957; Brock 1974) and *Achromatiaceae* (Van Niel 1948) that are based on distinct cell morphology and physiological characteristics and hold up well in the light of modern molecular taxonomy. For these reasons, the polyphyletic family designation *Thiotrichaceae* should no longer be used.

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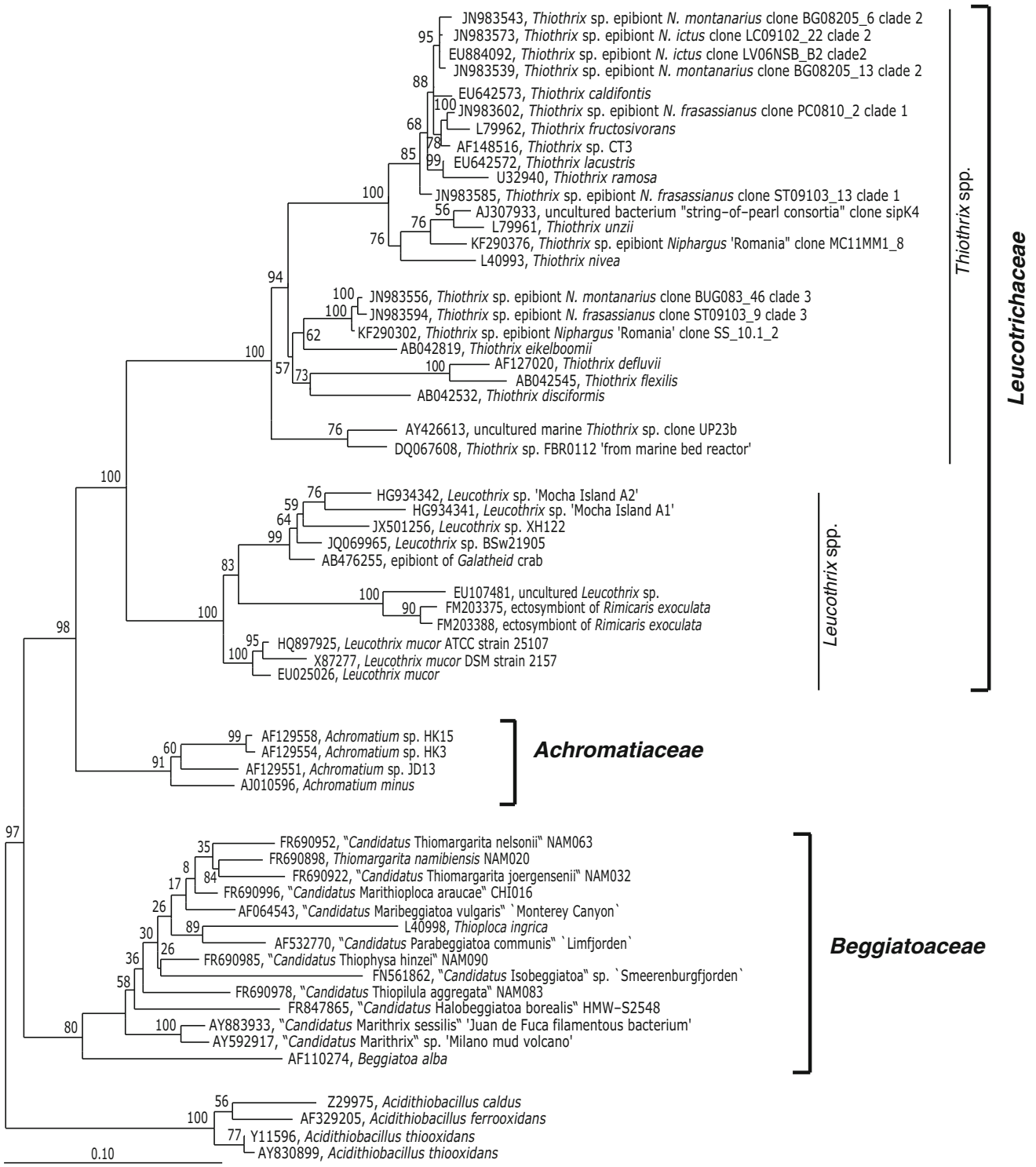

Fig. 20.1

Phylogeny of the family *Leucotrichaeceae*. The phylogenetic tree is based on near-complete 16S rRNA genes sequences, and shows the two sister genera *Leucothrix* and *Thiothrix* that constitute the *Leucotrichaceae* in relation to large sulfur bacteria of the sister families *Beggiatoaceae* and *Achromatiaceae*

This chapter provides an overview of the family *Leucotrichaecae* that synthesizes published taxonomic descriptions, physiology based on pure cultures and field samples, cell and filament morphology, and 16S rRNA gene sequences obtained from pure cultures and single cells.

Leucotrichaceae Buchanan 1957

Leu.co.trich.ac'e.ae. M.L. fem.n. *Leucothrix* type genus of the family; *-aceae* ending to denote a family; M.L. fem.pl.n. *Leucotrichaceae* the *Leucothrix* family.

Type genus: *Leucothrix* Oersted 1844.

Leu'co.thrix. Gr. adj. *leucus* clear, light; Gr. n. *thrix*, *trichis* hair; M.L. fem. n. *Leucothrix* colorless hair.

Molecular Analyses

Family *Leucotrichaceae*. The family *Leucotrichaceae* contains two genera of rosette-forming filamentous bacteria, the marine heterotroph *Leucothrix* that does not accumulate sulfur globules intracellularly, and the sulfur oxidizer *Thiothrix* that includes diverse autotrophic and heterotrophic representatives, forms intracellular sulfur globules and usually occurs in freshwater habitats. Based on 16S rRNA sequence analysis, both genera form a monophyletic lineage within the *Gammaproteobacteria* that is independent of the *Beggiatoaceae* (Howarth et al. 1999; *Fig. 20.1*). The *Leucotrichaceae* forms two major branches, one consisting of *Leucothrix mucor* and diverse *Leucothrix* phylotypes and environmental enrichments, the other including diverse *Thiothrix* species and strains that differentiate themselves into increasingly distal branches (*Fig. 20.1*). Due to historical precedent, *Leucothrix mucor* remains the type species and genus of the family (Brock 1974).

***Genus Leucothrix*.** The only described species and type species in the genus *Leucothrix*, *L. mucor* DSM 2157, has been placed at the basis of the *Leucothrix*/*Thiothrix* clade by 16S rRNA analysis (Ludwig et al. 1995; Howarth et al. 1999). Previously, *Leucothrix mucor* had been included in early 16S rRNA oligonucleotide surveys, and shown to be a member of the *Gammaproteobacteria* (Woese et al. 1985), but unrelated to filamentous *Cyanobacteria*, *Chloroflexi* (*Herpetosiphon*) and *Betaproteobacteria* (*Vitreoscilla*) (Reichenbach et al. 1986). In addition to the 16S and 23S rRNA genes of *L. mucor* str. DSM 2157 (X87277 [identical to NR_044870], X87285), the gyrB genes for gyrase subunit B (HQ897924), the soxB genes for sulfate thioesterase/sulfate thiohydrolase (EF618586), as well as an assembled genome (GB Projects PRJNA218889 and PRJNA81139) are available from Genbank. Functional gene data also exist for *Leucothrix mucor* strain DSM 621 (soxB gene, EF618580).

Although only the type species, *L. mucor*, is currently recognized, the genus *Leucothrix* does not necessarily lack diversity. Several strains of *L. mucor* isolated from different Atlantic and Pacific coastal locations had similar G+C% values (with a single exception, 48.0–49.5 %), indicating that the physiological requirements and the morphology of the organism are sufficiently specific to yield closely related strains of the same species as long as a consistent isolation procedure is maintained (Brock and Mandel 1966). However, it is very likely that diverse *Leucothrix*-like bacteria exist in nature, as shown by preliminary studies of isolated strains (Williams and Unz 1985, 1989), and by the G+C range of 46–51 mol% found among more than 30 different strains (Kelly and Brock 1969b). New 16S rRNA data from environmental studies, including epibionts of marine invertebrates, demonstrate expanding phylogenetic diversity within the genus (*Fig. 20.1*). The metadata of published database entries of *Leucothrix* environmental 16S rRNA gene clones show a preference of this bacterium for organic-rich marine habitats; examples include marine *Leucothrix* clones from a coastal algal bloom (AF195464; Kelly and Chistoserdov 2001), an arctic fjord (JQ069965), mangrove rhizosphere (JQ965724), ectobionts of cold seep and hydrothermal invertebrates, such as *Shinkaia crosnieri* and *Rimicaris exoculata* (EU107481, FM203375, FM203388, AB476255; Watsuji et al. 2010), and from larvae of the Chinese mitten crab, *Eriocheir sinensis* (EU025026). A fresh examination of *Leucothrix* laboratory strains and new isolates with modern physiological and molecular methods is overdue.

Genus *Thiothrix*. Numerous isolations, physiological studies, 16S rRNA sequencing surveys and species descriptions have greatly expanded the known species diversity within the genus *Thiothrix*. Currently, nine species are described, with the type species *Thiothrix nivea* (Larkin and Shinabarger 1983). The first comprehensive phylogeny of the genus *Thiothrix* (Howarth et al. 1999) included the type species *T. nivea* (L40993; Teske et al. 1995), the well-documented but not yet validated strain '*T. ramosa*' (U32940; Polz et al. 1996), the species *T. eikelboomii* str. AP3^T (L79965, also AB042819 and NR_024758), *T. unzii* str. A1^T (L79961), *T. fructosivorans* strains I and Q (L79962 [type strain Q] and L79963), and *T. defluvii* (AF217020; synonymous with *Thiothrix* I str. Ben57^T). The genus was subsequently enlarged with the species *T. disciformis* (AB042532 [type strain B3-1] to AB042538) and *T. flexilis* (AB042543 [type strain EJ2M-B] to AB042545), both isolated from wastewater treatment plants and capable of growth without reduced sulfur (Aruga et al. 2002), and with the two hot spring species *T. caldifontis* (EU642573, type strain G1) and *T. lacustris* (EU642572, type strain BL) which can grow as facultative lithoautotrophs with reduced sulfur sources (Chernousova et al. 2009).

Increasingly, the genus *Thiothrix* is being analyzed with functional protein-coding genes, including the gyrB gene for DNA gyrase subunit B (Chernousova et al. 2009), the cpn60 gene for the 60 kDa heat shock chaperonin (Dumonceaux et al. 2006), and the narG gene for dissimilatory nitrate reductase (Trubitsyn et al. 2013). Phylogenetic analysis based on deduced

amino acid sequences of the gyrase B subunit showed a monophyletic genus *Thiothrix*, a result that is consistent with 16S rRNA analysis (Chernousova et al. 2012). The species *T. lacustris* strains AS and BL^T, *T. caldifontis* G1^T, *T. unzii* A1^T, and *T. eikelboomii* AR3^T reduce nitrate to nitrite with thiosulfate as electron donor, and harbor the narG gene, which encodes the alpha subunit of respiratory nitrate reductase NarGHI; gene expression was demonstrated for *T. lacustris* (Trubitsyn et al. 2013). *Thiothrix nivea* (strain DSM 5205^T) and *Thiothrix* sp. strain CTD (DSMZ 12750) possess the genes (aprBA) encoding for dissimilatory adenosine-5′-phosphosulfate reductase; this key enzyme of the dissimilatory sulfate-reducing pathway is postulated to operate in the reverse direction in sulfur-oxidizing prokaryotes, oxidizing sulfite to adenosine-5′-phosphosulfate (Meyer and Kuever 2007; Meyer et al. 2007). The genome of *Thiothrix nivea* DSM 5205 has been fully sequenced at JGI (JGI Project ID 4086502; http://www.jgi.doe.gov) and is available on Genbank (Lapidus et al. 2011).

Species of the Genus *Leucothrix*

Leucothrix mucor. Oerstedt 1844. Leu'co.thrix. Gr. *leucus* clear, light; Gr. N. *thrix*, *trichis* hair; M.L. femn. *Leucothrix* colorless hair. mu'cor. L. n. *mucor* mold; M.L. n. *mucor* a genus of molds.

The type species of the genus *Leucothrix*, *Leucothrix mucor*, is the only recognized species of the genus. *L. mucor* grows in filaments of variable length, often longer than 100 µm, with a diameter of 2–3 µm. Sulfur granules are not formed. Filaments are colorless, unbranched, nonmotile, and are lacking a sheath. Filaments often grow intertwined or in dense tangles, and are attached to solid substrates by means of an inconspicuous holdfast. Individual cells within filaments round up and form ovoid to spherical gonidia, which when released acquire a jerking gliding motility. Gonidia frequently aggregate in cultures, probably chemotactically, to form rosettes. *L. mucor* is obligately aerobic and heterotrophic, does not require growth factors, grows on glutamate as sole source of carbon, nitrogen and energy, uses sugars, organic acids, and other amino acids as carbon and energy sources, and NH_4^+ as nitrogen source. Thiosulfate is used as auxiliary electron donor for lithoheterotrohic growth (Grabovich et al. 1999). Growth requires Na^+: optimal 1.5 % NaCl, minimum 0.3 %, maximal 7 %. The growth temperature optimum is 25–28 °C, the maximum is 32–35 °C; *L. mucor* also grows at 0 °C to form visible colonies within 1–2 weeks. After its first successful isolation by Harold and Stanier (1955), *L. mucor* has been reisolated by other investigators; currently nine pure culture strains are available at the American Type Culture Collection, and two strains at the German Type Culture Collection. The neotype strain is ATCC 25107/DSM 2157, isolated as an epiphyte from seaweed (*Monostroma*) in Friday Harbor, Washington (Brock 1969) and represented by 16S and 23S rRNA gene sequencing (Ludwig et al. 1995).

The mol% G+C of the type strain was estimated as 49 by buoyant density (Brock and Mandel 1966), and was determined as 47.8 by genome sequencing. The genome of *L. mucor* 2157 constitutes 5.19 Mb.

Type strain: ATCC 25107, DSM 2157.

Genbank accession numbers of 16S rRNA gene sequence: X87277.

Other taxa. The species *L. cohaerens* was isolated by Pringsheim (1957) but has been lost soon after and remains unvalidated. The more recently proposed but also unvalidated species *L. thiophila* remains to be placed by 16S rRNA gene sequencing (Dul'tseva et al. 1996).

Species of the Genus *Thiothrix*

Thiothrix nivea. (Rabenhorst 1865) Winogradsky 1888.

Thi'o.thrix Gr. neut. n. *theion* (Latin transliteration *thium*), sulfur; Gr. n. *thrix* hair; M.L. fem. n. *Thiothrix* sulfur hair; ni've. a. L. adj. *nivea*, snow-white.

The type species of the genus *Thiothrix* is mixotrophic, requires carbon substrates (acetate, pyruvate, malate, oxalacetate) for growth, and sulfide or thiosulfate as reduced sulfur source for energy supply. *T. nivea* is aerobic, but prefers reduced oxygen concentrations (ca. 10 % saturation), and can also reduce nitrate to nitrite. Rod shaped cells, about 1.0–1.5 µm in diameter, seriate in multicellular filaments (trichomes) of uniform diameter. Filaments are ensheathed and non-motile. Attachment with holdfast structure, form gonidia successively at filament end. Rosettes are formed by gonidia when in high density. Gonidia are motile by gliding, about 1–2 µm/min. Stores sulfur in the periplasm and PHA in the cytoplasm. Produces cytochrome oxidase but no catalase. Gram-negative. Optimum temperature for growth is 25–30 °C, maximum about 32–34 °C, minimum about 6–8 °C. The type strain was isolated from sulfide-containing well water in John Pennekamp State Park in Key Largo, Florida (Larkin and Shinabarger 1983).

The mol% G+C of the DNA is: 52 (T_m).

Type strain: JP2, DSM 5202.

Genbank accession numbers of 16S rRNA gene sequence: L40093.

Thiothrix caldifontis. Chernousova, Cridneva, Grabovich, Dubinina, Akimov, Rosetti and Kuever 2009.

cal.di.fon'tis. L. adj. *caldus* hot; L. n. fons, *fontis* a spring; N.L. gen. n. *caldifontis* from a hot spring, pertaining to the source of isolation of the first strains.

Mixotrophic, aerobic; chemoheterotrophic growth with a variety of organic acids, e.g. lactate, and amino acids used as carbon and energy sources; lithotrophic growth in the presence of reduced sulfur compounds, such as sulfide and thiosulfate. Rod-shaped cells with rounded ends, seriate in multicellular filaments (trichomes) with polysaccharide sheaths. Gram-negative. Cells are 0.9–2.2 µm in diameter and 3.2–6.5 µm long. Filaments are non-motile. Gliding gonidia are produced from the apical ends of the filaments. Gonidia can form rosettes. At early stages of exponential growth, a spiral form of filaments is often observed. The top cells of short filaments sometimes form pin-like bulges during the stationary growth phase. The temperature range for growth is

7–37 °C, with optimum growth at 25 °C. The pH range for growth is 7.0–8.6, with optimum growth at pH 8.0. The type strain was isolated from the sulfide spring Petushok at 33–40 °C in the Northern Caucasus region, Russia.

The mol% G+C of the DNA is 52 (T_m).

Type strain: GI, DSM21228, VKM B-2520.

Genbank accession numbers of 16S rRNA gene sequence: EU642573.

Thiothrix defluvii. Howarth, Unz, Seviour, Blackall, Pickup, Jones, Yaguchi and Head 1999.

de.flu'vi.i. L. neut. n. *defluvium* sewage; L. gen.n. *defluvii* of sewage.

Mixotrophic, aerobic; isolates are extremely slow growing and biochemical properties of this organism have not been determined. Rods, variable in shape (cylindrical, barrel-shaped, frequently elongate and swollen) in multicellular filaments (trichomes) with base to tip differentiation, apical cells are 1.0–2.0 μm in diameter and 5.0–10.0 μm in length, whereas cells at the base of trichomes are 2.0–4.0 μm in diameter and 0.5–10 μm in length. Trichomes are unsheathed but may form knots; rosettes, holdfasts, and gliding gonidia are present in some strains. Gram negative or gram-variable. Growth occurs in the temperature range of 10–30 °C but not at 4 °C or 37 °C. Deposits intracellular sulfur but no PHA. Isolated from an activated sludge treatment plant in Australia.

The mol% G+C of the DNA is: unknown.

Type strain: Ben57.

Genbank accession numbers of 16S rRNA gene sequence: AF127020.

Thiothrix disciformis. Aruga, Kamagata, Kohno, Hanada, Nakamura and Kanagawa 2002.

dis.ci.for'mis. L. masc. n. *discus* a disc; L.n. *forma* shape; N.L. adj. *disciformis* disc-shaped, the main cell form.

Mixotrophic, aerobic; reduced inorganic sulfur is not required for growth, but internal storage of sulfur and production of sulfuric acid are observed when present. Glucose, fructose, mannose, sucrose, maltose, trehalose, mannitol, succinate, pyruvate, acetate, malate, butyrate, hydroxy-butyrate, glutamate, glycerol and aspartate are utilized as sole carbon sources. Almost all strains utilize citrate and alanine. Growth is inhibited by 0.5 % (w/v) NaCl. Cells are rods and form slightly bent multicellular filaments. Filaments are more than 0.5 mm long and can reach several millimeters. Colonies are fingerprint-like. Cell morphology in filaments is variable, particularly for length, and discoid to ovoid shaped cells are often observed. Cells of the major form are 1.2–3.0 μm in diameter and 0.5–3.0 μm in length. Elongate and swollen cells are sometimes present in filaments. Gram-negative. A sheath is absent. Sudanophilic granules are observed. Growth occurs in the temperature range 14–32 °C and pH range 7.0–7.9, but there is no growth at 4 or 37 °C. Optimum temperature 25–30 °C. Oxidase-positive and catalase-positive (violent bubble generation). Nitrate is not reduced. The G+C content of the DNA is 44–45 mol% (HPLC). Isolated from activated sludge suffering from bulking.

The mol% G+C of the DNA is 43.9–44.7.

Type strain is B3-1^T = DSM 14473^T = JCM 11364^T.

Genbank accession numbers of 16S rRNA gene sequence: AB042532.

Taxonomic comment: Additional strains of *T. disciformis* are B4-1 (=JCM 11365), B2-7 (=JCM 11362), SCM-A (=JCM 11132), B5-1 (=JCM 11366), B2-8 (=JCM 11363) and OS-F (=JCM 11131); their 16S rRNA gene sequences are available from Genbank under accession numbers AB042532 to AB042538 (Aruga et al. 2002).

Thiothrix eikelboomii. Howarth, Unz, Seviour, Blackall, Pickup, Jones, Yaguchi and Head 1999.

ei.kel.boom'i.i. M.L. gen. n. *eikelboomii* of Eikelboom, named for D.H. Eikelboom, who pioneered morphological identification of filamentous bacteria in wastewater.

Mixotrophic, aerobic; uses the widest variety of organic compounds among *Thiothrix*. Forms internal sulfur deposits but does not require reduced sulfur for growth. Rods may vary in shape (cuboidal, barrel-shaped, cylindrical, discoid, bead-like) depending on location in filaments; apical cells are 0.6–0.8 × 1.0–1.5 μm and frequently bead-like, whereas cells at the base of filaments are discoid, with 1.0–3.0 μm in diameter and 0.4–0.7 μm in length. Filaments have no sheath but may form knots, rosettes and a holdfast. The type strain does not form rosettes. Gliding gonidia occur only in rosette-forming strains and have a tuft of monopolar fimbriae but lack flagella. Gram negative or gram variable. Growth occurs in the temperature range of 10–33 °C but not at 37 °C. Growth pH range is 6.5–8.5. Isolated from activated sludge treating domestic wastewater (Williams and Unz 1985).

The mol% G+C of the DNA is 44.1–46.1.

Type strain: AP3, ATCC 49788.

Genbank accession numbers of 16S rRNA gene sequence: AB042819.

Taxonomic comment: Numerous strains of *T. eikelboomii* have been isolated mostly from wastewater treatment plants, which resulted in a correspondingly large number of 16S rRNA sequences deposited in Genbank. Initially, *T. eikelboomii* consisted of a cluster of several mutually closely related strains (Genbank entries L79965, AF126148, AF126150, AF126151, AF126153, AF126154, AF126155) from the USA, Australia and Japan (Howarth et al. 1999). Additional isolates (strain Tl-4 [=JCM 11127], Genbank entry AB042540; strain Tl-2 [=JCM11128], Genbank entry AB042541; strain COM-A [=JCM11133], Genbank entry AB042542; and strain KR-A [=JCM11129], Genbank entry AB042539) have amended and enlarged the species to its current definition (Kanagawa et al. 2000; Aruga et al. 2002).

Thiothrix flexilis. Aruga, Kamagata, Kohno, Hanada, Nakamura and Kanagawa 2002.

Fle.xi'lis. L. adj. *flexilis* pliable.

Mixotrophic, aerobic; reduced inorganic sulfur is not required for growth. When reduced inorganic sulfur compounds are present, only few sulfur granules are deposited, and frequently absent in situ. No or only slight production of sulfuric acid. Glucose, fructose, mannose, sucrose, maltose, trehalose, mannitol, lactate, propionate, succinate, pyruvate, acetate, malate, hydroxybutyrate, glutamate and aspartate are utilized

as sole carbon sources. Almost all strains utilize citrate and alanine. Cells are rod-shaped and form slightly bent multicellular filaments. Filaments are more than 0.5 mm long and can reach several millimetres. Colonies are fingerprint-like. Cell morphology in filaments is variable, particularly for length, discoid to ovoid-shaped cells are often observed. Cells are 1.0–4.0 μm in diameter and 0.5–5.5 μm in length. Elongate and swollen cells are sometimes present in filaments. Gram-negative. A sheath is absent. Rosettes and holdfasts are observed in some but not all strains. Gliding gonidia are produced from the end of the filaments only in rosette-forming strains. Sudanophilic granules are observed. Growth occurs in the temperature range 14–37 °C and pH range 7.0–7.9, but there is no growth at 4 °C or 42 °C. Optimum temperature 20–30 °C. Oxidase and catalase-positive. Good growth occurs in 0.1 % NaCl and growth is inhibited slightly in 2 % (w/v) NaCl. Nitrate is reduced to nitrite. Isolated from activated sludge suffering from bulking.

The G+C content of the DNA is 44 mol%.

Type strain: EJ2M-B^{T} = DSM 14609^{T} = JCM11135^{T}.

Genbank accession numbers of 16S rRNA gene sequence: AB042545.

Taxonomic comment: Additional strains of *T. flexilis* are EJ1M-B (=JCM 11134; Genbank entry AB042544) and SNR-3 (=JCM 11130, Genbank entry AB042543) (Aruga et al. 2002).

Thiothrix fructosivorans. Howarth, Unz, Seviour, Blackall, Pickup, Jones, Yaguchi and Head 1999.

fruc.to.si.vor'ans. M.L. neut. n. *fructosum* fructose; L. part. pres. *vorans*, eating; M.L. adj. *fructosivorans* fructose-eating.

Mixotrophic, aerobic; no requirement for reduced sulfur compounds for growth, but deposits internal sulfur when present. Grows on fructose, sucrose, melezitose, pyruvate, succinate, malate, acetate, lactate, and propionate as carbon and energy source, and hydrolyses gelatin. Stores volutin inclusions, sudanophilic granules and PHA. Sole nitrogen sources are ammonia, nitrate, proline, and cys-glucosamine. Rod-shaped cells are 1.2–2.5 × 2.7–4.5 μm, in multicellular filaments. Gram-negative, ensheathed, forming rosettes and holdfast, gliding gonidia with monopolar fimbriae but no flagella. Oxidase positive and weakly catalase positive. Growth occurs in the temperature range of 4–28 °C but not at 33 °C. Growth at pH range 6.5–8.5. Isolated from activated sludge treating domestic wastewater (Williams and Unz 1985).

The mol% G+C of the DNA is 51.5 (Chernousova et al. 2009).

Type strain: Q, ATCC 49748.

Genbank accession numbers of 16S rRNA gene sequence: L79962.

Taxonomic comment: a second strain of *T. fructosivorans*, strain I (ATCC 49749; Genbank accession No. L79963) is closely related to the type strain and was isolated from the same wastewater treatment plant (Williams and Unz 1985).

Thiothrix lacustris. Chernousova, Cridneva, Grabovich, Dubinina, Akimov, Rosetti and Kuever 2009.

la.cus'tris. N.L. fem. adj. *lacustris* belonging to a lake, referring to the site from where the type strain was isolated.

Mixotrophic, aerobic; chemoheterotrophic growth with a variety of organic acids and amino acids used as carbon and energy sources; lithotrophic growth in the presence of reduced sulfur compounds produces sulfuric acid. Maximum cell counts obtained during mixotrophic growth in the presence of thiosulfate, lactate and other organic substrates. Oxidase positive, catalase negative. Nitrogen sources are peptone, yeast extract ammonium, nitrate and nitrite. Rod-like cells with rounded ends, 0.9–2.3 μm in diameter and 4.4–6.3 μm long, occur in sheathed, non-motile filaments. Gram-negative. Gliding gonidia are produced from the apical ends of the filaments. Gonidia can produce rosettes. At early stages of exponential growth, a spiral form of filaments is often observed. During the stationary growth phase, pin-like bulges can appear on the ends of short filaments. The temperature range for growth is 5–32 °C, with optimum growth at 24 °C. The pH range for growth is 6.2–8.2, with optimum growth at pH 7.0. The type strain was isolated from a low-temperature lake of the Blue Lake system (Kabardino-Balkaria).

The mol% G+C of the DNA is 51.4.

Type strain: BL, DSM 21227, VKM B-2521.

Genbank accession numbers of 16S rRNA gene sequence: EU642572.

Thiothrix unzii. Howarth, Unz, Seviour, Blackall, Pickup, Jones, Yaguchi and Head 1999.

Un'zi.i. M.L. gen.n. unzii of Unz, named for R.F. Unz.

Mixotrophic, aerobic; requires reduced inorganic sulfur for growth. Gelatin and casein are hydrolyzed. Carbon sources are pyruvate, succinate, acetate, lactate and propionate. Weak growth with malate. Stores sulfur, volutin, lipids and PHA. Oxidase positive, catalase negative. Nitrogen sources are ammonia, nitrate, asparagine, glutamine, aspartate, glutamate, and glucosamine. Nitrate is reduced to nitrite. Rods 0.7–1.5 × 1.5–3.0 μm, in multicellular filaments. Gram-negative; filaments without sheaths, rosettes formed, holdfast present, gliding gonidia with monopolar fimbriae but no flagella. Growth occurs in the temperature range of 4–33 °C but not at 37 °C. Growth at pH range 6.5–8.5. Isolated from activated sludge treating domestic wastewater (Williams and Unz 1985).

The mol% G+C of the DNA is 49.3 (Chernousova et al. 2012).

Type strain: A1, ATCC 49747.

Genbank accession numbers of 16S rRNA gene sequence: L79961.

Other taxa: a second strain of *T. unzii*, strain TN (not deposited in ATCC; Genbank accession No. KF720709) is closely related to the type strain and was isolated from a sulfide spring in the North Caucasus (Belouscova, Dubinina and Grabovich, unpublished).

"***Thiothrix ramosa***". Odintsova and Dubinina 1990a.

Ra.mo'sa. L. fem. adj. ramosa, full of boughs, having many branches, branching, branchy.

This isolate is physiologically and molecularly well-studied (Odintsova and Dubinina 1990a, b, 1993; Odintsova et al. 1993; Polz et al. 1996), but has not yet been included in the list of prokaryotic names with standing in nomenclature (Parte 2014).

"*Thiothrix ramosa*" is capable of aerobic, mixotrophic and autotrophic growth, and can grow in purely lithoautotrophic medium. Oxidizes sulfide, thiosulfate, tetrathionate and carbon disulfide, when lactate is present as carbon source, it also grows mixotrophically on the sulfur sources methanethiol and diethyl sulfide. Substituted thiotherpenes can also be used as sole substrates. Isolated from a spring in Latvia.

The mol% G+C of the DNA is 51–52 mol%.

Type strain: A1, ATCC 49747.

Genbank accession numbers of 16S rRNA gene sequence: U32940.

Additonal *Thiothrix* isolates have not yet been validated as new species: The facultatively autotrophic *Thiothrix* strain CT3, from activated sludge, is a close relative of *T. fructosivorans* (AF148516) (Rosetti et al. 2003). The isolate "*Thiothrix arctophila*" remains to be placed by 16S rRNA sequencing (Dul'tseva and Dubinina 1994).

Phenotypic Analyses

General characteristics of the genus *Leucothrix*. This short characterization of the genus *Leucothrix* follows the succinct chapter on *Leucothrix* by Brock (2006). *Leucothrix* is identified 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 (*Fig. 20.2c*). 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 filaments are colorless, unbranched, and of variable length; they may reach lengths of 0.1–0.5 cm. The filaments do not taper, but there may be variation in diameter along the length of the filaments. 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. Rosette formation is a key diagnostic characteristic; it is not possible to easily identify an isolate as *Leucothrix* without observation of the presence of rosettes. *Leucothrix* is found widely in the littoral zone in marine environments worldwide, growing primarily as an epiphyte of micro- and macroscopic algae or animals (*Fig. 20.2a, b*). Although all *Leucothrix* strains that have been isolated so far are marine and require NaCl for growth, the possible existence of freshwater strains should not be ruled out.

Life cycle of *Thiothrix* and *Leucothrix*. *Leucothrix*, as well as *Thiothrix*, are characterized by a dimorphic lifecyle that includes the transition from a static multicellular filamentous form to a motile unicellular form (*Fig. 20.3*). 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 either released individually, often from the tips of the filaments, or, as more typically, the entire filaments disintegrates (*Fig. 20.3*). The gonidia are able to glide in a jerky manner,

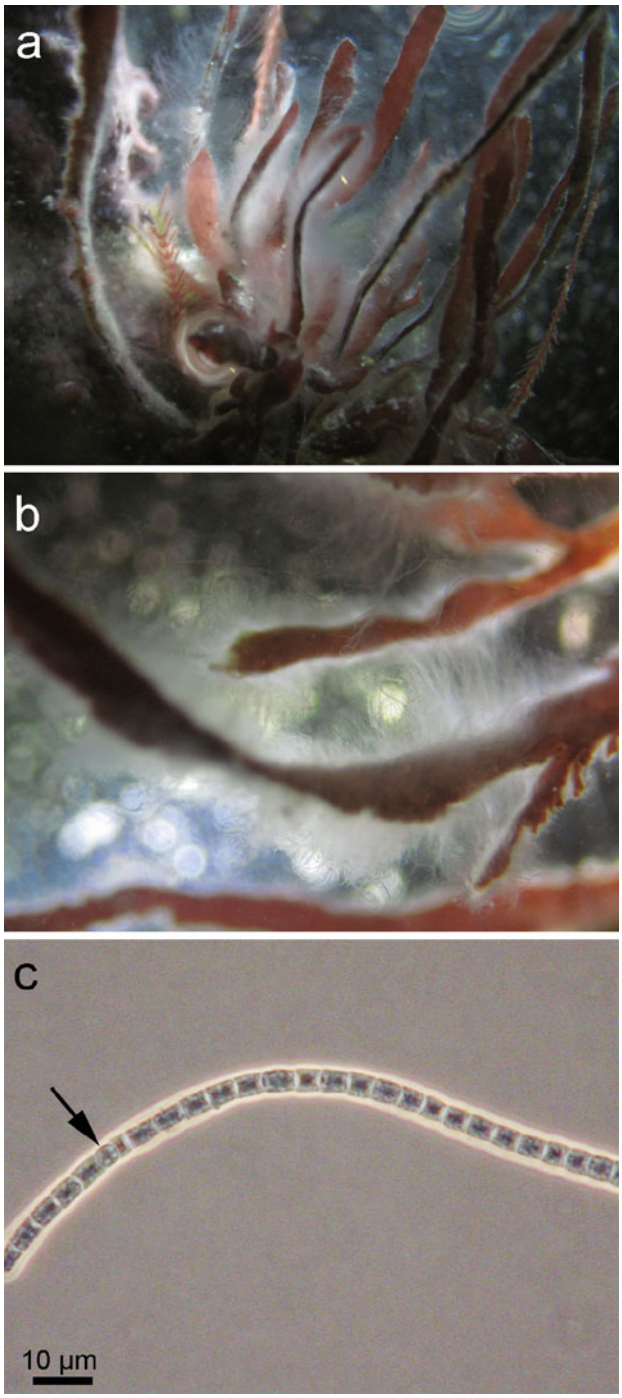

Fig. 20.2
Natural enrichment of *Leucothrix* spp. These *Leucothrix* enrichments are growing on red algae at shallow-water methane seeps of Mocha Island, Chile (Jessen et al. 2011; Sellanes et al. 2011). (**a**) *Leucothrix* filaments cover the algal fronds (*Gelidium lingulatum*) in a thick epiphyte layer. (**b**) Close-up view of the colorless *Leucothrix* filaments; the algal fronds are ca. 3–5 mm wide. (**c**). Individual *Leucothrix* filament; note the absence of sulfur globules, and the round shape of some cells (*arrow*) that most likely represent the initial stage of gonidia formation (Photos courtesy of Eduardo Tejos, Universidad de Concepcion, Chile (**a**, **c**), and adapted from Sellanes et al. 2011 (**b**))

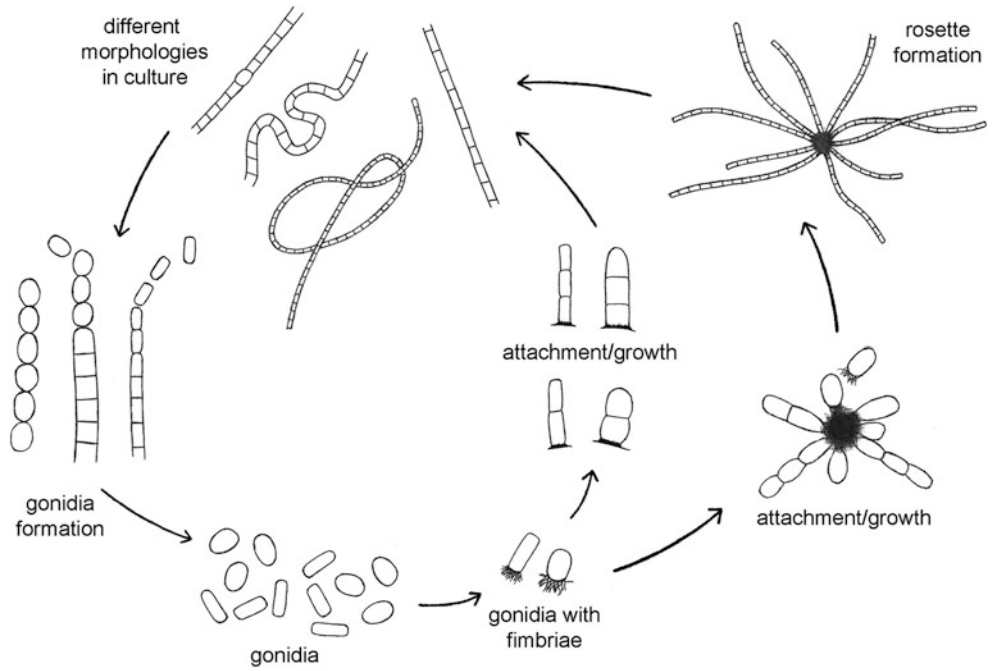

Fig. 20.3

Life cycle drawing of *Thiothrix* and *Leucothrix* as observed in culture studies. Attached filaments form apical gonidia that are released into the medium and are typically motile; they form fimbriae on one pole and attach to surfaces where they grow out into new filaments. Rosettes are formed when gonidia attach to each other when a high density of gonidia is present (Adapted from Brock 2006)

form fimbriae on one pole, and attach when they come into contact with a solid surface. They settle down on biotic or abiotic surfaces, synthesize a holdfast matrix, and form new filaments through growth and successive cell divisions. Attachment to other gonidia causes rosette formation (*Fig. 20.3*). The holdfast structure is inconspicuous, but fluoresces red when stained with primulin and viewed under blue light. In nature, the gonidia are presumably elements of dispersal and enable the organism to spread to other areas. Brock (2006) emphasizes that the term "gonidia," first used by Winogradsky (1888) for *Thiothrix*, should not be considered as the designation of some sort of unique structure; the gonidium of *Leucothrix* and *Thiothrix* corresponds functionally and structurally to the motile dispersal structure or hormogonium of the cyanobacteria, which is formed by the rounding up of a vegetative cell or group of cells in a filament.

Gonidia may form by terminal differentiation of a filament tip as in *Thiothrix*, or by division and dissociation of a fully formed filament, as in *Leucothrix*. Gonidia do not have a holdfast when first formed, but make it only in response to the proper environmental conditions. Individual gonidia may aggregate if they occur in sufficiently high concentrations. A new filament grows out from each gonidium, eventually resulting in a large and striking structure, a rosette (*Figs. 20.3* and *20.4a*). 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 (*Figs. 20.3* and *20.4b*; 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. 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. Larger structures, bulbs, may form in knotty cultures, probably as result of fusion of cells in the region of the knots (Brock 1964; Pringsheim 1957; Snellen and Raj 1970).

Physiology of *Leucothrix*. *Leucothrix* is chemoorganotrophic, strictly aerobic, and never deposits sulfur. It can use a wide variety of sugars and other organic compounds, particularly glutamate, mannose, and peptone, but not xylose as sources of nitrogen and/or carbon and energy (Bland and Brock 1973; Harold and Stanier 1955; Raj 1967). Most *L. mucor* strains require vitamin B12 for growth (Kelly and Brock 1969a, b). The deletion of calcium from the growth medium prevents rosette formation, but filaments tend to bundle (Snellen and Raj 1970). Lewin (1959) found that both Ca^{++} and K^{+} are required for growth. Ammonium can be used as nitrogen source. Na^{+} is required for growth, optimum is 1.5 % NaCl, minimum 0.3 %, maximum 7 %. Optimum temperature is 25–28 °C, maximum 32–35 °C, and it grows at 0 °C to form visible colonies within 1–2 weeks.

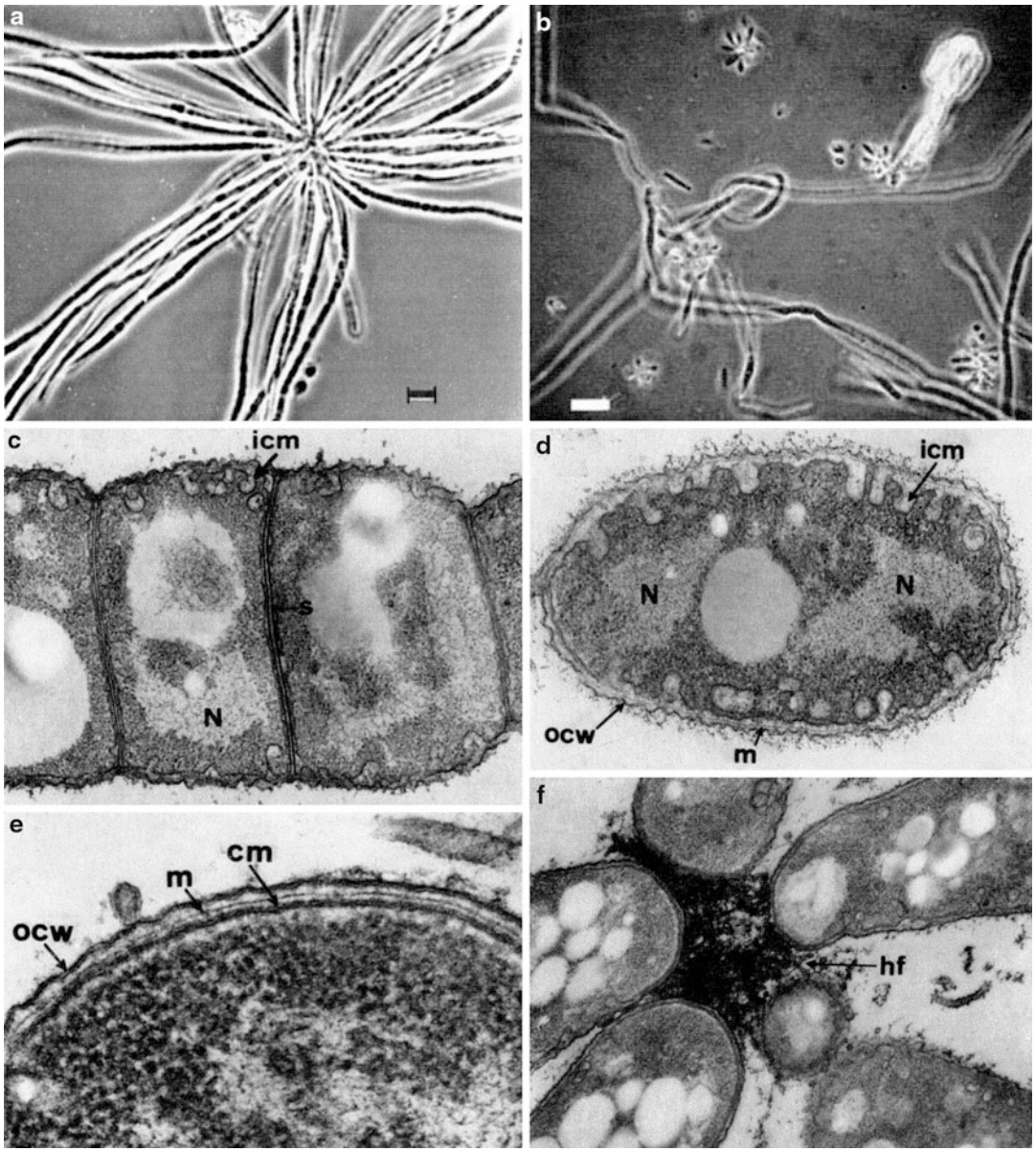

Fig. 20.4

Microscopic images of *Leucothrix spp*. (a) Rosette composed of several *Leucothrix* filaments. Phase-contrast photomicrograph. Bar = 10 μm. (b) Knots formed by filaments of *Leucothrix mucor*, and gonidia aggregates as rosettes. Phase-contrast photomicrograph. Bar = 5 μm. (c) Filament of *Leucothrix mucor* with prominent invagination of the cytoplasmatic membrane (icm) that occur on the periphery of the cells but can also be found along the cross walls that separate cells. *N* nuclear body, *s* cross wall septum, magnification ×55,000. (d) Cross sections of gonidial cell with numerous invaginations of the cytoplasmic membrane. The gonidia retain the cell envelope structure of the filaments and the fine structure of the cytoplasm. *ocw* outer cell wall, *m* middle layer of cell wall, most likely peptidoglycan, magnification ×84,000. (e) Electron micrograph of the cell envelope region illustrating that the middle layer (*m*) is single, whereas the outer cell wall layer (*ocw*) and cytoplasmic membrane (*cm*) are double unit membranes. Magnification ×155,500. (f) Cross sections through rosette of *Leucothrix mucor* filaments; the holdfast (*hf*) appears as the electron-dense material between the basal cells of the rosette. Magnification ×40,000. Photomicrographs (a) and (b), Brock 2006. Electron micrographs c–f, Brock and Conti 1969

While *Leucothrix* is characterized by a heterotrophic, aerobic metabolism, its growth is also stimulated by the presence of reduced sulfur sources that serve as auxiliary electron donors, as shown for L. *mucor* (Grabovich et al. 1999). In lithoheterotrophic growth, metabolic energy could be obtained by both substrate-linked phosporylation via adenosin-5′-phosphosulfate reductase (operating in oxidative direction from sulfite to sulfate), as well as through oxidative phosphorylation involving membrane-bound cytochromes that oxidize thiosulfate and sulfite (Grabovich et al. 1999). Yet, thiosulfate oxidation is not accompanied by the formation of intracellular sulfur globules (Grabovich et al. 1999). Informal reports of sulfur-accumulating

Leucothrix strains (as included in Brock 2006, and Bland and Brock 2005) have so far remained unconfirmed by published physiological studies or molecular analyses. Therefore, periplasmic sulfur inclusions remain a characteristic of the genus *Thiothrix*, but not *Leucothrix*.

Cell structure of *Leucothrix*. The ultrastructure of *Leucothrix* is typical prokaryotic (Brock and Conti 1969; Snellen and Raj 1970; Webster et al. 1968). The cell wall is typical gram-negative (*Fig. 20.4e*); it is multilayered, in contrast to gram-positive cell walls, running unparallel to its more nearly planar cytoplasmic membrane and the outer wall layer. The peptidoglycane layer is single and ca. 2 nm wide. The convoluted outer wall layer is double and ca. 9 nm wide, and consists of lipid, polysaccharide, and protein with the hydrophilic portion made of sugars and the hydrophobic portion of a unique lipid material (Brock 1974; Brock and Conti 1969). During the life cycle of the organism, little ultrastructural differentiation can be observed. There are no significant changes in cell wall structure during cell division, or when gonidia or rosettes are formed (*Fig. 20.4c, d*). However, the filaments in the process of knot formation show contorted cell walls and marked deformation of the cell wall septa (Brock and Conti 1969). Cell division is initiated by transverse septation, typically perpendicular to the long axis of the dividing cell. Every cell in the filament can initiate division independently. The formation and biological function of bulbs that are occasionally found along the filaments of *Leucothrix* are unknown until today. In rare cases "bulb-tubes" have been observed, describing a thin projection from one side of the bulb having a continuous connection of cell wall, cytoplasmic membrane and cytoplasm with the main body of the bulb (Snellen and Raj 1970).

All *Leucothrix* filaments show fimbrillar nuclear material, ribosomes, and some storage granules in the cytoplasmic matrix (*Fig. 20.4c–f*). Invaginations occur most frequently at the periphery of the cell, not at the septa. In electron micrographs from thin sections, the cytoplasm includes invaginations, cavities and vacuolar bodies (*Fig. 20.4c, d, f*), which are suggested to contain carbon storage compounds such as poly-ß-hydroxyalcanoates and lipids (Brock and Conti 1969). Thin sections through rosettes show the typical holdfast structure at the bottom of each attached filament as electron dense material (*Fig. 20.4f*), possibly consisting of polysaccharides that are peripheral to the outer cell wall layer (Brock 1966; Brock and Conti 1969).

General characteristics of the *genus Thiothrix*. The genus characterization by Unz and Head (2005) is amended here in the light of new species descriptions (Aruga et al. 2002; Chernousova et al. 2009) and physiological studies (Trubitcyn et al. 2013). *Thiothrix* typically occur in sewage water or attached as saprophytes to macroalgae, stones or other solid substrates in fast-flowing sulfidic streams or springs (*Fig. 20.5a*). All known species are mesophiles and neutrophiles. Depending on the species, *Thiothrix* cells are rod-shaped ranging from 0.7–4.0 µm width and 1.2–6.3 µm length, and seriate in rigid, unbranched, multicellular filaments of uniform or slightly tapering diameter. The filaments can reach lengths of over

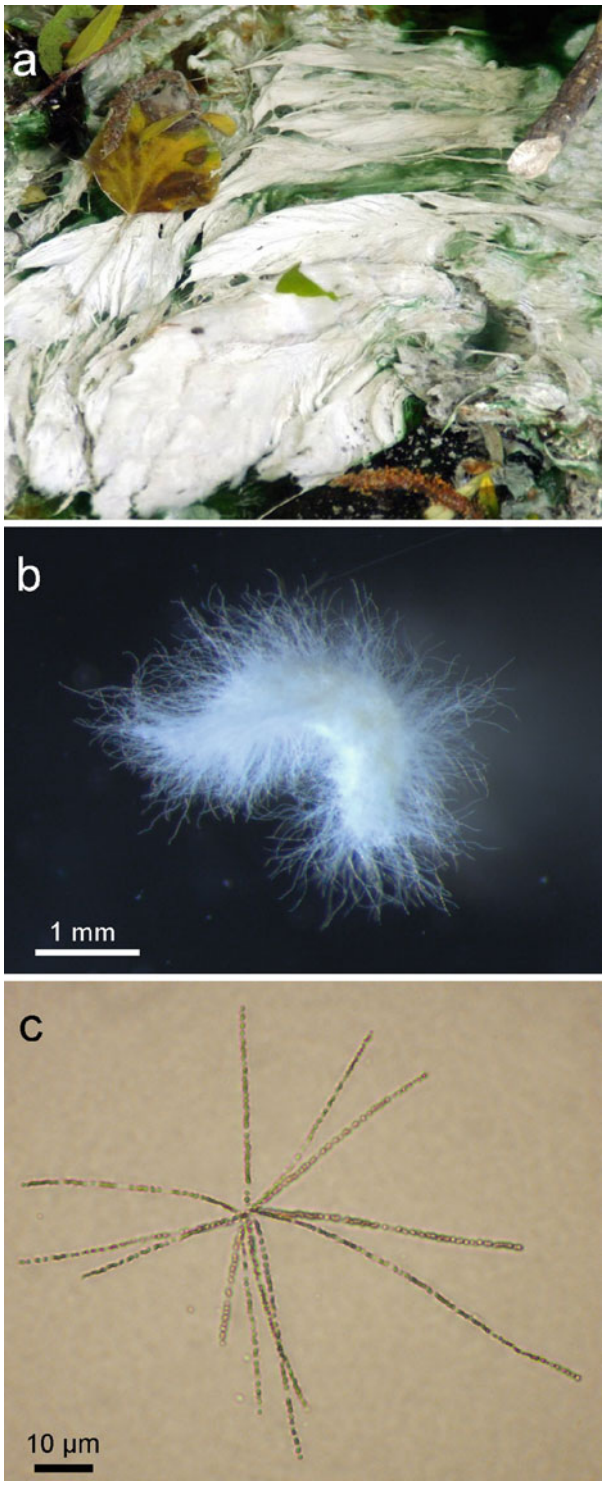

Fig. 20.5
Natural enrichment of *Thiothrix* spp. (a) White streamers of *Thiothrix* overgrowing leaf litter in the sulfidic stream near the entrance to Frasassi cave, Italy. (b) Floc of white *Thiothrix* filaments attached to small plant particle from this location. (c) Light microphotograph of rosette-forming *Thiothrix* filaments from the Frasassi location, with numerous sulfur inclusions (Photos courtesy of Lubos Polerecky, University of Utrecht, The Netherlands (a), and Verena Salman, UNC Chapel Hill (b, c))

500 µm (uniform) or 100–200 µm (tapered filaments), and can be ensheathed in some species. Rod-shaped gonidia with gliding motility are formed at the apical ends of filaments. Filaments often grow as rosettes and are anchored to a common holdfast (Fig. 20.5b, c). Only rosette-forming filaments produce gonidia. Resting stages are not known. Flagella are absent from the filaments, but the gonidia are motile due to a tuft of monopolar fimbriae.

All species grow as aerobes or microaerophiles, sometimes also by nitrate reduction to nitrite, and show facultatively autotrophic, chemoorganotrophic and mixotrophic metabolisms. The mixotrophic species require any of several small organic compounds, such as acetate, lactate, propionate, pyruvate, succinate, fumarate and oxalacetate, as well as reduced sulfur sources. Cells grown in the presence of an inorganic reduced sulfur source have intracellular sulfur globules. The sulfur globules appear to be internal by light microscopy, and electron microscopy indicates intracellular deposition within invaginations of the cytoplasmic membrane.

Physiology of *Thiothrix*. The genus *Thiothrix* includes filamentous, rosette-forming sulfur oxidizing bacteria that range in their physiology from predominantly heterotrophic species and strains (*T. eikelboomii*, *T. flexilis*, *T. disciformis*; Aruga et al. 2002) that use sulfide only as an auxiliary electron donor (Williams and Unz 1985, 1989), to species that are capable of sulfur-oxidizing autotrophic growth; the latter include '*T. ramosa*', (Odintsova et al. 1993) *Thiothrix* CT3 (Tandoi et al. 1994), *T. caldifontis* and *T. lacustris* (Chernousova et al. 2009), and a strain that was identified phenotypically as *T. nivea* (McGlannan and Makemson 1990). The neotype strain *Thiothrix nivea* has previously been described as an obligate mixotroph since pure cultures required both a reduced sulfur compound and an organic substrate for growth (Larkin and Shinabarger 1983; Strohl and Schmidt 1984; Nelson 1989); the results by McGlannan and Makemson (1990) suggest that autotrophic strains of *T. nivea* exist in nature. High rates of sulfide oxidation to sulfate (Strohl and Schmidt 1984) and oxidation of sulfur globules and sulfide to sulfate have also been reported for *T. nivea* (Schmidt et al. 1987).

The species of the genus *Thiothrix* can be divided into phylogenetically and physiologically defined clusters (Fig. 20.1) that show contrasting sulfur and carbon utilization patterns. The first is the "*T. nivea*" group, a monophyletic branch containing the species *T. nivea*, *T. unzii*, "*T. ramosa*", *T. fructosivorans*, *T. caldifontis*, and *T. lacustris*, where the first three require sulfur as electron donor for growth and the others prefer reduced sulfur compounds for mixotrophic growth.

The second major group is the "Eikelboom type 021N" filaments (Eikelboom 1975, 1977) represented by the species *T. eikelboomii*, *T. disciformis*, *T. defluvii*, and *T. flexilis*. The Eikelboom type 021N strains grow heterotrophically on a wide range of carbohydrates without requiring reduced sulfur (Aruga et al. 2002), while *T. nivea* JP2^T and *T. unzii* A1^T require reduced sulfur for growth (Larkin and Shinabarger 1983; Williams and Unz 1985). The filaments of Eikelboom type 021N bacteria are very long and reach several millimeters in length, while those of members of the *T. nivea* group are less than 1 mm long (Eikelboom 1975; Kanagawa et al. 2000; Williams and Unz 1985). The Eikelboom type 021N strains form fingerprint-like colonies, a characteristic different from members of the *T. nivea* group (Williams and Unz 1985; Kanagawa et al. 2000). In contrast to the Eikelboom type 021N strains, *T. nivea* JP2^T and *T. unzii* A1^T have no catalase activity (Larkin and Shinabarger 1983; Williams and Unz 1985). *T. nivea*, '*T. ramosa*' and *T. fructosivorans* form sheaths (Larkin and Shinabarger 1983; Odintsova and Dubinina 1990b; Williams et al. 1987), while the Eikelboom type 021N strains do not.

Cell structure of *Thiothrix*. The following summary of *Thiothrix* cell structure is based on the microscopic and ultrastructural studies of Bland and Staley (1978), and Williams et al. (1987) that should be consulted for further information. Multiple filaments, each consisting of several hundred seriate cells, are growing as a rosette emerging from a central branching point (Figs. 20.5c and 20.6e). Multicellular *Thiothrix* filaments consist of generally cylindrical cells; barrel-shaped, cuboidal, bead-like or discoid cell morphologies can also be found (Fig. 20.6a–d). The filaments are attached to each other and/or a solid surface (Fig. 20.5b) by an extracellular holdfast matrix that appears to be secreted by the basal cells of the rosette-forming filaments (Fig. 20.6e, f). The tips of individual filaments may divide into gonidial cells that, upon release, disperse, divide and then form the nucleus of new filaments, or aggregate into small rosettes of gonidia that grow into new rosettes of full-length filaments (Fig. 20.3). The gonidia have tufts of fimbriae that are located predominantly around one pole of the cell; these fimbriae attach to each other and establish intracellular contact (Larkin and Nelson 1987). The filaments of some *Thiothrix* species are surrounded by sheaths (Fig. 20.6b, g, h); in contrast to other sheathed sulfur-oxidizing bacteria, for example the genus *Thioploca*, each sheath contains only a single filament in *Thiothrix*. An unusual structural feature of *Thiothrix* filaments that requires additional documentation and follow-up study are extensive filamentous appendages that appear to grow on the fully developed filaments (sampled from a natural sulfur spring) like a coat of bristles (Morita and Burton 1965; Larkin 1980).

Thiothrix cells contain sulfur globules that are surrounded by two membranes; the vacuolar membrane immediately adjacent to the sulfur granule is surrounded by a distinct cytoplasmatic membrane that is sometimes connected to the cytoplasma membrane by invaginations (Fig. 20.6i). The sulfur globules are therefore topologically located in the periplasmic space. As in the family *Beggiatoceae*, sulfur globules in *Thiothrix* function as an electron donor reservoir; they are formed and consumed depending on the availability of reduced sulfur sources in the environment. Polyhydroxyalkanoates (Fig. 20.6a, d) and polyphosphates (Fig. 20.6f) form additional cytoplasmatic cell inclusions.

Each cell of *Thiothrix* is surrounded by a cytoplasmic membrane, followed by a peptidoglycan layer located within the periplasmic space, and an outer membrane of variable thickness due to additional membrane layers that appear in some species (Fig. 20.6j–m); in some species the sheath completes the cell envelope (Fig. 20.6m). Detailed chemical studies have

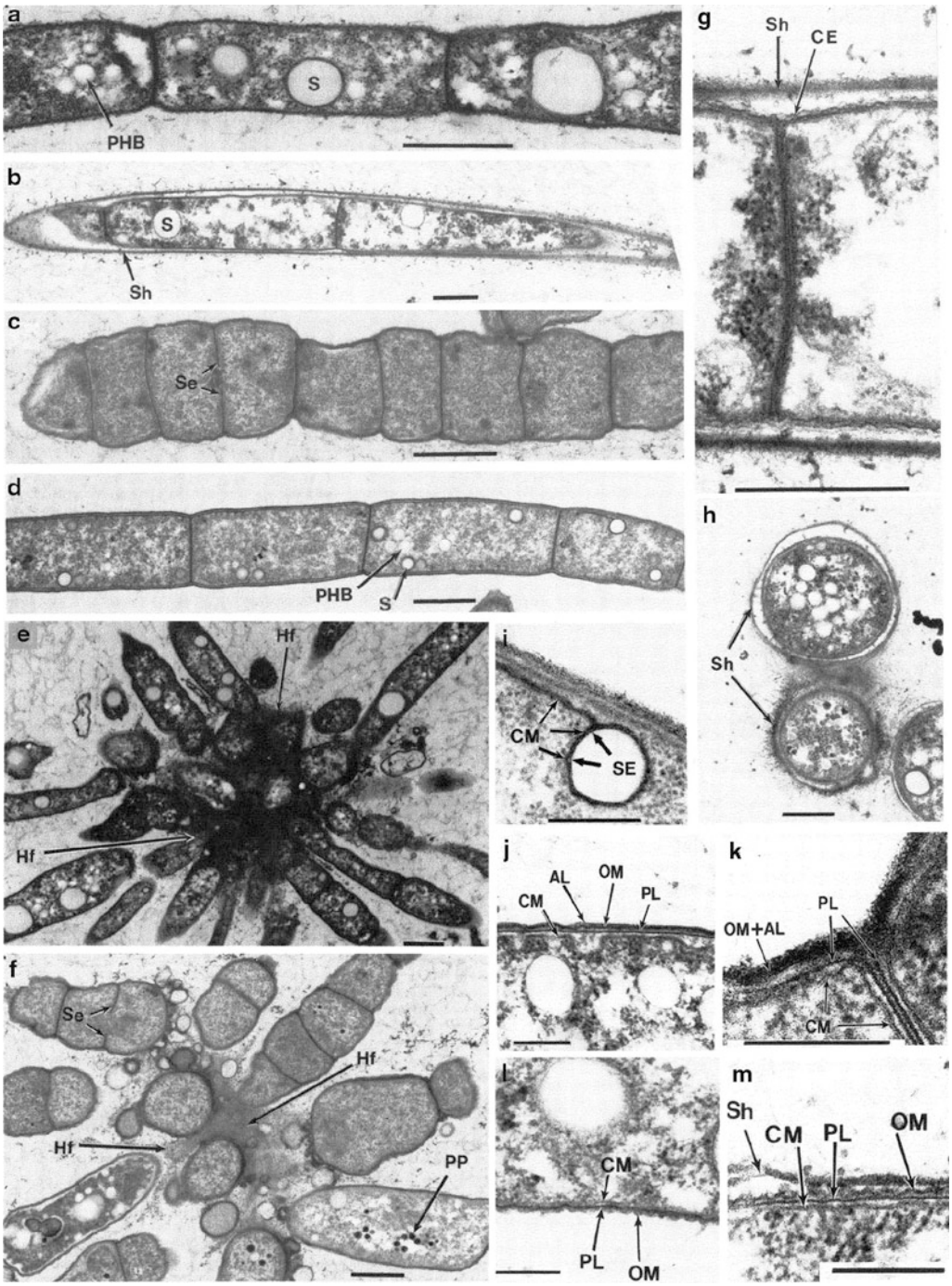

Fig. 20.6

Microscopic images of *Thiothrix*. General ultrastructural characteristics of filaments of *Thiothrix* and ''type 021N'' bacteria. (**a**) *Thiothrix unzii* strain A1. (**b**) *Thiothrix fructosivorans* strain Q. (**c**) *Thiothrix* ''type 021N'' strain N7. (**d**) *Thiothrix* ''type 021N'' strain N2. (**e**) Thin section through rosette in *Thiothrix unzii* strain A1. (**f**) Thin section through rosette in *Thiothrix* ''type 021N'' strain N7. (**g**) Sheath characteristics in *Thiothrix fructosivorans* strains Q; the sheath is visible along filament with septum separating two cells. (**h**) The sheath is shown surrounding a filament in cross section. (**i**) sulfur inclusions in *Thiothrix* ''type 021N'' strain N2, with two membrane layers consisting of sulfur inclusion membrane (*inside*) and cytoplasmic membrane (*outside*), the latter form extensions into the cytoplasm and are likely to continue towards the outer cytoplasmic membrane. Panels **j**–**m**: Cell membrane and envelope structure in *Thiothrix* ''Type 021N'' strain N7 (**j**) and strain N2 (**k**), *Thiothrix unzii* strain A1 (**l**) and *Thiothrix fructosivorans* strain Q (**m**). Abbreviations: *AL* additional outer layers, *CE* cell envelope, *CM* cytoplasmic membrane, *Hf* holdfast material, *PP* polyphosphate, *Se* cell septum, *PHB* polyhydroxybutyrate, *PL* peptidoglycan layer of cell wall, *PP* polyphosphate, *OM* outer membrane, *S* sulfur inclusion, *Se* septum, *SE* sulfur inclusion envelope, *Sh* sheath. Scale bars **a**–**f**, 1.0 μm. Scale bars (**g**, **h**), 0.5 μm. Scale bars (**l**–**m**), 250 nm (Electron micrographs modified from Williams et al. 1987)

investigated the complex polysaccharide structure of these sheaths in *T. nivea* (Takeda et al. 2012) and *T. fructosivorans* (Kondo et al. 2013). Cell septa consist of four distinct layers, the cytoplasmic membrane and the periplasmic layer on each side (Fig. 20.6k). During cell division within a filament, cell septa grow from opposite sides of the peripheral cell membrane into the cytoplasm until they meet in the center of the dividing cell and separate it into two daughter cells; as the new cell septum tightens and closes, electron-dense material that contrasts visually with the cytoplasm (potentially genomic DNA) congregates near the closing hole at the septa and appears to be redistributed between the separating daughter cells (Fig. 20.6c, f).

Structural similarities and differences among *Leucothrix and Thiothrix*. Most observations on *Thiothrix* morphology and cell structure also apply to *Leucothrix*. The gonidia-forming filaments and rosettes of *Leucothrix* (Fig. 20.4a, b) resemble those of *Thiothrix* (Fig. 20.5c). Individual filaments of *Leucothrix* often form knots, which is not observed in *Thiothrix*; this presumably results from asymmetrical growth on different sides of an individual filament that causes the filament tip to curve during filament elongation until genuine knots are completed (Figs. 20.3 and 20.4b). Also, in contrast to *Thiothrix*, sulfur globules are absent in cells of the genus *Leucothrix*; however, their filament cells and gonidial cells show extensive cytoplasmic invaginations (Fig. 20.4c, d). The multilayered cell envelope of cytoplasmic membrane, periplasmatic membrane (most likely a peptidoglycan layer) and outer cell wall resembles that of *Thiothrix* (Fig. 20.4e). The electron-dense holdfast structure surrounding the basal cells of rosette-forming *Leucothrix* filaments (Fig. 20.4f) is also closely reminiscent of its counterpart in *Thiothrix* (Fig. 20.6e).

Isolation, Enrichment and Maintenance Procedures

Enrichment and isolation of *Leucothrix*. Isolates of *Leucothrix* can be obtained using a basal salt medium (Brock 2006) with a low phosphate concentration, as this compound was reported to inhibit the growth of *Leucothrix*. The basal salt medium contains (per liter of deionized water) 11.75 g NaCl, 5.35 g $MgCl_2$ * 6 H_2O, 2.0 g Na_2SO_4, 0.75 g $CaCl_2$ * 2 H_2O, 0.35 g KCl, 0.5 g Tris(hydroxymethyl)aminomethane, 0.05 g $NaHPO_4$; the pH is adjusted to 7.6. Glutamate suffices as the sole source of carbon, nitrogen and energy for most strains, and—by adding 0.1 % monosodium glutamate (MSG) to basal salts and 2 % agar—allows the isolation of strains from environmental materials. Other useful supplements 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. The concentration of organic compounds should be kept low to avoid overgrowth by non-filamentous 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 for the characteristic coiled rope or thumbprint morphology of *L. mucor* colonies. Brock (2006) suggests a combination of a 12.5× eyepiece and 10× phase-contrast microscope objective for early identification of *Leucothrix* filaments, before nascent colonies are overgrown by bacterial epibionts. These colonies are picked by touching them with a sterile insect pin and transferring them to fresh agar plates of the same composition. This enrichment and microscopic monitoring strategy allows to isolate *Leucothrix* colonies directly from filaments still attached to seaweed fronds, and to identify the precise source habitat of an isolate; 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, the inoculum often grows best (overnight) when placed in a small (1–2 ml) volume of medium. These pre-cultures can serve as the inoculum for the buildup of large-volume cultures in large flasks. 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 suitable; the yeast extract provides growth factors needed by some strains, and the sodium lactate substantially increases the yield of most cultures.

Enrichment and Isolation of *Thiothrix*. Multiple protocols have been developed for the isolation of *Thiothrix* spp.: Enrichment in slide culture (Bland and Staley 1978), physical separation and plate streaking of *Thiothrix* tufts (two different procedures based on Strohl and Larkin 1978 or Williams and Unz 1985), and pre-enrichment in agar tubes (Williams and Unz 1985). The media formulations described here favor strains with heterotrophic capabilities; the enrichment and isolation of a broader spectrum of facultatively autotrophic, sulfur-oxidizing strains (Chernousova et al. 2009) may require different media (Armbruster 1969).

1. *Slide culture.* Slide culture has been used to enrich *Thiothrix* from natural sulfur spring habitats; *Thiothrix* rosettes and filaments growing on the glass slides provide suitable material for microscopy (Bland and Staley 1978). Slides are coated with the medium of Morita and Burton (1965) containing per liter of natural sulfur spring water: 2 g yeast extract, 0.1 g $CaCl_2$, 0.5 g sodium acetate, and 15 g agar. Slides inoculated with *Thiothrix* material were covered with a thin, gas-permeable Teflon membrane, placed in a desiccator containing water and 5 g of sodium sulfide, and incubated at 14 °C. The slides are periodically checked by phase contrast microscopy for filaments and rosettes that are developing attached to the agar-coated slide (Bland and Staley 1978).
2. *Physical separation and plate streaking I.* Fine-tipped forceps select for tufts of suspected *Thiothrix* filaments from *Thiothrix*-containing material contained in a Petri dish while under observation with a dissecting microscope and transmitted light (Larkin 1989). Bundles of filaments are

transferred to a second Petri dish containing about 5–10 ml of a salt solution SS-1 (Strohl and Larkin 1978), agitated by forceps, and then transferred to another petri dish containing salt solution SS-1. This salt solution contains (per liter) 200 mg NH_4Cl, 10 mg $MgSO_4$, 10 mg $CaSO_4$, and 5 ml trace element solution (per liter: 10 mg $ZnSO_4 * 7H_2O$, 20 mg $MnSO_4 * 4\ H_2O$, 0.5 µg $CuSO_4 * 5\ H_2O$, 10 mg H_3BO_3, 1 mg $Co(NO_3)_2$, 1 mg Na-Molybdate, 200 mg EDTA, 700 mg $FeSO_4 * 7\ H_2O$). The procedure is repeated through four or five transfers. A few drops from each dilution are transferred with a Pasteur pipette to either MP agar (SS-1 plus 0.01 % each of sodium acetate nutrient broth powder, and yeast extract, plus 0.03 % Na_2S, and 1.5 % agar; Strohl and Larkin 1978) or MY agar (SS-1 plus 0.01 % each of sodium acetate, nutrient broth agar, and yeast extract, plus 0.03 % Na_2S, and 1.5 % agar; Larkin 1980) in separate Petri dishes. Each dish is held at an angle so that the drops will flow across the agar surface. The excess is then withdrawn from the other side with the pipette. The dishes are incubated at about 20–30 ºC and are examined daily under transmitted light with the aid of a dissecting microscope. Colonies with a hairy or filamentous edge are transferred with sterile toothpicks to fresh media and are restreaked until pure.

3. *Physical separation and plate streaking II.* A washing-sonication pretreatment step helps to increase the density of filamentous bacteria in the inoculum and to reduce the content of adventitious microorganisms. Several loopfuls of enrichment culture surface film or activated sludge (10^{-1} dilution) samples are transferred to small glass Petri dishes containing 7 ml of sterile Mineral salts vitamin mix (MSV; see paragraph below). With the aid of a stereomicroscope at 15–45-fold magnification, approximately 40–50 single filaments or rosettes are individually transferred with sterile glass micropipettes through a series of six to seven washings in fresh MSV. The inoculum of washed filaments is transferred to 3 ml of MSV and both spread and streak-plated on glucose-sulfide or LT medium. Glucose-sulfide medium contains (per liter): 0.15 g glucose, 0.5 g $(NH_4)_2SO_4$, 0.01 g $Ca(NO_3)_2$, 0.05 g $K_2HPO_4 * 7\ H_2O$, 0.05 g KCl, 0.1 g $CaCO_3$, 0.187 g $Na_2S * 9\ H_2O$, 1.0 ml vitamin mix, 15 g agar; final pH is 7.5. LT medium contains per liter of MSV: 0.5 g sodium lactate, 0.5 g $Na_2S_2O_3 * 5\ H_2O$, and 12 g agar. All media are adjusted with NaOH to pH 7.2–7.5. Plates are incubated at 20–22 °C and examined periodically (15–45-fold magnification) for evidence of filamentous colonies. Suspect colonies are transferred three times on primary isolation media and once on SCY and CGY media (see Williams and Unz 1985) to ascertain purity.
4. *Enrichment in agar tubes.* This approach has been used for the isolation of *Thiothrix*, *Leucothrix*, and *Beggiatoa* spp. (Williams and Unz 1985). Enrichment cultures can provide inocula for pure culture isolation if *Thiothrix* strains cannot be isolated by direct plating of mixed liquid samples. 1 ml of activated sludge fluids are transferred to test tubes containing 5 ml of solid media (per liter: 1.5 g glucose and/or 0.6 g $Na_2S * 9\ H_2O$ plus 2 % Bacto-Agar) and an overlay of 15 ml of mineral salts-vitamin mix (MSV) containing the following ingredients per liter: 0.5 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4 * 7\ H_2O$, 0.05 g $CaCl_2 * 2\ H_2O$, 0.11 g K_2HPO_4, 0.085 g KH_2PO_4, 0.002 g $FeCl_3 * 6\ H_2O$, 0.003 g EDTA, and 1 ml vitamin solution (Eikelboom 1975). Cultures are incubated at final pH of 7.2–7.5 and at a temperature of 22–25 °C, and examined microscopically for evidence of *Thiothrix* trichomes in the surface biomass.
5. *Strain maintenance.* The type species can be maintained in semisolid (0.15 % agar) deeps of either MP or MY medium, with transfer intervals of ca. 3–4 weeks. Axenic cultures of other *Thiothrix* spp. can be maintained by cryopreservation at −83 °C to −90 °C, or at 10 °C by monthly transfers on LTH medium, which contains the following per liter of MSV: 1.0 g sodium lactate, 0.5 g $Na_2S_2O_3 * 5\ H_2O$, and 0.01 M HEPES buffer (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid). If required, the pH is adjusted to about 7.5 with NaOH (Unz and Head 2005).
6. *Testing for special characteristics.* MP broth or appropriate modifications are used to test sole carbon (0.05 %), nitrogen (0.02 %) and sulfur (0.03 %) sources in support of sustained growth of *T. nivea* strains through three successive transfers (Larkin 1989). Other *Thiothrix* species were nutritionally evaluated in MSV; optical density equal to more than four-fold greater than the controls counted as positive result (Williams and Unz 1989).

Ecology

Host associations of *Leucothrix*. In its marine habitat, *Leucothrix* filaments grow typically as epiphytes on marine macrophytes, and in association with marine invertebrates that offer suitable colonization surfaces for bacterial epibionts (reviewed in Raj and Ordal 1977). *Leucothrix* colonizes marine algae, arthropods, or rocky shores aerated by wave action (Brock 1966; Harold and Stanier 1955; Kelly and Brock 1969a; Pringsheim 1957). Due to its aerobic nature, *Leucothrix* is never found is slow-moving or stagnant waters, and the attachment to motile animals or flexible algal filaments constitutes an ecological advantage for the non-motile *Leucothrix*, interplaying winds, waves, currents and nutrients (Raj and Ordal 1977). *Leucothrix* overgrowth can cause 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 cultivation of lobsters.

Especially in temperate waters, *Leucothrix* growth density on seaweeds can be substantial (Kelly and Brock 1969a). *Leucothrix* occurs on red, green, and brown algae, and prefers intertidal rather than subtidal location (Bland and Brock 1973). In special cases host specificity can be observed; under laboratory conditions a preference of *L. mucor* for the red algae *Antithamnion sarniense* and *Bangia fuscopurpurea*, and the brown alga *Sphacelaria* sp., but not the red alga *Rhodochorton* sp., was observed (Bland and Brock 1973; Brock 1966). Several marine algae are known to produce significant amounts of fixed carbons

and other compounds, supporting growth of a number of different more or less associated heterotrophic bacteria, but some algal species also produce antimicrobial agents such as sulfuric acid or acrylic acid. Their cuticle or mucilaginous covering typically consists of mannans and mannose, with a wall composed of xylan. *L. mucor* cannot metabolize xylan but mannose, which is why it attaches only to the cuticle but does not penetrate to the wall. At night, when the alga stops the synthesis of its cuticle material, large numbers of *L. mucor* gonidia are released (Bland and Brock 1973).

Unusually dense growth on the filamentous red alga *Bangia fuscopurpurea* that is exposed to air for extended periods of time during low tide highlights the preference of *Leucothrix* for ample aeration, and the recruitment of larger structures as medium to reach full-air regions of the intertidal zone. At low tide, *Leucothrix* is found at the underside of the dry algae mats, maintaining moist conditions. Furthermore, there seems to be a correlation of *Leucothrix* density and algal age, indicating true growth on the alga and not incidental attachment. Brock (1966) showed that *Leucothrix* can grow on substances liberated by the alga, and a few years later Bland and Brock (1973), after conducting a variety of ecological studies, found that *Leucothrix* receives most of its nutrients from the algae and not from seawater. The attachment to its algal host is thus virtually obligate for survival, and it is the gonidium that initiates the association (Bland and Brock 1973; Harold and Stanier 1955).

Studies of the association of *Leucothrix* filaments with the red alga *Gelidium linguatum* at the methane seep area west of Mocha Island, off central Chile (*Fig. 20.2*), demonstrate that the direct exposure to methane seepage can result in assimilation of carbon derived from methane into *Leucothrix* (Jessen et al. 2011; Sellanes et al. 2011). The *Leucothrix* filaments show an unusually light carbon isotopic signature ($\delta^{13}C$ value of -39.2 ± 2.5 ‰) indicating that assimilated carbon was derived from local methane seepage. The filaments in turn provide an important source of methane-derived carbon for grazing invertebrates (Sellanes et al. 2011); the filaments are grazed by the tanaids *Nototanais dimorphus* and *Zeuxo marmoratus*, and by other amphipods (Sellanes et al. 2011, Sellanes personal observation). The filaments were identified by 16S rRNA sequencing and affiliate with other epiphytic filamentous *Leucothrix* spp. (*Fig. 20.1*).

Leucothrix does not only form associations with algae but also colonizes the eggs of commercially important fish (e.g. from cod fish and winter flounder), the larvae and carapaces of benthic marine crustacea; it overgrows the antenna of hermit crabs and the gills of horseshoe crabs, and forms heavy infestations on zooplankton, gravid rock crabs, small unidentified prawns and other benthic copepod and decapod crustaceans (Raj and Ordal 1977). Although *L. mucor* is not a pathogen in the sense that it affects human physiology, it can cause environmental damage; for example it triggers high crustacean mortalities by causing eggs to sink below the surface and by interfering with the filter apparatus of larvae. Details about the associations of *Leucothrix* and crustaceans remain to be studied, however, the frequent observation of feeding of their bacterial films by the host suggests a contribution of the epiphyte to the hosts daily nutrition (Johnson et al. 1971; Raj and Ordal 1977). Studies of the association of different kinds of bacteria, including *Leucothrix* filaments, with the septae of the Galatheid crab *Shinkaia crosnieri* showed that labeled bicarbonate was assimilated into the epibiotic microbial communities on the specialized tissues of the deepsea invertebrate. Interestingly, the incorporation of $^{13}HCO3^-$ into the epibiotic microbial communities was stimulated by the addition of sulfide and thiosulfate, but not by molecular hydrogen, strongly suggesting primary production by facultative thiotrophic (sulfur-oxidizing) energy metabolism (Watsuji et al. 2010), but whether this also includes the *Leucothrix* filaments needs to be verified.

Although *Leucothrix* attaches to a wide variety of substrates in nature, any association with abiotic surfaces such as rocks seems to be short-lived, and the filaments prefer a living host for permanent colonization. This observation is congruent with the requirement of most *Leucothrix mucor* isolates for vitamins (Kelly and Brock 1969a), which they might receive from the host or from other associated microorganisms.

The surface-attached lifestyle of *Leucothrix* suggests that this bacterium competes effectively with other epibionts, and might be a source as well as a recipient of signaling compounds and growth inhibitors. *Leucothrix* is sensitive to antibiotics and its growth can be inhibited by penicillin (0.1 mg/l), streptomycin (5.0 mg/l), or chloromycetin (0.7–0.9 mg/l) (Raj and Ordal 1977). *Leucothrix* strain N11, isolated from industrial chemical wastewater (Williams and Unz 1985), did not grow under exposure to >0.125 mg/l of streptomycin, gentamicin, tetracycline, ampicillin, and penicillin G. The same strain is resistant to sulfanilamide and lincymycin at >0.64 mg/l, and is sensitive to chloramphenicol (0.5 mg/l) and bacitracin (4.0 mg/l).

Host associations of Thiothrix. Its attached growth and filamentous morphology allows *Thiothrix* to form opportunistic or symbiotic associations with other microorganisms in aquatic habitats. An intriguing example are the "String-of-pearl" colonies that consist of *Thiothrix* filaments associated with uncultured archaea that grow attached to plant material in cold sulfidic freshwater springs (Rudolph et al. 2001; Moissl et al. 2002, 2003). The *Thiothrix* symbiont (Genbank accession number of 16S rRNA sequence, AJ307933; related to *T. unzii*) can be replaced by other, presumably sulfur-oxidizing filamentous bacteria, for example uncultured epsilonproteobacteria (Rudolph et al. 2004). The archaeal community is also variable and includes specific members of the euryarchaeota (the SM1 lineage) as well as diverse crenarchaeota (MG-1 and other lineages) (Koch et al. 2006). As a working hypothesis, the "String-of-pearl" colonies appear to remain viable in different taxonomic composition as long as biogeochemical functionality is maintained; however, the biochemical basis of this association and its possible symbiotic nature are not yet understood.

Members of the genus *Thiothrix* form associations with invertebrates that are host- and strain-specific, as shown by the best-studied example, the epibiotic association of *Thiothrix* with

the freshwater amphipod *Niphargus*. *Thiothrix* filaments growing attached to the exoskeleton of the amphipod *Niphargus* are alternately exposed to sulfide and oxygen and can assimilate CO_2, as the host amphipod spends most of its time in oxygenated waters but also dives to the sulfide/oxygen interface (Dattagupta et al. 2009). The amphipod host could also benefit from its chemosynthetic epibiont as a possible food source, but this symbiotic linkage remains to be proven. Molecular studies have shown that two different species of the amphipod *Niphargus* occur associated with three distinct populations of *Thiothrix* ectosymbionts in the sulfidic waters of the Frasassi cave system in Italy (Bauermeister et al. 2012); the ectobiont phylotypes are distinct from free-living *Thiothrix* sp. in the cave waters, and indicate intra- or interspecific inoculation among *Niphargus*. Similar results were found for *Thiothrix*/*Niphargus* associatons in sulfidic aquifers and the sulfidic Movile cave ecosystem in Romania; the *Niphargus*-specific *Thiothrix* ectobiont clusters did not overlap with free-living *Thiothrix* populations from Movile cave (Flot et al. 2013).

These *Thiothrix*/host associations may be to a large extent opportunistic. A *Thiothrix* population colonizes the outer surface of a motile aquatic host organism as long as this carrier shuttles between sulfidic and oxygenated waters and provides suitable redox conditions for *Thiothrix*. Without sulfide exposure, the host may not harbor any *Thiothrix* epibionts. For example, *Thiothrix* filaments cover the larvae of the mayfly (*Drunella grandis*) in a specific location where a sulfidic spring enters the freshwater creek habitat of the mayfly larvae. The *Thiothrix* filaments grow as epiphytes on the larvae, and appear to host intracellular bacterial parasites that remain so far unidentified (Larkin et al. 1990).

Under certain conditions, marine habitats can also harbor *Thiothrix*; their 16S rRNA sequences branch off at the base of the *Thiotrix* cluster (*Fig. 20.1*). For example, seasonal benthic sulfur-oxidizing mats on decaying plant material in a brackish coastal lagoon in Greenland contained abundant *Thiothrix* filaments; possibly, meltwater input in the arctic summer freshens the water in the lagoon sufficiently to allow the growth of *Thiothrix* mats (Glud et al. 2004). A marine amphipod crustacean harbored an epibiotic *Thiothrix* population that, by 16S rRNA gene sequence, constitutes a sister lineage to *T. eikelboomii* and *T. disciformis* (AY426613, Gillan and Dubilier 2004). A related *Thiothrix* population was forming flocs in the wastewater treatment system of a mariculture facility supplied with artificial seawater of 20 ppt salinity (DQ067608, Cytryn et al. 2006). Another marine occurrence could be the population of *Thiothrix*-like filamentous bacteria found as ectobionts on the cecum nodule of a deposit-feeding echnoid, *Echinocardium cordatum*, identified by filamentous morphology and by immunostaining with a *Thiothrix*-targeted antibody. Untypically, sulfur globules were not reported, and 16S rRNA sequencing would be required to substantiate the genus identification (Brigmon and de Ridder 1998). Similarly to *Leucothrix*, these marine *Thiothrix* populations could have relevance as a link in the food web that recycle secondary chemosynthetic production into higher trophic levels.

The oxidized niche of *Thiothrix*. Freshwater habitats characterized by mixed sulfidic and oxic fluids, including sulfur springs, vents, and irrigation ditches, provide suitable habitats for a variety of sulfur bacteria, but they constitute the primary habitat for *Thiothrix* (Bland and Staley 1978; Larkin and Strohl 1983; Strohl and Schmidt 1984; Macalady et al. 2008; Konkol et al. 2010). *Thiothrix*-dominated sulfur-oxidizing mats occur frequently in sulfidic waters of limestone caves (karst caves), where they oxidize reduced sulfur species to sulfate, and generate acidity that contributes to limestone dissolution (Brigmon et al. 1994; Engel et al. 2010; Steinhauer et al. 2010). A systematic comparison of the geochemical niches and growth forms of *Beggiatoa* and *Thiothrix* in a sulfidic karst cave stream (Frasassi cave complex, Italy) showed that *Thiothrix* filaments grow predominantly as streamers exposed to flowing water, and prefer lower sulfide (100–200 μM) and higher oxygen concentrations (up to 10–15 μM); in contrast, *Beggiatoa* filaments grow as stable biofilms under higher sulfide concentrations (mostly 150–500 μM) and lower oxygen concentrations (max. 5 μM; Macalady et al. 2008). Relatively oxidizing conditions and convective mixing of sulfidic and oxygenated waters select for the surface-attached rosette-forming growth of *Thiothrix* filaments, similar to the attached, rosette-forming sulfur oxidizer "*Candidatus* Marithrix" that forms a distinct phylogenetic lineage within the *Beggiatoaceae* (Teske and Salman 2014). Attached, rosette-forming, filamentous sulfur-oxidizing bacteria have therefore evolved twice—*Thiothrix* and *Leucothrix* in the *Leucotrichaecae*, and "*Candidatus* Marithrix" in the *Beggiatoaceae*—and have adapted to the same ecological niche of surface-attached growth sustained by well-mixed aerobic and sulfidic waters.

Applications

Role of Thiothrix in wastewater treatment. *Thiothrix* are conspicuous microbial community members in activated sludge in wastewater treatment plants; this man-made dynamic habitat is characterized by strong fluctuations in carbon substrates, sulfur sources, and oxygen and nitrate availability. Large numbers of *Thiothrix* filaments and flocs in the sludge interfere with sludge settling and lead to filamentous sludge bulking. *Thiothrix* filaments are well suited to the wastewater plant environment due to their versatile carbon assimilation patterns that can be examined using microautoradiography (Andreasen and Nielsen 1997) and FISH hybridization (Wagner et al. 1994; Nielsen et al. 1998, 1999). Several physiological findings stand out (Nielsen et al. 2000): (1) *Thiothrix* in activated sludge can grow heterotrophically as well as autotrophically and assimilate DIC as well as acetate; (2) DIC incorporation is strongly stimulated by acetate addition, indicating that acetate serves not only as a carbon source, but also as an energy source that facilitates autotrophic carbon fixation; (3) both uptake rates are increased further in the presence of the electron donor thiosulfate; (4) after thiosulfate addition and oxidation, sulfur accumulation in the cytoplasm proceeds not only aerobically,

but also with nitrate as electron acceptor; (5) under anaerobic conditions in the absence of nitrate, *Thiothrix* appears to use its intracellular sulfur globules as an electron acceptor reserve, similar to freshwater *Beggiatoa* spp. (Nelson and Castenholz 1981). Similarly, *Thiothrix*-dominated flocs accumulate transiently during sulfidic episodes within the wastewater treatment steps of a marine fish culture system and show high rates of sulfide oxidation under oxic as well as anoxic conditions in the presence of nitrate (Cytryn et al. 2006). These results from wastewater treatment facilities in different locations (Denmark and Israel) suggest that *Thiothrix* populations can persist as nitrate-reducing or sulfur-reducing facultative anaerobes.

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21 The Family *Methylococcaceae*

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Abstract
The family *Methylococcaceae* includes the type I methanotrophs, bacterial taxa belonging to class *Gammaproteobacteria* able to use methane and methanol as sole carbon and energy sources but are unable to use substrates containing carbon-carbon bonds. Phylogenetically the family is polyphyletic and includes three distinct clades. Nevertheless, all three clades have characteristics typical of type I methanotrophs including intracellular membranes arranged in lamellar stacks, possession of the particulate version but not usually the soluble version of methane monooxygenase, and utilization of the ribulose monophoshate pathway to assimilate C_1 carbon units. Members of the *Methylococcaceae* are found in any environment where methane and oxygen coexist including cold to thermal environments in both terrestrial and marine locations. Type I methanotrophs are efficient oxidizers of methane and have been applied as biofilters in industrial and remediation applications. Ecologically type I methanotrophs intercept much of the methane generated either biotically or abiotically and thus have a critical role in Earth's carbon cycles and natural homeostatic processes.

Taxonomy, Historical and Current Short Description of the Family *Methylococcaceae* Whittenbury and Krieg 1984 Emend. Bowman et al. 1993

The methanotrophs are a group of bacteria specialized in the utilization of methane and methanol and are a subset of the methylotrophs; organisms able to utilize C_1 compounds. Most methanotrophs are unable to grow on organic compounds possessing carbon-carbon bonds and are thus obligately methylotrophic. Methanotrophs are present in many natural ecosystems consuming much of the methane that is biogenically formed via methanogenesis or abiotically generated, for example, seeping from the ocean seabed, natural gas fields, and within coal mines. Methanotrophs are able to utilize methane as a carbon and energy source owing to possession of a number of unique features. Firstly, they possess the enzyme methane monooxygenase (MMO) which occurs in virtually all species as a copper-dependent membrane-bound protein and is referred to as particulate methane monooxygenase (pMMO). Some methanotrophs also possess an iron-dependent cytoplasmic equivalent referred to as soluble methane monooxygenase (sMMO). MMO carries out the first step of dissimilatory methane oxidation, where methane is oxidized to methanol.

There are several known clades of methanotrophs. The biology of these methanotrophic clades differs in terms of internal ultrastructure, carbon assimilation pathways, lipoquinone and fatty acid composition, and certain other physiological and biochemical aspects (Op den Camp et al. 2009). The differences between these groups of methanotrophs are summarized in *Table 21.1*. At present the type I methanotrophs are housed

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Table 21.1
Characteristics defining the different phylogenetic clades of methanotrophic bacteria[a]

Characteristics	Type Ia	Type Ib	Type Ic	Type II	Type II	"*Methylacidiphilium*"
Phylogenetic group	Class *Gammaproteobacteria*			Class *Alphaproteobacteria*		Phylum Verrucomicrobia
Family	*Methylococcaceae*			*Methylocystaceae*	*Beijerinckiaceae*	"*Methylacidiphiliaceae*"
Genera (valid names only)	*Methylococcus*	*Methylomonas*	*Methylohalobius*	*Methylocystis*	*Methylocella*	"*Methylacidiphilium*"
	Methylocaldum	*Methylobacter*	*Methylothermus*	*Methylosinus*	*Methylocapsa*	
	Methylogaea	*Methylomicrobium*				
		Methylosarcina				
		Methylovulum				
		Methylomarinum				
		Methylosoma				
		Methylosphaera				
		Crenothrix				
Intracytoplasmic membranes	Type I (lamellar stacks of membranes)			Type II (stacks of membranes parallel to cytoplasmic membrane)		Carboxysome-like structures or vesicular membranes
Resting cell type if present	*Azotobacter*-type cysts	"Immature" *Azotobacter*-type cysts	*Azotobacter*-type cysts	Exospores or cysts	Exospores or Azotobacter-type cysts	None
Primary form of methane monooxygenase	pMMO (sMMO rare)			pMMO and sMMO	pMMO or sMMO	pMMO
Primary formaldehyde assimilation pathway	Ribulose monophosphate pathway			Serine cycle		Serine cycle (modified)
Benson-Calvin cycle	Varies	Absent	Absent	Absent	Absent	Present
Major fatty acids present	$C_{16:0}$, $C_{16:1}$	$C_{14:0}$, $C_{16:1}$, $C_{16:0}$	$C_{16:0}$, $C_{18:1}$	$C_{18:1}$	$C_{18:1}$	$C_{18:0}$, $C_{15:0}$ anteiso, $C_{14:0}$ iso
Temperature preferences	Mesophilic to thermophilic	Psychrophilic to mesophilic	Mesophilic to thermophilic	Mesophilic	Mesophilic	Thermophilic
pH preferences	Neutrophilic	Varies	Varies	Varies	Acidophilic	Acidophilic
Species with salinity requirements	None	Varies	Varies	None	None	None
Obligate methylotrophy	Yes	Yes	Yes	Yes	Varies	Yes
Nitrogen fixation	Varies	Varies	None	Varies	Yes	Varies
DNA G + C ratio (mol%)	56–66	43–60	54–62	60–67	60–63	40–46

[a]This table is adapted from that of Op den Camp et al. (2009) and uses data shown in *Tables 21.2* through to 7 for the type I methanotrophs

within the family *Methylococcaceae* which is the subject of this chapter and includes, as of late 2012, 13 cultivated genera encompassing 34 validly described species and an additional uncultured taxon that is effectively of *Candidatus* status called *Crenothrix polyspora*. *Methylococcus* is the type genus of *Methylococcaceae*. The family *Methylococcaceae* was created by to include all known methanotrophs at one point (Whittenbury and Krieg 1984) and however was later limited to type I methanotrophs (Bowman et al. 1993). This review updates information in a prior review on methanotrophic bacteria originally published online for the prokaryotes and subsequently published in The Prokaryotes 3rd book edition (Bowman 2006).

Phylogenetic Studies

The use of 16S ribosomal RNA-based phylogenetic analysis (Bratina et al. 1992; Brusseau et al. 1994; Bowman et al. 1995) has helped to resolve some if not all of the nomenclatural problems which previously affected methanotrophs (Whittenbury and Krieg 1984; Bowman et al. 1993). It has become apparent that with the continual description of new taxa, the family *Methylococcaceae* is clearly separated into three clades (*Fig. 21.1*). The use of the pMMO A subunit gene *pmoA* correlates well with 16S rRNA gene data (Op den Camp et al. 2009), and three separate groups of cultured taxa can be defined (*Fig. 21.2*) but not taking into account uncultured lineages found in environmental samples. The three clades in this review are referred to as the types Ia, Ib, and Ic methanotrops (*Table 21.1*). The type Ia (also referred in the literature sometimes as the type X) methanotrophs contain mesophilic to mildly thermophilic terrestrial species. This clade includes the type genus *Methylococcus* (Bowman et al. 1993) and the genera *Methylocaldum* (Bodrossy et al. 1997) and *Methylogaea* (Geymonat et al. 2011). The type Ib methanotrophs contain mesophilic to psychrophilic terrestrial and saline ecosystem-derived gammaproteobacterial methanotrophs, including the genera *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylosphaera*, *Methylosoma*, *Methylosarcina*, *Methylomarinum*, *Methylovulum*, uncultured filamentous methanotrophs *Crenothrix* and "*Clonothrix*," and known methanotrophic mytilid endosymbionts. The type Ic methanotrophs form a deep-branching clade and includes the thermophilic genus *Methylothermus* (Tsubota et al. 2005) and the halophilic genus *Methylohalobius* (Heyer et al. 2005). The phylogenetically diverse nature of the type I methanotrophs suggests that methanotrophy emerged from an ancestor that diverged along multiple evolutionary tangents. Based on the sequence dissimilarities and the fact that thermophily usually is indicative of ancientness, molecular clock theory suggests type I methanotrophy diversified at the time of advanced atmospheric oxygenation of Earth, about ~1.0 Gya ago. This assumes an estimated evolutionary rate of 0.05 Gya per 1 % of 16S rRNA divergence (Ochman et al. 1999). Methanotrophs have also emerged in the *Alphaproteobacteria* as two separate but closely related families (*Methylocystaceae* and *Beijerinckiaceae*) and also in the phylum Verrucomicrobia (op den Camp et al. 2009) (*Table 21.1*). Since all of these lineages share a conserved three-gene pMMO cluster (usually arranged as *pmoCAB*), it suggests that methanotrophs evolved from a much more ancient ancestor perhaps at a time Earth only had low oxygen levels and plentiful methane. This is consistent with methanotrophs able to often grow at quite low oxygen tensions (see section "Metabolism and Physiology"). Despite the phylogenetic divergence the morphology and ultrastructural features of many type I methanotrophs are analogous (see *Figs. 21.3* and *21.4*); however unusual exceptions also occur such as the complex morphology of *Crenothrix* (see *Fig. 21.5*) that still suggest much remains to be learned about methanotrophs in terms of diversity, physiology, and evolution.

Type Ia Methanotrophs

Type Ia methanotrophs effectively represent family *Methylococcaceae sensu stricto* since *Methylococcus* is the type genus (*Tables 21.1–21.3*, *Figs. 21.1* and *21.2*). The known member species include mesophiles and moderate thermophiles that occur in terrestrial habitats. Traits that vary between the species are shown in *Table 21.2*. The major respiratory lipoquinone is a methylene-substituted version of ubiquinone-8, referred to as MQ-8 (Collins and Green 1985); however data is only available for *Methylococcus capsulatus*. The main fatty acids include mainly those with a chain length of 16, including $C_{16:0}$ (palmitic acid) and various isomers of $C_{16:1}$ (palmitic acid).

Genus *Methylococcus* Foster and Davis 1966 Emend. Bowman et al. 1993

The type species of *Methylococcus* is *Methylococcus capsulatus*, which was originally described by Foster and Davis (1966), isolated from sewage sludge based on ecological studies. *Methylococcus* strains can be found in many terrestrial soils and sediments (see section "Ecology"). Subsequent numerical taxonomic analyses suggested several of the methanotrophic species groups of Whittenbury et al. (1970b) were related to *Methylococcus*, and new *Methylococcus* species descriptions were later published (Romanovskaya et al. 1978) including the species *Methylococcus bovis*, *Methylococcus chroococcus*, *Methylococcus luteus*, *Methylococcus vinelandii*, and *Methylococcus whittenburyi*. Subsequent immunological, protein electrophoresis and fatty acid and genomic analyses (Andreev and Galchenko 1978, 1983; Galchenko and Nesterov 1981; Bezrukova et al. 1983; Meyer et al. 1986; Bowman et al. 1991a, b) clearly demonstrated the genus was made up of two groups. This nomenclatural problem was resolved when *Methylococcus capsulatus* and *Methylococcus thermophilus* were retained in *Methylococcus* with the description of *Methylococcus* emended to reflect this change (Bowman et al. 1993).

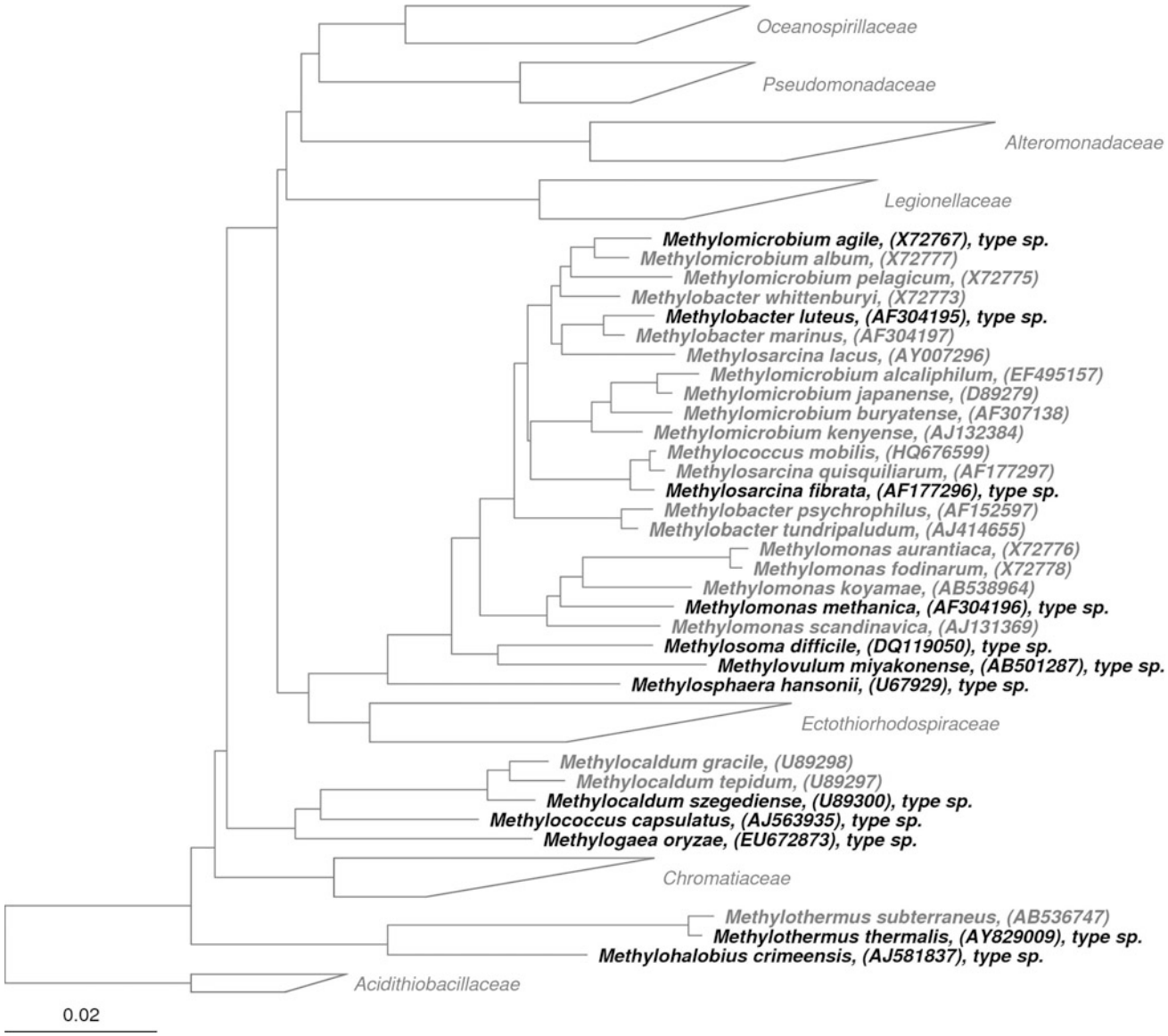

Fig. 21.1

Phylogenetic reconstruction of the family Methylococcaceae based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes-Cantor correction. The sequence datasets and alignments were used according to the All-Species Living Tree Project (LTP) database (Yarza et al., 2010; http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high quality type strain sequences proportionally distributed among the different bacterial and archaeal phyla. In addition, a 40% maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. Scale bar indicates estimated sequence divergence

Some anecdotal evidence suggests that *Methylococcus thermophilus* actually belongs to genus *Methylocaldum* (Bodrossy et al. 1997); however the type strains appear to be nonextant. Another still validly recognized species of the genus, *Methylococcus mobilis* (Hazeu et al. 1980) has also been lost. *Methylococcus chroococcus* is still retained as a valid species of the genus; however it is extremely likely it is merely a synonym of *Methylobacter whittenburyi* or a very close relative (Bowman et al. 1993). Another species described as belonging to *Methylococcus* called "*Methylococcus fulvus*" (Malashenko et al. 1972) also appears to be a subjective synonym of *Methylobacter luteus* (Romanovskaya et al. 1978). All other species of *Methylococcus* were transferred to the genus *Methylobacter* (Bowman et al. 1993), the original name coined for them by Whittenbury et al. (1970b). In the end only *Methylococcus capsulatus* remains as an extant and readily accessible representative of the genus, where it has represented a model for studying methanotrophy. Both the type strain Texas and the other well-studied strain Bath have genome sequences available (Boden et al. 2011; Kleiveland et al. 2012).

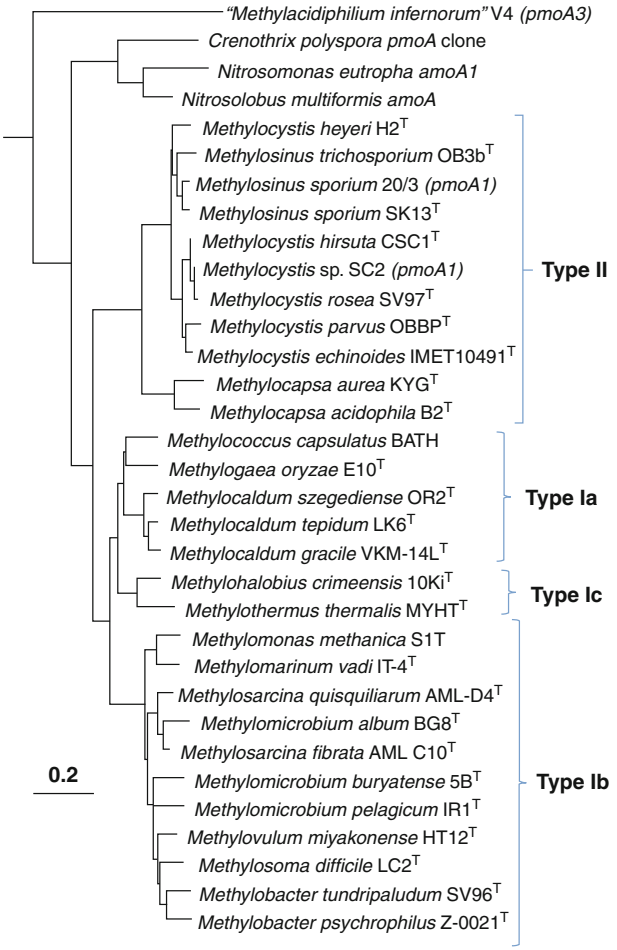

Fig. 21.2
Phylogenetic tree based on partial DNA sequences of the particulate methane monooxygenase subunit A genes (*pmoA*) for methanotrophs as well as closely allied ammonia monooxygenase subunit A genes (*amoA*) genes. The tree was clustered using the Maximum Likelihood algorithm and the three constructed using the Neighbour Joining method. A superscripted "T" denotes a type strain. The bar indicates a 0.2 change per position

Methylococcus capsulatus strains appear as nonmotile coccoidal cells (*Fig. 21.3e*) that, while unable to form sMMO are able to forms cysts, accumulate polyhydroxyalkanoate (PHA) granules and fix atmospheric nitrogen. Strains can take up several different compounds as nitrogen sources (Bowman et al. 1993). The species has a complete Benson-Calvin cycle and in the presence of methane can fix CO_2 (Baxter et al. 2002). The species is thermolerant growing between 25 °C and 55 °C and, optimally at 45 °C, prefers neutral pH conditions and is slightly tolerant to salt (*Table 21.2*). The DNA G + C composition of *Methylococcus* is 59–66 mol%. Strains contain several types of lipids including bacteriohopanepolyols, squalene, and sterols (Ourisson et al. 1987; Volkman 2003). Sterol synthesis is considered very rare in bacteria. *Methylococcus* phospholipids include mainly a phosphatidylethanolamine backbone (Fang et al. 2000), while the main fatty acids include $C_{16:0}$ and the $C_{16:1}$ isomers $C_{16:1}$ ω7c, $C_{16:1}$ ω6c, $C_{16:1}$ ω5c, and $C_{16:1}$ ω5t (*Table 21.3*). The possession of a series of $C_{16:1}$ double isomers including the unusual $C_{16:1}$ ω8c is a common property amongst the type I methanotrophs, though there is strong variation between the various taxa (Bowman et al. 1991a). The primary lipoquinone is also unusual being a 18-carbon position methylenated version of ubiquinone-8, referred to as MQ-8 (Collins and Green 1985).

Genus *Methylocaldum* Bodrossy et al. 1997

Moderately thermophilic to mesophilic methanotrophs from various terrestrial sites are included in the genus *Methylocaldum*, which currently includes three species, *Methylocaldum gracile*, *Methylocaldum szegediense*, and *Methylocaldum tepidum* (Bodrossy et al. 1997). *Methylocaldum tepidum* is the type species. The species *Methylocaldum gracile* was resurrected from "*Methylomonas gracilis*" NCIMB 11128, described previously by Russian microbiologists (Malashenko et al. 1975a; Romanovskaya et al. 1978). Additional *Methylocaldum*-related strains able to grow at 55 °C have been isolated from a variety of organic-rich environments heated by vigorous fermentation processes, including manure, silage, and compost (Eshinimaev et al. 2004; Jäckel et al. 2005).

Methylocaldum species appear as pleomorphic rod-shaped cells that are motile. The arrangement or number of flagella is unknown. Strains can encyst but are unable to fix atmospheric nitrogen. The species are mesophilic to thermolerant (*Table 21.2*) with *Methylocaldum szegediense* able to grow to 62 °C (*Table 21.2*). The DNA G + C composition of *Methylocaldum* species is 56–69 mol%. *Methylocaldum* species like several other type I methanotrophs contains triterpernoids of the hopane series, including aminobacteriohopanepentol and the 3-beta-methyl homologue. Some variation in hopane types occurs between species (Cvejic et al. 2000). It is unknown if they also synthesize sterols. The main fatty acids include $C_{16:0}$ and $C_{16:1}$ (Eshinimaev et al. 2004).

Genus *Methylogaea* Geymonat, Ferrando, and Tarlea 2011

Methylogaea contains only one species at present, *Methylogaea oryzae* (Geymonat et al. 2011), which originates from rice paddy soil obtained in Uruguay. The type strain was observed to contain glycogen granules. Cells appear as curved rods (*Fig. 21.3d*) that are nonmotile, lack cysts, are unable to fix nitrogen, and also lack sMMO (*Table 21.2*). *Methylogaea oryzae* can use a range of nitrogen sources including nitrate, ammonia, urea, lysine, peptone, and yeast extract (Geymonat et al. 2011). The lack of salt tolerance and preference for mesophilic and neutrophilic conditions suggests *Methylogaea oryzae* is limited to soil or freshwater ecosystems. The DNA G + C composition is 63 mol% while the main fatty acid present includes $C_{16:0}$ and $C_{16:1}$ ω7c (*Table 21.3*).

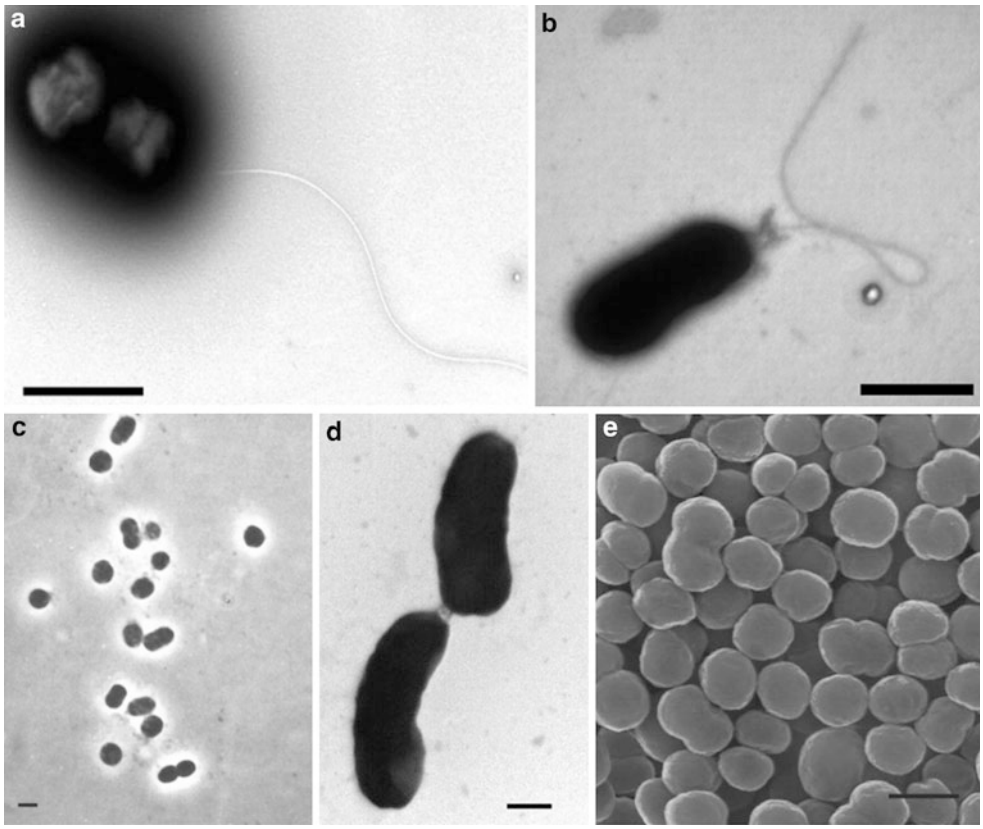

Fig. 21.3
Photographs of type I methanotrophs of family *Methylococcaceae* showing cellular morphology. (a) *Methylothermus subterraneus* cell with a single polar flagellum (Image from Hirayama et al. 2011), (b) *Methylomonas koyamae* cell with a single polar flagellum (Image from Ogiso et al. 2012), (c) cells of *Methylothermus thermalis* (Image from Tsubota et al. 2005), (d) cells of *Methylogaea oryzae* (Image from Geymonat et al. 2011), (e) cells of *Methylococcus capsulatus*. All *lines* equal 1 μm (All images are shown with permission from the copyright holder the Society of General Microbiology, UK)

Type Ib Methanotrophs

The type Ib cluster contains the taxonomically most diverse set of related methanotrophs including eight cultured genera and the two uncultured filamentous species: *Clonothrix polyspora* and "*Clonothrix fusca*." The type Ib methanotrophs also include on its periphery of its phylogenetic radiation the methanotrophic mytilid endosymbionts (data not shown). In general type Ib species are ecophysiologically diverse including psychrophiles, mesophiles, halophiles, and alkaliphiles. One species, *Methylomonas paludis* is mildly acidiphilic (Danilova et al. 2013). Species have been isolated from a wide range of terrestrial, athalassic, and marine environments (*Table 21.4*). The DNA base composition ranges from 43 to 60 mol%. The primary lipoquinones present are either ubiquinone-8 or the methylenated form of ubiquinone-8 (Collins and Green 1985). The main fatty acids include those with 14 and/or 16 carbon units including several $C_{16:1}$ isomers. The fatty acids composition varies between the different species groups considerably (*Table 21.5*). The presence of sMMO occurs in some species of genus *Methylomonas* (Koh et al. 1993; Auman and Lidstrom 2002) and *Methylomicrobium* (Fuse et al. 1998; Nakamura et al. 2007); however most species lack sMMO genes.

Genus *Methylomonas* (*ex* Leadbetter 1974) Whittenbury and Krieg 1984

The members of *Methylomonas* unlike almost all other methanotrophs synthesize carotenoids giving their colonies a pale to bright pink or bright orange pigmentation. The type species of the genus *Methylomonas* is *Methylomonas methanica*. The genus was revived by Whittenbury and Krieg (1984), before then the genus had been known for quite some time under a number of different guises. The species was first isolated by Sohngen (1906), who named it "*Bacillus methanica*," making it the first recorded methanotroph. Orla-Jensen (1909) subsequently renamed it "*Methanomonas methanica*." Morphologically similar pink-pigmented strains were isolated also from methane to air enrichments of aquatic plants and other freshwater habitat ampless and were referred to as "*Pseudomonas methanica*" (Dworkin and Foster 1956).

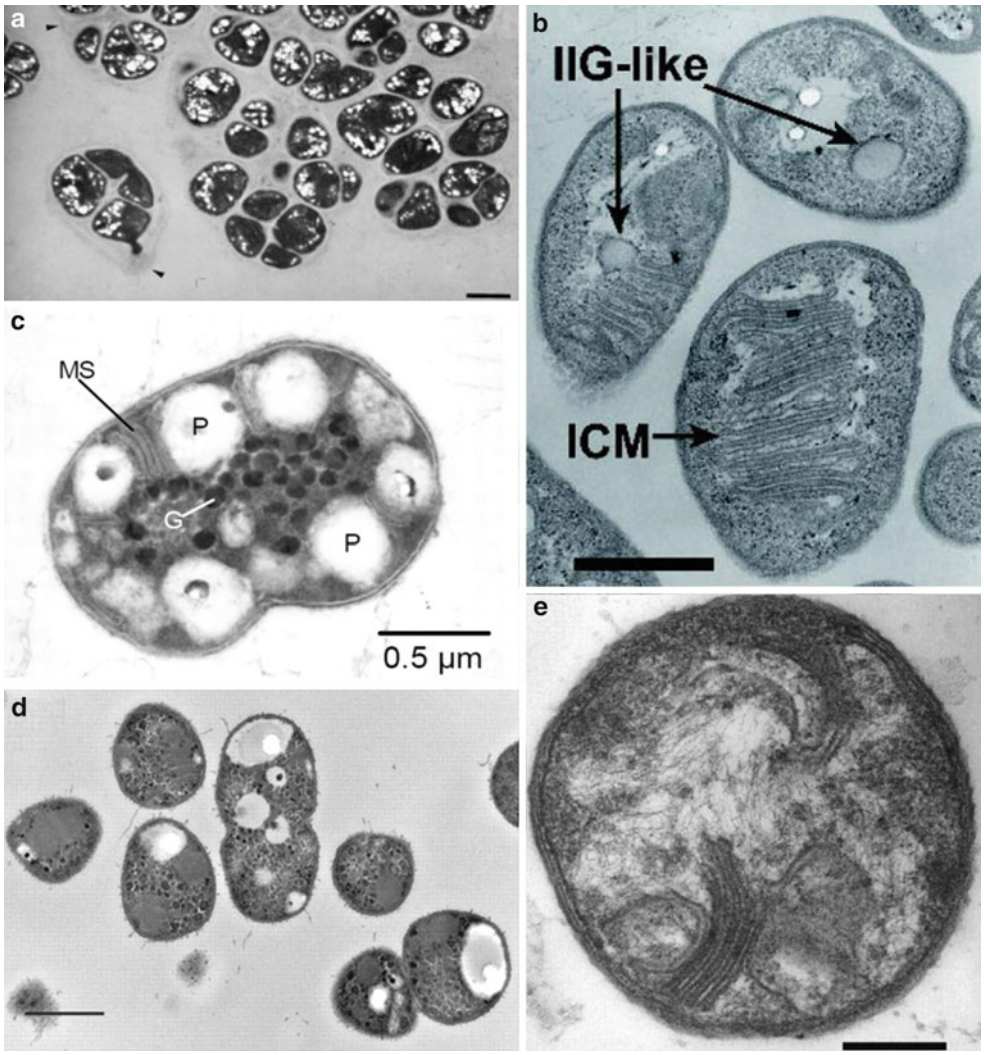

Fig. 21.4

Thin sections of type I methanotrophs of family *Methylococcaceae* showing internal ultrastructure of cells. (a) Thin sections of sarcinal packets of cells of *Methylosarcina quisquiliarum* showing internal PHA inclusions, which appears as *white* inclusions; the *arrow* points to external capsular material (Image from Wise et al. 2001). (b) Thin sections of cells of *Methylomarinum vadi* showing lamellar bundles of intracytoplasmic membranes (*ICM*) and PHA or glycogen inclusion bodies (IIG-like) (Image from Hirayama et al. 2012). (c) Thin section of a cell of *Methylosoma difficile* showing intracytoplasmic membranes (*MS*), PHA granules (*P*), and glycogen inclusions (*G*) (Image from Rahalkar et al. 2007). (d) Thin section of cells of *Methylovulum miyakonense* showing large PHA inclusion bodies present (Image from Iguchi et al. 2011). (e) Thin section of a cell of *Methylobacter tundripaludum* showing lamellar bundles of intracytoplasmic membranes; the bar here is 0.5 µm (Image from Wartiainen et al. 2006). Bars are equal to 1 µm unless otherwise specified (All images are reproduced here with permission from the copyright holder the Society of General Microbiology, Reading, UK)

The name *Methylomonas methanica* was eventually coined by Whittenbury et al. (1970b) for isolates very similar to "*Pseudomonas methanica*," which they enriched from freshwater sediment. Finally, the species was formally described by Romanovskaya et al. (1978), who at the same time described "*Methylomonas rubra*." Other pink- or red-pigmented groups of *Methylomonas* described by Whittenbury et al. (1970b) were regarded as variants of *Methylomonas methanica*.

During the 1970s, with interest in biotechnology of single-cell protein and industrial catalysis expanding (Anthony 1982; Hou 1984), many pink-pigmented methylotrophic bacteria were isolated, most of which were grouped in the genus *Methylomonas*. Though most of these species were able to utilize methanol and methylamine, methane in general was not used. Admittedly at that time, a formal taxonomy was unavailable for the classification of methylotrophic bacteria. Several of these invalid *Methylomonas* spp. are now recognized as belonging to the betaproteobacterium species *Methylobacillus glycogenes* (Urakami and Komagata 1986b) including "*Methylomonas* (or *Methanomonas*) *methylovora*" (Kuono et al. 1973), "*Methylomonas methanolica*," "*Methylomonas espexii*," "*Methylomonas methanocatalesslica*," and "*Methylomonas*

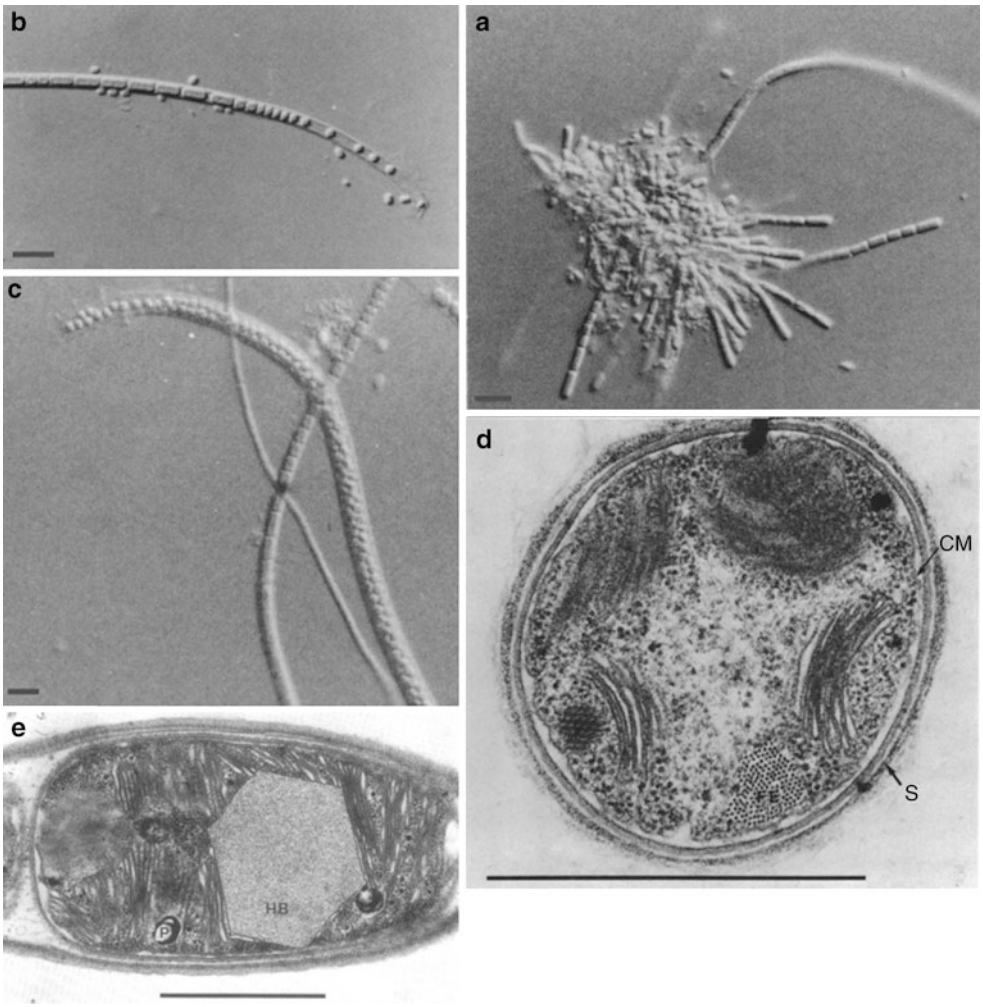

Fig. 21.5

Photographs showing morphological and ultrastructural features of the filamentous methanotroph *Crenothrix polyspora*. (a) A tuft of sheathed filaments containing septated cells. (b) A single filament dispersing spherical "macrogonidia" from the tip. (c) A single filament dispersing spherical "microgonidia" from the tip. (d) A thin section through a filament showing lamellar stacks of intracytoplasmic membrane (typical of type I methanotrophs), the cytoplasmic membrane (*CM*), the sheathe layer (*S*), and internal fibrillar elements (*FE*) of unknown nature. (e) Thin section of vegetative cells within a filament showing a large hexagonal body (*HB*) and possible polyphosphate granule (*P*). Bars for photographs a to c are 10 μm, while for d and e, they are 1 μm (All images are reproduced here with permission from the copyright holder, the American Society of Microbiology, Washington DC, USA)

methanofructolica" (Urakami and Komagata 1986a). "*Methylomonas clara*" (Faust et al. 1977) has been shown to belong to the species *Methylophilus methylotrophus* (Jenkins et al. 1987). A variety of methanotrophs that were also grouped in *Methylomonas* including "*Methylomonas methaninitrificans*" (Davis et al. 1964) and "*Methylomonas methanooxidans*" (Brown and Strawinski 1958) probably were actually members of genus *Methylosinus* (Whittenbury et al. 1970a). The species "*Methylomonas margaritae*" (Takeda et al. 1974) and "*Methylomonas flagellata*" (Morinaga et al. 1976) possess traits very similar to *Methylomicrobium agile*; however neither species have extant cultures to confirm this. The marine species *Methylomonas pelagica* (Sieburth et al. 1987) was initially transferred to the genus *Methylobacter* until it became part of genus *Methylomicrobium*.

Five additional species have been added to *Methylomonas* that are validly recognized and have characteristics and phylogeny that form the basis of *Methylomonas* as taxonomic entity. These include orange-pigmented species *Methylomonas fodinarum* and *Methylomonas aurantiaca*, isolated from coal-mine drainage water and from sewage sludge and marshy soils, respectively (Bowman et al. 1990). *Methylomonas scandinavica* was named for pink isolates obtained from deep subsurface groundwater (Kalyuzhnaya et al. 1999). A rice soil isolate which also forms a pink pigment was named *Methylomonas koyamae* (Ogiso et al. 2012). A comparatively acidophilic strain isolated from sphagnum peat soil with pale pink pigmentation was named *Methylomonas paludis* (Danilova et al. 2013).

Methylomonas species are distinctly rodlike, are motile with a single polar flagellum (see ❯ *Fig. 21.3b*), form

Table 21.2
Differential phenotypic properties separating members of the type Ia methanotrophs

Characteristics	*Methylococcus capsulatus*	*Methylocaldum gracile*	*Methylocaldum szegediense*	*Methylocaldum tepidum*	*Methylogaea oryzae*
Habitat	Freshwater mud	Freshwater mud, soil	Natural gas field hot spring	Agricultural soil	Rice paddy soil
Colony	White to pale brown	Brown	Brown	Brown	White 1–2 mm 1 week
Motility	−	+	+	+	−
Shape	Coccoidal-short rods	Thin rod to cocci	Pleomorphic rod	Pleomorphic rod	Curved rods
Size	0.5–2.0 × 1.0–2.0	0.4–0.5 × 1.0–1.5	0.6–1.2 × 1.2–1.5	1.0–1.2 × 1.0–1.8	2.0–2.2 × 0.5–0.7
Cysts	+	+	+	+	−
C1 assimilation pathway key enzymes	RuMP/serine	RuMP/serine	RuMP/serine	RuMP/serine	ND
Temperature growth range (optimal)	25–55 (45)	20–47 (42)	37–62 (55)	30–47 (42)	20–37 (30)
pH growth range (optimal)	5.5–8.5 (7.0)	ND	ND	ND	5–8 (6.5–6.8)
NaCl range for growth (%)	0–2.5	ND	ND	ND	0–0.5
nifH gene present	+	ND	ND	ND	+
N_2 fixation	+	−	−	−	−
PHA granules	+	ND	ND	ND	+
Utilization of methylamine, formate	V	ND	−	−	−

Abbreviations: + traits is positive for all or most strains of the species, − trait is negative for all or most strains of the species, *V* varies between strains of the same species, *RuMP* ribulose monophosphate, *PHA* polyhydroxyalkanoate, *ND* no data is available

desiccation-sensitive "immature cysts" (Whittenbury et al. 1970a), form polyhydroxyalkanoate granules, and are mesophilic showing no growth at 4 °C but grow well at 30 °C. Cells may occur singly, in pairs, or in short chains. The sphagnum peat species *Methylomonas paludis* is an exception to most of these traits lacking motility, cysts, and PHA granules and being acidophilic growing from approximately pH 4–7 and preferring growth at about pH 6 (Danilova et al. 2013). Most strains of *Methylomonas* are mildly salt tolerant growing with 1.5 % NaCl; however the peatland and rice soil species only tolerate at most 0.5 % (w/v) NaCl. The ecophysiological diversity within the genus indicates *Methylomonas* has a broad distribution in the environment. Ecological studies suggest *Methylomonas* spp. is found in estuarine, in coastal, and in most terrestrial ecosystems (Whittenbury et al. 1970b; Bowman et al. 1990, 1993; McDonald et al. 2005) and is capable of colonizing plants (Iguchi et al. 2012). The mild salt tolerance of *Methylomonas* species likely contributes to their wide-ranging distribution though none are strictly marine as such.

The DNA G + C composition of *Methylomonas* species ranges from 48 to 59 mol% (T_m). The polar lipids of *Methylomonas* species that have been tested include phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine, and phosphatidyldimethylethanolamine (Fang et al. 2000). The fatty acid profiles amongst the five species are very similar, with $C_{14:0}$ and different forms of palmitic acid ($C_{16:1}$) predominating including $C_{16:1}$ ω8c, $C_{16:1}$ ω7c, $C_{16:1}$ ω6c, $C_{16:1}$ ω5c, and $C_{16:1}$ ω5t. The outer membrane lipopolysaccharide hydroxy fatty acids have been analyzed in detail in some *Methylomonas* species and include mainly $C_{16:0}$ 3-OH (Bowman et al. 1991a). *Methylomonas methanica* contains methylenated ubiquinone-8 (MQ-8) as its major respiratory lipoquinone (Collins and Green 1985).

Genus *Methylobacter* Bowman et al. 1993 Emend. Bowman et al. 1995

As mentioned previously *Methylobacter* consists of species at one stage that were classified in *Methylococcus*, including *Methylobacter luteus*, which is the type species, *Methylobacter marinus*, and *Methylobacter whittenburyi* (Bowman et al. 1993). Two additional species, isolated from tundra soil, also belong to the genus *Methylobacter psychrophilus* (Omelchenko et al. 1996) and *Methylobacter tundripaludum* (Wartiainen et al. 2006). 16S rRNA gene sequence data (*Fig. 21.1*) demonstrates that *Methylobacter luteus*, *Methylobacter marinus*, and *Methylobacter whittenburyi* form a common cluster while *Methylobacter psychrophilus* and *Methylobacter tundripaludum* form an adjacent but quite distinct lineage. Between these lineages lie the remaining five halophilic *Methylomicrobium* species, represented by *Methylomicrobium pelagicum*, and the three

Table 21.3
Fatty acid and lipoquinone composition of type Ia methanotrophs

Fatty acids	*Methylococcus*	*Methylocaldum*[a]	*Methylogaea*
	Composition (%)		
$C_{12:0}$[b]		0–TR[c]	2
$C_{14:0}$	1–6	2–3	6
$C_{15:0}$	0–2	2–4	1
$C_{16:1}$	31–38	12–47	17
$C_{16:}$1 ω9c			7
$C_{16:1}$ ω7c	10–23		10
$C_{16:1}$ ω6c	4–12		
$C_{16:1}$ ω5c	3–9	0–TR	
$C_{16:1}$ ω5t	2–6	0–TR	
$C_{16:0}$	33–56	43–65	62
$C_{17:1}$ ω7c	0–2		
$C_{17:1}$ ω7t	0–2		
$C_{17:0}$		0–TR	
$C_{17:0}$ cyc	0–14	3–9	
$C_{18:0}$	0–2		
$C_{18:1}$ ω7c	0–6		
$C_{18:1}$ ω9c	0–3		
$C_{19:1}$ cyc	TR–2		
Lipoquinone	MQ–8	ND	ND

[a]Data from Eshinimaev et al. 2004; 16:1 double positions were not determined
[b]Fatty acid nomenclature: C_n carbon chain length, 2-OH or 3-OH, α-, and β-hydroxy fatty acids, *iso* iso-branched fatty acids, *:n* number of double bonds present, *ωnc cis*-isomer monounsaturated fatty acid with the bond located at the indicated number of carbon units from the methyl end of the molecule. In the case of polyunsaturated fatty acids, the first double bond is located at the third carbon unit from the methyl end
[c]*TR* trace fatty acid making up <1 % of total analyzed fatty acids

species of genus *Methylosarcina*. This taxonomic issue could be potentially resolved by conglomeration of all the taxa or perhaps better by classifying the tundra *Methylobacter* species and the halophilic *Methylomicrobium* species into distinct genera, thus creating a more consistent characteristics-based taxonomy. This second strategy is assumed here where phenotypic and chemotaxonomic traits for the separate type Ib methanotroph subclades are summarized (*Tables 21.4* and *21.5*).

The cells of M*ethylobacter* species possess a characteristic coccoidal to elliptical plump rodlike morphology (example *Fig. 21.4e*) with a width of 0.8–1.5 μm and a length of 1.2–3.0 μm and occur mostly singly or in pairs; however chain formation is prevalent in some strains in the late exponential growth phase. *Methylobacter luteus* is nonmotile, while *Methylobacter whittenburyi* strains usually are motile when first isolated but can spontaneously lose the ability after extensive subculture. Motility appears most pronounced in young cultures of *Methylobacter whittenburyi* and *Methylobacter marinus* with older cultures often devoid of motile cells. Motility is conferred by a single polar flagellum. Cells are surrounded by capsular material detectable by India ink staining, and cell walls are typical of gram-negative bacteria.

Methylobacter species form well-defined *Azotobacter*-type cysts which may give cells a refractile appearance (Whittenbury et al. 1970a). PHA granules tend to form in early log-phase cultures. Colonies on NMS agar are circular, convex, and smooth; have an entire edge; and possess a creamy consistency. The colonies of *Methylobacter luteus* are pigmented yellow, and some strains form a diffusible yellow pigment. Both *Methylobacter whittenburyi* and *Methylobacter marinus* colonies are tan and slowly exude tan to brown pigments into the agar media. *Methylobacter* strains are strictly aerobic obligate methanotrophs with carbon and energy substrates limited to methane and methanol. Methane appears to be oxidized only by particulate MMO. The presence of soluble MMO (see section "Metabolism and Physiology") has not been demonstrated in *Methylobacter* strains so far (Stainthorpe et al. 1991; Murrell et al. 1998). *Methylobacter* can utilize nitrate and ammonia salts, yeast extract, casamino acids, and various other amino acids as nitrogen sources (Bowman et al. 1993). High levels (>0.5 % w/v) of complex organic compounds are inhibitory to their growth. Some strains, particularly those of *Methylobacter luteus*, can produce a urease, but none are known to fix atmospheric nitrogen. *Methylobacter luteus* species cluster is mesophilic with most strains growing between 15 °C and 40 °C and optimally at about 30 °C. However, the tundra species *Methylobacter psychrophilus* (Omelchenko et al. 1996) and *Methylobacter tundripaludum* are much more cold adapted with growth optimal at about 10–25 °C. In addition, *Methylobacter psychrophilus* are able to form gas vesicles. *Methylobacter species* are by nature neutrophilic with the pH range for growth ranging from 5.5 to 9.0 and a pH optimum at about 7.0.

Methylobacter strains may require growth factors and salt for growth. The estuarine species *Methylobacter marinus* grows optimally with about 0.1 M NaCl in tap water or with half-strength seawater salts. Some strains of *Methylobacter marinus* also require nicotinic acid for growth (Lidstrom 1988). The mol% G + C of *Methylobacter* DNA ranges from 46 to 55 (T_m). *Methylobacter luteus cluster* species have very similar fatty acid profiles, with $C_{16:1}$ ω7c predominating and accompanied by lower levels of $C_{14:0}$, $C_{16:1}$ ω6c, $C_{16:1}$ ω5c, and $C_{16:0}$. The lack of $C_{16:1}$ ω8c and relatively low levels of $C_{14:0}$ and $C_{16:0}$ distinguish these *Methylobacter* spp. from other type Ib methanotrophs. The tundra species however have a quite different fatty acid composition and include substantial levels of $C_{16:1}$ ω8c and $C_{16:1}$ ω5t (*Table 21.5*). The outer membrane lipopolysaccharide hydroxy fatty acids have been analyzed in detail in *Methylobacter luteus* and *Methylobacter whittenburyi* (Bowman et al. 1991a). The major components found in *Methylobacter luteus* are $C_{10:0}$ 3-OH and $C_{16:0}$ 3-OH plus smaller quantities of $C_{12:0}$ 2-OH, $C_{14:0}$ 3-OH, and $C_{15:0}$ 3-OH, while the major component of *Methylobacter whittenburyi* is only $C_{16:0}$ 3-OH. *Methylobacter* spp. contain ubiquinone-8 (Q-8) as their major respiratory lipoquinone (Collins and Green 1985).

Table 21.4

Differential phenotypic properties separating members of the type Ib methanotrophs

Characteristics	*Methylomonas*	*Methylovulum*	*Methylosoma*	*Methylosphaera*	*Methylobacter luteus* cluster	*Methylobacter psychrophilus* cluster	*Methylomicrobium agile* cluster	Halophilic *Methylomicrobium* species cluster	*Methylomarinum*	*Methylosarcina*
Habitat	Coal mines, freshwater sediment, sewage, soil, groundwater	Soil	Lake sediment	Polar marine salinity lakes	Freshwater sediment, sewage, coastal sediment	Tundra soils	Sewage, soil, freshwater mud	Seawater, soda lakes, marine sediment	Marine sediment	Soil, lake sediment
Colony pigmentation	Pink or orange	Pale brown	Pale pink	No growth on agar	Yellow, white, brown	White to buff, pale pink	White to buff	White, cream, pale pink	Cream	White, buff-light brown
Metabolism	Aerobic	Aerobic	Microaerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic
Motility	+, single polar	–	–	–	V, single polar	–	+, single polar	+, single polar	+, single polar	V, single polar
Shape	Rods to coccobacilli	Coccoidal-short rods	Coccoid-short rods	Coccoidal	Coccoidal-oval	Coccoidal-rods	Cocci-rods	Cocci-rods	Short rod to oval	Short rods, fusiforms, sarcinal packets
Cysts	V	–	+	–	+	V	–	V	–	+
pMMO	+	+	+	+	+	+	+	+	+	+
sMMO	V	+	–	–	–	–	–	V	–	–
Growth at <4 °C	V	–	–	+	–	+	–	–	–	V
Growth at 30 °C	+	+	+	–	+	V	+	+	+	+
Growth at 45 °C	–	–	–	–	–	–	–	–	+	–
Growth at < pH 5	V	–	–	–	–	–	–	–	+	V
Growth at > pH 9	–	–	–	–	–	–	–	V	–	–
Growth with 0.5 % (w/v) NaCl	+	–	–	–	V	–	+	V	–	+
Growth with 2.5 % NaCl	–	–	–	+	V	–	–	+	+	–
Requires Na^+ ions	–	–	–	+	V	–	–	+	+	–
N_2 fixation	+	–	+	+	–	–	–	–	–	–
PHA granules	V	+	+	+	+	+	V	+	ND	+
DNA G + C ratio (mol%)	48–59	49	50	43–46	49–55	46–47	54–60	48–52	51–52	53–54

Abbreviations: + traits is positive for all or most strains of the species, *V* trait varies amongst species, – trait is negative for all or most strains of the species, *PHA* polyhydroxyalkanoate, *ND* no data is available

Table 21.5

Fatty acid and lipoquinone composition of type Ib methanotrophs

Fatty acid[c]	*Methylomonas*	*Methylovulum*	*Methylosoma*	*Methylobacter luteus* cluster	*Methylobacter psychrophilus* cluster	*Methylomicrobium agile* cluster	Halophilic *Methylomicrobium* species cluster[a, b]	*Methylomarinum*	*Methylosarcina*	*Methylosphaera*
	Composition (%)									
$C_{12:0}$			3					TR	0–4	
$C_{14:0}$	12–25	34	8	7–10		TR–2	5	2–4	1–2	2–3
$C_{14:0}$ 2-OH										
$C_{15:0}$	0–1	3	TR	0–4		0–7	TR–1	TR–2		1–2
$C_{15:0}$ iso	0–2			0–TR		0–TR				
$C_{16:1}$ ω11c			2							
$C_{16:1}$ω9c	0–TR									
$C_{16:1}$ ω8c	19–41				35	12–19			14–34	37–41
$C_{16:1}$ ω7c	7–15		60	56–58	23	14–20	51	51	18–32	16–19
$C_{16:1}$ ω7t								TR		2–3
$C_{16:1}$ ω6c	4–13		15	4–5		6–14		2	7–9	17–18
$C_{16:1}$ ω6t								TR		
$C_{16:1}$ ω5c	2–6			5–8		5–7	15	TR	0–TR	0–TR
$C_{16:1}$ ω5t	8–35			10–11	26	5–28		7	19–30	
$C_{16:0}$	4–9	47	8	8–9		11–18	21	32	10–20	14–15
$C_{16:0}$ 3-OH		8					TR–1	2		
$C_{16:1}$ 3-OH			1							
$C_{16:0}$ iso										
$C_{17:0}$						0–TR				
$C_{17:1}$ ω8c										TR–1
$C_{17:1}$ ω7c	0–TR									TR–1
$C_{17:1}$ ω6c		6								
$C_{17:0}$ cyc	0–2									
$C_{18:0}$	0–1					0–3	1	TR		TR
$C_{18:1}$ ω7c	TR–2			TR–3		0–26	TR	TR		1–2
$C_{18:1}$ ω9c	0–TR						1			0–1
$C_{18:0}$ 3-OH							1			
$C_{18:2}$ ω2,6c							1			
$C_{19:1}$ cyc	TR									
Lipoquinone	MQ–8			Q–8		Q–8	Q–8	MQ–8		

[a]Data available for *Methylobacter tundripaludum* only (Wartiainen et al. 2006)

[b]Data available for *Methylomicrobium buryatense* only (Kalyuzhnaya et al. 2001)

[c]Fatty acid nomenclature and abbreviations are shown in the footnote of *Table 21.3*

Genus *Methylomicrobium* Bowman et al. 1995 Emend. Kalyuzhnaya et al. 2008

The genus *Methylomicrobium* currently has seven species with *Methylomicrobium agile* as the type species (Bowman et al. 1995). As mentioned in the section on *Methylobacter*, 16S rRNA gene sequence data indicates the genus is actually two distinct clades. *Methylomicrobium agile* and *Methylomicrobium album* are closely related to *Methylosarcina lacus* (Kalyuzhnaya et al. 2008) based on more accurate sequence data, while the remaining species, all halophilic, deriving from marine samples or soda lakes, form a separate cluster. The halophilic species include *Methylomicrobium pelagicum* (Sieburth et al. 1987; Bowman et al. 1993), *Methylomicrobium alcaliphilum* (Khmelenina et al. 1997; Sorokin et al. 2000), *Methylomicrobium buryatense* (Kalyuzhnaya et al. 2001), *Methylomicrobium japanense*, and *Methylomicrobium kenyense* (Fuse et al. 1998; Kalyuzhnaya et al. 2008). Cells of *Methylomicrobium* spp. appear as single or paired regular short rods, 0.5–0.1.5 μm wide and 1.5–2.5 μm long. All species are actively motile, are propelled by a single polar flagellum, possess a standard gram-negative cell wall, and are surrounded by a thin slime capsule. Cells contain type I intracytoplasmic membranes typical of other *Methylococcaceae*; however they lack the ability to form cysts. Most strains contain PHA and polyphosphate granules. Cells are not heat- or desiccation resistant and are somewhat sensitive to methane starvation, losing viability in only a few days when exposed to a methane-free atmosphere. Colonies on NMS agar are nonpigmented, circular, convex flat, and smooth and usually possess a creamy consistency.

Methylomicrobium strains are strictly aerobic obligate methanotrophs with carbon and energy substrates limited to methane and methanol. Upon isolation most *Methylomicrobium* strains can tolerate and grow quite well on methanol. Methane appears to be oxidized predominantly by pMMO, but sMMO is usually absent though marine strains related to *Methylomicrobium buryatense* have been shown to possess sMMO (Fuse et al. 1998; Nakamura et al. 2007). *Methylomicrobium* spp. can utilize nitrate and ammonia salts, yeast extract, casamino acids, and various amino acids as nitrogen sources, though high levels (>0.5 % w/v) of complex organic compounds are inhibitory to growth. Urease and nitrogen fixation activities are generally absent. *Methylomicrobium* species are mainly mesophilic growing between 10 °C and 30 °C (*Table 21.4*). The main difference between the two separate *Methylomicrobium* clusters is salt requirement. Neither *Methylomicrobium agile* nor *Methylomicrobium album* require salt but can tolerate low levels. Isolates of these species come from mainly freshwater and soil (Whittenbury et al. 1970b) but potentially could be found in estuarine sediment. A fast-growing unnamed strain closely related to the *Methylomicrobium agile* cluster was isolated from manure effluent and was also only slightly salt tolerant (Kim et al. 2008). The marine-derived strains *Methylomicrobium pelagicum* and *Methylomicrobium japanense* on the other hand prefer seawater salinity levels and a pH about that of seawater (pH 8). The three soda lake species, *Methylomicrobium alcaliphilum*, *Methylomicrobium buryatense*, and *Methylomicrobium kenyense* prefer different levels of salt and alkaline pH. *Methylomicrobium alcaliphilum* is the most tolerant of all these species that grows in the presence of up to 10 % salts and can grow at pH 10 (Sorokin et al. 2000). *Methylomicrobium kenyense* has a much more narrow pH preference growing only between pH 9 and 11 and requiring about 2–4 % NaCl. *Methylomicrobium buryatense* has a lower salt requirement (0.5–0.7 % NaCl) but has a relatively broad pH range for growth (pH 6–11) (Kalyuzhnaya et al. 2008). The data suggests halophilic *Methylomicrobium* species are diverse and adapted to a wide range of saline habitats. Ecological data suggests strongly that halophilic *Methylomicrobium* species are common and sometimes dominate the methanotrophic community in marine sites where methane is present and also potentially act as endosymbionts or mutualists in deepwater coral (Jensen et al. 2008); however many uncultured methanotrophs are also present in marine samples that may represent undescribed genera adjacent to *Methylomicrobium*. Sequence data overall suggests the halophilic *Methylomicrobium* species should be placed into a new genus.

The DNA base composition is distinct for the two *Methylomicrobium* clades with 54–60 mol% recorded for the *Methylomicrobium agile* clade and 48–52 mol% for the halophilic clade. Fatty acid profiles of *Methylomicrobium* species are dominated by different versions of palmitic acid ($C_{16:1}$) with palmitic acid ($C_{16:0}$) present at lower levels (*Table 21.5*). The distribution of palmitic acid isomers in which double bond position differs varies considerably between the two *Methylomicrobium* clades. In *Methylomicrobium alcaliphilum* iso-branched $C_{16:0}$ unusually predominates, possibly an adaptation to its soda lake environment (Khmelenina et al. 1997). Overall the chemotaxonomic data is relatively scant for these groups, and for taxonomic revision more detailed analyses will need to be performed. The carbohydrate fraction of the outer membrane lipopolysaccharide in *Methylomicrobium album* was shown to be fairly conventional containing D-glucose, L-fucose, and D-heptose (Sutherland and Kennedy 1986). The hydroxy fatty acids from the lipopolysaccharide fraction, *Methylomicrobium album*, and *Methylomicrobium agile* (Bowman et al. 1991a) are predominantly $C_{16:0}$ 3-OH. The primary respiratory lipoquinone is Q-8, as found in *Methylobacter* spp. (Collins and Green 1985); however no data is available for the halophilic species.

Genus *Methylosphaera* Bowman et al. 1997

Methylosphaera includes a single species *Methylosphaera hansonii* that branches on the periphery of the type Ib methanotroph clade. The species is marine, requiring sea salts for growth, and is psychrophilic. It was isolated from saline meromictic lakes or lagoons of Eastern Antarctica, including Ace Lake and Burton Lake. In these lakes the species grows maximally at the oxic/anoxic interface below which the anoxic waters are methane saturated. *Methylosphaera hansonii* strains

appear as featureless spherical cells, which exhibit refractility by phase contrast microscopy possibly due to the presence of gas vesicles. Though cells may show signs of uneven binary division, evidence for budding division has yet to be confirmed. *Methylosphaera* cells possess standard gram-negative cell walls and type I ICM when grown under methane; however cells lack flagella, cysts, or other types of resting stages typically found in methanotrophic bacteria. The species is also intolerant to all forms of agar and so far has only been grown successfully in liquid seawater-NMS media. Methane monooxygenase activity is restricted to the particulate (pMMO) form with no soluble (sMMO) activity detected by the naphthalene oxidation assay. *Methylosphaera* strains can utilize nitrate, ammonia, and L-glutamine for nitrogen; however yeast extract and casamino acids are less suitable, causing partial inhibition of growth when tested at 0.05–0.1 % (w/v) and complete inhibition of growth at concentrations of 0.25–0.5 % (w/v). *Methylosphaera* strains can fix atmospheric nitrogen; unlike other methanotrophs this property is not especially oxygen sensitive. All strains are psychrophilic with optimum and maximum growth temperatures varying slightly between strains. Optimal growth occurs at 10–15 °C, while no growth occurs at 25 °C. A doubling time of 20–24 h was determined for strains growing at or close to their optimum temperature. In NMS-seawater liquid media, growth occurs at pH 6.0–8.0 and a pH of approximately 7.5 is optimal for growth. The DNA G+C composition values of *Methylosphaera hansonii* are amongst the lowest amongst the type I methanotrophs, ranging from 43 to 46 mol%. The major fatty acids of the genus include mainly $C_{16:1}$ typical of Ib methanotrophs including $C_{16:1}$ ω8c, $C_{16:1}$ ω7c, and $C_{16:1}$ ω6c. The species also contains high levels of several methylated derivatives of lanosterol (Schouten et al. 2000). Since the mid-2000s the type strain is no longer extant, thus the genus and species strictly speaking are of illegitimate status until a new type strain can be obtained to allow official reinstatement of the taxon.

Genus *Methylosarcina* Wise et al. 2001

The genus *Methylosarcina* was named for isolates from landfill soil that form packets of cells ("sarcina") connected by fibrillar material. *Figure 21.4a* shows a thin section through a typical sarcina. These strains formed the species *Methylosarcina fibrata*, which is the type species, and a second species *Methylosarcina quisquiliarum* (Wise et al. 2001). A third species called *Methylosarcina lacus*, isolated from freshwater lake sediment, was added subsequently; however this species lacks the sarcina morphological property of the other two species (Kalyuzhnaya et al. 2005). The species *Methylomicrobium agile* and *Methylomicrobium album* are related to *Methylosarcina lacus after* reappraisal of 16S rRNA gene sequence data (Kalyuzhnaya et al. 2008). Based on molecular ecological studies, members of *Methylosarcina* are most frequently found in soil. *Methylosarcina* species are mesophilic or psychrotolerant, preferring low salt, neutral pH conditions. Cells may be motile but all known species form cysts, but cannot fix nitrogen and only form the particulate version of methane monooxygenase (*Table 21.4*). The DNA G + C base composition ranges from 53 to 54 mol%. The major fatty acids present in *Methylosarcina* strains are $C_{16:1}$ isomers similar to that of *Methylomonas* spp., including large amounts of $C_{16:1}$ ω8c, $C_{16:1}$ ω7c, and $C_{16:1}$ ω5t (*Table 21.5*).

Genus *Methylosoma* Ralhalkar et al. 2007

Methylosoma includes one species, *Methylosoma difficile*, which comprises very slow growing, coccoidal cells, that prefer a microaerobic atmosphere when growing on methane. The typical generation time ranges from 5 to 8 days with best growth occurring under a 2 % (vol/vol) oxygen atmosphere. The type strain was obtained from freshwater lake sediment (Rahalkar et al. 2007). *Methylosoma* forms a subclade with other halophobic taxa including the forest soil-associated genus *Methylovulum*, the tundra soil-dwelling *Methylobacter* species, and the biofilm-forming *Crenothrix* and *Clonothrix*. *Methylosoma difficile* cells appear as short rods that contain PHA granules (*Fig. 21.4c*); are mesophilic, intolerant of NaCl, neutrophilic, and nonmotile; forms cysts; and fixes nitrogen but lacks sMMO (*Table 21.4*). The known distribution of *Methylosoma* includes freshwater sediment and soil ecosystems. The DNA G+C base composition is 50 mol%. Most of its fatty acid is made up of $C_{16:1}$ ω7c, $C_{16:1}$ ω6c, and $C_{16:0}$ (*Table 21.5*).

Genus *Methylovulum* Iguchi et al. 2011

The genus *Methylovulum* contains to date a single species called *Methylovulum miyakonense*, which was isolated from forest soil in Japan (Iguchi et al. 2011a). The species contains both particulate and soluble forms of methane monooxygenase (Iguchi et al. 2010). *Methylovulum* like its relatives is intolerant to saline conditions typical of bacteria found in soil and freshwater ecosystems. *Methylovulum miyakonense* forms cells that are cooccoidal or short rods in appearance that are nonmotile and unable to fix nitrogen and lack encystment but can form (some PHA granules *Fig. 21.4d*; *Table 21.4*). Ecological studies suggest that besides forest soil-related taxa, *Methylovulum*-related taxa have been detected in Arctic lakes (He et al. 2012b) and also sphagnum-dominated peat soil (Kip et al. 2011). An isolate from peat moss was noted to contain a remarkable series of bacteriohopanepolyols (van Winden et al. 2012b) including monounsaturated aminobacteriohopanepentols, aminobacteriohopanetetrols, and aminobacteriohopanetriols. Such lipids could be useful as signature lipids for detection of *Methylovulum*. The DNA G+C base composition is about 49 mol%. Most of the fatty acids are composed of $C_{16:0}$, $C_{14:0}$, and $C_{16:0}$ 3-OH (*Table 21.5*).

Genus *Methylomarinum* Hirayama et al. 2012

Related to the halophilic *Methylomicrobium* cluster species, *Methylomarinum* represents a marine-type Ib methanotroph.

Currently this genus contains one species, *Methylomarinum vadi*, isolated from surface sediments of a shallow submarine hydrothermal system (Hirayama et al. 2012). *Methylomarinum vadi* forms oval to coccoidal cells (Fig. 21.4b), lacks pigmentation, and is mesophilic able to grow up to 45 °C. The type strain requires Na^+ ions for growth and prefers a pH of around 6–7, is motile with a single polar flagellum, does not form cysts, fixes nitrogen, or forms sMMO (Table 21.4). The DNA G+C base composition is 51–52 mol%. The major fatty acids present in *Methylosarcina* strains are $C_{16:1}$ isomers and $C_{16:0}$ (Table 21.5). The main fatty acids present include $C_{16:0}$, $C_{16:1}$ ω7c, and $C_{16:1}$ ω5t, while its major lipoquinone is the methylenated version of ubiquinone-8 (MQ-8) (Hirayama et al. 2012).

Sheathed Filamentous Uncultured Methanotrophs: *Crenothrix* Cohn 1870, "*Clonothrix*" Roze 1896

Leptothrix-like yellow-brown sheathed filamentous-like microorganisms that form extensive biofilms and can potentially block the flow of wells and other waterwork systems (Howsam 1988; Taylor et al. 1997; Madoni et al. 2000) include varieties that are proven to be methanotrophic but not able to oxidize ammonia. Originally considered cyanophyte-like, though clearly non-photosynthetic, these iron- or manganese-encrusted filamentous microbes were classified as *Crenothrix polyspora* (Cohn 1870) and also "*Clonothrix fusca*" (Roze 1896), though the latter was considered to be a morphological intermediate of *C. polyspora* until only recently (Vigliotta et al. 2007b). Also since "*Clonothrix fusca*" was associated with the Botanical Taxonomic Code not the Bacterial Code, it has remained obscure for more than a century. Overgrowth of both *Crenothrix* and "*Clonothrix*" generally occurs during winter correlating to increased concentrations of Ca^{2+} and Mg^{2+} within water. Despite being known to the biofouling-related research field for decades, the detailed understanding of the biology of these bacteria has been hampered, since to date both have defied cultivation. Nevertheless they have highly distinctive and compared to their immediate relatives curiously complex morphological features and life cycle. Both *Crenothrix* and "*Clonothrix*" appear as bundles of sheathed filaments up to 2 cm in length (Fig. 21.5a), which propagate by septation and release small coccoidal cells ("gonidia") from the tips of individual filaments (Fig. 21.5). Gonidia can be of the "macrogonidia" type, with just a single filament of larger septated cells (Fig. 21.5b) or bundles of cells ("microgonidia") (Fig. 21.5c) (Volker et al. 1977). When cross-sectioned individual gonidia have an elaborate ultrastructure including several sheath layers, classic lamellar type I intracytoplasmic membranes (Fig. 21.5d, e), polyphosphate granules, mysterious hexagonal-shaped inclusions (Fig. 21.5e), as well as equally mysterious rod-shaped fibrillar inclusion bodies (Fig. 21.5d). *C. polyspora* was observed to have degree of 16S rRNA-gene diversity, forming four subclades (with >98 % sequence similarity overall) as well as possessing a divergent *pmoA* gene, the expression of which is stimulated 30-fold by the presence of methane (Stoecker et al. 2006). Microautoradiographic analysis also found *Crenothrix polyspora* was able to take up acetate and glucose, suggesting it may be facultatively methanotrophic. "*Clonothrix fusca*" is genetically distinct but phylogenetically related to *Crenothrix*. It has a more conventional *pmoA* gene that is clustered amongst the clade consisting of *Methylomicrobium* and its relatives (Vigliotta et al. 2007a). These fascinating microbes are sources of biogenic iron oxides and occur naturally in neutral pH springs, in subterranean waters, and in coal mines (Fru 2009; Han et al. 2009; Baskar et al. 2012). Clearly genome sequences for representatives of these bacteria would provide further insight into their biology and physiology.

Type Ic Methanotrophs

The type Ic methanotrophs include the deep-branching genera *Methylohalobius* and *Methylothermus*. Though phylogenetically distinct they possess typical type I methanotroph traits. In that respect all type Ic methanotrophic species described so far have typical gram-negative cell walls, possess a strictly aerobic metabolism, and possess type I intracytoplasmic membranes with a lamellar configuration. They possess particulate methane monooxygenase but not soluble methane monooxygenase and assimilate C_1 carbons via the ribulose monophosphate pathway but not the serine pathway. They can utilize methane and methanol as sole carbon and energy sources but not methylamine, formate, or compounds with carbon-carbon bonds. They also are unable to fix nitrogen nor possess *nifH* homologs but can use ammonia and nitrate as nitrogen sources, but not glycine. They do not form *Azotobacter*-like cysts. Other traits are detailed in the description papers (Heyer et al. 2005; Tsubota et al. 2005; Hirayama et al. 2011) and Table 21.6, which shows differential characteristics, the most useful being motility, and temperature and salinity requirements. DNA base composition ranges from 54 to 62 mol%. The primary lipoquinones present include ubiquinone-8, though only *Methylothermus thermalis* has been analyzed for this trait. The main fatty acids mainly include $C_{16:0}$ (palmitic acid), $C_{18:1}$ ω9c (oleic acid), or $C_{18:1}$ ω7c (Table 21.7).

Genus *Methylohalobius* Heyer et al. 2005

The genus *Methylohalobius* is a deep-branching type Ic methanotroph and was discovered in samples collected from two hypersaline lakes (Kirleutskoe and Krugloe) in Crimea in the Ukraine (Heyer et al. 2005). At this stage, only one species is known, *Methylohalobius crimeensis*, and however is clearly the most halophilic of any known methanotroph with optimal growth occurring at 1.0–1.5 M NaCl and tolerating up to 14 % (w/v) NaCl. The type strain is relatively slow growing with a generation time of 25–36 h. Molecular ecological studies indicate *Methylohalobius*-like taxa are prevalent in certain athalassic soda lake environments, such as Mono Lake in California (Lin et al. 2005).

Table 21.6
Differential phenotypic properties separating members of the type Ic methanotrophs

Characteristic	*Methylothermus thermalis*	*Methylothermus subterraneus*	*Methylohalobius crimeensis*
Habitat	Hot spring	Subsurface aquifer	Hypersaline lakes
Colony pigmentation	Light brown	No growth on agar	White to cream
Motility (flagella)	– [a]	+ (single polar)	+ (ND)
Shape	Coccoidal	Coccoidal	Coccoidal to oval
Size (μm)	0.6–0.8	0.8–1.4	0.5–1.6 × 1.6–3.2
RuBisCO (*cbbL* gene detected)	–	–	ND
Temperature growth range (optimum)	37–67 (57–59)	37–65 (55–60)	15–42 (30–35)
pH growth range (optimum)	6.5–7.5 (6.8)	5.2–7.5 (5.8–6.3)	6.5–7.5 (7.0)
NaCl growth range (w/v %)	0–3 (0.5–1)	0–1 (0–0.3)	1–14 (5.5–8)
PHA granules	–	+	+
DNA G + C ratio (mol%)	62–63	54–55	58–59

[a]Abbreviations: + traits is positive for all or most strains of the species, – trait is negative for all or most strains of the species, *PHA* polyhydroxyalkanoate, *ND* no data is available

Table 21.7
Fatty acids and major lipoquinone composition of type Ic methanotrophs

Fatty acid[a]	*Methylothermus*	*Methylohalobius*
	% composition	
$C_{12:0}$	0–TR	0–TR
$C_{14:0}$	TR–1	1–3
$C_{14:0}$ 2-OH	0–TR	
$C_{15:}0$	TR–2	TR
$C_{15:0}$ iso	0–TR	
$C_{16:1}$ ω7c (summed feature 3)	2–4	14–20
$C_{16:0}$	37–52	23
$C_{16:0}$ 2-OH	0–9	
$C_{16:0}$ 3-OH	0–3	
$C_{16:0}$ 3-OH iso	0–4	
$C_{17:0}$	1–3	TR
$C_{17:1}$ ω6c	0–TR	
$C_{17:0}$ cyc	2–5	0–TR
$C_{18:0}$	0–2	TR
$C_{18:1}$ ω7c	TR–35	52–61
$C_{18:1}$ ω9c	0–35	
$C_{19:1}$ cyc	2–4	
$C_{19:1}$	0–3	
Lipoquinone	Ubiquinone-8	ND

[a]Fatty acid nomenclature and abbreviations are given in the footnote for *Table 21.3*

Genus *Methylothermus* Tsubota et al. 2005

A methanotrophic strain designated HB isolated from a hot spring in Hungary was shown to be able to grow at 72 °C (Bodrossy et al. 1999). To date this is the most thermophilic methanotroph known. 16S rRNA gene sequence analysis indicated HB belonged to a distinct lineage of methanotrophs that diverges substantially from known type I methanotrophs, and the suggested name *Methylothermus* was coined though not described officially. Further isolations by Japanese microbiologists lead to the formal description of the genus *Methylothermus* (Tsubota et al. 2005), which now includes *Methylothermus thermalis* and *Methylothermus subterraneus* (Hirayama et al. 2011). *Figure 21.3a, c* shows the typical morphology of cells of the genus. *Methylothermus thermalis* is the type species. Both species grow relatively rapidly on methane (generation times 2.3–7 h). Strain HB almost certainly represents yet another species of *Methylothermus*; however it is doubtful the strain is now extant or available for further study. Characteristically, *Methylothermus* species are true thermophiles growing best at around 55–60 °C. *Methylothermus thermalis* is neutrophilic and halophilic deriving from a marine hydrothermal vent system; on the other hand *Methylothermus subterraneus* tends towards being more acidophilic (though its pH growth range is rather narrow) and is non-halophilic. Molecular ecological studies have detected *Methylothermus*-like *pmoA* sequences in oceanic hydrothermal vent field samples (Nercessian et al. 2005a) and hot springs in the Kamchatka Peninsula region (Kizilova et al. 2012) suggesting the genus has a widespread distribution in thermal ecosystems.

Isolation and Cultivation

Enrichment and Primary Isolation. Methanotrophs can be enriched, isolated, and cultivated in a mineral media containing an inorganic nitrogen source and high purity methane in the headspace. The nitrate mineral salts (NMS) medium as described by Whittenbury et al. (1970b) in various modified forms is generally used with only minor modification of the trace element mineral constituents. Nitrate salts are the usual

nitrogen source for methanotroph cultivation; however a low concentration of ammonia salts (<10 mM) can be substituted or used in combination with nitrates. High levels of ammonia are inhibitory to methanotrophs as it competitively inhibits MMO and may be oxidized to toxic hydroxylamine by MMO activity. Adding low levels of copper levels ensures good growth of all methanotrophs, as the membrane-bound form of MMO (see section "Physiology") is a copper-containing enzyme. However, if copper is removed from the medium, the isolation of methanotrophs able to form soluble MMO can be potentially enhanced. Phosphates are also necessary for the growth of methanotrophs and in general require 10–100 mM. Unless otherwise specified, methanotrophs are always grown under methane since methanol much more strongly favors the growth of non-methanotrophic methylotrophs that can grow much more rapidly. A small amount of sample is added to a liquid NMS medium (see below) in serum vials or in cotton-wool-stoppered flasks and placed within airtight containers. Agar plates can be incubated easily in containers, such as most desiccators and anaerobic jars, with an inlet tap or valve. An attached pressure gauge can be used if accurate methane additions are needed. Methane can then be added directly to vials and containers by first removing a portion of the headspace. The best methane to air ratio to use is equivocal but should be in a range of 1:10–1:1, as no dramatic difference in growth rates or yields occurs with the different ratios. Methane must be of high purity as natural gas could contain acetylene which is a suicide substrate of MMO and will prevent growth even at very low concentrations (Prior and Dalton 1985b). Static incubation proceeds at an appropriate incubation temperature. Most known species of methanotrophs can be enriched and subsequently purified and cultivated at 25–30 °C. However, the temperature can be adjusted depending on the temperature of the source material. For example, the psychrophiles *Methylobacter psychrophilus* and *Methylosphaera hansonii* were isolated from enrichments incubated at 2–10 °C. All media used for psychrophiles should be prechilled before use. Enrichments take several days to several weeks. Some slow-growing species, such as *Methylosoma difficile*, may take several weeks to develop visible growth. Growth from the enrichments can then be directly plated onto NMS agar plates, which are then incubated under a methane to air or transferred to fresh liquid media for serial dilution. If samples contain large numbers of methanotrophs, turbidity in the enrichments will develop fairly rapidly (usually within 7 days), and static cultures often can develop a well-defined pellicle of growth, especially if *Methylomonas* species are enriched. For enrichments that rapidly develop turbidity, plating cultures early to achieve maximal biodiversity (if desired) is prudent, owing to the possible eventual domination of the culture by a single genotype. Unfortunately early plating does not make purification any easier.

The basic nitrate mineral salts (NMS) medium (in 1,000 ml distilled water) typically used for enrichment and cultivation of most neutrophilic, non-halophilic methanotrophs include 1 g $MgSO_4 \cdot 7H_2O$, 1 g KNO_3, 0.717 g $Na_2HPO_4 \cdot 12H_2O$, 0.272 g KH_2PO_4, 0.2 g $CaCl_2 \cdot 6H_2O$, 5 mg ferric ammonium EDTA 5 mg, and 1 ml trace element solution 1 ml. The trace element solution (in 1,000 distilled water) consists of 0.5 g disodium EDTA, 0.2 g FeSO4 · $7H_2O$ 0.2 g, 0.03 H_3BO_3, 0.02 g CoCl2 · 6H2O, 0.03 g CuSO4 · 5H2O 0.01 g $ZnSO_4 \cdot 7H_2O$, 3 mg $MnCl_2 \cdot 4H_2O$, 3 mg $Na_2MoO_4 \cdot 2H_2O$, and 2 mg $NiCl_2 \cdot 6H_2O$. The trace element solution pH is neutralized with KOH to form a stable solution. The pH of the NMS medium is adjusted to pH 6.8, and if solid media are desired, agar (or noble agar or agarose) is added to a concentration of about 1.5 % (w/v) and boiled gently to dissolve the agar. The final medium is autoclaved at 15 psi pressure (121 °C) for 15 min to sterilize.

Media modifications needed for growth of specific methanotrophs can be easily achieved. For the growth of marine methanotrophs, such as *Methylomicrobium pelagicum*, the NMS medium is prepared with artificial or natural seawater instead of distilled water, and pH is adjusted to about 8. To do this NMS medium can be replaced with 0.1 or 0.2 μ-filtered natural raw seawater. Artificial sea salts solution can also be added to achieve the desired salinity, typically 35 psi. An artificial sea salts solution (in 1,000 ml distilled water) has the following formula: 24.32 g NaCl, 5.143 g $MgCl_2$, 4.06 g Na_2SO_4, 1.14 g $CaCl_2$, 0.69 g KCl, 0.2 g $NaHCO_3$, 0.1 g KBr, 0.027 g H_3BO_4, 0.026 g $Sr(NO_3)_2$, 3 mg NaF, 2 mg Na_2SiO_3, 2 mg $FePO_4$, and 2 mg NH_4NO_3. Sea salts can also be purchased from a chemical or aquarium supply company.

Some methanotrophs prefer saline, alkaline media rather than seawater media including *Methylomicrobium alcaliphilum* and *Methylomicrobium kenyense*. To grow such species the NMS medium formula is amended with 2 % NaCl, and the pH is adjusted to about 9.5 or 10. The growth of *Methylobacter marinus* requires preparation of NMS medium with tap water (or some source natural water) and emendation with 1–2 % NaCl and, after autoclaving, addition of 1 ml of vitamin solution. Oddly this species will not grow in media prepared with distilled water, even with added NaCl and vitamins. A vitamin ×10 stock solution typically contains (per 100 ml distilled water): 10 mg pyridoxine HCl, 5 mg calcium pantothenate, 5 mg nicotinamide, 5 mg nicotinic acid, 5 mg riboflavin, 5 mg thiamine HCl, 2 mg biotin, 2 mg folic acid, and 0.1 mg cobalamin. The solution once dissolved is filtered, sterilized, and for long-term use, stored frozen, shielded from light.

Purification of Cultures. One of the most problematic areas of methanotroph study is obtaining pure cultures. In practically all situations methanotroph enrichments are heavily contaminated by non-methanotrophic (often methylotrophic) bacteria, which can easily overgrow cultures and stubbornly persist when methanotrophs are growing quite well due to their ability crossfeed on methanol and formate leaking from cells. Predatory bacteria and/or protists can also consume methanotrophic bacteria in enrichment cultures, potentially hampering isolation. Because methanotrophs may at times be relatively slow growers when plates are incubated in high humidity, fungal contamination is a frequent problem unless steps are taken to ensure containers are thoroughly cleaned with ethanol before each use. Combinations of fungicides such as cycloheximide and nystatin added to the medium are usually quite effective in reducing this problem. Cycloheximide and a suspension of

nystatin can be added in a minimal amount of methanol (up to 0.25 ml per liter) to yield final concentrations of 200 U and 100 μg per ml of media, respectively. One approach to obtain pure cultures of methanotrophs is to use a plate microscope to observe colonies at an early stage of development (within 1–3 days). Colonies, well separated from others, can be picked with a sterile needle or loop and transferred to clear section of the plate – well away from other developing microcolonies. Several colonies may have to be transferred in this way to obtain one successfully growing colony. It is necessary to make sure that the agar plates are fairly dry and excess liquid enrichment is not transferred onto the plate. This helps restrict the spreading of oligotrophic gliding bacteria and hyphomicrobia, both of which are a particular nuisances. An alternative, straightforward approach to the isolation of methanotrophs utilizes NMS agar media containing a small amount of yeast extract (0.025 % w/v) and/or methanol (0.025 % v/v) (Malashenko et al. 1975a, b) with incubation under a methane to air atmosphere. The enrichment cultures are serially diluted onto the media to the point of extinction. The yeast extract and methanol allow contaminants to grow and be more visible without affecting the growth of methanotrophs; indeed methanotroph growth may be considerably stimulated. Thus, methanotroph and contaminating bacterial colonies are more clearly distinguishable. Single colonies on the spread plates are then transferred to liquid media. A number of passages from liquid media to spread plates and back to liquid media may be necessary. Several methanotrophs do not grow on agar (e.g., *Methylothermus subterraneus*). In some cases, highly purified agars, such as noble agar, used at lower concentrations may allow growth. Alternatively, silica gel may be used (Galchenko et al. 1975, 1977); however preparation is often difficult and time consuming. For direct purification of these strains, a useful approach involves serially diluting in NMS liquid media in 96-well plastic titer trays (Bowman et al. 1997; Escoffier et al. 1997) as is done in most-probable-number counting experiments. Several strains can be purified in the same tray simultaneously. After sufficient incubation, the wells with the highest dilutions showing growth are examined microscopically. A number of separate transfers and dilutions are required to obtain morphologically homogeneous cultures. Some simple checks also help assure the purity of methanotroph cultures. The checks include incubating methanotrophs on NMS agar or in liquid NMS media without methane; no growth should occur. In addition, strains should be plated on a complex organic media such as nutrient agar (Oxoid or Difco) or R2A agar (Oxoid) and incubated with and without methane; again no growth should occur. The final test is to sequence the 16S rRNA gene of the methanotrophic strain in question. Mixed cultures will yield a mixed-template sequence chromatogram, which will require further rounds of dilution. It is possible contaminants are at low levels; thus for complete confirmation the aforementioned nutrient media and methane-free conditions need to be assessed assiduously.

Cultivation with Methanol. If methanol is desired as a growth substrate for strains, it should be added only after the liquid medium has cooled to about room temperature after autoclaving. Agar media should be cooled to about 50 °C before addition of methanol. The methanol must be filter sterilized before use. A similar approach is used for other volatile substrates. Otherwise substrates besides methanol can be added to the medium before autoclaving if not heat labile or added after autoclaving if heat labile. Methanol can be inhibitory to many freshly isolated methanotrophs due to rapid buildup of formaldehyde and must be instead provided in the vapor phase within a jar or container. Otherwise most methanotrophs can readily grow on concentrations up to 0.5 % (vol/vol) or more.

Metabolism and Physiology

Methanotrophs possess a strictly aerobic metabolism. Most methanotrophs are limited to methane and methanol as substrates; however a few species can utilize other C_1 compounds including formate and methylamine, though they appear to be exceptional (Bowman et al. 1993). Most methanotrophic strains can grow over a wide range of oxygen concentrations (<0.5–60 % v/v). Some strains or species have been described as having a preference for microaerophilic conditions (e.g., *Methylosoma difficile* (Rahalkar et al. 2007)) though this seems unusual. Nevertheless most strains show pronounceably less growth yield only once oxygen tension drops below 0.5 % (vol/vol) (Ren et al. 1997), while at least some strains still demonstrate significant growth at only 0.1 % (vol/vol) oxygen. Methanotrophs can readily survive under anoxic conditions for extended durations and can rapidly respond when methane and oxygen once again become available (Takeda 1988; Roslev and King 1994, 1995). As a result methanotrophs have the metabolic capacity to access methane beyond narrow gradients that may not be stable in many ecosystems.

Resting Stage Formation. Cyst formation amongst members of *Methylococcaceae* varies from species to species, and detection and acknowledgement of cysts may be subject to interpretation due to most strains having the propensity to accumulate large amounts of polyhydroxylalkanoate. Where cysts have been studied in more detail, they appear as single- or multibodied spherical inclusions, similar to those observed in *Azotobacter* species. Cysts develop in stationary growth phase cultures (Whittenbury et al. 1970a; Malashenko et al. 1975b; Hazeu et al. 1980). Increased cyst formation is usually associated with increasing cell refractility and increased tan pigmentation of colonies possibly indicative of melaninogenesis. The cysts appear to confer some advantages to cells experiencing deprived conditions especially lack of methane availability and desiccation; thus encysted cells are able to survive without methane or desiccation for several weeks (Whittenbury et al. 1970a). However, the cysts do not confer any heat resistance such as that provided by the exospores of *Methylosinus* species. In other type I methanotrophs (*Methylomonas*, *Methylococcus*, and *Methylocaldum* species), cyst formation is not as profuse as has been found in species of *Methylobacter* nor are cysts as well defined and are resultantly less effective in providing resistant to methane deprivation or desiccation. Moreover, cysts contain

glucan-type polysaccharides, which may act as an endogenous source of carbon and energy (Sutherland and MacKenzie 1977). Cysts can be visualized by light microscopy by using a stain developed for *Azotobacter*-type cysts (Vela and Wyss 1964).

Intracytoplasmic Membranes. Methane oxidation usually takes place in methanotrophs within membrane systems referred to as intracytoplasmic membranes (ICM) which appear as a series of elaborate intracellular membrane folds that can be readily observed by electron microscopy. Intracytoplasmic membranes occur in two major ultrastructural forms. In type I methanotrophs, ICM appears as a series of laminations or vesicular arrangements of the cytoplasmic membrane crossing the cell horizontally. In type II methanotrophs, the ICM occurs along the periphery of the cell wall and encloses a distinct lumen. Intracytoplasmic membranes are formed best when methanotrophs grow on methane, but ICM form to a lesser extent when methanotrophs grow on methanol (Best and Higgins 1981). The amount of ICM increases in proportion to methane oxidation rates, acting to increase available surface area for the oxidation of methane. The affinity of methanotrophs to methane appears to vary with growth conditions, changing by greater than an order of magnitude (Km 0.05–1 μm). The shifts in affinity are linked to the concentration of MMO within cells and changes in the relative levels of ICM (Dunfield et al. 1999). Methanotrophs in natural habitats experiencing low fluxes of methane are thus able to cope by maximizing methane-oxidizing efficiency. Methanotrophs experiencing oxygen limitation exhibit a reduction in ICM (Scott et al. 1981), while ICM synthesis is inhibited by removing copper from the medium (Prior and Dalton 1985a) which is linked to the concomitant repression in the synthesis of pMMO (see below).

Dissimilatory Methane Oxidation. The methane oxidation pathway results in the oxidation of methane to CO_2 and is used by methanotrophs to generate reducing equivalents, energy, and obtain carbon for biosynthesis. The first step of the pathway involves the oxidation of methane to methanol. Methanol is then oxidized to formaldehyde via methanol dehydrogenase, a pyrroloquinoline quinone-dependent protein that donates electrons to cytochrome *c* to power electron transport. Formaldehyde is then incorporated as cellular carbon via specialized metabolic pathways. Since formaldehyde is toxic, excess is rapidly oxidized to CO_2 via formate. This section of the methane oxidation pathway also provides cells with reducing equivalents and also helps drive electron transport and thus provide a chemiosmotic gradient to power cell functions.

Methane Monooxygenase. Methane monooxygenase (MMO; E.C.1.14.13.25) is the enzyme responsible for the oxidation of methane to methanol. It does this by incorporating a single atom of oxygen and the reaction has the following stoichiometry (Dworkin and Foster 1956):

$$CH_4 + NAD(P)H_2 + O_2 \rightarrow CH_3OH + NAD(P)^+ + H_2O$$

Two types of MMO have been found in methanotrophs; however almost all methanotrophs possess pMMO – a copper-containing enzyme which is tightly bound within the ICM. Particulate MMO has an active site which includes copper (Lieberman et al. 2003), and in the presence of copper limitation, pMMO synthesis is repressed resulting in reduced growth yields on methane (unless the methanotrophs possess sMMO) and in reduced ICM development. Previously it was found that the *amoA* gene, which codes for the active subunit of ammonia monooxygenase, hybridizes to methanotroph DNA (Semrau et al. 1995), and the genes are believed evolutionarily related (Holmes et al. 1995). Using *amoA* as a probe, the genes for pMMO were isolated and sequenced (Semrau et al. 1995). In both type I and II methanotrophs, pMMO contains three subunits, coded by the *pmoA*, *pmoB*, and *pmoC* genes (coding proteins 27, 45, and 28 kDa in size, respectively), and is present as a single or as duplicate copies in methanotrophs. In the case of sMMO, the enzyme can be readily detected by the naphthalene oxidation assay due to its unusually broad substrate specificity (Brusseau et al. 1990; Graham et al. 1992). The genes for both pMMO and sMMO are completely distinct and as a result sMMO can be readily detected by PCR of the *mmoX* gene (Martin and Murrell 1995). Both pMMO and sMMO have a common copper-inducible regulatory pathway (Nielsen et al. 1997). The importance of copper to methane oxidation has lead to the finding that some methanotrophs possess a fluorescent chromopeptide called methanobactin that acts as an chalkopore, chelating copper (Kim et al. 2004) as well as having superoxide-dismutase and peroxidase-like activities. The latter activities remove oxygen-reactive species generated by the copper ions (Choi et al. 2008). Type I methanotrophs seem to also possess a range of chalkopore-like compounds as shown in a rapid assay designed to detect copper chelation (Yoon et al. 2011); however these compounds may be quite different to methanobactin (Graham and Kim 2011). Due to the importance of copper to methanotrophs, it has been considered that methanotrophs play an important role in copper biogeochemistry (Fru 2011). More details on pMMO and sMMO biochemistry are covered in a number of reviews (e.g., Hakemian and Rosenzweig 2007; Lieberman and Rosenzweig 2004).

Carbon Assimilation Pathways. Methanotrophs fix carbon in the form of formaldehyde, which is rapidly cycled owing to its high toxicity. Formaldehyde is fixed by two different pathways in methanotrophs: the ribulose monophosphate (RuMP) pathway used mainly by type I methanotrophs and the serine pathway used by type II methanotrophs (Anthony 1982; Hanson and Hanson 1996) and the verrucomicrobial methanotrophs (Op den Camp et al. 2009). By assaying the key enzymes of these pathways, methanotrophs may be distinguished. In the case of the RuMP pathway, the key enzyme is hexulose phosphate synthase, whereas in the serine pathway the key enzyme is hydroxypyruvate reductase. *Methylococcus* and *Methylocaldum* species appear to contain both pathways, though the serine pathway may not be fully intact. A functional RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase), required for fixation of CO_2, is found in both *Methylococcus* and *Methylocaldum* species but not in tested *Methylomonas*, *Methylomicrobium*, *Methylobacter*, and type II methanotroph species. When grown on agar in the presence of methane, *M. capsulatus* could fix CO_2, suggesting RuBisCO could be

allowing CO_2 to be incorporated as a supplementary form of carbon during cellular metabolism (Baxter et al. 2002). In some strains, complex carbon sources can be used as a source of carbon when they are grown in the presence of methane leading to better growth yields (Whittenbury et al. 1970b; Whittenbury and Dalton 1981); however this activity still remains poorly studied.

Nitrogen Metabolism. Most methanotrophs assimilate ammonia and nitrate by the glutamine synthetase-glutamine 2-oxoglutarate aminotransferase system (Murrell and Dalton 1983a; Toukdarian and Lidstrom 1984b). As mentioned previously ammonia at high levels can be toxic due to possible oxidation to hydroxylamine or competition with MMO. Some type I methanotrophs possess hydroxylamine oxidase, a cytochrome-linked enzyme that could detoxify hydroxylamine by conversion to nitrite. Nitrite may be further oxidized to nitrous oxide (Campbell et al. 2011). Some strains can use hydroxylamine and nitrite as a nitrogen sources (Bowman et al. 1993) possibly by a variation of this pathway. Methanotrophs also can assimilate nitrogen from urea, amino acids and related compounds, and complex organic sources such yeast extract. In addition, many methanotrophs are able to fix atmospheric nitrogen including *Methylomonas* spp., *Methylococcus capsulatus*, and *Methylosphaera hansonii*. In most cases the nitrogenase formed is oxygen sensitive (Murrell and Dalton 1983b; Takeda 1988; Zhivotchenko et al. 1995). In the case of methanotrophs with oxygen-sensitive nitrogenase, reducing the oxygen partial pressure in the headspace enhances growth on agar under nitrogen-free conditions; however oxygen concentrations over 10 % will abolish nitrogenase activity almost completely (Zhivotchenko et al. 1995). A number of homologs of *nifH* have been shown in strains such as *Methylomonas methanica*, which appeared unable to fix nitrogen in vitro (Oakley and Murrell 1988). Southern blotting indicates the *nif* genes of various methanotrophs are homologous with each other and genes of *Klebsiella pneumoniae* (Toukdarian and Lidstrom 1984a; Oakley and Murrell 1988).

Sterols and Bacteriohopanepolyols. Methanotrophs are unusual in that they have the capacity to synthesize membrane lipids that include those of the sterol and hopane series. Sterols are formed by most eukaryotes but are only present in bacteria at very low levels and when detected thought to be contaminants (Volkman 2003). *Methylococcus capsulatus* and *Methylosphaera hansonii* were found to form substantial levels of 4-methylated sterols that are believed to be synthesized by demethylation and hydrogenation of the precursor lanosterol (Lamb et al. 2007). These species also contained squalene, steradienes, and steratrienes (Bouvier et al. 1976; Schouten et al. 2000). Bacteriohopanepolyols are membrane-associated pentacyclic triterpenoids and found in methanotrophs, methylotrophs, cyanobacteria, and various anaerobic and aerobic heterotrophic bacteria. Bacteriohopanes are of interest since due to their resistance to degradation represent important paleoenvironmental biomarkers (Talbot and Farrimond 2007) and thus could be used potentially to trace methanotrophs in environmental samples (e.g., Coolen et al. 2008; van Winden et al. 2012b).

Genome Studies

Genome sequences are available for *Methylococcus capsulatus* type strain Texas (Kleiveland et al. 2012) and strain Bath (Ward et al. 2004), *Methylomonas methanica* strain MC09 (Boden et al. 2011), and *Methylomicrobium alcaliphilum* type strain 20Z (Vuilleumier et al. 2012). Draft sequences are also available for *Methylobacter tundripaludum* type strain SV96 (Svenning et al. 2011) and *Methylomicrobium album* type strain BG8 (Lucas et al. unpublished). As of writing the sequencing of the *Methylocaldum szegediense* type strain O-12, two phylogenetically distinct but unspeciated *Methylomonas* strains, *Methylobacter luteus* type strain IMV-B-3098, *Methylobacter marinus* type strain A45, *Methylomicrobium buryatense* type strain 5G, and *Methylovulum miyakonense* type strain M12 are in progress.

Based on the data so far, type Ib methanotroph genomes are considerably larger (4.4–5.0 Mbp vs. 3.3 Mbp) than those of type Ia, though the sample size is still small. All sequenced strains have complete sets of genes for the Embden-Meyerhof-Parnas (glycolysis) pathway, the Entner-Doudoroff pathway, and the pentose phosphate pathway; *Methylococcus capsulatus* strains Texas and Bath and *Methylomicrobium alcaliphilum* 20Z also have complete sets of enzymes for the tricarboxylic acid cycle, which contradicts earlier work done on *M. capsulatus* (Higgins et al. 1981), which suggested 2-oxoglutarate dehydrogenase was absent. In *Methylomonas methanica* MC09 its TCA cycle lacks a fumarase gene homolog. *M. capsulatus* Bath and Texas strains also have complete Benson-Calvin cycles present confirming earlier functional studies (e.g., Baxter et al. 2002). The serine cycle for C_1 compound incorporation appears to be incomplete in most strains. A complete urea cycle was found in *M. tundripaludum* SV96 but not in the other strains. *Methylomonas methanica* MCO9 contains a soluble methane monooxygenase gene cluster consistent with some *M. methanica* strains having this property (i.e., Koh et al. 1993; Auman et al. 2000). *Methylomicrobium alcaliphilum* contains a pathway for ectoine synthesis, confirming data from Eshinimaev and colleagues (2007) who indicated halophilic type I methanotrophs tested had up to 12 % of their cell mass as ectoine when grown with 6 % NaCl. The pathway is also found in other halophilic methylotrophs of the genera *Methylophaga* and *Methylarcula* and could have been obtained by strain 20Z via lateral gene transfer (Reshetnikov et al. 2011). Strain 20Z also possesses the means for synthesizing and accumulating sucrose (Vuilleumier et al. 2012) and like ectoine likely acts as a compatible solutes allowing growth or survival in highly saline and alkaline soda lake habitats. *M. tundripaludum* contained an unusually large number of cold shock protein homologs (20 in total) that may aid its growth in polar soils (Svenning et al. 2011) (*Table 21.8*).

Ecology

Several specific culture-independent approaches have been applied to specific detection of methanotrophs to not only

Table 21.8
Summarized genome data for type I methanotroph strains that have genome sequence data available

Genome-sequenced strain	Status	Size (Mb)	G + C (mol%)	Protein-coding gene no.	pMMO gene cluster	Benson-Calvin cycle	sMMO gene cluster	Hydroxylamine oxidase	Nif gene clusters
Methylococcus capsulatus Bath	Complete	3.30	63.6	2,956	1 copy	+	–	–	+
Methylococcus capsulatus Texas	Draft	3.26	63.4	2,975	2 copies	+	–	–	+
Methylomicrobium alcaliphilum 20Z	Complete	4.67 + 128 Kb plasmid	48.75	4,083	1 copy	–	–	+	–
Methylomonas methanica MC09	Complete	5.05	51.3	4,494	1 copy	–	1 copy	+	+
Methylobacter tundripaludum SV96	Near finished	4.85	49.5	4,241	1 copy	–	–	+	+
Methylomicrobium album BG8	Near finished	4.49 + 47.2 kb plasmid		3,749	2 copies	–	–	–	–

avoid the hugely laborious process of cultivation but also detect uncultured taxa. These approaches include development of 16S rRNA gene-based probes adapted for fluorescent in situ hybridization; probes can be either type or genus specific (Eller et al. 2001; Carini et al. 2005; Kalyuzhnaya et al. 2006; Kallistova et al. 2007; Dianou et al. 2012). The novel application of stable isotope probing of DNA using ^{13}C-labelled methane allowed pinpointing biological active methanotroph communities (Morris et al. 2002). Specific PCR oligonucleotides adapted for standard PCR (Gulledge et al. 2001), quantitative PRC (Kolb et al. 2003; Tavormina et al. 2010; He et al. 2012a, b; Kim et al. 2012b), and functional gene microarray analysis (Bodrossy et al. 2006; Stralis-Pavese et al. 2004, 2011) allowed direct detection and sequence analysis of pMMO and sMMO genes. Some methods have pitfalls, for example, stable isotope probing procedure relies on the uptake and incorporation of the ^{13}C-moiety of the labelled methane into DNA, which occurs at a slow, leading to some label incorporated into non-methanotrophs via crossfeeding. When nutrients are added to speed the process up, fewer methanotrophs are actually detected due to some taxa growing rapidly at the expense of others (Cébron et al. 2007). This problem can be overcome by probing mRNA instead of DNA, which has more rapid and specific accumulation of ^{13}C label (Dumont et al. 2011). Despite certain limitations the molecular ecological understanding of methanotrophs has thus advanced tremendously in the last decade with methanotrophs studied in virtually all environments where methane accumulates or is present.

Thermal Terrestrial Ecosystems. High-temperature ecosystems in which methanotrophs have been found range from hot springs to organic-rich environments in which metabolic heating occurs (e.g., compost) (Trotsenko et al. 2009). In hot springs with high populations of methanotrophs, mostly only type I methanotrophs have been isolated or detected via *pmoA* sequence analysis (Malashenko et al. 1975a, b; Bodrossy et al. 1997, 1999; Tsubota et al. 2005; Hirayama et al. 2011). For example, *Methylothermus*-related taxa occur in specific hot springs located throughout the Kamchatka Peninsula, while phylotypes related to *Methylococcus*, *Methylomonas*, and *Methylobacter* that likely include novel species have been detected in various neutral pH and acidic springs (Zelenkina et al. 2009; Kizilova et al. 2012).

Alkaline and Athalassic Ecosystems. Methanotrophs have been discovered in various athalassic saline and soda lakes that have varying degrees of salinity and alkaline pH. A number of soda lake-derived strains have been classified including *Methylomicrobium alcaliphilum* (Khmelenina et al. 1997; Sorokin et al. 2000), *Methylomicrobium buryatense*, *Methylomicrobium kenyense* (Kalyuzhnaya et al. 2001, 2008), and *Methylohalobius crimeensis* (Heyer et al. 2005). Based on analysis using the stable isotope probing method, soda lakes sampled in south eastern Siberia that are pH 9–10 and with 0–4 % salinity were dominated by type I methanotrophs, though a wide diversity of types I and II methanotrophs were also present. Dominant taxa (based on *pmoA* sequences) included those related to *Methylomicrobium buryatense*, *Methylothermus*/*Methylohalobius*, and *Methylobacter psychrophilus* (Lin et al. 2004). The well-studied alkaline Mono Lake in California has a high methane oxidation rate. Based on *pmoA* sequence analysis, dominant methanotrophs present also include mainly type I methanotrophs similar to those found in the Siberian soda lakes. Community structural composition correlated to some extent with methane oxidation rates (Lin et al. 2005). In Lonar Lake, a slightly salinity, alkaline (pH 9.5–10) waterbody located in a 52,000-year-old meteorite crater in Maharashtra, India, most methanotrophs found were related to halophilic *Methylomicrobium* species, such as *Methylomicrobium buryatense*. Methanol and methylamine were mainly used

instead by *Methylophaga* and *Bacillus methanolicus* related taxa, respectively (Antony et al. 2010). In alkaline (pH 9.3–9.4) soil collected in a Chinese coal mine, a diverse range of methanotrophs were found including both type I and type II varieties. Using SIP and microarray analysis, the type I methanotrophs present were mainly related to *Methylococcus* and *Methylosoma*/"*Clonothrix*"-related unclassified taxa, as well as lower numbers of other genera (Han et al. 2009).

Low-Temperature, Wetland, and Acidic Ecosystems. About 5 % of the global methane flux into the atmosphere emerges from high-latitude waterbodies. Lipid biomarker and sequence analysis showed methanotrophs are present in saline, meromictic Antarctic lakes (Schouten et al. 2001; Coolen et al. 2004), the bottom waters of which are methane saturated. This data confirms previous isolation and biomarker analysis results for *Methylosphaera hansonii* that is present in these lakes at concentrating at oxycline, though additional yet uncultivated methanotrophs may also be present (Bowman et al. 1997; Schouten et al. 2000). Most polar lakes are however freshwater, and fluxes of methane are greater in these lakes since methanogenesis is more active. In a freshwater lake on the north slope of Alaska (Lake Qalluuraq), type I methanotrophs were the major methane oxidizers in surface sediments with *Methylosoma*, *Methylomonas*, and *Methylobacter* predominating. *Methylobacter* was also detected within the sediment layer. The methanotrophs in sediment microcosms were active over a range of temperatures 4–21 °C with most activity occurring in the surface 0–1 cm layer (He et al. 2012a, b). A high proportion of methane emerges from littoral wetland areas in lake ecosystems. Type I methanotrophs (*Methylomonas*, *Methylobacter*, and uncultured type Ib taxa) were found to become more common when the water table rose, while type II methanotrophs became more important in stagnant, drier conditions and during winter (Siljanen et al. 2012).

Another even larger source of methane to the atmosphere (about 10 %, Cicerone and Oremland 1988) is tundra wetlands. In waterlogged Arctic permafrost and surface soil layers, methane oxidation can be very active, and it was observed the predominant taxa include type Ib methanotrophs related closely to *Methylobacter psychrophilus* and *Methylobacter tundripaludum*, both isolated from tundra soils (Omelchenko et al. 1996; Berestovskaya et al. 2002; Wartiainen et al. 2006) as well as genus *Methylosarcina* (Liebner et al. 2009; Martineau et al. 2010). The taxa present did not always seem very cold adapted; indeed methane oxidation was much greater at 25 °C than 4 °C in Canadian High Arctic soil, indicating strong seasonality in methane oxidation occurs (Martineau et al. 2010). This also may suggest that with increased warming and resultant melting of the permafrost, enhanced methane oxidation could occur to at least partly compensate the expected increase in methane emissions (Whalen and Reeburgh 1990).

Peatlands are another important source of biogenic methane to the atmosphere due to high levels of accumulated plant material deposited over millennia. Though peatland soils are typically rather acidic, acidophilic methanotrophs are highly abundant and act to limit the methane flux into the atmosphere; however temperature increases may lead to increases in methane flux since methanotrophic populations may not expand enough to compensate (van Winden et al. 2012a). Diversity analyses demonstrate the majority of peatland methanotrophs are type II methanotrophs belonging to genera such as *Methylocella* and *Methylocapsa*, which can be also found in much less acidic soil types (Rahman et al. 2011). Type I methanotrophs present tend to be related to *Methylomonas*, *Methylosoma*, and *Methylovulum* (Morris et al. 2002; Chen et al. 2007; van Winden et al. 2012b). An acidophilic *Methylomonas* species *Methylomonas paludis* that prefers to grow at about pH 6 and can grow down to less than pH 4 has been described from peat soil (Danilova et al. 2013).

Soil Ecosystems. Most active ecological research on methanotrophs has been conducted in soils. The reason is that soil ecosystems globally contribute a high proportion of methane gas emissions especially rice-growing soils and landfill sites (Wise et al. 1999). Also, natural soils have been suspected to harbor oligotrophic methanotrophs that can oxidize atmospheric methane (Bender and Conrad 1992). Molecular data suggests that soils contain methanotrophs related to known and unknown genera with the types present influenced by soil chemistry and methane availability (Hoffmann et al. 2002). Temperature is also an important factor, and thus methanotrophs found in different climactic regions may differ (Kalyuzhnaya et al. 2002; Mohanty et al. 2007). Type I methanotrophs found in diverse soils include those related to *Methylomonas*, *Methylosarcina*, *Methylobacter*, *Methylococcus*, and *Methylocaldum* (Bourne et al. 2001; Reay et al. 2001; Knief et al. 2003; Kolb et al. 2003) as well as more divergent uncultured taxa. These uncultured taxa may include those that are methane oligotrophs (Knief and Dunfield 2005). In agricultural soil experiments using *pmoA* gene-based microarrays and other methods, *Methylocaldum* or *Methylosarcina* predominated with type II methanotrophs more common in deeper soil layers (Stralis-Pavese et al. 2004; Henneberger et al. 2012). Type I methanotrophs were most common in surface soil where oxygen levels were high; *Methylocystis* out competed *Methylocaldum* under conditions of lower methane tension (Stralis-Pavese et al. 2004). Other studies suggest this could be due to *Methylocystis* strains having a higher affinity to methane (Dunfield and Conrad 2000), while others so far uncultured type II methanotrophs may depend more on atmosphere-derived methane. Type I methanotrophs seem dependent on methanogenic sources of methane instead (Knief et al. 2006).

In waterlogged soils and wetland sediments, methane levels are quite high due to active methanogenesis. Molecular analyses revealed such locations have diverse communities of methanotrophs of both type I and type II taxa (Hoffmann et al. 2002; Dubey et al. 2003; De Journett et al. 2007; Vishwakarma et al. 2009; Reim et al. 2012; Dianou et al. 2012), which though not substantially influenced by the types of plants present (De Journett et al. 2007) are nevertheless very dynamic. Type I methanotrophs were observed to be more numerous on rice plant roots than in the rhizosphere (Wu et al. 2009). Thus, the rhizoplane of plants growing in muddy soils could be an excellent site for isolation of

type I methanotrophs and for conducting functional studies on methanotrophy. Subsets of the methanotroph community are affected by soil chemical perturbations, for example, application of urea as a nitrogen fertilizer may be selective for methanotrophs able to form urease (Noll et al. 2008). More fundamentally methanotrophs are affected by soil moisture and conductivity. In riparian soils, which experience only periodic moisture, increases in methane oxidation were found upon wetting to demonstrate highly dynamic responses by methanotrophs presumably in tandem with increased methanogenesis. Most of the responsive methanotrophs were *Methylobacter* and *Methylosarcina*, while type II methanotrophs appeared to be less responsive possibly because they prefer continually moist soil environments (Bodelier et al. 2012). This is also true of rice paddies; when constantly flooded, type II methanotrophs eventually predominate in the muddy soil, while type I methanotrophs instead predominate in well-drained soils (Mayumi et al. 2010). Ma and Lu (2011) obtained contradictory results showing that type II methanotrophs predominate in rice paddy soils examined after they became well drained. Close examination of rice paddy soils using a raft of molecular techniques showed most methanotrophic activity is actually concentrated at a very narrow oxic to anoxic interface less than 1 mm thick (Reim et al. 2012). Here type Ib methanotrophs related to *Methylobacter* predominated and were actively oxidizing methane at low (mM-level) oxygen tensions. Meanwhile outside this active zone, other methanotrophs including those related to *Methylosarcina* eke out an existence at low methane and high oxygen tensions, while type II methanotrophs were numerous but in general less active and mainly occur as resting cells (Reim et al. 2012). Coculture studies also demonstrate that methanotrophs can be stimulated by cobalamin excreted by other soil bacteria (Iguchi et al. 2011b). The overall findings suggest a dynamic, very focused structure occurs in methanotroph communities and that prevailing physicochemical and nutrient conditions as well as presence of non-methanotrophic partners likely dictate what methanotrophs are active at any given time and the methane oxidation capacity of the soil system. Methanotrophs are clearly very important in these ecosystems since ^{13}C-labelled methane used to enrich methanotrophs makes it way via crossfeeding and metabolism into amoeba, ciliate, and flagellate protistans suggesting methanotrophs are being grazed and thus an integral part of a methane-supported food web (Murase and Frenzel 2007). Relatively little is still known about methanotroph physiology and the exact details of biological interactions require detailed research on methanotrophic isolates in tandem with studies performed using natural communities. In the end connecting efficient methane oxidation rates to community structure may allow for manipulation of soil ecosystems, especially rice soils, to reduce methane fluxes to the atmosphere.

Aquatic Ecosystems: Lakes, Rivers, Streams, Ponds, and Groundwater. In all ecosystems methanotrophs concentrate at locations where methane and oxygen are simultaneously available. In stratified lakes this is typically the oxycline, especially in lakes whose bottom waters are methane saturated. Methanotrophs also congregate in surface sediments. Since methanogenesis can be quite intense in some systems, populations of *Methylococcaceae* often can but not always dominate the entire microbial community in freshwater pond, river, and lake sediments (Costello et al. 2002; Bussmann et al. 2004; Nercessian et al. 2005b; Rahalkar et al. 2007, 2009); thus they play integral roles in freshwater ecosystem food webs and carbon and nitrogen cycles. The populations of methanotrophs in sediment of Lake Constance in Central Europe were found to peak at a depth of 2–3 cm where the sediment was anoxic and no methane oxidation being present suggesting accumulation of potentially viable cells (Rahalkar et al. 2009). Specific communities present in freshwater sediments likely reflect the idiosyncrasies of the given waterbodies, especially salinity, and populations are also likely seasonally dynamic in terms of what might affect rates of methanogenesis. The major taxa found in the water column and the surface sediment of low-salinity waterbodies include a range of different taxa including *Methylomonas*, *Methylobacter*, *Methylosoma* (and its relatives), and *Methylosarcina* (Auman et al. 2000; Sundh et al. 2006; Tsutsumi et al. 2011).

Groundwater ecosystems also support methanotrophic populations when provided with enough methane (Kotelnikova 2002). Type I methanotroph clones obtained from groundwater aquifers or the overlying vadose zone include *Methylomonas*, *Methylocaldum*, *Methylosarcina*, and *Methylobacter psychrophilus* as well as a distinct 16S rRNA phylogenetic lineage grouped near *Methylobacter*, which with reanalysis likely comprises *Crenothrix* and "*Clonothrix*" (Koh et al. 1993; Newby et al. 2004; Urmann et al. 2008). Stable isotope probing of methanotroph communities in the groundwater of a cave that has a 1–2 % (vol/vol) methane level in its atmosphere indicated dominant methanotrophs present, which included *Methylomonas*- and *Methylococcus*-related taxa. The labelling experiments also suggest that methanotrophs are integral in the cave ecosystem helping to sustain biological activity and supporting a diverse microbial communities due to production of organic biomass (Hutchens et al. 2004).

Marine Ecosystems and Methanotroph Endosymbioses. Methanotrophs abound in the surface sediments of oceanic methane seeps (Inagaki et al. 2004; Tavormina et al. 2008), gas hydrate areas (Yan et al. 2006), hydrothermal vent areas (Elsaied et al. 2004; Nercessian et al. 2005a; Hirayama et al. 2007), and mud volcanoes (Losekann et al. 2007). Methanotrophs have been also detected in open ocean waters (Sieburth et al. 1987; Holmes et al. 1995; Hayashi et al. 2007) possibly in areas that have high local rates of benthic methanogenesis, such as estuaries (McDonald et al. 2005). Accumulated molecular analysis data suggests that type I methanotrophs predominate in marine or saline terrestrial ecosystems, though some other studies suggest type II methanotrophs may be important in some situations (e.g., Carini et al. 2005); however no halophilic type II methanotrophs have been cultured so far. Many of the *pmoA* lineages found are of uncultured methanotrophs as shown in an analysis of *pmoA* sequences in methane seeps sediments obtained in the tropical

Timor Sea. Two primary clusters were observed with one cluster distantly related to *Methylococcus* and *Methylocaldum*, while a second was related to *Methylomicrobium pelagicum* and relatives (Wasmund et al. 2009). Similar studies using different approaches (Redmond et al. 2010; Tavormina et al. 2010) detected either one or both of these clusters (called in the Tavormina study study OPU1 and OPU3) in sediment and seawater collected in sites where methane was detectable in the water column (including the Gulf of Mexico and sites of California and near Fiji) due to overlying possible or known active methane seepage sites. Neither of the uncultured methanotroph clusters was detected in an area lacking methane, in this case oligotrophic waters near Bermuda (Tavormina et al. 2010). In Saanich Inlet (Canada), which has high levels of benthic methane production, sequences related to halophilic *Methylomicrobium* species (and similar to the OPU3 clade of Tavormina et al. 2010) were found to predominate when methane was used to enrich bacteria in microcosms (Sauter et al. 2012).

Methane coming from seeps or vents can support food webs via grazing of methanotrophs and via endosymbiotic associations (DeAngelis et al. 1991; Stewart et al. 2005; Roberts et al. 2006). For example, bacterial mats on *Alvinell*a-colonized hydrothermal vent fragments and sediments collected from areas in the Mid-Atlantic Ridge and East Pacific Rise were also found to include free-living, possibly thermophilic, methanotrophs related to *Methylothermus*. In sediment from the Mid-Atlantic Ridge, putative uncultured methanotrophs with divergent *pmoA* sequences were also detected. These samples also had methanogenic communities present as well (Nercessian et al. 2005a). Metagenomic sequence-based genome assembly and metatranscriptome analysis revealed some of these uncultured methanotrophs were an active part of the larger lithotrophic community both in hydrothermal vent plumes and in the background water column (Lesniewski et al. 2012). The best studied methanotroph endosymbioses include those of the bivalve molluscan genus *Bathymodiolus*, which are common around oceanic hydrothermal vents and various methane seeps (Stewart et al. 2005; Duperron et al. 2006). These molluscs rely on methane and sulfur as their major sources of carbon and energy. This is possible due to endosymbiotic populations of methanotrophic and thiotrophic bacteria dwelling in *Bathymodiolus* gills. Type I methanotrophs, so far uncultured, have been detected in gill samples by various means including FISH, biomarker, and molecular analysis. Analysis of specimens at the Rainbow and Logachev hydrothermal fields indicated methanotrophs related to *Methylosphaera* and *Methylobacter psychrophilus* were present in tissue samples (Pimenov et al. 2002). At sites at Mid-Atlantic Ridge, different *Bathymodiolus* species had near-identical methanotroph and thiotrophic symbionts; however relative abundance changed based on location near hydrothermal vents suggesting responses to methane and sulfur levels in the water (Duperron et al. 2006).

Deepwater corals occur below the photic zone in areas of the sea including fjords and also below the margins of continental shelves and despite total darkness are still oases of life. Like *Bathymodiolus* these reefs are apparently dependent on methane seeps (Roberts et al. 2006) and thus could possess endosymbiotic relationships with methanotrophs. Stable isotope probing suggested such endosymbionts could be *Methylomicrobium* species related to *Methylomicrobium pelagicum* and *Methylomicrobium japanense* (Jensen et al. 2008).

Applications

The main role *Methylococcaceae* members play in practical applications in recent years has been as biological filters of methane. Methanotrophs have also been considered as cheap sources of single-cell protein (Anthony 1982) and the natural plastic-like polymer poly-beta-hydroxybutyrate (Hou 1984; Wendlandt et al. 1998). Many biotechnologically directed studies of methanotrophs have focused on the capability of sMMO from such methanotrophs as *Methylosinus trichosporium* OB3b to co-oxidize a wide range of carbon substrates, for example, in the production of epoxides for plastics manufacture (Hou 1984) and within the bioremediation field (Koh et al. 1993); however most *Methylococcaceae* lack sMMO, so they have been less studied for such applications. The efficient ability to oxidize methane at a range of different conditions, however, makes type I methanotrophs or consortia of methanotrophs with other bacteria provide potential low-cost solutions for scrubbing methane from air flows, soil matrices, and liquid effluent (Hatamoto et al. 2011). Also some type I methanotrophs, such as *Methylomicrobium* spp., from soda lakes grow rapidly and are tolerant to a wide range of physiochemical stresses making them potentially amenable as methane oxidation catalysts with enhancement of cellular properties possible by genetic manipulation (Ojala et al. 2011).

Methylomonas spp. produce carotenoids including the rarely observed 30-carbon 4,4′-diapolycopene dialdehyde and the equivalent carboxylic acid. A novel protein called CrtNb, which codes a carotenoid oxidase, was found to be responsible for conversion of 4,4′apolycopene to the aldehyde derivative. The carotenoid compound once extracted has a dark red to purple color and could be responsible for the characteristic pink color of various *Methylomonas* strains. The application of this compound as a coloring agent in food or cosmetics has been considered (Tao et al. 2005). A strain of *Methylomonas* has also been engineered via insertion of promoterless transposons carrying carotenoid biosynthesis genes to produce the pink-colored C_{40} carotenoids canthaxanthin and astaxanthin (Sharpe et al. 2007). The authors of the study proposed engineered methanotrophs could be cheap sources of carotenoid pigments for various applications (Das et al. 2007).

Landfill soils are a major source of biogenic methane. To remediate such site the use of biocovers that have a structure and material content that fosters methanotrophic populations are being devised and tested. An example that included earthworm casts added to rice paddy soil in a 3:7 ratio increased methane oxidation rates fivefold relative to that of normal paddy soil (Moon et al. 2010). Indeed certain plants (Wang et al. 2009) and earthworm activity can enhance methane-oxidizing

activity by type I (bit not type II) methanotrophs in landfill soils (Binet et al. 1998; Héry et al. 2008; Kumaresan et al. 2011) without otherwise effecting the nature of the methanotrophic community. This may occur by stable provision of methane and nutrients via bioturbation of the soil matrix. In biocovers and in phytostimulated landfill soils, it has been found type Ia and Ib methanotrophs typically predominate, especially *Methylocaldum*- and *Methylosarcina*-related taxa (Chen et al. 2007; Kallistova et al. 2007; Wang et al. 2009; Moon et al. 2010; Chi et al. 2012). Provision of certain volatile compounds such as dimethyl sulfide seems to boost type I (but not type II) methanotroph populations and could be applied to enhance biocover methane filtration (Kim et al. 2012b).

Biofilms of methanotrophs can be potentially used in several applications as methane scrubbers, removing methane from effluent gases and liquids before environmental discharge. Downward flow sponge reactors were tested as a means to scrub methane from anaerobic digester wastewaters in order to prevent excessive methane emissions. It was found that *Methylocaldum* dominated in the reactor community and that with reduction in oxygen flow rates, ammonia oxidation was first reduced, followed by methane oxidation, and finally sulfide oxidizers also present were the last to be affected suggesting in anaerobic gradient zones methanotrophs may interact with microaerophilic and more strictly aerobic communities (Hatamoto et al. 2011). Closer examination of a wastewater methanotroph biofilm filter revealed a complex community in which methanotrophs made up about a quarter of the biomass with the remainder consisting of many types of methylotrophs, heterotrophs, and facultative autotrophs, such as *Hyphomicrobium*, *Flavobacterium*, and *Hydrogenophaga* (Kim et al. 2012a). The data suggests that a successful methanotroph filter likely consists of complex consortia where many species interact to create a stable but active assemblage. Contaminants in waste streams may impact on the performance of biocovers and biofilm filters. It was found the polyaromatic hydrocarbon pyrene represses methane oxidation rates in soil possibly due to DNA intercalation. It was however observed that members of genus *Methylocaldum* tolerated pyrene exposure (Deng et al. 2011) suggesting manipulated biocovers can potentially withstand irregular pollutant contamination.

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22 The Family *Moraxellaceae*

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Abstract
The family *Moraxellaceae* is a member of the order *Pseudomonadales*, class *Gammaproteobacteria*, currently encompassing the genera *Acinetobacter*, *Moraxella* (the type genus), and *Psychrobacter*, and the more recently proposed genera *Alkanindiges*, *Paraperlucidibaca*, and *Perlucidibaca*. Several of the microorganisms included in this family have a history of debate and changes in their names over the times, generating difficulties in the interpretation of early literature. The family is composed by a heterogeneous group of bacteria distributed over a wide variety of natural habitats and having diverse ecological and clinical significances. The family includes species that colonize mucosal membranes or the skin of humans and animals, and can occasionally cause a variety of infections, as well as apparently harmless species occurring in the environment, including water, soil, and foodstuffs. Most of them are considered saprophytes of little clinical significance, while a few represent important infectious agents. In recent years, certain species, notably those included in the so-called *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex, have emerged as important agents of opportunistic infections in the scenario of difficult-to-treat healthcare-associated infections. Two species, *Moraxella catarrhalis* and *Moraxella bovis*, play an important role in human and veterinary medicine, respectively. *M. catarrhalis* is recognized as an exclusively human pathogen causing lower and upper respiratory tract infections, and *M. bovis* is the primary etiologic agent of infectious bovine keratoconjunctivitis, the most important ocular disease of cattle worldwide, causing significant economic impact. The genus *Psychrobacter* is mainly represented by psychrophilic bacteria particularly found in natural cold saline environments. Some species are associated with terrestrial and marine animals, and with foodstuff. They are rarely isolated from human sources, and little is known about their clinical significance. On the other hand, some *Psychrobacter* are sources of cold-adapted proteins and enzymes which have broad applicability in industrial processes, modification of heat-labile substances, and in energy conservation. Members of the genus *Acinetobacter* may also have a wide range of industrial applications, such as bioremediation of waste waters and effluents, degradation of petrochemicals, production of biopolymers and biosurfactants, biomass production, and production of immune adjuvants.

Taxonomy: Historical and Current

Mo.ra.xel.la'ce.ae. M.L. fem. n. *Moraxella*, type genus of the family.; *-aceae*, ending to denote family; M.L. fem. pl. n. *Moraxellaceae*, the *Moraxella* family (Rossau et al. 1991).

The family *Moraxellaceae* is a member of the order *Pseudomonadales* (Orla-Jensen 1921), class *Gammaproteobacteria* and rRNA superfamily II (Garrity et al. 2005), phylum Proteobacteria (Stackebrandt et al. 1988; Gao et al. 2009). The family *Moraxellaceae* was originally proposed to accommodate the genera *Moraxella*, *Acinetobacter*, and *Psychrobacter* and related microorganisms, on the basis of results of DNA-DNA hybridization and DNA-rRNA hybridization studies (Rossau et al. 1991). Subsequently, analysis of 16S rDNA gene sequences was found to constitute an important part of the framework for taxonomic studies of the members of this family (Pettersson et al. 1998; Juni and Bøvre 2005). Currently, besides encompassing the type genus *Moraxella* (Lwoff 1939; emended by Henriksen and Bøvre 1968; Juni and Bøvre 2005), as well as the genera *Acinetobacter* (Brisou and Prévot 1954; Juni and Bøvre 2005) and *Psychrobacter* (Juni and Heym 1986), the family is also considered to include the more recently proposed genera *Alkanindiges* (Bogan et al. 2003), *Perlucidibaca* (Song et al. 2008), and *Paraperlucidibaca* (Oh et al. 2011). The genus *Enhydrobacter* (Staley et al. 1987) has also been considered in the family *Moraxellaceae* in certain communications and databases (e.g., http://www.bacterio.net/ and http://www.ncbi.nlm.nih.gov/ taxonomy, as of May 2013) or as a genus incertae sedis in the *Bergey's Manual of Systematic Bacteriology*, 2nd edition) (Staley and Brenner 2005). However, evidence and a proposal for this genus to be transferred to the family *Rhodospirillaceae* within the class *Alphaproteobacteria* has already been published (Kawamura et al. 2012).

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Several of the microorganisms included in this family have a history of debate and changes in their names over the times. A variety of denominations have been used by different authors 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 (Bergogne-Bérézin and Towner 1996; Juni and Bøvre 2005; Bowman 2006; Hays 2006; Towner 2006; Vaneechoutte et al. 2011). Nowadays, although the larger application of molecular approaches has contributed with important insights into the phylogeny and taxonomy of many members of this group of bacteria (Gao et al. 2009), some of them may still deserve reevaluation in order to reach consensus. For that, application of polyphasic approaches to analyze key representative strains within each taxon will be helpful to resolve their relationships, and to clarify their classification and phylogenetic standing.

These microorganisms are nonfermentative, due to their inability to metabolize carbohydrates by the fermentative pathway. On Gram staining, bacterial cells appear as Gram-negative rods, coccobacilli, or diplococci, and there may be a tendency to resist destain. They usually occur in pairs or short chains, often becoming more coccoid as the cultures age (stationary phase). The cells are nonmotile in liquid media, but surface-bound motility may be observed. Capsules and fimbriae may be present. Chemo-organotrophic and aerobic. Mesophilic or psychrophilic. Strains belonging to the genera *Moraxella*, *Perlucidibaca*, *Paraperlucidibaca*, and *Psychrobacter* are oxidase-positive, while strains of *Acinetobacter* and *Alkanindiges* are oxidase-negative. They are usually catalase-positive. Indole is usually not produced. True waxes may be present. The fatty acid profiles show the presence of mainly unbranched, saturated, and mono- or di-unsaturated fatty acids with predominantly 16 and 18 carbon atoms. G+C content usually ranges from 38 to 50 mol% (Rossau et al. 1991; Juni and Bøvre 2005). Rarely found species belonging to the genera more recently described have higher G+C contents, in the range of 61–63 mol% (Bogan et al. 2003; Song et al. 2008; Oh et al. 2011).

The phylogenetic relationships among the members of the family *Moraxellaceae* based on the comparison of the 16S rRNA gene sequences are shown in ❯ *Fig. 22.1*. Sequencing of the 16S rRNA gene is a powerful tool to establish the differentiation among genera included in this family. It allows separation of the six clusters comprising the multi-species genera *Acinetobacter*, *Moraxella*, and *Psychrobacter*, and the three additional genera (*Alkanindiges*, *Paraperlucidibaca*, and *Perlucidibaca*) that are composed by a single species each, up to date. Discrimination among species within each genus is also achievable by comparative analysis of the 16S rRNA gene sequences. Some species, however, possess very similar 16S rRNA gene sequences, and such analysis can only provide a preliminary assignment of species identity within the genus. In these cases, the application of additional molecular tools, in conjunction with phenotypic testing, is necessary for reliable identification.

Mo.rax.el la. M.L. dim. *-ella* ending; M.L. fem. n. *Moraxella* named after V. Morax, a Swiss ophthalmologist who pioneered the recognition of the type species (Lwoff 1939; Henriksen and Bøvre 1968).

The taxonomy of the genus *Moraxella* has undergone several revisions resulting in considerable changes, since the original proposal by Lwoff (1939). Several members of this family had previously been allocated in different genera, as exemplified by *Moraxella catarrhalis*, an important pathogen in the group, that has received different names since its first discovery in 1896 (Frosch and Kolle 1896; see references Hays 2006 and Juni and Bøvre 2005 for details). In 1979, K. Bøvre (1979) proposed to divide the genus *Moraxella* into two subgenera: subgenus *Moraxella* (Lwoff 1939) Bøvre 1979 and subgenus *Branhamella* (Catlin 1970) Bøvre 1979. However, these subgenera designations were not included in the *Approved Lists of Bacterial Names* (Skerman et al. 1980, 1989), and even though their valid publication was requested (Euzeby 2001), the Judicial Commission of the International Committee on Systematics of Prokaryotes (Tindall 2008; Garrity et al. 2011) considered that these names were not in accordance with the Rules of the Code at the time the Approved Lists were drawn up and they could not have been added to the lists. Consequently, such subgenera division is no longer in use (Garrity et al. 2011; also see http://www.bacterio.net/ for details). Currently, this genus comprises approximately 19 species that have been validly named, including *Moraxella atlantae, Moraxella boevrei, Moraxella bovis, Moraxella bovoculi, Moraxella canis, Moraxella caprae, Moraxella catarrhalis, Moraxella caviae, Moraxella cuniculi, Moraxella equi, Moraxella lacunata, Moraxella lincolnii, Moraxella nonliquefaciens, Moraxella oblonga, Moraxella osloensis, Moraxella ovis, Moraxella pluranimalium, Moraxella porci*, and *Moraxella saccharolytica*. All the respective 16S rRNA gene sequences are available, except for *M. saccharolytica*. In fact, recent information available at the BCCM/LMG bacteria catalog (http://bccm.belspo.be/db/lmg_taxon_browser.php?startwith=m; as of May 2013) indicates that strain LMG 1039 (ATCC 19248), the type strain for *M. saccharolytica*, has been identified as a member of the genus *Chryseobacterium* according to 16S rDNA gene sequence analysis. Species of *Moraxella* have DNA G+C contents ranging from 40 to 49.6 mol%.

A.ci.ne to.bac.ter. Gr. adj. *akinetos* unable to move; M.L. n. *bacter* the masculine form of the Gr. neut. n. *bactrum* a rod; M.L. masc. n. *Acinetobacter* nonmotile Rod (Brisou and Prévot 1954).

The taxonomy of the genus *Acinetobacter* has also experienced extensive changes (Juni and Bøvre 2005; Towner 2006; Dijkshoorn and Nemec 2008; Peleg et al. 2008; Vaneechoutte et al. 2011; Alvarez-Pérez et al. 2013). The first documentation of a strain representative of this genus is credited to Beijerink, in 1911, by isolating a microorganism from the soil and naming it *Micrococcus calcoaceticus* (apud Baumann 1968). The generic designation as *Acinetobacter* was proposed by Brisou and Prévot (1954), after several names had been used to denominate these microorganisms (Henriksen 1973; Bergogne-Bérézin and Towner 1996). They remain as a heterogeneous group of

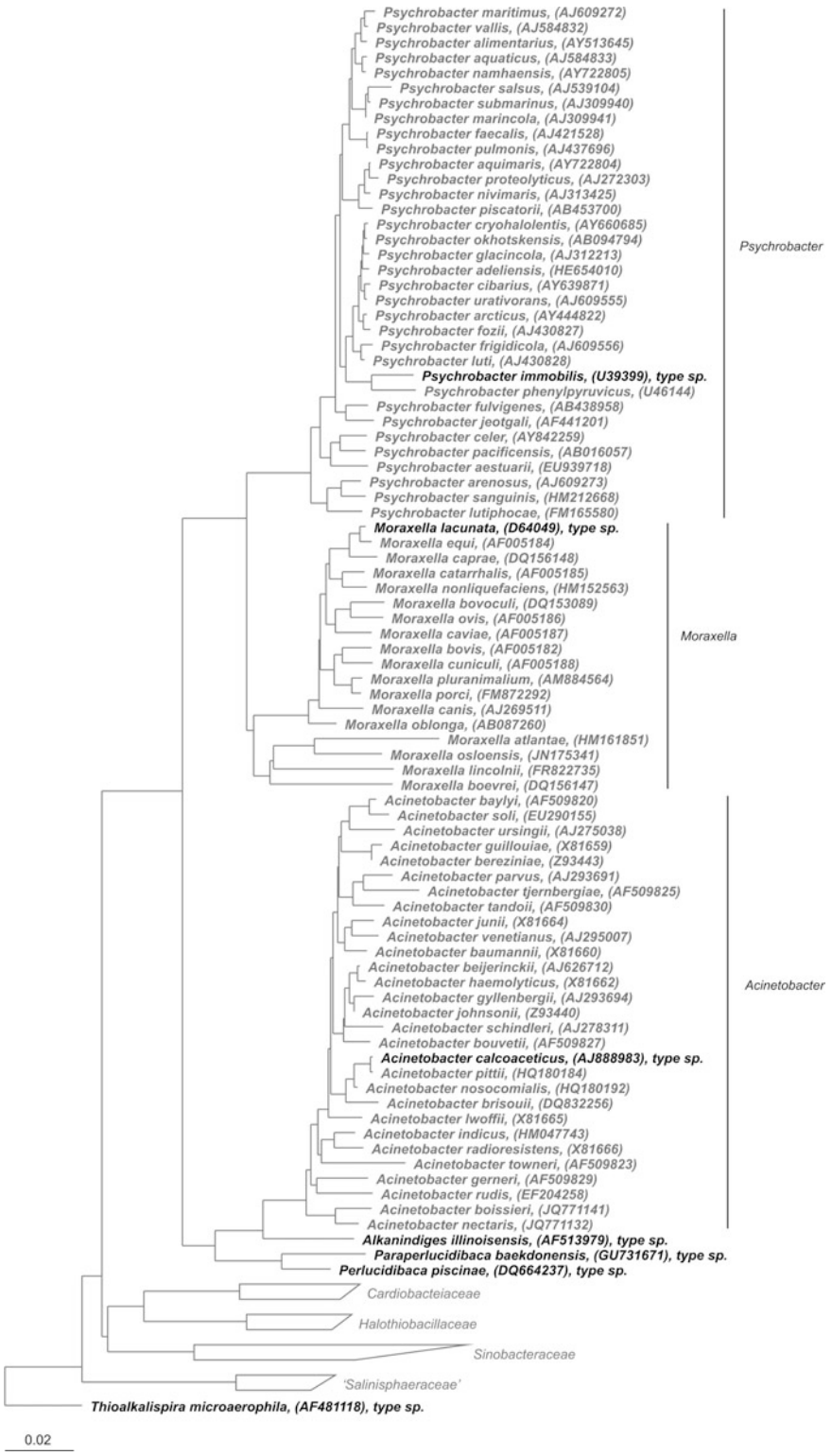

Fig. 22.1

Phylogenetic reconstruction of the family *Moraxellaceae* based on 16S rRNA gene and created using the neighbor-joining algorithm with the Jukes-Cantor correction. The sequence datasets and alignments were used according to the All-Species Living Tree Project (*LTP*) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high quality type strain sequences proportionally distributed among the different bacterial and archaeal phyla. In addition, a 40 % maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. Scale bar indicates estimated sequence divergence

oxidase-positive and oxidase-negative bacteria, until the proposal of moving the oxidase-positive strains to the genus *Moraxella* (Baumann 1968). The Subcommittee on the Taxonomy of *Moraxella* and Allied Bacteria subsequently proposed that the genus *Acinetobacter* should include only the oxidase-negative strains (Lessel 1971), a division that has been supported by the use of transformation tests (Juni 1978). Until today, the transformation assay is a basic tool in *Acinetobacter* taxonomy and the ability to transform *Acinetobacter* strain BD413 still constitutes a prerequisite for inclusion of isolates in the genus *Acinetobacter* (Juni 1984; Weyant et al. 1996; Peleg et al. 2008).

The genus was previously classified in the family *Neisseriaceae* (Juni 1984), but the results of molecular studies indicated that *Acinetobacter* should be classified in the family *Moraxellaceae*, as proposed by Rossau et al. (1991). Of major importance in the long and complicated history of the genus were the DNA-DNA reassociation studies that resulted in the recognition of several genomic species or genospecies (Bouvet and Grimont 1986; Nishimura et al. 1988; Tjernberg and Ursing 1989; also see following references for details: Juni and Bøvre 2005; Dijkshoorn and Nemec 2008; Peleg et al. 2008). Some of them, however, are difficult to differentiate on the basis of phenotypic characteristics and even at the light of molecular testing may still be referred as complexes of species. Although phenotypic identification is challenging, various molecular methods have been developed in an attempt to provide precise identification for taxonomic and epidemiological applications. Sequencing techniques have been helpful in discriminating these microorganisms, and in supporting the description of several of the newer species in the genus, but their use needs to be intensified in order to clarify the diversity of species constituting this genus. ❯ *Table 22.1* lists the species and/or genomic species recognized as members of the genus *Acinetobacter*. Currently, approximately 38 species are considered to belong to the genus *Acinetobacter*. They include 29 valid named species (*Acinetobacter baumannii, Acinetobacter baylyi, Acinetobacter beijerinckii, Acinetobacter bereziniae, Acinetobacter boissieri, Acinetobacter bouvetii, Acinetobacter brisouii, Acinetobacter calcoaceticus, Acinetobacter gerneri, Acinetobacter guillouiae, Acinetobacter gyllenbergii, Acinetobacter haemolyticus, Acinetobacter indicus, Acinetobacter johnsonii, Acinetobacter junii, Acinetobacter lwoffii, Acinetobacter nectaris, Acinetobacter nosocomialis, Acinetobacter parvus, Acinetobacter pittii, Acinetobacter radioresistens, Acinetobacter rudis, Acinetobacter schindleri, Acinetobacter soli, Acinetobacter tandoii, Acinetobacter tjernbergiae, Acinetobacter towneri, Acinetobacter ursingii*, and *Acinetobacter venetianus*) and nine that still remain as unnamed genomic species with provisional designations (6, 13BJ/14TU, 14BJ, 15BJ, 15TU, 16, 17, Between 1 and 3, and Close to 13TU), as shown in ❯ *Table 22.1*. All of the already named species have 16S rRNA gene sequences deposited in the GenBank. Several additional species denominations have been mentioned in the last 5–6 years, but they have not been validated yet. Species of *Acinetobacter* have DNA G+C contents ranging from 38 to 47 mol%.

Table 22.1
Genomic (DNA-DNA hybridization groups) and named species of *Acinetobacter*

Genomic species	Named species	References[a]
1	*A. calcoaceticus*	1, 4
2	*A. baumannii*	1, 4
3	*A. pittii*	1, 4, 14
4	*A. haemolyticus*	1, 4
5	*A. junii*	1, 4
7	*A. johnsonii*	1, 4
8/9	*A. lwoffii*	1, 4
10	*A. bereziniae*	1, 4, 13
11	*A. guillouiae*	1, 4, 13
12	*A. radioresistens*	1, 2, 4
13TU	*A. nosocomialis*	4, 14
6		1, 4
13BJ/14TU		4, 3
14BJ		3
15TU		4
15BJ		3
16BJ		3
17BJ		3
"Between 1 and 3"		5
"Close to 13 TU"		5
	A. baylyi	7
	A. beijerinckii	10
	A. boissieri	17
	A. bouvetii	7
	A. brisouii	12
	A. gerneri	7
	A. gyllenbergii	10
	A. indicus	16
	A. nectaris	17
	A. parvus	8
	A. rudis	15
	A. schindleri	6
	A. soli	9
	A. tandoii	7
	A. tjernbergiae	7
	A. towneri	7
	A. ursingii	6
	A. venetianus	11

[a]1, Bouvet and Grimont 1986; 2, Nishimura et al. 1988; 3, Bouvet and Jeanjean 1989; 4, Tjernberg and Ursing 1989; 5, Gerner-Smidt and Tjernberg 1993; 6, Nemec et al. 2001; 7, Carr et al. 2003; 8, Nemec et al. 2003; 9, Kim et al. 2008; 10, Nemec et al. 2009; 11, Vaneechoutte et al. 2009; 12, Anandham et al. 2010; 13, Nemec et al. 2010; 14, Nemec et al. 2011; 15, Vaz-Moreira et al. 2011; 16, Malhotra et al. 2012; 17, Alvarez-Pérez et al. 2013

Psy.chro bac.ter. Gr. adj. *psychros* cold; M.L. masc. n. *bacter* rod; M.L. masc. n. *Psychrobacter* a rod that grows at low temperatures (Juni and Heym 1986).

The genus *Psychrobacter* was proposed by Juni and Heym (1986), along with the description of the species *Psychrobacter immobilis*, and includes microorganisms that are cold-adapted and osmotolerant. The genus has become better known more recently due to the intensification of environmental studies associating bacterial isolation and 16S rRNA gene sequencing. The number of species composing the genus has also grown rapidly, mostly from the increasing exploration of marine and polar ecosystems. Nevertheless, some of the species included in this genus have been recovered from a variety of clinical sources (see section on "Pathogenicity: Clinical Relevance"). Presently, the genus *Psychrobacter* consists of 34 species: *Psychrobacter adeliensis, Psychrobacter aestuarii, Psychrobacter alimentarius, Psychrobacter aquaticus, Psychrobacter aquimaris, Psychrobacter arcticus, Psychrobacter arenosus, Psychrobacter celer, Psychrobacter cibarius, Psychrobacter cryohalolentis, Psychrobacter faecalis, Psychrobacter fozii, Psychrobacter frigidicola, Psychrobacter fulvigenes, Psychrobacter glacincola, Psychrobacter immobilis, Psychrobacter jeotgali, Psychrobacter luti, Psychrobacter lutiphocae, Psychrobacter marincola, Psychrobacter maritimus, Psychrobacter namhaensis, Psychrobacter nivimaris, Psychrobacter okhotskensis, Psychrobacter pacificensis, Psychrobacter phenylpyruvicus, Psychrobacter piscatorii, Psychrobacter proteolyticus, Psychrobacter pulmonis, Psychrobacter salsus, Psychrobacter sanguinis, Psychrobacter submarinus, Psychrobacter urativorans*, and *Psychrobacter vallis*. Species of *Psychrobacter* have DNA G+C contents ranging from 42 to 50 mol%.

The genus *Alkanindiges* is represented by the species *Alkanindiges illinoisensis*, proposed to accommodate an alkane-degrading bacterium isolated from chronically crude oil–contaminated soil from an oilfield in southern Illinois, USA (Bogan et al. 2003). The proposal was supported by 16S rRNA gene sequencing analysis and results of growth testing in the presence of a range of substrates. A second species was proposed for inclusion in the genus *Alkanindiges*, named "*Alkanindiges hongkongensis*," represented by a clinical isolate recovered from the abscess pus of a 72-year-old patient with Warthin's tumor and parotid abscess (Woo et al. 2005). The denomination, however, has not been validly published, so far. The DNA G+C content of the A. *illinoisensis* type strain is 46.2 mol%.

In 2008, the new genus *Perlucidibaca* was proposed for inclusion in the family *Moraxellaceae*, corresponding to a single species, named *Perlucidibaca piscinae* (Song et al. 2008). This novel species designates a freshwater bacterium isolated from an artificial freshwater pond in Korea. Phylogenetic trees generated using 16S rRNA gene sequences showed that the novel isolate belonged to the family *Moraxellaceae* and formed a distinct lineage within the family. The DNA G+C content of the *P. piscinae* type strain is 63.1 mol%. Interestingly, sequence comparisons showed that the most closely related cultured species with respect to this strain was "*Alkanindiges hongkongensis*," described by Woo et al. (2005) but not yet validated as mentioned above.

The genus *Paraperlucidibaca* is the most recently proposed for inclusion in the family (Oh et al. 2011). It is composed by the novel species *Paraperlucidibaca baekdonensis*, created to accommodate a Gram-negative, non-spore-forming, rod-shaped bacterial strain isolated from seawater of the East Sea in Korea. Phylogenetic trees based on 16S rRNA gene sequence analysis showed that this new species forms a cluster with *Perlucidibaca piscinae* and various uncultured and unidentified *Gammaproteobacteria*. The DNA G+C content of the type strain of *P. baekdonensis* is 61.3 mol%.

Overall, the genera included in the family *Moraxellaceae* comprise a diverse group of microorganisms presenting a variety of phenotypic and genotypic traits and having diverse ecological and clinical significances. They may be represented by species that colonize mucosal membranes or the skin of humans and animals, and can occasionally cause a variety of infections, as well as apparently harmless species occurring in the environment, including water, soil, and foodstuffs. Availability of information about these microorganisms is also diverse, ranging from vast, for the oldest and better known genera, to very poor for the most recently described genera and species. In fact, some of the more recently described members, mostly from environmental sources, included only one or a few strains at the time of the publication. Accordingly, most of the data presented in this chapter is related to species belonging to the three predominant genera of *Moraxellaceae*: *Acinetobacter*, *Moraxella*, and *Psychrobacter*.

Molecular Analyses

DNA-DNA hybridization analysis and 16S rRNA gene sequencing are two of the main procedures that have been used to obtain data for establishing phylogenetic relationships among the members of the family *Moraxellaceae* (Pettersson et al. 1998; Juni and Bøvre 2005; Peleg et al. 2008; Vaneechoutte and De Baere 2008; Murayama et al. 2010). The relationships among members of the *Moraxellaceae* were originally established by genetic transformation experiments (Bøvre and Hagen 1981). DNA-DNA hybridization studies of the family *Neisseriaceae*, as this family was previously constituted, led to the conclusion that organisms presently considered to be members of the *Moraxellaceae* are not related to the *Neisseriaceae* (Rossau et al. 1989, 1991). Although DNA-DNA hybridization is a useful procedure for establishing membership in the *Moraxellaceae*, in some cases, the results do not necessarily provide the means for distinguishing genera, or species of a particular genus, within the family (Juni and Bøvre 2005). In general, the usefulness of 16S rRNA gene sequence analysis is similar to DNA-DNA hybridization in that it enables the recognition of strains as members of the *Moraxellaceae*. By using these two procedures, it was possible to demonstrate that the genera *Acinetobacter*, *Moraxella*, and *Psychrobacter* are not members of the *Neisseriaceae* (Rossau et al. 1991; Enright et al. 1994), and

that they constituted monophyletic taxa, forming a distinct line of descent within the *Gammaproteobacteria*.

The difficulties generally encountered for precise identification of the diverse members of the family *Moraxellaceae* based on biochemical characteristics, especially at the species level, have generated a major interest in the development and application of a variety of molecular methods. More recently, several molecular techniques have been applied not only for taxonomic studies of members of the family *Moraxellaceae*, but also for identification and epidemiological purposes.

The genus *Acinetobacter* is possibly the major example, within the family *Moraxellaceae*, on the use of a wide array of molecular tools for characterization at different levels. Three comprehensive pioneer studies based on DNA-DNA hybridization experiments have laid the foundation for the current taxonomy of the genus *Acinetobacter* (Bouvet and Grimont 1986; Tjernberg and Ursing 1989; Gerner-Smidt et al. 1991). Since then, various genotypic methods have been described for the identification of species of *Acinetobacter*, although DNA-DNA hybridization continues to be a reference method that has been used for the description of most of the *Acinetobacter* species. Amplified 16S ribosomal DNA restriction analysis (ARDRA), a method that is based on restriction analysis of the amplified 16S rRNA gene, has been applied for the identification of these microorganisms, by using a panel of five endonuclease restriction enzymes: *Cfo*I, *Alu*I, *Mbo*I, *Rsa*I, and *Msp*I (Vaneechoutte et al. 1995; Dijkshoorn et al. 1998). Multiple profiles were found to occur in some species, while identical profiles occur in others, and additional phenotypic testing is required for definitive identification (Nemec et al. 2000). Amplified Fragment Length Polymorphism (AFLP) analysis, by using *Eco*RI and *Mse*I as restriction enzymes, has been used for the identification of *Acinetobacter* strains. The results correlated generally well with those obtained by DNA-DNA hybridization experiments, and the method has been considered as an alternative tool to identify isolates to the species level and to delineate novel species (Janssen et al. 1996; Dijkshoorn and Nemec 2008).

More recently, several sequencing-based methods have been applied for the identification of *Acinetobacter*. Among them, sequencing of the 16S rRNA gene is possibly the method more frequently used, and a detailed description of the method and application for *Acinetobacter* identification is available (Vaneechoutte and De Baere 2008). Although 16S rDNA sequence analysis is a valuable method, the intra- and interspecies similarity values are very close, and the grouping of some species of *Acinetobacter*, including those belonging to the Acb complex, obtained by this method does not correspond well with the grouping obtained by DNA-DNA hybridization (Vaneechoutte and De Baere 2008). Sequence analysis of *rpoB* gene has also been evaluated in detail for the identification of several species of *Acinetobacter*: Two polymorphic zones ("zones 1 and 2"), within or flanking the gene, were found to be particularly promising (La Scola et al. 2006). Zone 1 alone was further shown to be sufficient for the identification of the four major species belonging to the Acb complex of species (Gundi et al. 2009). The combined zones 1 and 2 were also useful for identification of these species and to delineate several other novel *Acinetobacter* species (Vaneechoutte et al. 2009; Nemec et al. 2010, 2011). Although not yet validated for all the validly described species, the *rpoB* sequence is currently the most promising sequence for the differentiation of *Acinetobacter* species. Multiplex PCR methods for detection of species-specific gene sequences have also been developed for a few members of the genus *Acinetobacter*. In one of the methods, the intrinsic carbapenemase gene $bla_{\text{OXA-51-like}}$ was found to be specific for *A. baumannii*, as detected in a multiplex PCR also targeting $bla_{\text{OXA-23-like}}$ and the class 1 integrase gene (Turton et al. 2006). More recently, however, the presence of the gene $bla_{\text{OXA-51-like}}$ was detected in Asian strains of *A. nosocomialis*, indicating that it may not be specific for *A. baumannii* (Lee et al. 2009; Koh et al. 2012). Multiplex PCR identification based on specific sequences in the *gyrB* gene has also been developed for the identification of species belonging to the Acb complex (Higgins et al. 2010).

The increasing documentation of *Acinetobacter* as important agent of opportunistic infections in the scenario of difficult-to-treat healthcare-associated infections (HAIs), has generated a high demand for strain typing and epidemiological studies. The introduction of molecular techniques has substantially improved the ability to discriminate *Acinetobacter* isolates within a given species, and extensive reviews are available (Dijkshoorn et al. 2007; Seifert and Wisplinghoff 2008; Peleg et al. 2008, 2012). Plasmid typing was one of the first techniques applied for molecular typing for these microorganisms, allowing the detection of a high intraspecific diversity of plasmids among isolates belonging to the Acb complex (Gerner-Smidt 1989; Towner 1991; Seifert et al. 1997; Nemec et al. 1999). More recent studies showed that most of the plasmids that are indigenous in *Acinetobacter* may have a long history of exchange with other plasmids and chromosomes, and have also indicated the spread of plasmid-associated carbapenem-resistance genes (Chen et al. 2009; Lu et al. 2009; Fondi et al. 2010). A special application of plasmid typing is PCR-based replicon sequence typing to characterize A. *baumannii* strains with a $bla_{\text{OXA-58}}$ or $bla_{\text{OXA-23}}$ carbapenemase gene (Bertini et al. 2010). AFLP analysis has also been applied to investigate the epidemiology and diversity among species of *Acinetobacter* (Dobrewski et al. 2006; van den Broek et al. 2006, 2009; Dijkshoorn and Nemec 2008; Nemec et al. 2008), apart from being used for species identification. This method has been useful in delineating clusters of isolates within *A. baumannii* that are considered to represent clonal lineages (Dijkshoorn et al. 1996; Da Silva et al. 2007; Dijkshoorn and Nemec 2008). Ribotyping also represents a method that was primarily developed for species identification (Gerner-Smidt 1992; Brisse et al. 2000; van Dessel et al. 2004), but was shown to be useful for typing *Acinetobacter*, especially strains included in the Acb complex. An automatic ribotyping system has been used in several studies for typing *Acinetobacter*. A variety of restriction enzymes, including *Eco*RI, *Cla*I, *Sal*I, *Hind*III, and *Hinc*II, have been used in different ribotyping protocols proposed. In general, results obtained by ribotyping correlate with those obtained by AFLP analysis, but are less

discriminatory than AFLP and PFGE, another technique that is now widely used, as it will be mentioned ahead (Dijkshoorn et al. 1996; Nemec et al. 2004; van Dessel et al. 2004). Different PCR-based fingerprinting systems have been designed for *Acinetobacter* (Grundmann et al. 1997). A protocol, using primers DAF4 and M13, appeared useful for local studies of strain relatedness (Wroblewska et al. 2004). Another protocol, using the repetitive (GTG)5 primer, also allowed for differentiation of *A. baumannii* strains (Huys et al. 2005). However, these methods are not considered useful for longitudinal comparisons. An automated commercially available rep-PCR system, the Diversilab™, was found to be more robust, as fingerprints generated were highly discriminatory and comparable to AFLP analysis of A. *baumannii* strains (Carretto et al. 2008, 2011). Analysis of chromosomal DNA macrorestriction profiles by pulsed-field gel electrophoresis (PFGE) has now been extensively evaluated for epidemiological typing of *Acinetobacter*. A multicenter study of strains of the Acb complex has shown that PFGE (using *Apa*I as restriction enzyme) provides profiles that are comparable between laboratories (Seifert et al. 2005). This technique is able to detect the intra- and inter-hospital bacterial transmission, providing a method especially suited for local short-term outbreak investigations. PFGE results have shown improved discrimination, and this technique has been considered as the "gold standard" for fine-scale typing of *A. baumannii* isolates (Hamouda et al. 2010), although some difficulties in exchangeability between different electrophoresis platforms and laboratories are considered a limitation in its use. On the other hand, Multilocus Sequence Typing (MLST), based on the comparative sequence analyses of loci from housekeeping genes, offers the possibility to transfer typing data between laboratories and via the internet, making it an appropriate technique for global and long-term epidemiological studies (Hamouda et al. 2010). Currently, two MLST schemes are available for typing of *A. baumannii* strains. The Bartual scheme (http://pubmlst.org/abaumannii/) is based on DNA sequence comparisons of the internal fragments of seven housekeeping genes (*gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD*) (Bartual et al. 2005). Similarly, the Pasteur scheme (http://www.pasteur.fr/recherche/genopole/PF8/mlst/) is based on DNA sequence comparisons of the internal fragments of seven housekeeping genes including three genes from the Bartual scheme (*gltA*, *recA*, and *cpn60*) plus four other genes (*fusA*, *pyrG*, *rpoB*, and *rplB*) (Diancourt et al. 2010). The two schemes have generally showed compatible results although a recent study has reported a higher resolution of the Bartual MLST scheme, providing a better association between epidemiological features, occurrence of acquired OXA genes, and temporal distribution of the isolates (Da Silva et al. 2007; Mugnier et al. 2010; Grosso et al. 2011). In general, PFGE and MLST should be considered as complementary tools to each other in epidemiological studies (Villalon et al. 2011). Schemes for Variable Number of Tandem Repeat (VNTR) analysis of *Acinetobacter* have been proposed. A pioneer approach proposed for *A. baumannii* allowed the identification of two loci that were useful in combination with PFGE to determine patient-to-patient transmission (Turton et al. 2009). Recently, Pourcel et al. (2011) presented a scheme based on allele differences at eight loci. Results indicated that the MLVA grouping corresponded well with grouping by PFGE and PCR-based profiling of *ompA*, *csuE*, and $bla_{\text{OXA-51-like}}$ genes to identify European (EU) clones, and it was more discriminatory than the latter two methods. MLVA data can be stored in a database and exchanged between institutes (http://mIva.u-psudir), representing a promising method.

Studies using a combination of various typing methods have revealed a large diversity within A. *baumannii*. Three major groups of highly similar strains were distinguished among isolates from different hospitals, and designated as EU clones I, II, and III (Orskov and Orskov 1983; Dijkshoorn et al. 1996; Nemec et al. 2004; van Dessel et al. 2004). EU clones I and II have been found in association with outbreaks worldwide, and several additional clones have been reported (Nemec et al. 2007; Carretto et al. 2011; Petersen et al. 2011). Although, these clones were originally delineated by a polyphasic approach using a combination of techniques (including cell-envelope protein analysis, ribotyping, and AFLP analysis), more recently, MLST has been used as a universal method to identify *Acinetobacter* clones (Diancourt et al. 2010). A multiplex PCR targeting three genes for rapid identification of the three EU clones has also been described (Turton et al. 2007).

A variety of *Acinetobacter* genomes are listed on the NCBI site (http://www.ncbi.nlm.nih.gov/sites/entrez) at different stages of analysis and some have their sequencing already complete. A number of these, including the type strain of *A. baumannii* (ATCC 19606^{T}), *A. pittii* (SH024), *A. nosocomialis* (RUH2624), and *A. calcoaceticus* (RUH2202), have been sequenced as part of the Human Microbiome Project ("*Acinetobacter* group Sequencing Project", Broad Institute of Harvard and MIT (http://www.broadinstitute.org). The genome sizes are in the range of 3.1–4.0 Mb.

Apart from application in taxonomy, the use of molecular methods for identification and epidemiological studies with members of the genus *Moraxella* is relatively recent. PCR-based methods have been designed for a few species of *Moraxella*, most of them aiming the detection of the pathogenic species in clinical specimens. In this context, different formats of multiplex PCR assays have been proposed for the detection of *M. catarrhalis* and other upper respiratory tract pathogens (Post et al. 1996; Kunthalert et al. 2013). A multiplex PCR-based reverse line blot hybridization (mPCR/RLB) assay has been used for the detection of *M. catarrhalis* in respiratory specimens obtained from children with pneumonia (Wang et al. 2008). A quantitative real-time PCR (qPCR) method has also been developed to measure the occurrence of *M. catarrhalis* and other pathogens in nasopharyngeal specimens collected from children (Dunne et al. 2012). In the veterinary area, a multiplex real-time PCR assay was developed for the detection and differentiation of *M. bovis*, *M. bovoculi*, and *M. ovis* and applied in pure culture isolates and clinical specimens collected from cattle (Shen et al. 2011; O'Connor et al. 2012).

Several genetic typing methods have been employed in recent years to further elucidate the molecular epidemiology of infections associated with members of the genus *Moraxella*. Most of the studies were performed with *M. catarrhalis*, as commented below.

RFLP analysis has been widely used in the past for the molecular typing of *M. catarrhalis*. While a variety of restriction enzymes have been tested, *Hae*III and *Hin*fI were considered as giving superior results (Beaulieu et al. 1993; Faden et al. 1994; Christensen et al. 1995). PCR-RFLP of *M. catarrhalis*, using primers for M46, a *M. catarrhalis* specific DNA fragment which encodes genes for glycyl tRNA synthetase, and *Hae*III and *Rsa*I to digest the resulting amplicons, demonstrated a moderate level of discrimination between isolates (Walker et al. 1998; Verduin et al. 2000; Wolf et al. 2000). M46 PCR-RFLP was found to be appropriate for short-term epidemiological studies, while multilocus PCR-RFLP could be used for more long-term epidemiological studies, as the degree of discrimination between isolates would be similar to using a multiprobe-RFLP approach (Walker et al. 1998). Probed RFLP (involving either conventional RFLP or PCR-RFLP followed by Southern blot hybridization with labeled probes) was also evaluated for *M. catarrhalis* (Beaulieu et al. 1993). Probed RFLP has also been used to successfully distinguish between complement-resistant and -sensitive *M. catarrhalis* lineages (Wolf et al. 2000). A comparison between single locus PCR-RFLP and multiprobe-RFLP showed the latter was more discriminatory (Walker et al. 1998). Ribotyping, both manual (with a combination of *Hind*III and *Pst*I as restriction enzymes) and automated (using either *Eco*RI or *Pst*I), has been applied to *M. catarrhalis* (Brygge et al. 1999; Verduin et al. 2000). The degree of discrimination seen between *M. catarrhalis* isolates when using ribotyping is somewhat dependent on whether the isolates are complement-sensitive or -resistant, and a higher level of discrimination is seen with complement-resistant isolates.

Several techniques based on random amplified polymorphic DNA (RAPD-PCR) analysis have been evaluated for *Moraxella*, showing varying levels of discrimination. A rep-PCR format was used to examine *M. bovis* isolates collected from cattle with infectious bovine keratoconjunctivitis (Prieto et al. 1999). In another study, a combination of rep-PCR and RAPD primers was found to distinguish between complement-resistant and sensitive *M. catarrhalis* strains (Verduin et al. 2000). In a study of *M. catarrhalis* isolates comparing RAPD with PFGE, one of the primers tested gave results with the same level of discrimination as PFGE (Vu-Thien et al. 1999). However, results of later studies indicated the lower power of RAPD techniques when compared with results obtained by PFGE and automated ribotyping (Vu-Thien et al. 1999; Pingault et al. 2007; Yokota et al. 2007). Results of the pioneer study on the application of PFGE for typing *M. catarrhalis* indicated that *Not*I and *Sma*I were restriction enzymes suitable for the analysis of this microorganism (Kawakami et al. 1994). Further studies confirmed that *Not*I, *Spe*I, and *Sma*I are the most appropriate restriction enzymes for typing *M. catarrhalis* (Yano et al. 2000; Verduin et al. 2000; Masaki et al. 2003). Since then, PFGE has become the preferred molecular method for typing *M. catarrhalis*. A MLST scheme for *M. catarrhalis* was launched in 2003. The MLST database and methodology is accessible at http://mlst.ucc.ie/mlst/dbs/Mcatarrhalis. In this MLST scheme, eight housekeeping genes are target (*glyRS*, *ppa*, *efp*, *funC*, *trpE*, *mutY*, *adk*, and *abcZ*) that are distributed around the *M. catarrhalis* genome (Wirth et al. 2007).

Several *Moraxella* genomes are listed on the NCBI site (http://www.ncbi.nlm.nih.gov/genome/?term=moraxella), as of May 2013, mostly incomplete or with no data available yet. The first report of a *Moraxella* genome completely assembled and annotated was published in 2010 (de Vries et al. 2010; Zomer et al. 2012). It is the genome sequence of an isolate of *M. catarrhalis*, strain RH4, which originally was isolated from blood of an infected patient. The RH4 genome consists of 1,863,286 nucleotides (1.86 Mb).

The intensive use of molecular techniques, especially 16S rRNA gene sequencing, to study bacterial isolates from various environments, has been crucial to improve the conceptions about the microorganisms included in the genus *Psychrobacter*. 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.

Other than for taxonomic applications, however, studies using molecular techniques for the identification and epidemiological approaches are still rarely applied to members of the genus *Psychrobacter*. Recently, denaturing gradient gel electrophoresis (DGGE)-based methods have been developed for the analysis of *Psychrobacter* populations. In a study on seafood quality analysis, DGGE was applied for molecular identification of dominant microbiota present after ice storage on several general growth media. The results revealed that many microorganisms potentially associated with spoilage were overlooked by using only one single growth medium (Broekaert et al. 2011). Members of the genus *Psychrobacter* were included among the microorganisms overlooked, and were identified by both partial 16S rRNA gene and *gyrB* gene sequencing. In another study, aiming identification of microbiota present on the surface of Taleggio cheese sampled at various times during ripening, brines, swabs of wooden shelves used for cheese dry-salting, PCR-DGGE was used, allowing the detection of *Psychrobacter cibarius* (Feligini et al. 2012). More recently, a DGGE-based method was developed for the analysis of *Psychrobacter* populations in aquatic systems. DGGE profiles inferred that *Psychrobacter* populations analyzed were very stable, representing a strong indication for the presence of well-adapted phylotypes (Azevedo et al. 2013).

Several *Psychrobacter* genomes are listed on the NCBI site (http://0-www.ncbi.nlm.nih.gov.elis.tmu.edu.tw/genome/?term=psychrobacter), as of May 2013, mostly at early stages of analysis. The genome sequence of *Psychrobacter arcticus*, strain 273–4, a psychroactive Siberian permafrost bacterium, has been completely sequenced, revealing mechanisms for adaptation to low-temperature growth (Ayala-del-Río et al. 2010). The size of strain 273–4 genome is 2.65 Mb.

Also, the draft genome sequence of *Psychrobacter* sp. PAMC 21119, which was isolated from permafrost soil of Antarctica, was recently presented (Kim et al. 2012). It is expected that these data could provide insights into adaptation and evolution strategies under extreme environmental conditions.

Phenotypic Analyses

The microorganisms described in this chapter are catalase-positive (except for those included in the newly recognized genera *Perlucidibaca* and *Paraperlucidibaca*), nonfermenting bacteria that grow significantly better under aerobic rather than anaerobic conditions, and some are strictly aerobic. *Acinetobacter* and *Moraxella* are mesophilic, unable to grow at 4 °C, and are not halotolerant or halophilic, while members of the genus *Psychrobacter* are psychrotolerant and osmotolerant. Fastidious growth requirements are common among most species of *Moraxella*, variable among *Psychrobacter* and rarely observed among *Acinetobacter* strains. The oxidase test is positive for *Moraxella* and *Psychrobacter* and negative for *Acinetobacter*. Colonies are usually grayish-white, although some strains may appear pale yellow. Most *M. canis* strains may produce a brown pigment when grown on starch-containing media.

Table 22.2 provides the phenotypic characteristics that are helpful in distinguishing the six genera presently recognized as members the family *Moraxellaceae*, while *Tables 22.3–22.5* list the phenotypic characteristics that can be used to differentiate the diverse species that compose the three major genera, *Acinetobacter*, *Moraxella*, and *Psychrobacter*, respectively. However, the data listed in these Tables are compiled from various pieces of literature, whereby different authors may have used different media and/or protocols. Potential differences in reactivity and/or timing for final reading may be observed according to the procedures used.

The members of the genus *Acinetobacter* are strict aerobes, nonfermenting Gram-negative coccobacillary bacteria with a positive catalase reaction and a negative oxidase reaction. The negative oxidase reaction is quite helpful to distinguish the genus from most of the other related bacteria. Bacterial cells are short, plump, Gram-negative rods, typically 0.9–1.6 μm in diameter and 1.5–2.5 μm in length, in the logarithmic phase of growth, but often becoming more coccoid (coccobacillary) in the stationary phase. The cell wall ultrastructure is typical of Gram-negative bacteria in general, but the cells are occasionally difficult to destain, remaining with a slightly Gram-positive coloration. They are usually coccoid and occur in pairs, resembling members of the genus *Neisseria*, although this characteristic may vary according to the species or strain. Chains of variable length may also be observed. No spores are formed, and flagella are absent. Many strains are encapsulated, and the capsule may be readily seen in India wet ink mounts. Colonies are usually non-pigmented, but some strains form white- to cream-colored colonies, which vary in consistency from butyrous to smooth and mucoid, and from 1 to 2 mm in diameter. They grow at a wide range of temperatures: Most strains will grow at 33 °C, but some environmental isolates grow better if incubated at temperatures from 20 °C to 30 °C. Clinically important species commonly grow well at 37 °C or at higher temperatures up to 44 °C, except for *A. pitti* (Juni and Bøvre 2005; Nemec et al. 2011; Vaneechoutte et al. 2011). They also have the ability to transform the mutant strain BD413 from auxotrophy to prototrophy, a feature that can be used for genus identification (Juni 1972). Tween 80 esterase activity is frequently present, gelatinase production varies, and nitrate reductase is mostly absent. Motility test (evaluated by the hanging drop method) is negative, and these microorganisms are generally considered to be nonmotile, although "twitching" or "gliding" motility on semisolid media has been occasionally reported. Unlike other members of the family *Moraxellaceae*, most strains are resistant to penicillin and many clinical isolates are resistant to cephalosporins.

Phenotypic differentiation of this genus from similar bacteria should be based on a combination of characteristics. The nonfastidious nature and wide biochemical activities of the members of the genus makes them 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. The following characteristics are commonly used for genus level identification of *Acinetobacter* isolates: Gram-negative coccobacilli, aerobic (nonfermentative), nonmotile, and oxidase-negative (*Table 22.2*).

Identification to the species level based on phenotypic characteristics is more problematic and time-consuming. At least two major systems have been proposed for the phenotypic identification of the species included in the genus *Acinetobacter*. One was proposed by Bouvet and Grimont (1987) and has been updated by Vaneechoutte et al. (2011). It was based on physiological (biochemical and growth temperature) characteristics, and nutritional characteristics. Another scheme of 22 phenotypic tests (Kämpfer et al. 1993) has also been described that differentiates most of the genomic species known at the present time but this scheme is also laborious and time-consuming. Furthermore, delineation of some of the species within the genus *Acinetobacter* is still difficult. Several highly related species cannot be clearly distinguished from each other by these approaches, and are frequently referred as complexes of species when identification is based on phenotypic backgrounds only. For example, *A. baumannii* and its closely related species of medical importance, and *A. calcoaceticus*, an environmental species, are generally not differentiated on the basis of phenotypic characteristics and are frequently referred as the *A. calcoaceticus-A. baumannii* (Acb) complex composed of glucose-oxidizing non-hemolytic closely related species (Towner 2006; Peleg et al. 2008). In addition, the need for in-house preparation of most of the tests precludes the use of this identification scheme in most diagnostic laboratories. The phenotypic characteristics that can be useful to differentiate the valid species of *Acinetobacter* are listed in *Table 22.3*.

Table 22.2
Characteristics useful for differentiating the genera included in the family *Moraxellaceae*[a]

Characteristic	*Acinetobacter*	*Alkanindiges*	*Moraxella*	*Paraperlucidibaca*	*Perlucidibaca*	*Psychrobacter*
Anaerobic growth	–	–	–	–	+	(–)
Growth at:						
4 °C	–	ND	–	–	–	(+)
37 °C	+	+	+	–	+	(–)
42 °C	V	ND	V	–	–	–
Motility (Flagella)	–[b]	–	–	–	+	–
Catalase test	+	+	+	–	–	+
Oxidase test	–	–	+	+	+	+
Growth in the presence of 6 % NaCl	–	ND	–	–	–	+
Hydrolysis of Tween 80	(+)	+	V	+	+	(+)
Indole production	ND	–	–	ND	+	–[c]
Nitrate reduction	(–)	+	(+)	–	w	V
Occurrence in marine habitats	–	–	–	+	–	+
DNA G+C content (%)	38–47	46.2	40–49.6	61.3	63.1	42–50

[a]Data from Bogan et al. 2003; Juni and Bøvre 2005; Song et al. 2008; Oh et al. 2011. + positive, – negative, (+) most species are positive, (–) most species are negative, *V* variable (~50 % of the species are positive and ~ 50 % are negative), *w* weak reaction, *ND* no data available
[b]Twitching motility on semisolid agar occasionally occurs
[c]*Psychrobacter okhotskensis* is indole positive

Phenotypic identification of *Acinetobacter* species in the clinical microbiology laboratory by commercial identification systems is also difficult, as most commercial rapid identification systems are inaccurate (Bernards et al. 1996). This may result from the small number of relevant characteristics tested in the systems and/or from the insufficient quality of reference data in the identification matrices. Again, one important example is represented by the species included in the *A. calcocoacetius-A. baumannii* complex that are generally not differentiated by these systems. On the other hand, these systems can be useful for genus level identification and, when supplemented with aerobic acidification of glucose testing (oxidation-fermentation test), hemolysis, and growth at 44 °C, also for the presumptive identification of *A. baumannii*. Promising results have been obtained with the automated Biolog system, which involves the detection of oxidation with 95 different carbon sources (Dijkshoorn 1996), and with the API ZYM system, based on the detection of enzymatic activities (Poh and Loh 1985).

Serological identification can be applied for members of genus *Acinetobacter*. A large number of capsular types have been identified, resulting in the delineation of a large number of different serovars in two of the main genomic species (two and three, now constituting the species *A. baumannii* and *A. pittii*, respectively) associated with infection in humans (Traub and Leonhard 1994). Also, lipopolysaccharide (LPS) molecules from clinical *Acinetobacter* isolates have been investigated and identified as being of the smooth phenotype, indicating the serotyping scheme for identification of clinically important members of the genus may be possible (Pantophlet et al. 1998, 2002).

The microorganisms included in the genus *Moraxella* are coccoid or coccobacillary organisms (plump rods), occurring predominantly in pairs and sometimes in short chains, and also tend to resist decolorization in the Gram stain. *M. canis* and *M. catarrhalis* are *Neisseria*-like diplococci, and they can be distinguished from other *Moraxella* or other coccoid species by performing Gram staining on cells cultured in the vicinity of a penicillin disk: Cells of *M. canis* and *M. catarrhalis* remain spherical diplococci of 0.5–1.5 μm in diameter, although of irregular size, whereas coccobacilli show obviously rod-shaped and filamentous cells. Most *Moraxella* species are susceptible to penicillin and its derivatives, as well as to cephalosporins (Das et al. 1997; Juni and Bøvre 2005; Vaneechoutte et al. 2011).

Colonies of *Moraxella* grown on blood or chocolate agar media are generally gray to white, opaque, and smooth, measuring about 1–3 mm after 24 h of incubation. Characteristically, the colonies may be nudged intact across the plate with a bacteriological loop like as "hockey puck," and can be removed from the agar entirely, being very consistent. Colony morphology however may substantially vary according to the species. Most *M. canis* colonies are large and smooth, resembling those of the *Enterobacteriaceae*, and may produce a brown pigment when grown on starch-containing Müeller Hinton agar (Jannes et al. 1993). Some strains may also produce very slimy colonies resembling colonies of *Klebsiella pneumoniae* (Jannes et al. 1993). *M. nonliquefaciens* forms smooth, translucent to semiopaque colonies 0.1–0.5 mm in diameter after 24 h and 1 mm in diameter after 48 h of growth on sheep blood agar plates. Occasionally, these colonies spread and pit the agar.

Table 22.3

Phenotypic characteristics of the species included in the genus *Acinetobacter*[a]

Characteristic	*A. baumannii*	*A. baylyi*	*A. beijerinckii*	*A. bereziniae*	*A. bouvetii*	*A. brisouii*	*A. calcoaceticus*	*A. gerneri*	*A. guillouiae*	*A. gyllenbergii*	*A. haemolyticus*	*A. indicus*	*A. johnsonii*	*A. junii*	*A. lwoffii*	*A. nosocomialis*	*A. parvus*	*A. pittii*	*A. radioresistens*	*A. rudis*	*A. schindleri*	*A. soli*	*A. tandoii*	*A. tjernbergiae*	*A. towneri*	*A. ursingii*	*A. venetianus*
Growth at:																											
37 °C	+	+	+	+	D	+	(+)	+	V	(+)	+	+	V	+	+	+	+	+	+	+	+	+	+	–	+	(+)	+
41 °C	+	V	–	–	–	–	(–)	–	–	–	–	+	–	V	–	+	–	+	+	–	(+)	+	–	–	+	–	–
44 °C	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	(+)	–	(–)	–	–	–	–	–	–	–	–	–
Hemolytic activity	–	–	+	–	–	ND	–	–	–	+	+	–	–	V	–	–	–	–	–	–	–	–	–	–	–	–	+
Acid from D-Glucose	+	+	–	(+)	–	–	(+)	+	–	–	V	–	–	–	(–)	+	–	(+)	–	–	–	+	–	–	–	–	–
Utilization of:																											
Adipate	(+)	+	–	V	–	–	+	+	+	+	ND	–	–	–	(+)	(+)	–	+	+	–	V	+	–	–	–	+	V
β-Alanine	+	–	–	+	–	+	(+)	+	(+)	+	–	–	–	–	–	(+)	–	(+)	–	ND	–	–	–	–	–	–	–
4-Aminobutyrate	+	+	+	+	–	–	+	+	(+)	(–)	+	D	V	V	(+)	+	–	+	+	ND	–	ND	+	–	–	–	+
L-Arabinose	V	–	–	–	–	–	V	–	–	–	–	D	–	–	–	+	–	(+)	–	–	–	–	–	ND	–	–	–
L-Arginine	+	+	–	–	–	–	+	–	–	+	+	–	V	+	–	+	–	+	+	–	–	–	+	+	–	–	+
L-Aspartate	+	+	+	+	–	ND	+	+	+	–	V	–	V	V	–	+	–	+	–	–	–	+	+	V	–	(+)	–
Benzoate	V	+	–	+	+	V	+	+	(+)	+	–	+	+	+	(+)	(+)	–	(+)	+	+	(+)	ND	+	V	+	V	+
2,3 Butanediol	+	+	–	+	–	ND	+	+	+	–	–	D	V	–	–	(+)	–	(+)	+	+	V	ND	+	–	V	–	–
Citrate (Simmons)	+	+	+	+	+	–	(+)	+	+	+	(+)	–	V	V	(–)	+	–	+	–	+	V	+	+	–	–	+	+
Ethanol	(+)	+	+	+	+	ND	(+)	+	+	V	(+)	+	+	V	+	+	+	+	+	+	(+)	ND	+	ND	+	+	+
L-Histidine	(+)	–	+	(+)	+	–	+	–	(+)	+	(+)	–	–	V	–	+	–	+	–	+	–	ND	+	+	–	–	+
DL-Lactate	+	+	–	+	+	+	+	+	+	+	–	+	+	+	+	+	–	+	+	ND	+	+	+	–	+	+	–
D-Malate	(+)	D	+	(+)	–	+	D	–	+	+	(+)	–	–	V	(–)	+	–	(+)	–	ND	(+)	+	+	+	V	(+)	+
L-Ornithine	V	–	–	–	–	ND	+	–	–	V	–	–	(–)	–	–	(+)	V	(+)	–	ND	–	–	+	–	–	–	–
Phelnylacetate	V	–	–	V	–	+	+	+	V	+	–	+	–	–	(+)	(+)	–	V	+	+	–	+	+	–	–	–	–
Putrescine	(+)	–	–	–	–	ND	+	–	–	–	ND	–	–	–	–	(+)	–	+	V	–	–	ND	+	–	–	–	–

[a]Data from Nemec et al. 2011; Malhotra et al. 2012; Alvarez-Pérez et al. 2013. Two species (*A. boissieri* and *A. nectaris*) were not included in this table, because information is not available for most of the tests listed. + positive, – negative, (+) most strains are positive, (–) most strains are negative, *V* variable (~50 % of the strains are positive and ~50 % are negative), *ND* no data available, *D* different data reported in literature

Table 22.4

Phenotypic characteristics of the species included in the genus *Moraxella* [a]

Characteristic	*M.atlantae*	*M.boevrei*	*M.bovis*	*M.bovoculi*	*M.canis*	*M.caprae*	*M.catarrhalis*	*M.caviae*	*M.cuniculi*	*M.equi*	*M.lacunata*	*M.lincolnii*	*M.nonliquefaciens*	*M.osloensis*	*M.ovis*	*M.pluranimalium*	*M.porci*
Growth at 42 °C	(−)	−	−	−	+	−	V	+	+	−	−	−	V	V	+	−	+
Growth on MacConkey agar	+	−	−	−	V	−	−	−	−	ND	−	ND	−	V	−	−	−
Growth on minimal medium containing ammonium and acetate	−	−	−	−	(+)	−	−	−	−	ND	−	ND	−	(+)	−	ND	ND
Hemolysis	−	+	+	(+)	+	+	−	+	−	−	−	−	−	−	(+)	−	−
Nitrate reduction	−	+	(−)	(+)	(+)	+	(+)	+	−	−	+	−	+	V	(+)	−	V
Tolerance of 3 % NaCl	w	+	+	+	+	+	+	+	−	+	−	−	+	−	+	−	V
Activity of:																	
Acid phosphatase	+	−	w	−	−	−	−	−	w	−	w	−	−	+	−	−	−
Alkaline phosphate	(+)	−	−	(−)	+	−	+	+	D	+	+	−	−	+	+	V	−
DNase	−	−	−	V	(+)	−	+	−	−	−	−	−	−	−	(−)	−	−
Esterase	+	+	+	+	+	D	+	D	+	+	+	+	+	+	+	+	+
Phenylalanine deaminase	−	−	−	+	−	−	D	−	−	ND	D	−	−	V	−	ND	ND
Hydrolysis of:																	
Gelatin	D	+	+	w	−	−	−	−	−	+	+	−	−	−	−	−	−
Tween 80	−	+	(+)	(+)	−	+	−	−	−	ND	−	−	−	−	−	−	−

[a]Data from Rossau et al. 1991; Jannes et al. 1993; Vandamme et al. 1993; Kodjo et al. 1995, 1997; Xie and Yokota 2005; Angelos et al. 2007; Vela et al. 2009, 2010. *M. oblonga* was not included in this table, because information is not available for most of the tests listed. + positive, − negative, (+) most strains are positive, (−) most strains are negative, *V* variable (~50 % of the strains are positive and ~50 % are negative), *w* weak reaction, *ND* no data available, *D* different data reported in literature

Table 22.5

Phenotypic characteristics of the species included in the genus *Psychrobacter*[a]

Characteristic	*P. adeliensis*	*P. aestuarii*	*P. alimentarius*	*P. aquaticus*	*P. aquimaris*	*P. arcticus*	*P. arenosus*	*P. celer*	*P. cibarius*	*P. cryohalolentis*	*P. faecalis*	*P. fozii*	*P. frigidicola*	*P. fulvigenes*	*P. glacincola*	*P. immobilis*	*P. jeotgali*	*P. luti*	*P. lutiphocae*	*P. marincola*	*P. maritimus*	*P. namhaensis*	*P. nivimaris*	*P. okhotskensis*	*P. pacificensis*	*P. phenylpyruvicus*	*P. piscatorii*	*P. proteolyticus*	*P. pulmonis*	*P. salsus*	*P. sanguinis*	*P. submarinus*	*P. urativorans*	*P. vallis*
Growth at (°C):																																		
30	+	+	+	+	+	−	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V	+
37	−	+	−	−	−	−	+	+	−	−	D	−	−	+	−	−	−	−	+	−	+	+	−	V	−	+	−	−	+	−	+	−	−	−
Growth with NaCl (%; w/v):																																		
0	+	+	+	+	+	−	+	+	−	+	+	D	+	+	V	+	+	+	+	−	+	+	+	+	−	+	+	+	+	−	+	−	+	+
10	+	−	+	w	+	+	+	+	+	+	+	+	−	+	+	V	+	−	−	+	+	+	+	+	−	−	+	+	ND	+	−	+	−	+
15	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	ND	−	−	+	−	−
Nitrate reduction	+	ND	−	+	−	V	+	−	+	−	+	−	−	+	V	V	+	+	+	−	+	−	−	D	−	D	w	−	+	−	−	−	V	−
Acid production from:																																		
L-Arabinose	−	−	+	−	+	−	+	+	−	−	−	−	−	−	−	+	−	−	−	−	−	+	D	−	+	−	+	+	−	−	−	+	−	−
D-Galactose	ND	−	+	−	+	−	+	+	−	−	−	−	−	−	−	+	−	−	ND	−	−	+	+	−	+	−	+	+	ND	ND	−	−	−	−
D-Glucose	−	−	+	−	+	−	+	+	−	−	−	−	−	−	−	+	−	−	−	−	−	+	+	−	+	−	+	+	−	−	−	−	−	+
Lactose	−	−	+	−	+	−	+	+	−	−	−	−	−	−	−	V	−	−	−	−	−	+	D	−	+	−	−	+	−	−	−	−	−	−
L-Rhamnose	ND	−	+	ND	+	−	+	+	−	−	−	−	−	−	−	+	−	−	ND	−	−	+	D	ND	+	−	+	+	ND	ND	−	−	−	ND
D-Xylose	−	ND	+	−	+	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	+	D	−	+	−	+	+	−	−	−	−	−	−
Activity of:																																		
Acid phosphatase	−	−	w	ND	−	+	−	−	−	−	−	+	−	−	−	V	−	−	−	D	ND	−	ND	−	−	−	ND	D	−	−	V	−	D	ND
Alkaline phosphatase	−	−	+	+	+	+	+	+	V	+	+	+	−	+	D	+	+	+	−	+	ND	+	ND	+	+	(+)	ND	+	−	−	+	+	D	+

Table 22.5 (continued)

Characteristic	*P. adeliensis*	*P. aestuarii*	*P. alimentarius*	*P. aquaticus*	*P. aquimaris*	*P. arcticus*	*P. arenosus*	*P. celer*	*P. cibarius*	*P. cryohalolentis*	*P. faecalis*	*P. fozii*	*P. frigidicola*	*P. fulvigenes*	*P. glacincola*	*P. immobilis*	*P. jeotgali*	*P. luti*	*P. lutiphocae*	*P. marincola*	*P. maritimus*	*P. namhaensis*	*P. nivimaris*	*P. okhotskensis*	*P. pacificensis*	*P. phenylpyruvicus*	*P. piscatorii*	*P. proteolyticus*	*P. pulmonis*	*P. salsus*	*P. sanguinis*	*P. submarinus*	*P. urativorans*	*P. vallis*
β-Galactosidase	–	–	–	–	–	–	ND	–	–	–	–	–	–	–	–	–	+	–	–	–	ND	–	ND	–	–	–	ND	–	–	ND	–	–	–	ND
β-Glucosidase	ND	–	–	ND	–	–	ND	–	–	–	–	–	–	ND	–	–	+	+	–	–	ND	–	ND	–	–	–	ND	–	–	ND	–	–	V	ND
Leucine arylamidase	ND	+	+	ND	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	ND	+	ND	+	ND	+	+	ND	–	+	+	ND
Urease	–	–	–	+	–	–	–	–	–	–	–	+	–	+	V	V	+	–	+	–	+	–	–	D	+	+	w	+	–	–	+	–	V	–
Valine arylamidase	ND	–	–	–	–	+	–	–	V	–	D	–	–	–	D	D	–	–	–	+	ND	–	ND	–	+	–	ND	w	+	ND	V	D	–	ND
Hydrolysis of:																																		
Gelatin	–	–	–	–	–	–	–	–	–	–	ND	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
Tween 80	ND	+	+	+	+	+	+	+	+	+	ND	–	–	–	+	+	+	+	ND	+	+	+	+	+	D	(+)	+	w	–	ND	ND	+	–	+
Utilization of:																																		
Acetate	–	+	+	–	+	+	+	+	+	+	+	V	+	+	+	+	+	+	ND	–	+	+	+	+	V	+	+	–	ND	–	ND	–	V	–
Citrate	–	+	+	–	–	–	+	–	–	+	+	–	–	+	V	V	V	+	–	–	–	–	–	–	–	+		+	–	–	–	–	–	–
Malate	ND	+	+	ND	+	–	+	+	+	–	+	+	D	+	–	D	V	+	+	–	V	+	–	+	+	+	+	–	–	ND	ND	–	V	ND
Pyruvate	–	+	+	–	+	+	–	+	+	ND	+	+	–	ND	+	+	D	–	ND	–	–	+	+	+	+	+	+	+	ND	–	ND	ND	+	+

[a]Data from Juni and Heym 1986; Bowman et al. 1996, 1997a; Maruyama et al. 2000; Denner et al. 2001a; Kämpfer et al. 2002; Romanenko et al. 2002, 2004, 2009; Bozal et al. 2003; Yoon et al. 2003, 2005; Heuchert et al. 2004; Shivaji et al. 2004, 2005; Jung et al. 2005; Bakermans et al. 2006; Yassin and Busse 2009; Baik et al. 2010; Yumoto et al. 2010; Deschaght et al. 2012

+ positive, – negative, (+), most strains are positive, *V* variable (~50 % of the strains are positive and ~50 % are negative), *w* weak reaction, *ND* no data available, *D* different data reported in literature

The colonial morphologies of *M. lincolnii* (Vandamme et al. 1993) and *M. osloensis* are similar, but pitting is rare. On the other hand, pitting is common among *M. lacunata* strains, whose colonies are smaller and form dark haloes on chocolate agar. Rod-shaped *Moraxella* species, especially *M. atlantae* and *M. lincolnii*, are more fastidious and display smaller colonies on sheep blood agar (usually less than 1 mm in diameter after 24 h). Colonies of *M. atlantae* are small (usually 0.5 mm in diameter) and show pitting and spreading (Bøvre et al. 1976). The growth of *M. atlantae* is stimulated by bile salts, which explains its growth on MacConkey agar. *M. nonliquefaciens* and *M. osloensis* produce colonies that are somewhat larger than those of *M. atlantae* and that are rarely pitting. Colonies of *M. nonliquefaciens* may be mucoid. The species of *Moraxella* are asaccharolytic and strongly oxidase-positive.

M. catarrhalis and *M. canis* may also be easily distinguished from the commensal species of *Neisseria* which are also frequently isolated from respiratory tract clinical specimens, as the commensal species of *Moraxella* are, by the ability to produce DNase. The alkalinization of acetate test is helpful in distinguishing *M. catarrhalis* (most strains are negative) from *M. canis* (most strains are positive). There are few biochemical differences between *M. catarrhalis* and *M. nonliquefaciens*, which can be differentiated from each other mainly on the basis of morphological characteristics and by nitrite reductase, alkaline phosphatase, and DNase activity of *M. catarrhalis*. A few tests are also useful to differentiate three species of veterinary importance, associated with infectious bovine keratoconjunctivitis (*M. bovis*, *M. bovoculi*, and *M. ovis*): The phenylalanine deaminase and nitrate reduction tests help to distinguish *M. bovis* from *M. bovoculi*, while the tests based on growth at 42 °C and production of alkaline phosphatase help to separate *M. ovis* from both *M. bovis* and *M. bovoculi*.

Table 22.4 lists some of the phenotypic characteristics that can be useful to differentiate the species of *Moraxella*. However, several tests have not been extensively evaluated for all the species or have not been tested at all, making comparisons between identification schemes difficult. In general, phenotypic distinguishing between the different *Moraxella* species tends to be difficult, because of the asaccharolytic nature of the genus. To circumvent this, sequencing of the 16S rRNA gene has been reported as a useful adjunct to biochemical testing (Enright et al. 1994; Pettersson et al. 1998; 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 Bøvre (1984).

Most of the bacteria that compose the genus *Psychrobacter* are strictly aerobic (exceptions are *P. aquimaris*, *P. celer*, and *P. namhaensis*), showing no growth under anaerobic conditions and are almost always strongly catalase- and oxidase-positive. They 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, possibly owing to accumulated cytochrome proteins (Bowman et al. 1997b; Juni and Bøvre 2005). When grown under optimal conditions, cells are coccobacilli (typically 0.4–1.8 μm long and 0.4–1.6 μm wide). Differences in cell morphology, including rod-like cells in chains, with the rods having either rounded or pointed ends, have been described (Juni and Heym 1986).

As the other members of the family *Moraxellaceae*, they are Gram-negative but in some circumstances can retain the crystal violet dye and occasionally will stain slightly Gram-positive. Also, they are classified as nonmotile, although they may express "twitching" motility. They may possess numerous short fimbriae. Cells can be lysed 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 neutrophilic, growing best at pH 6.0–8.0 but not below pH 5.5 or above pH 9.0. They are distinctive as they can grow well at low temperature and can tolerate a wide range of salt concentrations. 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 to distinguish *Psychrobacter* species. *Table 22.5* lists some biochemical and nutritional characteristics that are useful for distinguishing the species of *Psychrobacter*.

In general, most species of *Psychrobacter* are 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). They do not break down complex substrates such as polysaccharides, and some may hydrolyze proteins (Denner et al. 2001). Preferred carbon sources are organic acids and amino acids. Species of *Psychrobacter* often produce lipases and can break down a few substrates common in nature but not normally catabolized by most Gram-negative bacteria. Two among those substrates are acetate and pyruvate, compounds that can serve as the sole source of carbon, nitrogen, and energy for many *Psychrobacter* species (Bowman et al. 1996; 2006).

Some characteristics are helpful in the recognition of some clinically important species of *Psychrobacter*. *P. faecalis* and *P. pulmonis* are coccoid gram-negative rods growing on the surface of TSA as large, creamy colonies. *P. faecalis* is saccharolytic and acidifies glucose and xylose, while *P. pulmonis* is asaccharolytic. Both species produce acid from ethylene glycol. They are Tween 80 esterase and tributyrate esterase positive. They are nitrate reductase positive and, unlike the type strain of *P. immobilis*, are urease negative and nitrite reductase positive. *P. phenylpyruvicus*, formerly *Moraxella phenylpyruvica* (Bowman et al. 1996), has the morphological and cultural appearance resembling a *Moraxella* but is urease and phenylalanine deaminase positive. A unique feature of the species is its marked growth improvement by Tween 80. Colonies on TSA with 1 % Tween 80 have a size two to three times larger than on sheep blood agar. The other *Psychrobacter* species, in contrast to *P. phenylpyruvicus*, grow abundantly on ordinary media such as TSA, and their growth is not promoted by Tween 80. *Psychrobacter* species are resistant to penicillin and susceptible to most of the other antibiotics (Gini 1990; Lloyd-Puryear et al. 1991). Among the six species of *Psychrobacter*

already recognized as having some medical importance (*P. faecalis*. *P. immobilis*, *P. phenylpyruvicus*, *P. pulmonis*, and *P. sanguinis*), only *P. immobilis* produces acids from carbohydrates, while the other five species do not produce acids from L-arabinose, D- galactose, D-glucose, lactose, L-rhamnose, and D-xylose. Most of them do not grow in the presence of NaCl concentrations higher than 10 %, and only *P. pulmonis* do not produce alkaline phosphatase. On the other hand, *P. alimentarius*, *P. celer*, *P. namhaensis*, *P. pacificensis*, *P. piscatorii*, and *P. proteolyticus* produce acids from different carbohydrates. *P. proteolyticus* is the only species of *Psychrobacter* that hydrolyzes gelatin.

Overall, the use of conventional identification schemes has now been replaced in many laboratories by commercial kits or automated phenotypic identification systems, like the API 20NE, the Vitek 2, and the Phoenix (Bernards et al. 1996; O'Hara and Miller 2003; Bosshard et al. 2006; Vaneechoutte et al. 2011). However, the ability of commercial systems to identify the microorganisms included in the family *Moraxellaceae* is variable, being frequently very poor, and, in several cases, has not been extensively evaluated. Therefore, the application of commercially available phenotypic identification systems for the identification of members of the family *Moraxellaceae* often results in identification to the genus or group level only, requiring the use of supplemental biochemical testing for accurate species identification.

Macromolecules like proteins, fatty acids, lipopolysaccharides, or enzymes are considered useful taxonomic markers that may constitute important identification tools, depending on the microorganism. Methods based on the analysis of some of different chemotaxonomic markers have also been applied for the identification of members of the family *Moraxellaceae*. Automated cellular fatty acid analysis has been applied (Veys et al. 1989; Osterhout et al. 1991), but in view of the difficulties inherent to this approach (Osterhout et al. 1991), it is recommended that fatty acid profiles be used only in conjunction with conventional or commercial identification systems. The fatty acid profiles for several species of these microorganisms have been published (Weyant et al. 1996). On the other hand, proteins are molecules of special interest for use as targets in identification and/or typing systems because of the frequently high correlation of the results of tests based on the analysis of protein profiles and results of genetic testing. Cell-envelope protein profiling using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has shown that the profiles comprising certain protein bands could be used for identification of several species of *Acinetobacter* (Dijkshoorn et al. 1990). However, the use of this molecular phenotypic approach decreased after the introduction of DNA-based methods. Currently, protein fingerprinting using a matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometer is revolutionizing microbial diagnostics. The number of studies is still limited, but the results are promising, indicating the differentiation of diverse species of *Acinetobacter* (Nemec et al. 2010; Sedo et al. 2011; Dubois et al. 2012). The method certainly deserves to be further evaluated for the identification of the diverse members of the family *Moraxellaceae*, including for the differentiation of members of the Acb complex (Alvarez-Buylla et al. 2012; Dubois et al. 2012; Espinal et al. 2012).

Isolation, Enrichment, and Maintenance Procedures

The source (for example, if environmental, clinical, foodstuff) and kind of the specimens to be tested for the presence of members of the family *Moraxellaceae* influence the procedure to be used, including the type of medium needed for primary isolation. In general, isolation of most of these microorganisms can be accomplished by using standard laboratory media such as Trypticase Soy Agar, Brain Heart Infusion Agar, and Blood Agar Base (or many of the other agar bases commonly used) containing 5 % sheep blood. Different media have also been devised for selective isolation of some of these microorganisms (especially for *Acinetobacter* and *Moraxella*), but, in several cases, their usefulness remains to be widely assessed, as none have been proven to be highly specific, and not all strains grow on these selective media.

As most other bacteria, these microorganisms can be maintained indefinitely when lyophilized. Cultures frozen at −70 °C or less (freezing in the vapor phase of liquid nitrogen, at temperatures of −130 °C and lower, frequently around −180 °C, is one of the best options) can be stored for several years as heavy bacterial cell suspensions made directly in a skim milk (10 %) solution containing glycerol (10 %) or other cryopreservative media commonly used for maintenance of bacteria. These are the preferable methods for preservation of strains of bacteria included in the family *Moraxellaceae*. They can also be preserved for many years at −20 °C as bacterial suspensions prepared in cryopreservative media. Many strains of *Moraxellaceae* can also survive for several months at 4 °C on agar slants prepared with ordinary agar bases, such as Brain Heart Infusion Agar, and Trypticase Soy Agar supplemented or not with blood. Certain strains of the less well-known species, however, may not be as resistant to adverse conditions and may not survive long if more adequate preservation procedures are not used.

Several aspects of the procedures for isolation (including media and temperature of incubation) and maintenance of these microorganisms may be peculiar for each separate genus and, eventually for a given species or group of species within a genus, as discussed below.

Although isolation of *Acinetobacter* can be accomplished by using standard, non-selective media as mentioned above, the use of a differential medium such as MacConkey agar may be helpful in recognizing colonies of *Acinetobacter* on primary isolation. The optimum growth temperature for most *Acinetobacter* 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 (Towner 2006; Vaneechoutte et al. 2011).

Based on the fact that most strains of *Acinetobacter* can grow in a simple mineral medium containing a single carbon and energy source such as acetate, an enrichment culture procedure for isolating members of this genus from soil and water has been described (Baumann 1968). For that, samples (5 ml sample of water, or a filtered 10 % soil suspension) are inoculated in 20 ml of Baumann's Enrichment Medium, and incubated at either 30 °C or room temperature, under vigorously aeration. After incubation for 24 or 48 h, cultures are streaked onto suitable isolation media. Vigorous aeration at a pH of 5.5–6.0 favors the enrichment of *Acinetobacter* strains. On the other hand, selective liquid enrichment was rarely used for isolation of *Acinetobacter* from clinical specimens, and direct plating on general purpose media such as blood agar or MacConkey agar was usually preferred because of their broad bacterial coverage. However, the results of studies more recently undertaken due to the increasing need of screening for colonization are indicating that the inclusion of an enrichment step substantially increased the sensitive of the procedures for isolation of *Acinetobacter* (Marchaim et al. 2007; Doi et al. 2011). Also, in certain circumstances, it may be preferable to use a more selective and differential medium that suppresses the growth of other bacteria, and may be indicative of the presence of these microorganisms. Such a medium can also be used for plating-out liquid enrichment cultures. An antibiotic-containing selective medium (Leeds *Acinetobacter* Medium, LAM, containing vancomycin, cefsulodin, and cephradine) has been proposed, and it was found to be useful for the recovery of *Acinetobacter* from clinical and environmental sources (Jawad et al. 1994). *Acinetobacter* strains typically produce circular, convex, smooth, pink to mauve colonies after 24 h of incubation in this medium. Modified versions of this medium have recently been proposed for selective screening of *A. baumannii* colonization on human body surfaces, by replacing cefsulodin and cephradine with either aztreonam or ceftazidime (Doi et al. 2011). Additionally, more selective, modified versions of MacConkey agar have also been tested (Ajao et al. 2011).

The increasing incidence of multidrug-resistant *Acinetobacter baumannii* as an important nosocomial pathogen has raised the importance of using methods for selective isolation of this microorganism, and stimulated the development of other alternative selective media and procedures. A selective medium containing chromogenic substrates, the CHROMagar *Acinetobacter*, has been proposed for the isolation and presumptive identification of *Acinetobacter*. It contains agents which inhibit the growth of most gram-positive organisms as well as carbapenem-susceptible gram-negative bacilli, and it also incorporates substrates enabling color-based preliminary identification of colonies recovered within 18–24 h of inoculation. This media has been evaluated for detection of enteric carriage of multidrug-resistant *A. baumannii* in critically ill patients and in samples collected from hospital environments (Gordon and Wareham 2009; Akers et al. 2010; Ajao et al. 2011). Although CHROMagar *Acinetobacter* was found to be sensitive and specific for multidrug-resistant *A. baumannii*, it was unable to distinguish carbapenem-resistant from carbapenem-susceptible *A. baumannii*. Recently, promising modified versions of CHROMagar *Acinetobacter* were proposed to improve the ability of this medium for better selection for multidrug-resistant *A. baumannii* (Wareham and Gordon 2011; Thom et al. 2012; Barsoumian et al. 2013; Song et al. 2013), and deserve further evaluation.

Most *Acinetobacter* strains can be stored for few weeks on nutrient agar slants kept at room temperature, but many strains may not survive for more than short periods in such conditions or even under refrigeration at 4 °C. For long-term preservation, lyophilization should be used, or heavy suspension of cells in Brain Heart Infusion broth supplemented with 20 % v/v glycerol or in skim milk (10 %) solution supplemented with glycerol (10 %) can be frozen at −70 °C or less.

Members of the genus *Moraxella* can also be grown on standard laboratory media such as Trypticase Soy Agar or Brain Heart Infusion Agar (Hays 2006; Vaneechoutte et al. 2011). Most detailed information on isolation procedures is available for *M. catarrhalis* due to the importance of this species as a human pathogen. Optimum growth conditions for *M. catarrhalis* include a temperature of 35–37 °C in an atmosphere containing 3–7 % CO_2, though the organism is also capable of growing at a larger range of temperatures (20–42 °C) and in ambient air. Cultures of *M. catarrhalis* may be grown on standard laboratory media including Müeller Hinton agar, Columbia blood agar, and Brain Heart Infusion or Trypticase Soy Agar, though the addition of supplements, such as boiled blood plus vitamin and amino acid enrichment (chocolate agar), may result in an enhancement of growth (Doern and Morse 1980; Vaneechoutte et al. 2011). *Moraxella catarrhalis* does not grow on Modified Thayer-Martin medium. A defined medium for *M. catarrhalis* culture has been described (Juni et al. 1986), but the growth of *M. catarrhalis* from clinical specimens may be complicated by the presence of the normal bacterial microbiota, 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. Several types of selective media have been described for *M. catarrhalis* and reported to enhance recovery (Vaneechoutte et al. 1988). These media are supplemented with antimicrobials, including trimethoprim, vancomycin, and an antifungal agent, with the further addition of acetazolamide (a synthetic sulfonamide). Of interest, *M. catarrhalis* isolated on modified in New York City 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).

Preservation of most *Moraxella* strains may follow the general recommendations mentioned at the beginning of this section. For long-term storage (several years) of *M. catarrhalis* strains at −70 °C or less, STGG (skimmed milk, tryptone, glucose, glycerol) medium has been reported to yield good results (Kaijalainen et al. 2004).

Most species of *Psychrobacter* can be grown on various standard frequently used culture media, including Trypticase Soy Agar and Brain Heart Infusion Agar. Marine Agar is also

a common media for routine growth of *Psychrobacter*, as most species grow very well on this medium. 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 most of them grow rapidly at 20–25 °C. Excellent growth for most species occurs on nutrient and Trypticase Soy Agar, especially when these media are supplemented with extra NaCl (Bowman 2006; Vaneechoutte et al. 2011). Some clinically important species, such as *P. phenylpyruvicus* and *P. pulmonis*, include fastidious strains that may show poor or no growth on the media mentioned. Instead, these can be grown readily on brain heart infusion, Columbia blood agar, or nutrient and Trypticase Soy Agar supplemented with 5 % horse serum, after incubation in a humid atmosphere at 30–37 °C. CO_2 is not required. *Psychrobacter* can be isolated by direct plating of the specimen onto appropriate media, such as Marine Agar for marine samples (seawater, sea-ice, sediment etc.), nutrient agar or Trypticase Soy Agar (supplemented with 6 % NaCl to exclude non-halotolerant species) for terrestrial samples, and Trypticase Soy Agar or Brain Heart Infusion Agar supplemented with animal blood or serum for clinical specimens. Incubation at lower temperatures for samples from more temperate climes will help the selective growth of *Psychrobacter* strains. Increasing NaCl concentration in media to about 0.5–1.0 M or greater can also increase selectivity. 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 *Psychrobacter* isolates from permafrost and sea-ice samples (Bowman et al. 1997b; Vishnivetskaya et al. 2000; Bowman 2006). Largish, cream-colored, smooth colonies with a buttery consistency appearing on plates are characteristics presumptive of *Psychrobacter* strains.

Psychrobacter strains can be maintained on agar plates or slants at normal growth temperature for 1–3 weeks or at 2–4 °C for several months. Temperatures lower than 4 °C (around 2 °C) are better, as fungal contamination is reduced and survival for cold-adapted species is enhanced. Marine agar is an excellent media for agar storage for most species. Other media, such as Trypticase Soy Agar and Brain Heart Infusion Agar, can be used for species not able to grow well on Marine Agar. Heavy cell suspensions of *Psychrobacter* strains in one of the cryopreservation media previously mentioned for other *Moraxellaceae* can also be used for maintenance for several years in cryopreservation freezers or in liquid nitrogen. Lyophilization can also be employed using a variety of desiccants including skim milk, horse serum, or fetal bovine serum (Bozal et al. 2003; Bowman 2006).

Ecology

Some members of the family *Moraxellaceae* are widespread in nature and can be found in soil, plants, water, food, and on the skin and mucous membranes of humans and animals. The occurrence of the different genus or species may vary according to the kind of environment or host (Dijkshoorn and Nemec 2008; Visca et al. 2011; Deschaght et al. 2012; Azevedo et al. 2013).

Some of the species of *Acinetobacter*, such as *A. johnsonii*, *A. lwoffii*, and *A. radioresistens*, seem to be natural inhabitants of human skin, and can be readily isolated from moist skin areas, such as toe webs, the groin, and the axilla (Seifert et al. 1997; Peleg et al. 2008). Representatives of the genus *Acinetobacter* have also been found in feces of non-hospitalized individuals (Dijkshoorn et al 2005; Peleg et al. 2008). Other potential reservoirs of these microorganisms may include a range of both moist and dry surfaces and equipment within the hospital environment (such as table tops, dust, ventilatory equipment, humidifiers, mattresses, urinals, and wash basins), as well as the patients and staff (Bergogne-Bérézin and Towner 1996; Peleg et al. 2008). They are also ubiquitous microorganisms in soil, water, and sewage (Towner 1996), and have being referred to constitute as much as 0.001 % of the total heterotrophic aerobic population of soil and water (Baumann 1968). They can be isolated from heavily polluted water, such as that in wastewater treatment plants, but are more frequently found near the surface of fresh water (Droop and Jannasch 1977).

Members of the genus *Acinetobacter* can also be recovered from a variety of foodstuffs, including eviscerated chicken carcasses, various poultry and other meats, milk products, and vegetables, and are known to be 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; Peleg et al. 2008).

Differences in the distribution of the various species according to the sources have been recognized, especially if clinical, food, or environmental (Gennari and Lombardi 1993; Narciso-da-Rocha et al. 2013), but few studies are available comparing isolates from different sources by using recent taxonomic criteria for identification. Many studies within the genus *Acinetobacter* have been performed with clinical isolates, and indicate that the vast majority of clinically significant isolates belong to the *A. calcoaceticus–A. baumannii* complex (Dijkshoorn and Nemec 2008; Peleg et al. 2008; Vaneechoutte et al. 2011). On the other hand, *A. johnsonii* and *A. lwoffii* seem to predominate in foods and the environment (Gennari and Lombardi 1993). The ubiquitous occurrence of *A. johnsonii* in wastewater treatment plants has also been demonstrated (Kämpfer et al. 1992; Knight et al. 1993). Other species appear to comprise minority components of the different bacterial populations investigated, up to date, but they may have evolved to acquire a selective advantage in as-yet unrecognized specialized ecological niches. Some genetic interchange between populations is 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 (Towner 2006).

Members of the genus *Moraxella* are inhabitants of mucous membranes of humans (mainly of the upper respiratory tract, conjunctiva, and genital tract) and a variety of other warm-blooded animals. The distribution of species seems to be variable

according to the host and anatomic area (Ringvold et al. 1985; Vaneechoutte et al. 2000). *M. nonliquefaciens* is most commonly isolated from the human upper respiratory tract, while *M. osloensis* is a common resident of the human genital tract. *M. atlantae*, *M. lacunata*, and *M. lincolnii* are also part of the normal microbiota of the human respiratory tract. *M. catarrhalis*, a species that is considered an important cause of infections in humans, can be recovered from the oropharynx of healthy adults and children, at different rates, as discussed in the "Pathogenicity: Clinical Relevance" section. Most of the other species of *Moraxella* have been firstly or only isolated from healthy or diseased animals, and such association was frequently considered to name these species, including *M. boevrei* and *M. caprae* (isolated from goats), *M. bovis* and *M. bovoculi* (cattle and other animals including horses), *M. canis* (dogs, cats, and camels), *M. caviae* (guinea pigs), *M. cuniculi* (rabbits), *M. equi* (horses), *M. ovis* (sheep), *M. pluranimalium* (pigs and sheep), and *M. porci* (pigs) (Jannes et al. 1993; Kodjo et al. 1995, 1997; Angelos et al. 2007; Tejedor-Junco et al. 2010; O'Connor et al. 2012). The actual range of natural habitats of members of this genus, however, still remains to be elucidated in the light of updated taxonomic recommendations and the use of reliable procedures for species identification.

The genus *Psychrobacter* is found in a wide range of habitats, mostly in cold, saline environments, such as sea-ice and icy coastal seawater (Bowman et al. 1997a, 1997b; Bozal et al. 2003; Brinkmeyer et al. 2003; Yumoto et al. 2003). Some species of *Psychrobacter* 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; Bakermans et al. 2006), and accreted ice at the base of deep ice cores above seawater (Bowman et al. 1997a; Ponder et al. 2008).

Species of *Psychrobacter* are also readily isolated from conventional marine environments including seawater (Venkateswaran et al. 1991; Kisand et al. 2002; Romanenko et al. 2002; Yoon et al. 2005), marine sediment, and salt marshes (Ansede et al. 2001; Baik et al. 2010) and sea depths of up to 6,000 m (Maruyama et al. 1997; Maruyama et al. 2000) and subseafloor sediments (Inagaki et al. 2003). *Psychrobacter* strains have also been shown to occur on unusual calcium carbonate column formations (called "ikaite tufa" in Greenland fjords; Stougaard et al. 2002). Some species of *Psychrobacter* are also frequently found as components of the microbiota of food, a finding that may be associated with their combined psychrotolerance and osmotolerance. They are commonly found on salted fish, chilled fish flesh, some shell fish, fermented seafood, chilled meat products of all varieties, and even cheese and raw milk (Gennari et al. 1992, 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; Jung et al. 2005). Some strains of *Psychrobacter* can spoil food, but they are considered relatively minor spoilers compared to other bacterial species. In fish, *Psychrobacter* spoilage results in a musty off-odor, usually after storage in the cold for 7–10 days. Unlike most spoilers, *Psychrobacter* tolerate salting and can survive storage in 25 % NaCl for prolonged periods (Bjorkevoll et al. 2003). *Psychrobacter* appears to be part of the microbiota of fish skin and the skin of various other animals. It can be found in the tissues of marine animals and sponges and on seaweeds and algae (Denner et al. 2001b; Pukall et al. 2001; Romanenko et al. 2002, 2009). *Psychrobacter* representatives are also common in uric acid–rich soil like the Antarctic ornithogenic soils (Bowman et al. 1996) and in fecal bioaerosols (Kämpfer et al. 2002). Otherwise, these microorganisms seem to be absent from most soil environments unless they are exposed to low temperatures and intermittent freezing (Wery et al. 2003). Although little is still known about the ecological impact of the genus *Psychrobacter*, this genus is likely to play a role primarily as a commensal, degrading various organic carbon compounds other than sugars. The genus has evolved to tolerate low temperatures and has a highly developed osmotolerance which allows it to be highly competitive and prevalent in many cold ecosystems.

The type strains that represent the three genera more recently incorporated into the family *Moraxellaceae* have been isolated from environmental sources, either aquatic environments (*Perlucidibaca* from freshwater and *Paraperlucidibaca* from seawater) or soil (*Alkanindiges*) (Bogan et al. 2003; Song et al. 2008; Oh et al. 2011).

An extensive evaluation of the natural habitats of the different members of the family *Moraxellaceae* has been impaired by several aspects, including methodological differences and difficulties, as well as lack of consensus in the taxonomy of these microorganisms. The availability and use of more discriminatory molecular methodologies for microbial identification will allow a better exploration of the diversity of different microbiomes and will certainly contribute with important insights on the ecological role and impacts of these microorganisms.

Pathogenicity: Clinical Relevance

The genus *Acinetobacter* comprises a heterogeneous group of bacteria that were long considered saprophytes of little clinical significance, and have recently became a focus of major attention due to the emergence of certain species as important opportunistic pathogens that contribute significantly to patient morbidity and mortality (Visca et al. 2011).

Members of the genus *Acinetobacter* are the second nonfermenters most commonly isolated from human clinical specimens, after *Pseudomonas aeruginosa*, and their incidence is increasing. The success of opportunistic species of *Acinetobacter* can be attributed to the ability to long-term survival on inanimate surfaces, due to desiccation resistance and biofilm development (i.e., medical devices), and their large repertoire of antibiotic resistance mechanisms (Jawad et al. 1998; Catalano et al. 1999; Braun 2008; Wroblewska et al. 2008).

Together, these features have favored the emergence of *Acinetobacter* in hospital settings as one of the most important nosocomial pathogen recognized nowadays.

Most of the nosocomial infections and hospital outbreaks worldwide have been attributed to *A. baumannii*, particularly in intensive care units (ICU), followed by *A. nosocomialis* and *A. pitti* (previously known as genomic species 13TU and 3, respectively), and *A. lwoffi*. Nosocomial infections caused by other named species such as *A. berezinae*, *A. guillouiae*, *A. haemolyticus*, *A. johnsonii*, *A. junii*, *A. parvus*, *A. radioresistens*, *A. schindleri*, *A. soli*, and *A. ursingii* are rare and usually related to a better clinical course. Infections caused by *Acinetobacter* spp. include ventilator-associated pneumonia (VAP), bacteremia, meningitis, urinary tract infections, cholangitis, peritonitis, skin and wound infections, ventriculitis, and infective endocarditis. *Acinetobacter* spp. are also increasingly involved in superinfections in burn patients (Gaynes and Edwards 2005; Dijkshoorn et al. 2007; Peleg et al. 2008; Sebeny et al. 2008; Kim et al. 2009).

VAP is the most frequent clinical manifestation of hospital-acquired *A. baumannii* infections affecting ventilated patients in ICUs. In addition, *A. baumannii* bloodstream infections, although less common, are associated with considerable morbidity and high mortality rates. Risk factors for a fatal outcome are severity-of-illness markers, such as septic shock at the onset of the infection and fatal underlying disease (Dijkshoorn et al. 2007; Visca et al. 2011).

Hand carriage seems to be related to the primary reservoir of *A. baumannii*, but air-borne spread can contribute to the diffusion of the organisms throughout the wards and ICUs. Digestive tract colonization has been documented in infants and in adults, and may participate in the dissemination routes in the hospital setting (Markogiannakis et al. 2008; Peleg et al. 2008; Manchanda et al. 2010).

Members of the genus *Acinetobacter* have been reported occasionally as causative agents of community-acquired infections. *A. baumannii* was found to be associated with a series of fatal cases of community pneumonia, and *A. haemolyticus* with endocarditis and bloody diarrhea as a result of verotoxin production (Castellanos et al. 1995; Chen et al. 2001; Anstey et al. 2002; Grotiuz et al. 2006; Leung et al. 2006).

The pathogenic mechanisms of *Acinetobacter* spp. are little understood. *A. baumannii* is the most studied species; however, precise mechanisms involved in the establishment and progression of infections by this species are still unclear (Peleg et al. 2008; Visca et al. 2011).

Cell-surface hydrophobicity is an important determinant of cell adhesion. In *Acinetobacter*, the presence of protein protrusions on cell surface confers hydrophobicity, being complementary to fimbriae and other cell wall components that facilitate the adhesion to host cells. Also, the surface hydrophobicity protects the microorganisms from phagocytosis and appears to play a role in its attachment to various polymers (Doughari et al. 2011).

The presence of genes encoding biofilm functions is widespread among *A. baumannii* strains. Several virulence determinants contribute to bacterial biofilm formation. Pili assembly and production of biofilm-associated protein (Bap) are considered essential factors in *A. baumannii* attachment to particular abiotic surfaces such as catheters, prostheses, and ventilators. The *csu* operon, containing six genes (*csuA/BABCDE*), encodes a chaperone-usher secretion system that acts in pilus assembly (De Breij et al. 2009). Despite its importance in biofilm formation on abiotic surfaces, they are less important for adherence in mammalian cells (De Breij et al. 2010). Bap is a surface exposed protein and, besides being involved in bacterial adherence to eukaryotic cells, is important for maintaining the mature biofilm architecture (Loehfelm et al. 2008; Brossard and Campagnari 2012).

Adherence on biotic surfaces is a fundamental step for colonization, and outer-membrane proteins (Omp) in Gram-negative bacteria are known to have essential roles in pathogenesis (Lee et al. 2006). OmpA (previously known as Omp38) is a trimeric outer-membrane porin that is associated with several aspects of *Acinetobacter* virulence. This protein is considered as a key virulence factor of *A. baumannii*. OmpA binds to a range of eukaryotic cells, is important for cellular invasion, and it targets both the nucleus and mitochondria (Cerqueira and Peleg 2011). OmpA can be secreted via outer-membrane vesicles (OMVs), and also participates in their biogenesis (Kwon et al. 2009). Host cells exposed to OMVs or purified OmpA triggers apoptosis (Choi et al. 2005, 2008). Additionally, OmpA partially contributes to serum resistance and biofilm formation, is immunomodulatory, and activates dendritic cells through TLR2 and MAPK and NK-κB pathways, which results in stimulation of $CD4^{+}$T cells (Erridge et al. 2007; Gaddy et al. 2009). High homology between the OmpA gene sequence of different *Acinetobacter* species, such as *A. baumannii*, *A. baylyi*, *A. radioresistens*, and *A. junii*, has been identified (Vila et al. 2007; Poirel et al. 2011; Weber et al. 2013).

Other virulence factors that have been associated with the members of the genus *Acinetobacter* are siderophores (acinetobactins), several enzymes (e.g., esterases, certain aminopeptidases, urease, and acid phosphatase), slime, and verotoxins (Mihara et al. 2004; Jacobs et al. 2010). Verotoxins are associated with bloody diarrhea promoted by *A. haemolyticus* infection. The mechanism by which *A. haemolyticus* produces this toxin is not well understood, and its emergence is worrying, given the high transformability capacity of *Acinetobacter* (Doughari et al. 2011).

The clinical impact of *Acinetobacter* is determined by virulence factors coupled with increasing resistance to the major antimicrobial agents. Antimicrobial resistance is a cause of concern, particularly for this group of microorganisms that exhibit a great propensity for nosocomial cross-transmission, and seem to have a remarkable ability to develop resistance to new antimicrobial agents.

Acinetobacter expressed a wide variety of intrinsic and acquired mechanisms of resistance to major antibiotic classes. *A. baumannii* which is highly predominant in infections is also the most resistant to drugs, and multiresistant epidemic clones have been extensively reported from numerous countries around the world (Diancourt et al. 2010).

A. baumannii produces an intrinsic AmpC-type cephalosporinase which is normally expressed at a low level and it is not inducible (Bou and Martinez-Beltran 2000). The presence of IS*Aba-1*, an insertion sequence belonging to the family of IS*4*, upstream of the bla_{ampC} results in gene overexpression by providing promoter sequences, and produces a resistant phenotype characterized by resistance to ampicillin, cephalotin, piperacillin, cefotaxime, and ceftazidime (Corvec et al. 2003; Héritier et al. 2006). *A. baumannii* also produces another naturally occurring β-lactamase, which is an oxacillinase, OXA-51 and its point-mutant variants (Turton et al. 2006). These enzymes have low level of carbapenemase activity, but also may be overproduced when supplied with efficient promoters by IS*Aba-1* or IS*Aba-9* (Smith et al. 2007; Figueiredo et al. 2009).

Only a few examples of acquired narrow-spectrum clavulanic acid penicillinases, the Ambler class A TEM-1 and TEM-2 and the carbenicillinase CARB-5 (conferring high-level resistance to aminopenicillins and carbenicillin, respectively), and clavulanic resistant oxacillinases, OXA-20 and OXA-21 have been observed in *Acinetobacter* (Potron et al. 2009). However, the clavulanate-inhibited penicillinase SCO-1 has been identified in various *Acinetobacter* species, including *A. baumannii*, *A. baylyi*, *A. junii*, and *A. johnsonii*. SCO-1 hydrolyzes significantly penicillins, and at a low level cephalothin, ceftazidime, and cefepime (Poirel et al. 2007; de Vries et al. 2009).

The class A of the so-called clavulanic acid–inhibited extended-spectrum β-lactamases (ESBLs) is clinically relevant in *A. baumannii*, since they confer resistance to several expanded-spectrum cephalosporins, such as ceftazidime or cefotaxime (Rodriguez-Martinez et al. 2010). In *A. baumannii*, PER-1 was the first to be reported (Nordmann and Naas 1994; Vahaboglu et al. 1997). The bla_{PER-7} gene encodes higher resistance to third-generation cephalosporins and monolactams than PER-1, due to the insertion of the element IS*CR1* (Bonnin et al. 2011; Opazo et al. 2012). However, the most important ESBL in *A. baumannii* is VEB-1 (Naas et al. 2006; Pasterán et al. 2006). The emerging group of ESBLs that occurs in *Enterobacteriaceae*, such as TEM and SHV, has been rarely identified in *Acinetobacter*, located either on the chromosome (e.g., bla_{SHV-5}) or on plasmids (e.g., bla_{TEM-92}, $bla_{TEM-116}$, bla_{SHV-12}) (Endimiani et al. 2007; Naas et al. 2007). Likewise, CTX-M-type, such as CTX-M-2, CTX-M-43, and CTX-M-15, also exhibits very low prevalence among *Acinetobacter* strains (Nagano et al. 2004; Poirel et al. 2011).

Acquired carbapenem-hydrolyzing β-lactamases have been identified as a source of carbapenem resistance in *A. baumannii*. They belong to class D (oxacillinases), class B (metallo-β-lactamases), and class A. Carbapenem-hydrolyzing class D β-lactamases (CHDLs) are often involved in carbapenem resistance in *A. baumannii*. The most frequent CHDLs usually identified are OXA-23, -25, -26, -40 (also name OXA-24), and −58 like lineage (Mugnier et al. 2010; Poirel et al. 2010). Metallo-β-lactamases (MBLs) confer high-level resistance to carbapenem (Fu et al. 2010; Higgins et al. 2010; Mugnier et al. 2010; Towner et al. 2011). Among MBLs, IMP-like, VIM-like, SIM-1 type, and the most recent NDM-1 (and its variant NDM-2) have already been described in *A. baumanni* (Göttig et al. 2010; Kaase et al. 2011; Poirel et al. 2012). KPC enzymes and GES-type representing class A are still rare among *Acinetobacter* isolates (Perez et al. 2010; Robledo et al. 2010; Bonnin et al. 2011).

Resistance to β-lactams, particularly carbapenems, may occur by nonenzymatic mechanisms, including porin loss and active drug efflux. Active efflux mediated by several efflux pumps has been described in *Acinetobacter*. A*cinetobacter* drug efflux pumps—AdeABC and AdeIJK—are the most prominent in *A. baumannii*, and the overexpression of these systems can pump out β-lactams (such as cefepime, cefpirome and cefotaxime), as well as aminoglycosides, chloramphenicol, erythromycin, tetracyclines, fluoroquinolones, and trimethoprim (Vila et al. 2007; Coyne et al. 2011).

Aminoglycoside-modifying enzymes are also responsible for resistance to aminoglycosides in clinical isolates of *Acinetobacter*. The three types of aminoglycoside-modifying enzymes (acetylases, adenylases, and phosphotransferases) have been detected in clinical isolates of *A. baumannii*. Resistance to chloramphenicol based on the expression of chloramphenicol acetyltransferase (CAT) and specific mutations in quinolone-resistance-determining-region (QRDR) are also known to impact the susceptibility to these antimicrobial agents in isolates belong to the genus *Acinetobacter* (Poirel et al. 2011). Emergence of colistin-resistant *A. baumannii* has already been notified in North Korea (Ko et al. 2007; Adams et al. 2009).

In general, most of the members of the genus *Moraxella* are environmental or skin and mucosal inhabitants that rarely cause infections in humans or other animals. Among them, the species *M. atlantae*, *M. canis*, *M. lacunata*, *M. lincolnii*, *M. nonliquefaciens*, and *M. osloensis* can be isolated occasionally from clinical specimens (Shah et al. 2000; Christensen et al. 2001; De Baere et al. 2002; Han and Tarrand 2004; Woodbury et al. 2009; Dien Bard et al. 2011; Rafiq et al. 2011). However, *M. catarrhalis* and *M. bovis* play important role in human and veterinary medicine, respectively (Postma et al. 2008; Murphy and Parameswaran 2009; Sano et al. 2010; Gupta et al. 2011; Bernhard et al. 2012).

Moraxella catarrhalis besides being a human commensal is recognized as an exclusively human pathogen, causing lower and upper respiratory tract infections. *M. catarrhalis* has been recognized as a cause of mucosal infections in children, mainly acute otitis media (AOM) and sinusitis, and contributes to a worse clinical course in cases of chronic obstructive pulmonary disease (COPD) in adults, especially in the elderly, triggering approximately 10 % of acute inflammatory exacerbations (Wald 1992; Faden et al. 1994; Bakri et al. 2002; Verduin et al. 2002; de Vries et al. 2009; Bernhard et al. 2012). *M. catarrhalis* is now considered as the third most common bacterial pathogen of the respiratory tract, responsible for approximately 20 % of all AOM episodes (Broides et al. 2009). Its frequency of isolation from the nasopharynx or the middle ear cavity has increased since changes in epidemiology occurred, secondary to the

introduction of vaccination against other pathogens sharing the same niche (such as the heptavalent pneumococcal conjugate vaccine) (Brook et al. 2006; Spijkerman et al. 2012).

Many reports have indicated the seasonal incidence of *M. catarrhalis* infection, with winter and spring months showing the greatest rates of isolation. There is some speculation that seasonal viral respiratory tract infection (e.g., respiratory syncytial virus but not adenoviruses) may be a predisposing factor in the seasonality of *M. catarrhalis* infection (Pettigrew et al. 2011). In addition to viral infections that pave the way for subsequent secondary bacterial infections, cold shock determined by the rapid downshift of temperature (to about 26 °C) induces adaptive events in the upper respiratory tract flora that may lead to the transition from asymptomatic colonization to infection (Spaniol et al. 2009).

Invasive infections (e.g., bacteremia, endocarditis, neonatal meningitidis, septic arthritis, ventriculitis) are very rare. Only few cases have been reported in recent years, almost exclusively associated with immunocompromised individuals (Ahmed et al. 2008). However, no clear association between *M. catarrhalis* and a particular immunodeficiency has been identified.

Nosocomial infections may also occur (Cook et al. 1989; Denamur et al. 1989; Richards et al. 1993). Person-to-person and contaminated environmental sources have been reported in nosocomial transmission, especially in multiple-bed respiratory wards and during the winter months, suggesting the aerosol-mediated mode of dissemination. Because of the mildness of the disease, nosocomial spread can be neglected (Masaki et al. 2003; Levy et al. 2009; Murphy and Parameswaran 2009). From a clinician perspective, it is important to consider that *M. catarrhalis* may be isolated as part of a mixed population of pathogens and the determination of its role in the disease process is valuable, particularly with respect to antibiotic therapy choice.

The recognition of *M. catarrhalis* as an important human pathogen has stimulated active investigation into the molecular mechanisms of pathogenesis. It is generally accepted that this species is composed of two major phylogenetic lineages (based on 16S RNA gene sequence), one of only moderate virulence, the so-called serosensitive subpopulation (type 2), and a second, the seroresistant one (type 1), which is enriched among strains that harbor two major virulence traits: complement resistance and adherence to epithelial respiratory tract cells (Bootsma et al. 2000; Verduin et al. 2000; Wirth et al. 2007). These virulence factors allow *M. catarrhalis* to evade the host immune defense and thus to establish infection. Moreover, numerous putative virulence factors have been identified, enhancing the interaction with the host and promoting disease (Vidakovics and Riesbeck 2009).

This microorganism expresses a variety of different adhesins, including the ubiquitous surface protein family (UspA), *Moraxella* immunoglobulin D-binding protein (MID) also named human erythrocyte agglutinin (Hag), the outer-membrane protein CD (OMP CD), *M. catarrhalis*-adherence protein (McaP), and lipoligosaccharide (LOS) (Forsgren et al. 2003; Lipski et al. 2007; Mawas et al. 2009; Aebi 2011).

The UspA family consists of at least three proteins: UspA1, UspA2, and the close related hybrid protein designated UspA2H. They are oligomeric adhesins with multifunctional binding sites, including domains attaching to complement and extracellular matrix proteins (such as fibronectin, laminin, and vitronectin (Tan et al. 2005; Singh et al. 2010). Interestingly, UspA1 also binds carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) which are expressed on the surface of respiratory tract epithelial cells (Hill et al. 2005). UspA1-mediated binding to host cells varies upon phase variation, which is regulated at the level of transcription by variation in a homopolymeric poly (G) tract located upstream of the *uspA1* open reading frame (Lafontaine et al. 2001). During DNA replication, these repeats are prone to slipped-strand mispairing, resulting in the removal of one or more G residues and thereby influencing the level of UspA1 expression (Brooks et al. 2008; Bernhard et al. 2012).

It was noted that cold shock facilitates *M. catarrhalis* adherence to pharyngeal and laryngeal epithelial cells by increasing expression and/or function of UspA1 and UspA2 (Heiniger et al. 2005). The exact mechanism is not yet fully elucidated, but cold shock may upregulate adhesin expression by prolonging the mRNA half-life and enhancing binding to fibronectin, and it also increases the OMP-mediated proinflammatory activity of pharyngeal epithelial cells and enhances sIgA binding on the bacterial surface (Spaniol et al. 2011).

MID/Hag is a highly conserved OMP that binds immunoglobulin D (IgD) in a nonimmune manner (Forsgren et al. 2003). This protein is a multimeric complex that experimentally mediates the adhesion of *M. catarrhalis* to different cell lines (e.g., Chang conjunctival cells, A549 cells). MID/Hag is able to induce the activation of human B-lymphocyte. Expression of MID/Hag is subject to translational phase variation via slipped-strand mispairing in a poly(G) tract, as well as have been described for UspA1 (Mollenkvist et al. 2003; Bullard et al. 2005).

M. catarrhalis express at least eight outer-membrane proteins (OMPs A to H), all exhibiting a relatively high degree of interstrain similarity: 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 still unclear. OMP B2 or CopB is largely conserved between *M. catarrhalis* isolates and its expression increases under iron-limiting conditions, suggesting that CopB may be involved in *M. catarrhalis* iron-acquisition (Aebi et al. 1996; Liu et al. 2006).

Originally considered as two distinct proteins, OMPs C and D actually represent two different stable forms of the same protein—OMP CD. This protein 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 (Akimana and Lafontaine 2007; Liu et al. 2007). The presence of antibodies against OMP CD correlates with protection against otitis media in children (Liu et al. 2007).

Cold shock also increases OMP-mediated release of the proinflammatory cytokine IL-8 in pharyngeal epithelial cells

and reduces the expression of the porin M35, which may also affect the outer-membrane permeability for aminopenicillins, leading to clinically relevant implications (Jetter et al. 2009; Slevogt et al. 2008; Spaniol et al. 2009).

Three serotypes of LOS (A, B, and C) have been identified in *M. catarrhalis* strains, based on chemical structure differences. Each serotype contains a common polysaccharide core, but shows differences in terminal sugar groups in one of the molecule branches (Holme et al. 1999; Akgul et al. 2005; Peng et al. 2007). Serotype B was only identified in seroresistant type 1 lineage, and it is predominant among strains isolated from adults. Furthermore, LOS, UspA1, and probably other OMPs are implicated in host cell invasion. However, the molecular mechanisms involved are still poorly understood (Peng et al. 2007; Bernhard et al. 2012).

One mechanism that *M. catarrhalis* uses to subvert innate host immune response is complement inactivation by multiple ways, suggesting that seroresistant strains have potential virulence properties higher than that of serosusceptible strains. The most important OMPs involved in serum resistance are the UspAs. These proteins are able to interfere with the deposition of C3b on the bacterial surface by absorbing C3 from serum, thereby preventing activation of the alternative complement pathway. The binding of the complement inhibitor C4bp (C4-binding protein) on the surface of *M. catarrhalis* enables the bacteria to inhibit the classical pathway. C4bp affinity of UspA2 seems to be higher than that of UspA1 (Nordstrom et al. 2004; Vidakovics and Riesbeck 2009). *M. catarrhalis* has also been shown to inhibit the direct formation of the membrane attack complex by binding vitronectin to UspA2 (Attia et al. 2005, 2006; Singh et al. 2010).

M. catarrhalis releases outer-membrane vesicles (OMVs), which carry some of the underlying periplasm, together with OMPs (including UspA family), porins, receptors, and LOS. UspA-expressing OMVs specifically bind C3 and hence counteract the complement cascade. Most importantly, *Moraxella* OMVs also increase the survival of the extraordinary serum susceptible non-typeable *Haemophilus influenzae* when exposed to human serum (Tan et al. 2007). As *Moraxella* strains are often isolated as a copathogen with *H. influenzae*, this phenomenon could thus explain how these two respiratory pathogens collaborate (Verhaegh et al. 2011).

M. catarrhalis forms biofilms in vitro and have been identified in middle ear effusion obtained from children (Hall-Stoodley et al. 2006). The UspA family and MID/Hag are involved in the regulation of biofilm formation (Pearson et al. 2006). The role of biofilms in the pathogenesis of AOM is an area of active investigation (Hall-Stoodley and Stoodley 2009). The better comprehension of the biofilm formation in middle ear of children with otitis media could contribute to the understanding of the pathogenesis of recurrent/chronic AOM.

Since *M. catarrhalis* is a strict human pathogen, major obstacles in vaccine development have been the lack of a completely satisfactory animal model that reflects the features of the human disease and, consequently, the absence of correlates of protection against this pathogen. Nevertheless, targeting adhesion molecules represents a logical approach toward finding a suitable vaccine candidate which undoubtedly will determinate a substantial human and economic impact (Tan and Riesbeck 2007; Mawas et al. 2009).

Moraxella bovis is the primary etiologic agent of infectious bovine keratoconjunctivitis (IBK), the most important ocular disease of cattle worldwide, that although rarely fatal causes significant financial loss (Baptista 1979; Postma et al. 2008). *M. bovis* have been classified in non-cross-reacting serogroups based on differences in antigenic determinants, and also on the analysis of OMP profiles and DNA fingerprints (Moore and Lepper 1991). IBK is a highly contagious multifactorial disease, and the virulence of *M. bovis* is influenced by both host and environmental factors, such as breed and age of the animal, host immune system, UV light exposure, face fly population, concurrent pathogens, climate, and pasture conditions (Gerhardt et al. 1982; Kopecky et al. 1986). *M. bovis* is transmitted by animal handlers or direct animal-to-animal contact with nasal and ocular discharges, contact with fomites, and most commonly by mechanical vectors, the female face flies (Van Geem and Broce 1985).

M. bovis exhibits several virulence factors, but only the presence of pili on bacteria surface and the expression of a β-hemolytic cytotoxin are determinants in disease. The pili are required for the adherence of *M. bovis* to bovine cornea, causing clinical signs of IBK. Two types of pili, Q and I (previously named β and α pili), have been identified. *M. bovis* strains expressing Q pili type are significantly more virulent, although a single strain is capable of producing both types (Marrs et al. 1985).

Hemolytic *M. bovis* strains produce a pore-forming cytotoxin (cytolisin/hemolysin) that promotes the development of corneal ulcers by lysis of epithelial cells. *M. bovis* cytotoxin may be related to RTX (repeats in structural toxins) family that gathers bacterial pore-forming exoproteins encoded by *mbxA* gene (Angelos et al. 2003, 2007). Characterization of regions flanking the RTX operon of cytotoxin genes has revealed an organization that supports the hypothesis that they are located on a mobile genetic element resembling a pathogenicity island (Hess and Angelos 2006). In addition, hydrolytic enzymes identified in *M. bovis* culture supernatant, such as C4 esterase, C8 esterase–lipase, C14 lipase, phosphoamidase, hyaluronidase, and phosphatase, and proteolytic enzymes (leucine and valine aminopeptidases and gelatinase), may participate in the production of corneal ulcers (Frank and Gerber 1981; Farn et al. 2001; Postma et al. 2008)

Most species of *Moraxella*, other than *M. catarrhalis*, are susceptible to penicillin and ampicillin, cephalosporins, tetracyclines, macrolides, aminoglycosides, and fluoroquinolones (Verduin et al. 2002). *M. catarrhalis* is almost uniformly resistant to penicillin, ampicillin, and amoxicillin due to the production of three isotypes of β-lactamases BRO-1, BRO-2, and BRO-3, whereas the latter is uncommon (Eliasson et al. 1992). BRO-1 is the most common, being present in about 90 % of β-lactamase-producing strains, and it is associated with MICs higher than those obtained for BRO-2 as a consequence of the higher

transcriptional activity of the gene (Esel et al. 2007; Khan et al. 2010). Both enzymes appear to be constitutively expressed, are chromosome- or plasmid-encoded proteins, and are inactivated by β-lactamase inhibitors (McGregor et al. 1998).

BRO enzymes of *M. catarrhalis* can confer protection from β-lactam antibiotics to other coexisting respiratory pathogens in the same host, such as *Streptococcus pneumoniae* or *Haemophilus influenzae*. This phenomenon which has been referred to as the indirect pathogenicity of *M. catarrhalis* may lead to antibiotic failure, when treating a mixed infection (Verduin et al. 2002). Besides *M catarrhalis*, β-lactamase producers have been identified among few other species, such as *M. lacunata* and *M. nonliquefaciens* (Wallace et al. 1989; Eliasson et al. 1992).

The BRO β-lactamases resistance phenotype is unique and could be used to guide optimal antimicrobial therapy (Khan et al. 2010). However, *Moraxella* antimicrobial susceptibility patterns may be altered genetically over time. Mechanisms underlying *M. catarrhalis* resistance to β-lactams, macrolides, and sulfonamides have been reported. These include downregulation of the M35 porin; mutations at A2982T, A2796T, and A2983T in 23S rRNA (conferring high-level resistance to macrolides and licosamides); and secretion of β-lactamases containing OMVs (Courter et al. 2010; Saito et al. 2012).

The genus *Psychrobacter* is mainly represented by psychrophilic microorganisms particularly found in natural environments. Most of *Psychrobacter* species have been isolated from surface and deep seawater, sea-ice, Antarctic ornithogenic soil, and Arctic permafrost (Bowman et al. 1996; Bowman et al. 1997b; Maruyama et al. 2000; Denner et al. 2001a; Romanenko et al 2002; Shivaji et al. 2005; Bakermans et al. 2006). Some species have been associated with terrestrial and marine animals, such as fish, marine crustacean, seals, pigeon, poultry, and lambs (Kämpfer et al. 2002; Vela et al. 2003; Yassin and Busse 2009). *Psychrobacter* have already been obtained from food, especially processed meat and poultry products (Fontana et al. 2010).

These bacteria are rarely isolated from human sources, and little is known about their true clinical significance. Moreover, the mechanisms of pathogenesis and epidemiology of pathogenic strains are essentially unknown. Transmission appears to be mainly in the form of environmental contact or nosocomial. *P. immobilis*, *P. faecalis*, *P. phenylpyruvicus*, and *P. sanguinis* are the species that have already been reported as causes of infections in humans. Although these microorganisms may be associated with severe infections, *Psychrobacter* are considered as opportunistic pathogens that have been associated with patients at extremes of age, with serious underlying diseases and other severely ill immunocompromised patients (Wirth et al. 2012).

P. immobilis has been recognized as a cause of chronic peritonitis, ocular infections such as keratinoconjunctivitis, and in a fatal infection in an acquired immunodeficiency syndrome (AIDS) patient (Gini 1990; Lozano et al. 1994; Daley et al. 1997). *P. phenylpyruvicus* has been associated with cases of infant meningitis and bacteremia, as well as endocarditis in the elderly, surgical wound infection, and infective arthritis (Lloyd-Puryear et al. 1991; Deschaght et al. 2012). *P. faecalis* and *P. sanguinis* have been isolated from infected wounds and bacteremia in elderly patients, respectively (Wirth et al. 2012).

The susceptibility of *Psychrobacter* strains to antimicrobial agents is largely unknown. In general, strains recovered from nonclinical sources were found to be resistant to most penicillin derivatives, such as ampicillin and benzylpenicillin, and susceptible to various aminoglycosides, including kanamycin, neomycin, and streptomycin (Romanenko et al. 2002).

Application

Members of the genus *Acinetobacter* have been used in a wide range of industrial applications, such as bioremediation of waste waters and effluents, degradation of petrochemicals, production of biopolymers and biosurfactants, biomass production, and production of immune adjuvants. They have been used for bioremediation of numerous hazardous and unpleasant waste and residue pollutant compounds produced as by-products of commercial processes. Aromatic compounds which are toxic to most microorganisms, such as salicylate, halogenated aromatics, and phenol, are capable of being degraded by members of the genus *Acinetobacter* (Schirmer et al. 1997). Some strains of *Acinetobacter* are also capable of elaborating and excreting polymers, such as a heteropolysaccharide named emulsan, that have been used to emulsify hydrocarbons, such as oils (Foght et al. 1989), thus making these substrates available for degradation in an aqueous environment (Navon-Venezia et al. 1995; Barkay et al. 1999; Gutnick and Bach 2000), and reducing the viscosity during pipeline transport and storage, enhancing the biodegradability following accidental spillage (Gutnick et al. 1991). Two other important polysaccharides are produced by *Acinetobacter*, termed biodispersan and alasan. The first one has been shown to bind to inorganic materials such as calcium carbonate and is capable of dispersing limestone in water (Rosenberg et al. 1988). 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. Alasan is another bioemulsifying polysaccharide produced by *Acinetobacter radioresistens* (Navon-Venezia et al. 1995, 1998; Barkay et al. 1999).

Some members of the genus *Psychrobacter* are sources of cold-adapted proteins and enzymes which have broad applicability in industrial processes, modification of heat-labile substances (such as various foods), and in energy conservation. Several cold-active enzymes produced by *Psychrobacter* strains have already been described, including metalloprotease (Denner et al. 2001), lipase (Arpigny et al. 1997; Ascione et al. 2012), glutamate dehydrogenases (Camardella et al. 2002), restriction endonuclease (Rina et al. 1997), and uracil-DNA glycosylase (Lee et al. 2009), among others. Certain *Psychrobacter* strains have been used as plant-growth-promoting bacteria, increasing the tolerance to Ni stress in contaminated soils, indicating that they may play an important role in phytoextraction of

metal-contaminated sites (Ma et al. 2011). *Psychrobacter*, especially *P. cibarius* and *P. celler*, have also been used in dairy products manufacturing to enhance the flavor development of farmhouse or industrialized ripened cheese (Deetae et al. 2007; Feligini et al. 2012; Irlinger et al. 2012; Gori et al. 2013).

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23 The Family *Moritellaceae*

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Abstract
Moritellaceae is a family that belongs to the order *Alteromonadales* within the class *Gammaproteobacteria*. It embraces the genera *Moritella* and *Paramoritella*. Currently seven species and one species are known in the genera *Moritella* and *Paramoritella*, respectively. All species were phenotypically characterized as halophilic facultative anaerobes and isolated from marine environments. The genus *Moritella* has been known to consist solely of psychrophilic species, while *Paramoritella* species is mesophilic. The difference of temperature adaptation between two genera within the same family is quite similar to the evolutionally pattern observed in the two genera, *Colwellia* and *Thalassomonas*, within the family *Colwelliaceae*. The 16S rRNA gene sequences indicate that both genera are related with 93 % similarity level. The entire family is phylogenetically closely related to the family *Shewanellaceae*. *Moritella* species have been isolated from seawater, sediment, and fish samples, which were collected only from cold marine environments. *Moritella* species have been studied as model microorganisms of low-temperature-adapted enzymes, piezophilic adaptation of marine bacteria to the deep sea, and an economically severe fish pathogen. *Moritella* species are also known as producers of polyunsaturated fatty acids (PUFAs) such as docosahexanoic acid (DHA). The genus *Paramoritella* is now comprised of a single species *Paramoritella alkaliphila* isolated from hard coral and marine sand from tropical marine environments. These two genera are clearly differentiated by their habitats, growth temperature properties, G + C mol%, and lower levels of 16S rRNA gene sequence similarity (<93 %).

Taxonomy, Historical, and Current

Short Description of the Family and Its Genera

History of the Family and Its Genera

Until the establishment of genus *Moritella*, the type strain of this genus *Moritella marina* was classified as *Vibrio marinus*, one of the most well-studied psychrophilic microorganisms isolated from cold marine environments (Morita and Haight 1964; Morita and Albright 1965; Albright 1969; Felter et al. 1969).

E. Rosenberg et al. (eds.), *The Prokaryotes – Gammaproteobacteria*, DOI 10.1007/978-3-642-38922-1_227,

V. marinus was originally described by Russell (1891), but the strain was subsequently lost (Colwell and Morita 1964). Later this species was reisolated from seawater off the coast of Oregon at a depth of 1,200 m (Colwell and Morita 1964). A comparative 5S rRNA sequencing study indicated that *V. marinus* was significantly different from previously known *Vibrio* species (MacDonell and Colwell 1984). However, a new taxon was not proposed because only single strain was available at that time (MacDonell and Colwell 1985). *V. marinus* was later proposed to be the type species of a new genus *Moritella* based on DNA homology studies, but was awaiting further validation (Steven 1990). Subsequent comparative 16S rRNA sequencing studies demonstrated that *V. marinus* is more closely related to the genera *Shewanella* and *Pseudoalteromonas* than to the genus *Vibrio* (Kita-Tsukamoto et al. 1993; Gauthier et al. 1995). Additional *Moritella* strains were isolated and used for phylogenetic analysis (Urakawa et al. 1998). The phylogenetic data clearly demonstrated the independency of *V. marinus* from the genus *Vibrio* and the justification of the proposal of new genus. *V. marinus* was transferred to a new genus *Moritella* gen. nov. as *Moritella marina* comb. nov. The genus was proposed by Urakawa et al. (1998) as an effective publication and officially validated in the validation list no. 69 (1999). The genus *Moritella* presently comprises seven species: the type species *M. marina*, which is psychrophilic and non-piezophilic; *Moritella viscosa*, originally described as *Vibrio viscosus*, a marine fish pathogen that causes skin ulceration; *Moritella yayanosii*, an obligate piezophilic species; *Moritella japonica*, a piezophilic bacterium isolated from the Japan Trench; *Moritella profunda*, a piezophilic species isolated from the deep Atlantic sediment; *Moritella abyssi*, a piezophilic bacterium isolated from the deep-sea sediment with *M. profunda*; *Moritella dasanensis*, a psychrophilic and ice-active substance-forming species isolated from the Korean Arctic Dasan station.

In 2004, the genus *Moritella* was officially embraced in the family *Moritellaceae* (Ivanova et al. 2004). The original description of the family was given by Ivanova et al. (2004). Later the description was amended by Hosoya et al. (2009) as a consequence of the discovery and proposal of a new genus *Paramoritella* within the family *Moritellaceae*. The amended description of the family includes the change of 16S rRNA gene signature nucleotides consisting of G/A at position 399, C/T at position 858, G at position 1311, and C at position 1326. The family comprises the type genus *Moritella* Urakawa et al. (1999 [validation list no. 69]) and the genus *Paramoritella* Hosoya et al. (2009).

Short Description of the Family

Original description of *Moritellaceae* (Ivanova et al. 2004) was made before the discovery of *Paramoritella alkaliphila*. Hosoya et al. (2009) emended the description of the family *Moritellaceae* with the minor modification of 16S rRNA gene signature nucleotides. However, since various phenotypic and genotypic features are different between two genera *Moritella* and *Paramoritella*, additional emendation of the family description is needed.

***Moritellaceae* fam. nov.** (modified from Ivanova et al. 2004 emend. Hosoya et al. 2009).

Moritellaceae (Mo.ri.tel.la'ce.ae. N.L. fem. n. *Moritella* type genus of the family, -aceae ending to denote a family, N.L. fem. pl. n. *Moritellaceae* the *Moritella* family). Cells are chemoorganotrophic, halophilic, facultatively anaerobic Gram-negative curved or straight rods motile by a single polar flagellum. Do not form endospores or microcysts. Usually do not denitrify. Arginine dihydrolase is absent. The major isoprenoid quinone is Q-8. The major fatty acids are 14:0, 16:0, and 16:1. As additional major fatty acids, *Moritella* species produce 22:6 and *Paramoritella* species produce 18:1. Members of the family have been isolated solely from marine environments. The family is a member of the order *Alteromonadales* in the class *Gammaproteobacteria* with the following 16S rRNA gene signature nucleotides consisting of G/A at position 399, C/T at position 858, G at position 1311, and C at position 1326. The family comprises the type genus *Moritella* Urakawa et al. (1999) (validation list no. 69) and the genus *Paramoritella* Hosoya et al. (2009).

Moritella Urakawa et al. (1999) (validation list no. 69), gen. nov. (Type genus of the family *Moritellaceae* Ivanova et al. 2004).

Type species: *Moritella marina* (Baumann et al. 1984) Urakawa et al. (1999) (validation list no. 69).

Etymology: N.L. fem. dim. n. *Moritella*, named after Richard Y. Morita to honor his work in marine microbiology.

References: validation list no. 69 (1999) and Urakawa et al. (1998) as an effective publication.

The changes have been made for the descriptions of major quinone and fatty acid components. Habitats were expanded. The order information was added. The reference of type genus was amended from the effective publication to the validation list.

Short Description of the Genera

Description of *Moritella* gen. nov. (Modified from Urakawa et al. 1998).

Moritella (Mo.ri.tel'la. M.L. dim. ending -ella. M.L. fem. n. *Moritella*, named after Richard Y. Morita to honor his work in marine microbiology). Cells are chemoorganotrophic, halophilic, facultatively anaerobic Gram-negative curved or straight rods motile by a single polar flagellum. Colonies are circular, convex, opaque, and nonpigmented. Cells are isolated from cold marine habitats and grow at 4 °C. The optimum growth temperature is below 20 °C. Cells are oxidase and catalase positive, mostly negative for Voges-Proskauer and H_2S production. Do not produce arginine dihydrolase. Utilize *N*-acetyl glucosamine. Acid but no gas is produced from D-glucose. G + C mol% is 40–45. The major isoprenoid quinone is Q-8. The major fatty acids are 14:0, 16:0, 16:1, and 22:6 (docosahexanoic acid [DHA]). In the 16S rRNA gene primary structure, two-base

insertion of thymine presents (between bases 206 and 207 *Escherichia coli* numbering position). The genus *Moritella* is a member of the order *Alteromonadales* in the class *Gammaproteobacteria*. The type species is *Moritella marina* (formerly *Vibrio marinus*).

This modification was made by the critical reviews of original description and expanded knowledge mainly added by the descriptions of six new species and the proposal of the order *Alteromonadales* (Nogi et al. 1998; Nogi and Kato 1999; Benediktsdóttir et al. 2000; Xu et al. 2003; Ivanova et al. 2004; Kim et al. 2008).

Description of *Paramoritella* gen. nov. (Hosoya et al. 2009).

Paramoritella (Pa.ra.mo.ri.tel'la. Gr. prep. *para* beside; N.L. fem. n. *Moritella* a bacterial genus name; N.L. fem. n. *Paramoritella* beside *Moritella*). Cells are Gram-negative, chemoorganotrophic, facultatively anaerobic, require seawater for growth, motile by means of subpolar flagella, and positive for oxidase and catalase. The predominant fatty acids are 14:0, 16:0, 16:1v7c, and 18:1v7c, and the respiratory quinone is Q-8. This genus belongs to the class *Gammaproteobacteria*, and the type species is *Paramoritella alkaliphila*.

Table 23.1
Families within the order *Alteromonadales*

Family	Genus
Moritellaceae	*Moritella, Paramoritella*
Colwelliaceae	*Colwellia, Thalassomonas*
Psychromonadaceae	*Psychromonas*
Ferrimonadaceae	*Ferrimonas, Paraferrimonas*
Shewanellaceae	*Shewanella*
Idiomarinaceae	*Idiomarina, Aliidiomarina*
Pseudoalteromonadaceae	*Algicola, Pseudoalteromonas, Psychrosphaera*
Alteromonadaceae	*Aestuariibacter, Agarivorans, Aliagarivorans, Alishewanella, Alteromonas, Bowmanella, Catenovulum, Glaciecola, Haliea, Marinimicrobium, Marinobacter, Marinobacterium, Melitea, Microbulbifer, Saccharophagus, Salinimonas*

Phylogenetic Structure of the Family and Its Genera

Groups of bacteria belonging to the order *Alteromonadales* are commonly isolated from marine environments (Bowman et al. 1997). Their abundance and importance are also confirmed on the basis of culture-independent molecular techniques (Bowman and McCuaig 2003). Currently eight families are included in this order (Table 23.1). Comparative 16S rRNA gene sequence analysis clearly demonstrates that *Moritella* species are tightly related to each other (>97.8 % sequence similarity) and distinguishable from other members among the order *Alteromonadales* (Fig. 23.1). The phylogenetic position of *Paramoritella* is solitary; it forms an independent genus separated from other genera among the order *Alteromonadales*.

The difference of temperature adaptation between two genera, *Moritella* and *Paramoritella*, within the same family

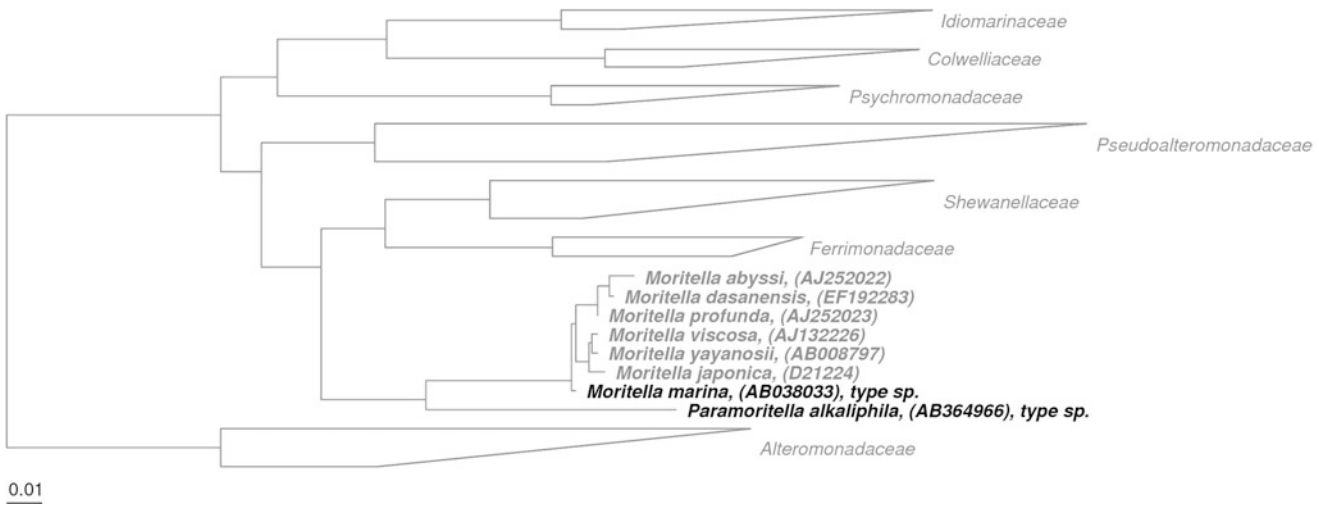

Fig. 23.1
Maximum likelihood phylogenetic tree, based on 16S rRNA gene sequences, showing the family *Moritellaceae* and related members within the order *Alteromonadales*. Scale bar indicates estimated sequence divergence

Moritellaceae is quite similar to the evolutionally pattern observed in two genera, *Colwellia* and *Thalassomonas*, within the family *Colwelliaceae* (Macian et al. 2001). The 16S rRNA gene sequences indicate that the two genera *Moritella* and *Paramoritella* are related with 93 % similarity level. The entire family is phylogenetically closely related to the family *Shewanellaceae* and *Ferrimonaceae* (Fig. 23.1; Ivanova et al. 2004; Hosoya et al. 2009).

```
                    200                     220
                     .....|....  |....|....  |
E.coli               GGGGGAC--C  TTCGGGCCTC  T
M.marina             GGGCCTCTTC  TTGAAAGCTC  T
M.viscosus           GGGCCTCTTC  TTGAAAGCTC  T
M.japonica           GGGCCTCTTC  TTGAAAGCTC  T
M.yayanosii          GGGCCTCTTC  TTGAAAGCTC  T
M.profunda           GGGCCTCTTC  TTGAAAGCTC  T
M.abyssi             GGGCCTCTTC  TTGAAAGCTC  T
M.dasanensis         GGGCCTCTTC  TTGAAAGCTC  C
P.alkaliphila        GGGGGAC--C  TTCGGGCCTC  G
S.putrefaciens       AGGGGAC--C  TTCGGGCCTT  C
F.balearica          AGGGGCT--C  TTCGGACCTT  G
C.psychroerythraea   GGGGGAT--T  TTCGGACCTC  T
I.abyssalis          GGGGGAC--C  TTCGGGCCTC  A
A.macleodii          GGG------C  TTCGGCTCCG  G
P.espejiana          GGG------C  TTCGGCTCCG  G
P.antarctica         TGGCCTCTAT  TTATAAGCTA  T
P.aquimarina         TGGCCTCTAT  TTATATGCTA  T
P.leiognathi         GGGGGAC--C  TTCGGGCCTC  T
```

Fig. 23.2
16S rRNA gene alignment of *Moritella* species and related taxa. Nucleotide numbering corresponds to the 16S rRNA gene of *Escherichia coli*. Unique T-T insertions at position between 206 and 207 among *Moritella* species are colored as red. The bacteria used in comparison with seven *Moritella* species are as follows: *Escherichia coli*, *Paramoritella alkaliphila*, *Shewanella putrefaciens*, *Ferrimonas balearica*, *Colwellia psychroerythraea*, *Idiomarina abyssalis*, *Alteromonas macleodii*, *Pseudoalteromonas espejiana*, *Psychromonas antarctica*, *Psychromonas aquimarina*, *Photobacterium leiognathi*

Molecular Analyses

The 16S rRNA Gene Signature Nucleotides

The signature nucleotides of the genus *Moritella* are A, T, G, and C at positions 399, 858, 1311, and 1326. Thymine-thymine insertion is found between bases 206 and 207 (*Escherichia coli* numbering). It was originally proposed by Urakawa et al. (1998) with the comparison of *M. marina* and 11 marine isolates. The robustness of these signature nucleotides has not been changing even after six new species have been reported. Although majorities of related taxa in the class *Gammaproteobacteria* do not have these insertions, *Psychromonas* species have AT insertion between bases 206 and 207 (Fig. 23.2).

In the family level, 16S rRNA gene signature nucleotides were amended: G/A at position 399, C/T at position 858, G at position 1311, and C at position 1326 (Hosoya et al. 2009).

DNA-DNA Hybridization

Comparative 16S rRNA analysis revealed that all *Moritella* species are tightly related to each other (>97.8 % sequence similarity). Since the similarity level among the genus is more than >97 %, DNA-DNA hybridization is essential for the discrimination of species (Wayne et al. 1987). The DNA-DNA relatedness among the genus *Moritella* is listed (Table 23.2). No obvious DNA-DNA hybridization groups were found, and the values ranged between 30 % and 60 %.

Table 23.2
DNA-DNA relatedness among members in the genus *Moritella*

		1	2	3	4	5	6	7
1	*M. marina*	–						
2	*M. japonica*	39[a]	–					
3	*M. yayanosii*	40[b]/30[c]		–				
4	*M. viscosa*	43[d]		57.5[b]	–			
5	*M. profunda*	41.5[b]				–		
6	*M. abyssi*			60[b]		55[b]	–	
7	*M. dasanensis*	43.5[e]					45.7[e]	–

Data adopted from:
[a]Nogi et al. (1998)
[b]Xu et al. (2003)
[c]Kato et al. (1998)
[d]Lunder et al. (2000)
[e]Kim et al. (2008)

Genome Analyses

Whole Genome Analysis

Whole genome sequence analyses have been done for *Moritella* sp. PE36 and *M. dasanensis* ArB 0140^T. *Moritella* sp. PE36 was selected because of its piezophilic nature. *M. dasanensis* was analyzed because of an interest of ice-pitting and hexagonal ice crystal formation activities. Currently, *M. viscosa* is also sequenced at Trust Sanger Institute because of its pathogenicity against marine fish.

Genome Analysis of PE36

Moritella sp. PE36 was isolated from 288 km offshore of San Diego in the Pacific Ocean at a depth of 3,584 m. This strain was characterized as deep-sea piezophile heterotroph, adapted to high pressure, and grows in a minimal medium with single carbon sources. Its optimum growth pressure was approximately 41.4 MPa, close to the pressure of its isolation depth (DeLong et al. 1997). The closest neighbor of this strain is *M. abyssi* based on the 16S rRNA gene sequence similarity (99.3 %). As well as other *Moritella* species, this strain contains polyunsaturated fatty acids (PUFAs) (DeLong and Yayanos 1985, 1986). The genome is 5,236,340 bp long, contains a plasmid (49,993 bp) and approximately 4,726 protein-encoding genes, 127 tRNA and 10 *rrn* operons, and the mol % G + C of DNA is 41.03 % (Kerman 2008). The value of mol% G + C is quite similar to the report of *M. abyssi* (41.6 %) (Xu et al. 2003).

Genome Analysis of *Moritella dasanensis*

The draft genome sequence of *M. dasanensis* ArB 0140^T was reported (Lee et al. 2012). The draft genome is 4,889,582 bp long, contains 4,293 protein-encoding genes, 91 tRNA genes and 10 rRNA operons, and the mol% G + C of DNA is 40.82 %. *Moritella* sp. strain PE36 and *Shewanella violacea* strain DSS12^T are the closest neighbors of strain ArB 0140^T. Further analysis of the *M. dasanensis* genome will be conducted to identify the genes involved in the cold adaptation mechanism and the ecological roles of this organism in the Arctic Ocean.

Phenotypic Analyses

Physiology and Identification Keys

Moritella and *Paramoritella* species are Gram-negative, chemoorganotrophic, halophilic, and facultative anaerobic motile rods. In a conventional phenotypic identification, strains belonging to the family *Moritellaceae* were classified into the members of the family *Vibrionaceae*. Thus, there is no wonder why many strains previously reported as *Vibrio* species were later deemed or reclassified as *Moritella* species (Benediktsdóttir et al. 2000; Colwell and Morita 1964; Morita 1975; DeLong et al. 1997; Hamamoto et al. 1995; Rüger and Tan 1992). Phenotypic comparison of *Moritella* and *Paramoritella* species is listed in *Table 23.3*.

The species of the genus *Moritella* are characterized as their low optimum and maximum growth temperatures. Three strains are facultative piezophiles and one strain, *M. yayanosii* is known as an obligate piezophile. Phenotypic differentiation can be achieved based on the acid production from sugars and the utilization patterns of carbon sources (*Table 23.3*). All strains are catalase and oxidase positive. Major isoprenoid quinone is Q-8. All strains reduce nitrate to nitrite without producing gas. None of the strains form pigmented colonies.

Currently *P. alkaliphila* is a solitary species in the genus *Paramoritella*. Since only single species is known, it is unclear whether alkaliphilic nature of *P. alkaliphila* is a common feature among the genus. If it were a common feature among the genus, the members of family *Moritellaceae* would be characterized as their extremophilic natures such as psychrophilly, piezophilly, and alkaliphilly.

Fatty Acid Composition

The high occurrence of the polyunsaturated fatty acid is a common feature among psychrophilic and piezophilic bacteria. The occurrence of 22:6 (docosahexanoic acid [DHA]) in fatty acid profiles is one of the most conspicuous properties of the members of the genera *Moritella* and *Colwellia* (DeLong et al. 1997; Kato et al. 1998). The fatty acid profiles of *Moritella* are different from the deep-sea *Shewanella* species that produce 20:5 (eicosapentaenoic acid [EPA]), but no DHA (Kato and Nogi 2001).

Isoprenoid Quinone Profile

The major isoprenoid quinone of *Moritella* species is Q-8. *Paramoritella alkaliphila* also has Q-8 as a major isoprenoid quinone. Thus, having Q-8 as a major isoprenoid quinone is a common feature among the family *Moritellaceae*. On the other hand, members of the genus *Shewanella* produce Q-7 and Q-8 together as the isoprenoid quinones (Kato and Nogi 2001). Thus, they are distinguishable from the species belonging to the family *Moritellaceae*.

Isolation, Enrichment, and Maintenance Procedures

Isolation and Enrichment

The most common medium used for both isolation and cultivation of *Moritella* species is ZoBell 2216 medium, which contains peptone and yeast extract (ZoBell 1941); now this medium is commercially available as Difco Marine Broth 2216 and Marine Agar 2216 (BD). All *Moritella* species can be cultured with this medium.

All *Moritella* species show psychrophilly and not tolerant for ambient laboratory temperatures. Thus, sample preparation and treatments must be cautioned so that microorganisms are kept

Table 23.3
Phenotypic comparison of *Moritella* and *Paramoritella* species

	M. marina	*M. japonica*	*M. yayanosii*	*M. profunda*	*M. abyssi*	*M. dasanensis*	*M. viscosa*	*P. alkaliphila*
Optimum growth temperature at atmospheric pressure	15	10	NG	2	4–6	9	15	30
Maximum growth temperature	<20	<20	ND	12	14	18	21–24	37–39
Optimum pressure (MPa) at 10 °C	0.1	50	80	22	30	ND	ND	ND
G + C content (mol%)	42.5	45.0	44.6	41.4	41.6	40.8	42.5	57
Growth at 20 °C	–	–	–	–	–	–	+	+
Gelatinase	+	+	+	–	–	+	+	+
Indole production	–	–	–	–	+	–	–	–
Acid production from								
Cellobiose	+	–	–	–	+	–	–	ND
D-Galactose	+	–	–	±	+	–	+	ND
Glycerol	+	+	–	–	–	–	–	–
Maltose	+	–	+	–	+	+	ND	ND
D-Mannitol	–	–	+	–	+	–	–	–
D-Mannose	–	–	+	–	–	–	–	ND
Xylose	–	–	+	–	–	–	–	–
Utilization as carbon source								
D-Arabinose	–	–	–	–	–	+	ND	ND
Cellobiose	+	–	–	–	+	–	ND	+
D-Galactose	+	–	–	+	+	–	ND	+
Glycerol	+	+	–	+	+	+	ND	ND
Maltose	+	–	–	–	+	–	ND	+
Trehalose	–	–	–	–	–	+	ND	+
Xylose	–	–	+	–	–	–	ND	+

Data are from Morita and Haight (1964), Nogi et al. (1998), Nogi and Kato (1999), Lunder et al. (2000), Benediktsdóttir et al. (2000), Xu et al. (2003), and Kim et al. (2008)
NG no growth, *ND* not determined

in cold and never exposed warm temperature for any extended period of time. For example, Rüger and Tan (1992) reported that agar plates and all solutions were chilled to 4 °C on a cold tray during the whole inoculation procedure.

For the selective enrichment of psychrophiles, low-temperature incubation ranged between 2 °C and 4 °C is widely used and recommended (Kato et al. 1998; Kim et al. 2008; Rüger and Tan 1992). Although, psychrophiles grow fast under the low-temperature conditions, incubation periods should be longer than typical mesophiles (Rüger and Tan 1992).

Four currently known *Moritella* species (*M. japonica*, *M. profunda*, *M. abyssi*, *and M. yayanosii*) are piezophilic and grow better under the pressure than atmospheric pressure. Thus, the use of a pressure vessel may require obtaining piezophilic strains from the deep sea (*Fig. 23.3*). Especially, obligate piezophiles such as *M. yayanosii* are only attained by using a pressure vessel (Kato et al. 1998). A sterilized plastic pouch is used for the isolation and cultivation of piezophilic bacterium. After inoculating the strain, the pouch is sealed without bubbles and stored in water filled and pressurized in the vessel.

Maintenance

To store bacterial cultures, low-temperature preservation is often used. That is because typical mesophilic bacteria only grow slowly or halt growing under the low-temperature condition. However, this rule cannot be applied in the case of *Moritella* species due to their psychrophilic nature. Since these psychrophilic bacteria grow well at low temperature, the transfer of cultures should be more frequent than mesophilic cultures. For example, *M. viscosa* is maintained on marine agar at 4 °C and reinoculated every 1 or 2 months (Benediktsdóttir et al. 2000). For short-term preservation, stab cultures in semisolid

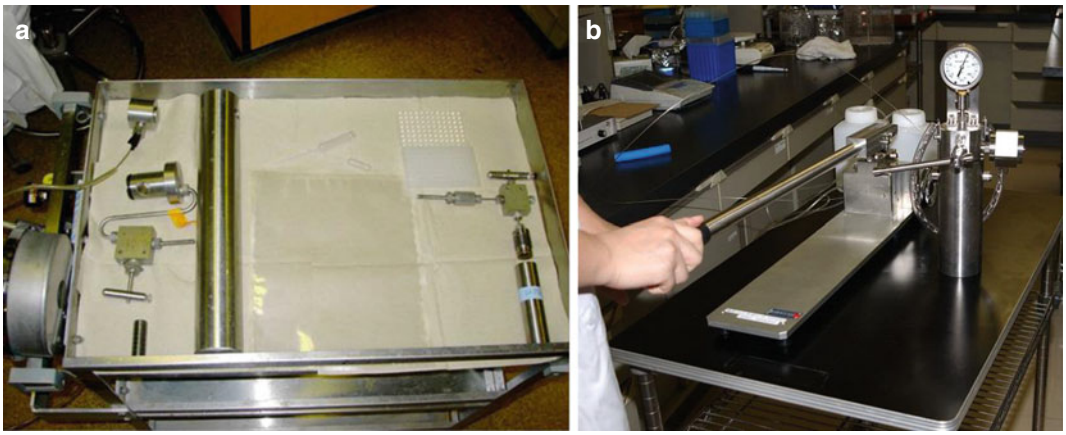

Fig. 23.3
High-pressure cultivation system consisted of a pressure-resistant container (a) and a hand pump with a coupler to inject water into the container (b). Various plastic cultivation units are also shown in a panel (a). This type of culture apparatus is essential to grow *Moritella yayanosii* (Courtesies of D. Bartlett, Scripps Institution of Oceanography, University of California and Y. Oshida, Japan Collection of Microorganisms, RIKEN BioResource Center)

medium are available for quarter to half year at low temperature (<4 °C). For long-term storage, preparation of glycerol stocks in an appropriate medium (10–20 % glycerol [v/v]) is the best preservation method. In a laboratory, a deep freezer is commonly used at −80 °C to preserve glycerol cultures. For more stable long-term storage, glycerol stocks in liquid nitrogen or lyophilization can be used. Culture collections maintain *Moritella* species as glycerol stocks or freeze-dried samples. Availability of lyophilization is confirmed in the case of *M. marina* strain ATCC 15381^T at American Type Culture Collection. *M. yayanosii* is an obligate piezophilic bacterium; this species cannot grow at atmospheric pressure. Thus, the maintenance of this culture requires a unique pressure vessel (*Fig. 23.3*). This strain is cultured in marine broth at 10 °C and 70 MPa. *M. yayanosii* is capable to survive under atmospheric pressure for a few hours. Thus, one can inoculate and collect the cells under atmospheric pressure. *M. yayanosii* JCM 10263^T is preserved as a glycerol stock (10 % glycerol in marine broth [v/v]) in a liquid nitrogen tank (vapor phase) at Japan Collection of Microorganisms, RIKEN BioResource Center.

Ecology

Habitats

Although two genera *Moritella* and *Paramoritella* form the family *Moritellaceae*, their growth temperature properties are quite different; members of *Moritella* genus are characterized as psychrophiles, but *P. alkaliphila* is a mesophilic bacterium. Judging by their growth temperature properties, their habitats do not likely overlap each other; one is isolated from a tropical ocean, while others are isolated from permanently cold marine environments. Thus, habitats will be separately discussed in the following sections.

Habitats for *Moritella* Species

Approximately 71 % of the surface area in our planet is covered by the oceans. As oceans present an average depth of 3,800 m and more than 97 % of the ocean water locates below epipelagic zone, cold-temperature environments (<4 °C) represent the most common marine habitats for millions of creatures. One of the hypotheses of microbial evolution is that thermophiles were the first to evolve, followed by the mesophiles and then the psychrophiles (Morita 1975). Thus, ancestors of *Moritella* species have set out to adapt this vast last frontier. Now they successfully inhabit throughout the depth of the oceans (Kato et al. 1998). Though quantitative study of distribution pattern of *Moritella* species is quite limited, members of *Moritella* species occupy large portion of culturable facultative anaerobic isolates below the thermocline. Approximately 62 % (19/29 strains) of facultative anaerobic isolates from the deep Pacific Ocean were identified as *Moritella* species (Urakawa et al. 1999). *Moritella* species have been found only from deeper than 500 m depth, in contrast with the distribution of *Photobacterium* isolates, which were isolated from both deep and shallow (<500 m) waters (Urakawa et al. 1999). Bowman and colleagues (1997) isolated many psychrophilic bacteria from the Antarctic sea ice. Interestingly majority isolates were identified as members of *Alteromonadaceae* such as *Colwellia*, *Shewanella*, and *Marinobacter*; however, no *Moritella* strains were isolated. Since majority of species have been isolated from the deep sea, the major habitats for *Moritella* species are likely marine environments below the thermocline, where it is permanently maintained cold. Although quantitative measurements have not been done, at least two species, *M. viscosa* and *M. dasanensis*, were isolated from cold shallow waters. Thus, some *Moritella* species likely inhabit cold shallow waters.

Moritella species have been isolated from seawater, seafloor, and marine fish (Colwell and Morita 1964; Urakawa et al. 1998, 1999).

One of the best-studied marine bacteria *Vibrio* are known as one of the major components of surface microbial flora of marine life forms (Thompson et al. 2006). They are also frequently found in intestines of marine fish. Since *Moritella* species are also facultatively anaerobes, there is no wonder *Moritella* strains have been isolated from the intestines of deep-sea fish (Nakayama et al. 2005). *Moritella* species may share similar ecological niche characterized as hypoxia or anoxia with other facultative anaerobes such as *Vibrio*, *Photobacterium*, *Colwellia*, and *Shewanella* species in various cold marine environments, such as ocean floor (Bowman and McCuaig 2003; Bowman et al. 2003), digestive tracts of marine organisms (Nakayama et al. 2005), and microscale anaerobic environments (i.e., marine snow) (Alonso and Pernthaler 2005).

Although majority of *Moritella* species likely establish mutualistic relationships with marine organisms, one of the members of *Moritella* species, *M. viscosa*, is known as a fish pathogen, which causes winter ulcer for sea-farmed Atlantic salmon (Fig. 23.4). The mortality is limited, but the disease has economic significance due to lowered quality of the fish (Lunder et al. 2000).

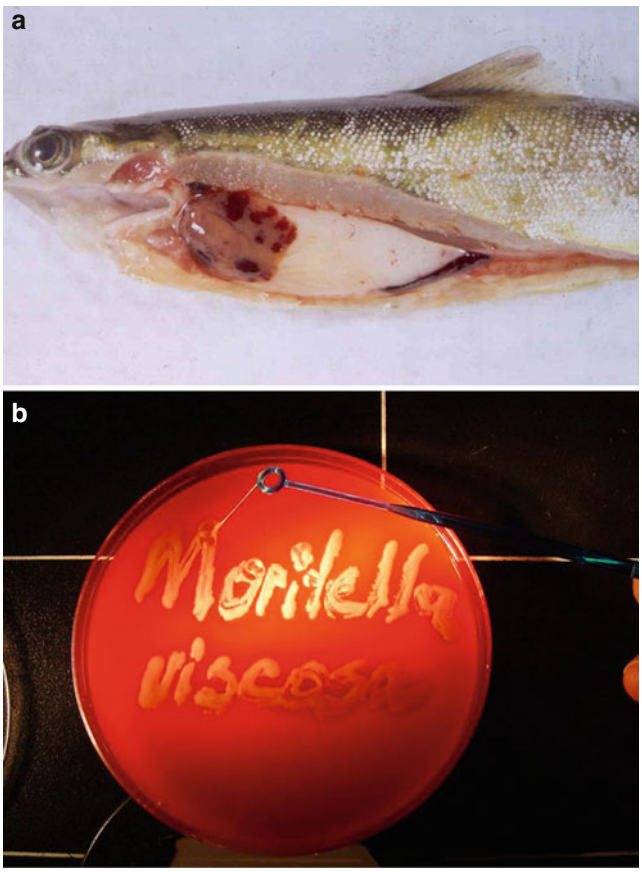

Fig. 23.4
***Moritella viscosa*. (a) Atlantic salmon with skin ulcer disease caused by *Moritella viscosa*. Internal symptoms include petechial hemorrhages on the liver. (b) Colonies of *Moritella viscosa*. The name of this species was originated from this viscous colony formation (Courtesy of B. Guðmundsdóttir, University of Iceland)**

Habitats for *Paramoritella* Species

Two strains (A3F-7^T and ssthio04PA2-7c) of marine heterotrophic alkaliphilic bacterium *P. alkaliphila* were isolated from hard coral (*Favites complanata*) and marine sand collected from the Republic of Palau, respectively. It suggests that *P. alkaliphila* may distribute widely in tropical marine environments.

Short Description of the Species

Moritella marina (Baumann, Furniss and Lee 1984) Urakawa, Kita-Tsukamoto, Steven, Ohwada, and Colwell 1999, comb. nov. (Type species of the genus)

Type strain: strain MP-1 = ATCC 15381 = CIP 102861 = NCCB 79030 = NCIMB 1144.
GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the type strain: AB038033.
Basonym: *Vibrio marinus* (Russell 1891) Baumann et al. 1984.
Other synonym: *Vibrio marinus* (Russell 1891) Ford 1927.
Etymology: L. fem. adj. *marina*, of or belonging to the sea, marine.
References: validation list no. 69 (1999); effective publication (Urakawa et al. 1998).

V. marinus was first isolated from the Gulf of Naples and described as *Spirillum marinus* by Russell (1891). Later 16 of *Vibrio marinus*-like strains were reisolated (Colwell and Morita 1964). However, only one strain, *V. marinus* MP-1 ^{T}, which was isolated from seawater 125 miles off the Oregon coast at a depth of 1,200 m, is still available as ATCC 15381^T (Morita and Haight 1964). The description and detailed characteristics of *Moritella marina* comb. nov. (ma.ri'na. L. adj. *marina*, of the sea, marine) are based on the data from Colwell and Morita (1964) and Colwell (1965). It should be noted that one of the strains, *V. marinus* strain PS 207, which was isolated from the skin of a Pacific cod and can grow at 30 °C, was later reclassified as *Vibrio logei* (= ATCC 15382) (Colwell and Morita 1964; Colwell 1965; Margaret et al. 1971).

Moritella japonica Nogi, Kato, and Horikoshi 1999, sp. nov.

Type strain: strain DSK1 = CIP 106291 = DSM 14879 = JCM 10249.
GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the type strain: D21224.

Etymology: N.L. fem. adj. *japonica*, pertaining to the Japan Trench, where this strain originated.
References: validation list no. 69 (1999); effective publication (Nogi et al. 1998).

M. japonica was isolated from the Japan Trench at a depth of 6,356 m. It was reported as a first barophilic species among *Moritella* genus (Fig. 23.5). This strain is able to grow in pressure vessels under hydrostatic pressures in a range of 0.1–70 MPa and at temperatures in a range of 4–15 °C. This species is not able to grow at temperatures above 20 °C. The optimum temperature and pressure conditions for growth are 15 °C and 50 MPa, respectively.

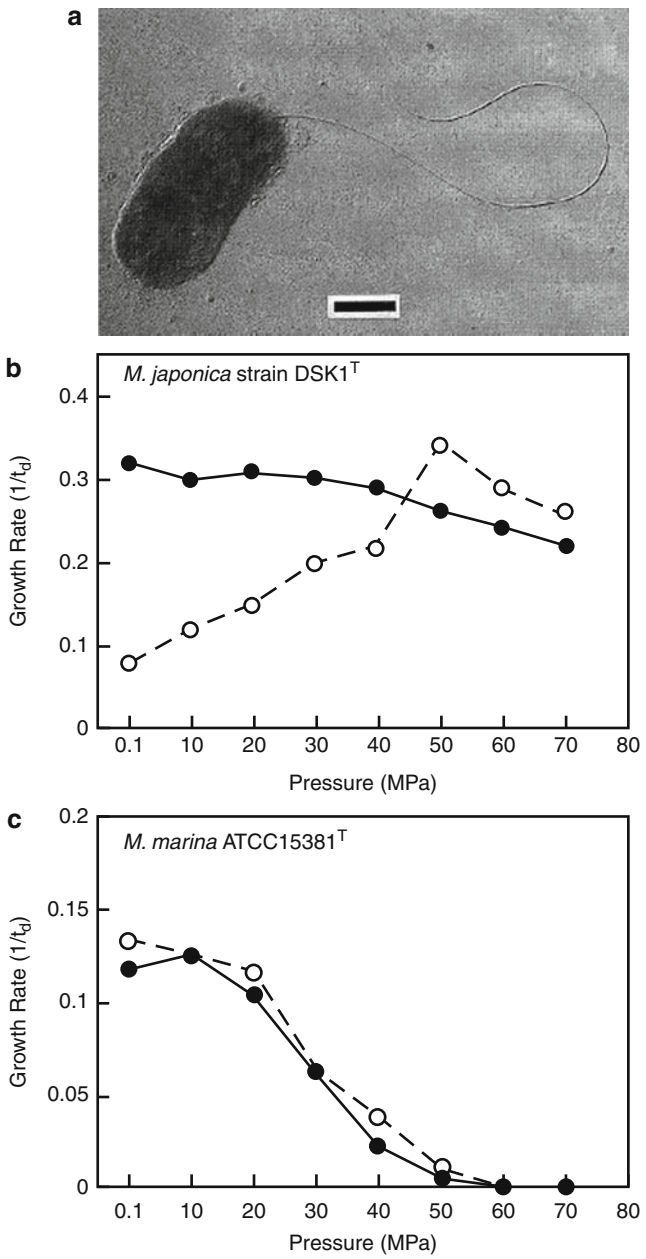

Fig. 23.5
***Moritella japonica*. Electron micrograph of stained, shadow-cast cell of strain DSK1^T (a). Growth rate comparison between *M. japonica* (b) and *Moritella marina* (c) under the pressure conditions at 10 °C (*closed circles with solid line*) and 15 °C (*open circles with dashed line*). Bar, 500 nm. t_d indicates doubling time (Data from Nogi et al. (1998))**

Moritella yayanosii Nogi and Kato 1999, sp. nov.

Type strain: strain DB21MT-5 = JCM 10263.
GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the type strain: AB008797.
Etymology: N.L. gen. masc. n. *yayanosii*, of Yayanos, named in honor of American deep-sea biologist Aristides Yayanos.
References: validation list no. 71 (1999); effective publication (Nogi and Kato 1999).

M. yayanosii was isolated from a sediment sample collected from the Mariana Trench, Challenger Deep at a depth of 10,898 m. The sediment was pressurized at approximately 100 MPa in a pressure vessel placed in a refrigerator (2–4 °C). For single-colony isolation, cultures were incubated under a pressure of 100 MPa in plastic bags (Fig. 23.3). Growth of cells under conditions of 0.1–100 MPa at 10 °C in pressure vessels was tested in marine broth (Kato et al. 1998). *M. yayanosii* is an obligate piezophilic bacterium that can grow at 100 MPa. The optimal pressure condition for growth is 80 MPa, and no growth is detected at pressures of less than 50 MPa. All physiological tests were performed in pressure vessels at 70 MPa at 10 °C (Nogi and Kato 1999). Acid is produced from D-mannose and xylose, which are effective characteristics to distinguish this species from other *Moritella* species (Table 23.3).

Moritella viscosa (Lunder, Sørum, Holstad, Steigerwalt, Mowinckel and Brenner 2000) Benediktsdóttir, Verdonck, Sproer, Helgasön, and Swings 2000, comb. nov.

Type strain: strain NVI 88/478 = ATCC BAA-105 = NCIMB 13584.
GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the type strain: AJ132226.
Basonym: *Vibrio viscosus* Lunder et al. (2000).
Etymology: L. fem. adj. *viscosa*, viscous, sticky, because of its thread-forming, adherent colonies.
Reference: Benediktsdóttir et al. (2000).

The type strain of *Moritella viscosa* NVI 88/478^T was originally isolated in 1988 in Norway from an Atlantic salmon with winter ulcer and proposed as *V. viscosus* (Lunder et al. 2000). This species has been reported as a pathogen that causes winter ulcer of Atlantic salmon and other marine fish from Norway, Iceland, and Scotland (Benediktsdóttir et al. 2000). Colonies are viscous and can form long threads when removed from the agar surface (Fig. 23.4). The maximum growth temperature of *M. viscosa* is

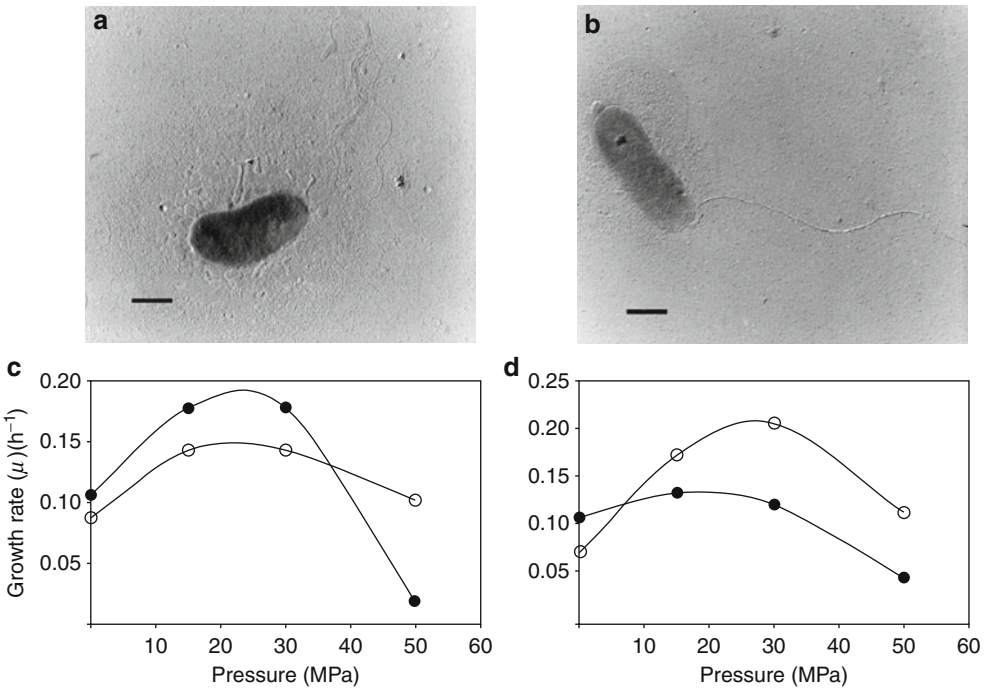

Fig. 23.6

Electron micrographs of stained, shadow-cast cells of *Moritella profunda* 2674^T (a) and *Moritella abyssi* 2693^T (b) and growth response of *M. profunda* (c) and *M. abyssi* (d) under different pressures at 6 °C (*closed circles*) and 10 °C (*open circles*). Bars, 1 μm. Growth rates were calculated as 1/td [t_d is doubling time (h)] (Data from Xu et al. (2003))

likely the highest among currently known *Moritella* species but different according to the literature. Lunder et al. (2000) reported that the growth occurred at 4–25 °C, but not at 30 °C. However, Benediktsdóttir et al. (2000) refuted that no strain was able to grow at 25 °C, but all grew at 21 °C and 4 °C after careful inspection.

Moritella profunda Xu, Nogi, Kato, Liang, Rüger, De Kegel, and Glansdoff 2003, sp. nov.

Type strain: strain 2674 = JCM 11435 = LMG 21259.
GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the type strain: AJ252023.
Etymology: L. fem. adj. *profunda*, from the deep.
Reference: Xu et al. (2003).

Moritella profunda is a psychropiezophilic bacterium isolated from a deep-sea sediment collected at a depth of 2,815 m in the Sierra Leone Rise region of the eastern tropical Atlantic (Fig. 23.6). The sample was cultured at 2 °C on a chilled seawater agar plate prepared with a medium containing 1.5 g peptone, 0.5 g yeast extract, 0.01 g $FePO_4 \cdot 4H_2O$, 750 mL sea water, and 250 ml distilled water. The maximum growth rate is given at 2 °C or possibly lower temperature; thus, this species has the lowest optimum temperature among the family *Moritellaceae*. Cells can grow at atmospheric pressure, but the piezophilic growth is stimulated with a maximum of 20–24 MPa at 6 °C and slightly higher at 10 °C. At 6 °C, the maximum pressure is between 50 and 60 MPa; it is increased considerably by raising the temperature to 10 °C. Elongated cells are occasionally found in the high-pressure incubated cultures.

Moritella abyssi Xu, Nogi, Kato, Liang, Rüger, De Kegel, and Glansdoff 2003, sp. nov.

Type strain: strain 2693 = JCM 11436 = LMG 21258.
GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the type strain: AJ252022.
Etymology: L. gen. n. *abyssi*, of/from the abyss.
Reference: Xu et al. (2003).

The type strain, strain 2693^T, was collected from the upper layer of deep Atlantic sediments (2,815 m) off the West African coast in 1983 (Fig. 23.6). *M. abyssi* is a piezophilic bacterium; cells can grow at atmospheric pressure, but the optimum pressures are 19–20 MPa at 6 °C and 30 MPa at 10 °C. The strain was isolated by the same manner with *M. profunda*. Cells are often elongated and show irregular forms under atmospheric pressure. Positive for indole test.

Moritella dasanensis Kim, Park, Lee, Park, Jung, Kang, Joo, Seo, and Kang 2008, sp. nov.

Type strain: strain ArB 0140 = JCM 14759 = KCCM 42845 = KCTC 10814.

GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the type strain: EF192283.
Etymology: N.L. fem. adj. *dasanensis*, pertaining to the Korean Arctic Dasan station where the type strain was isolated.
Reference: Kim et al. (2008).

M. dasanensis KCTC10814^T was isolated from surface seawater off the near shore of Kongsfjorden in the Svalbard Archipelago, Norway. The strain was isolated on a marine agar at 3 °C and maintained at the same temperature. Anaerobic growth of *M. dasanensis* was not tested in the original publication (Kim et al. 2008) but later confirmed (H. -J. Kim, personal communication). Strain ArB 0140^T grows between −3 °C and 18 °C. The optimal growth temperature is 9 °C. Based on the Ratkowsky growth model analysis (Ratkowsky et al. 1983), the notional minimum, optimum, and maximum growth temperatures were estimated as −11.9 °C, 9 °C, and 17.8 °C, respectively (Fig. 23.7). The unique feature of this species is the ability to secrete ice-active substances, which are macromolecular substances that affect the shape of ice crystals by binding to the growing ice crystals (Fig. 23.8). Ice-modifying activity was not observed in *M. marina*, *M. japonica*, and *M. abyssi*. The G + C mol% of this species must be amended. The G + C mol % reported in Kim et al. (2008) was 46.9 %, which was the highest G + C content value in the family. However, the G + C content obtained from the draft genome sequence of this species was 40.82 %, which was the lowest G + C value among the family.

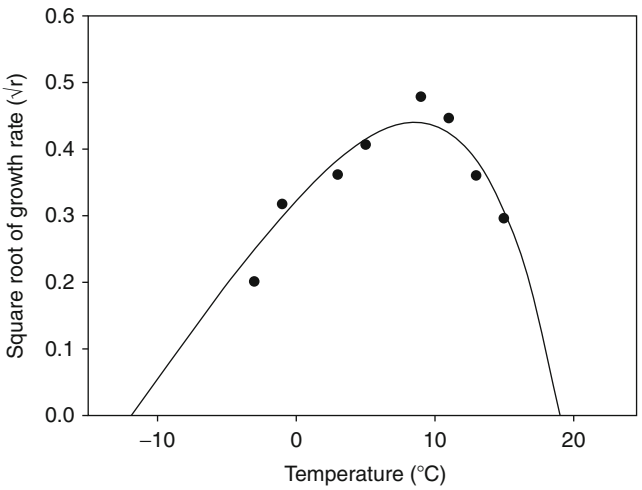

Fig. 23.7
Growth rate of *Moritella dasanensis* ArB 0140^T determined by using a temperature gradient incubator. The growth of culture at −3 °C and −1 °C was determined in a water bath. Fitted line was calculated by the Ratkowsky model (Ratkowsky et al. 1983) (Data from Kim et al. (2008))

***Paramoritella alkaliphila* Hosoya, Suzuki, Adachi, Matsuda, and Kasai 2009, gen. nov.**

Type strain: strain A3F-7 = MBIC 06429 = DSM 19956.
GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the type strain: AB364966.
Etymology: Gr. prep. para, beside; N.L. fem. n. *Moritella*, a bacterial genus name; N.L. fem. n. *Paramoritella*, beside *Moritella*.
Reference: Hosoya et al. (2009).

Two strains of marine, heterotrophic, alkaliphilic bacteria, designated A3F-7^T and ssthio04PA2-7c, were isolated

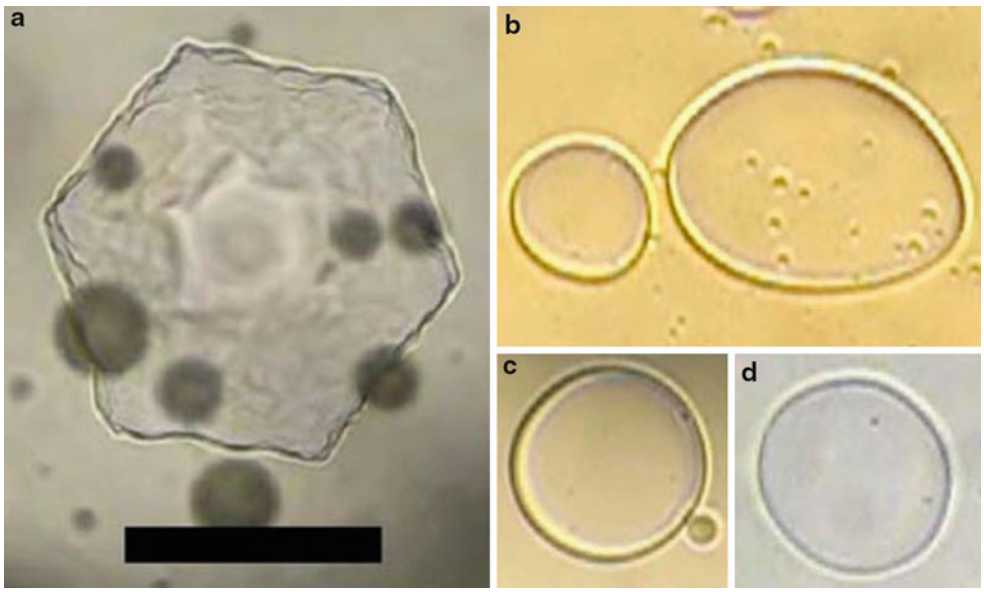

Fig. 23.8
Ice-modifying activity of *Moritella dasanensis* ArB 0140^T and other *Moritella* species. This activity was observed by using a nanolitre osmometer. (a) *M. dasanensis*, (b) *M. marina*, (c) *M. japonica*, and (d) *M. abyssi*. Bar, 100 μm (Data from Kim et al. (2008))

from hard coral (*Favites complanata*) and marine sand collected from the Republic of Palau, respectively. Strain A3F-7^T was isolated by using marine agar adjusted to pH 11 with Na_2CO_3 and $NaHCO_3$. Strain ssthio04PA2-7c was isolated from marine sand collected from the Republic of Palau in 2004. Strain ssthio04PA2-7c was isolated by using thio medium. A high level of DNA-DNA relatedness indicated that these two isolates were the same species. Phenotypic features resemble to other *Moritella* species except for the growth temperature and pH properties. The optimum temperature is 30 °C; growth occurs at 15 °C and 37 °C and growth is not observed at 8 or 40 °C. The pH range for growth is 7.0–11.0, and the optimum pH for growth is 9.0.

Application

Moritella species have been studied as model microorganisms of low-temperature-adapted enzymes (Morita 1975 and references therein; Deming 2002 and references therein), barophilic adaptation of marine bacteria to the deep sea (Lauro and Bartlett 2008 and references therein). *Moritella* species are also known as producers of long-chain fatty acids such as DHA (DeLong and Yayanos 1985, 1986).

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24 The Family *Oceanospirillaceae*

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Abstract
Oceanospirillaceae, a family within the order *Oceanospirillales*, currently consists of 17 genera including *Amphritea*, *Balneatrix*, *Bermanella*, *Marinomonas*, *Marinospirillum*, *Neptuniibacter*, *Neptunomonas*, *Nitrincola*, *Oceaniserpentilla*, *Oceanobacter*, *Oceanospirillum* (type genus), *Oleibacter*, *Oleispira*, *Pseudospirillum*, *Reinekea*, *Spongiispira*, and *Thalassolituus*, though recent phylogenetic analyses suggest a taxonomic realignment may be necessary as the inclusion of several genera has been shown dependent on the algorithm used to calculate their respective positions. Nearly all species inclusive to this aerobic family are Gram negative, motile rods, or helical shaped with positive oxidase and catalase reactions. All have DNA GC content of 41–63 mol% with the genome size of a member *Marinomonas* species having been reported at approximately 4.7 Mb through whole-genome sequence analysis. Most species, save those in the genus *Balneatrix*, are halophilic, requiring sodium ions for growth, and are widely distributed in marine environments, including marine organisms, seaglass, seawater, and sea sediment. The non-halophilic genus *Balneatrix* inhabits freshwater and has been identified as a human pathogen.

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Numerous *Oceanospirillaceae* species have unique characteristics applicable to industrial fields, including the capability for degrading petroleum compounds and secretion of bactericidal compounds or melanin pigment.

Taxonomy, Historical and Current

Short Description of *Oceanospirillaceae*

Oceanospirillaceae (O.ce.an.o.spi.ril.la'les. M. L. neut. n. *Oceanospirillum* type genus of the family;-aceae ending to denote family; M. L. fem pl. n. *Oceanospirillaceae* the *Oceanospirillum* family) was established by Garrity et al. (2005) on the basis of phylogenetic analysis of 16S rDNA sequences. The family *Oceanospirillaceae* belongs to the order *Oceanospirillales* of the class *gammaproteobacteria*; the order contains the families *Oceanospirillaceae*, *Alcanivoraceae*, *Hahellaceae*, *Halomonadaceae*, *Oleiphilaceae*, and *Saccharospirillaceae*. *Oceanospirillaceae* contains the genera *Amphritea*, *Balneatrix*, *Bermanella*, *Marinomonas*, *Marinospirillum*, *Neptuniibacter*, *Neptunomonas*, *Nitrincola*, *Oceaniserpentilla*, *Oceanobacter*, *Oceanospirillum* (type genus; Hylemon, Wells, Krieg and Jannasch 1973, 361$^{AL.}$), *Oleibacter*, *Oleispira*, *Pseudospirillum*, *Reinekea*, *Spongiispira*, and *Thalassolituus*, though this taxonomy must be reevaluated as the inclusion of *Balneatrix*, *Marinospirillum*, *Nitrincola*, *Pseudospirillum*, and *Reinekea* is dependent on the algorithm used to calculate their respective phylogenetic positions (e.g., positions vary between NJ and ML methods such that, at minimum, *Marinospirillum* and *Reinekea* should be transferred to other families) (*Fig. 24.1*). Prior to the establishment of the family *Oceanospirillaceae*, many of these genera and bacterial groups were categorized as lesser known members of the γ-Proteobacteria.

Almost all genera are halotolerant or halophilic marine bacteria, the exception being *Balneatrix* which has been isolated solely from freshwater and human clinical samples. Cells are primarily rod shaped, though some genera display helical or S-shaped morphologies, and all are motile by polar flagella. Physiologically, members of the *Oceanospirillaceae* are aerobic and strictly respiratory, save for *Neptunomonas*, which can perform weak fermentation reactions. All members are oxidase positive. Several species in the genera *Bermanella*, *Neptuniibacter*, *Neptunomonas*, *Oceaniserpentilla*, *Oceanobacter*, *Oleibacter*, *Oleispira*, *Spongiispira*, and *Thalassolituus* have been shown to be involved in petroleum degradation, and some *Marinomonas* strains have been shown to secrete bactericidal compounds and produce melanin. In most species, the primary isoprenoid quinones are Q8, while the majority of fatty acids are 14:0, 16:1ω7, 16:0, and 17:1ω6. The mol% GC of all *Oceanospirillaceae* DNA is 41–63. Genome size of a *Marinomonas* species has been determined to be approximately 4.7 Mb through whole-genome sequence analysis, although it remains to be seen if this is representative of the family as a whole. Summary of phenotypic information in *Oceanospirillaceae* is shown in *Table 24.1*.

Taxonomic History

As stated previously, *Oceanospirillaceae* was established in 2005 by Garrity et al. Prior to its establishment, many marine genera and bacterial groups were categorized as the lesser known marine genera of the class *γ-Proteobacteria;* in fact, several marine bacterial groups have been recognized in the literature as "*Oceanospirillum* and related genera," e.g., the 3rd edition of the Prokaryotes. The oldest genus of this family, *Oceanospirillum*, was established officially in 1973 (Hylemon et al. 1973), although several reclassifications and renamings for this bacterial group's members have since occurred. Presented here is a description of the history for this genus and related bacteria subsequently followed by a description of the second oldest (and largest) genus, *Marinomonas*, and other selected genera.

The genus *Oceanospirillum* was originally created to distinguish the marine species of the genus *Spirillum* from those found in freshwater (Hylemon et al. 1973). The *Spirillum* genus has a long history, originally encompassing all of the known aerobic and microaerophilic spirilla, including both marine and freshwater species (Ehrenberg 1832; Watanabe 1959; Hylemon et al. 1973). As originally defined, the DNA base composition for the genus ranged from 38 to 65 mol% G+C, unusually broad for a bacterial genus (Krieg 1974). Moreover, three distinct groups were evident within the genus: (a) aerobic, freshwater spirilla unable to tolerate 3 % NaCl (mol% G+C 50–65); (b) aerobic, marine spirilla requiring seawater for growth (mol% G+C 42–48); and (c) large, microaerophilic spirilla that belong to the species *S. volutans* (mol% G+C =38). To make the genus more consistent with other taxa, Hylemon et al. (1973) divided it into three genera: the genus *Aquaspirillum* containing the aerobic freshwater spirilla, the genus *Oceanospirillum* containing the marine species, and the genus *Spirillum* which was comprised solely of the single species, *S. volutans*. The *Oceanospirillum* as described by Hylemon et al. (1973) contained six species: *O. linum, O. minutulum, O. beijerinckii, O. maris, O. japonicum*, and interestingly an organism known as "*Spirillum lunatum*" (Williams and Rittenberg 1957), though this last inclusion poses taxonomic problems. The characteristics of the type strain (ATCC 11337 or NCMB 54) of "*Spirillum lunatum*" did not fit the original description of the species, and Linn and Krieg (1978) found that NCMB strain 54 actually consisted of a mixture of two dissimilar organisms. One was a short, vibroid rod that possessed a single polar flagellum, grew in either the presence or absence of seawater, catabolized sugars, did not form coccoid bodies, and had a mol% G+C of 63–64. The other was a larger, helical organism that possessed bipolar flagellar tufts, required seawater for growth, failed to utilize sugars, formed coccoid bodies, and had a mol% G+C of 45. The smaller organism did not appear to belong to either *Oceanospirillum* or *Aquaspirillum* and to date remains unclassified. The larger organism had characteristics more in accord with the original description of "*S. lunatum*" but differed in certain respects; it has since been classified as a new subspecies of *O. maris*: *O. maris* subsp. *williamsae*. Terasaki later added four more species: *O. hiroshimense*, *O. pelagicum*, *O. pusillum*, and *O.*

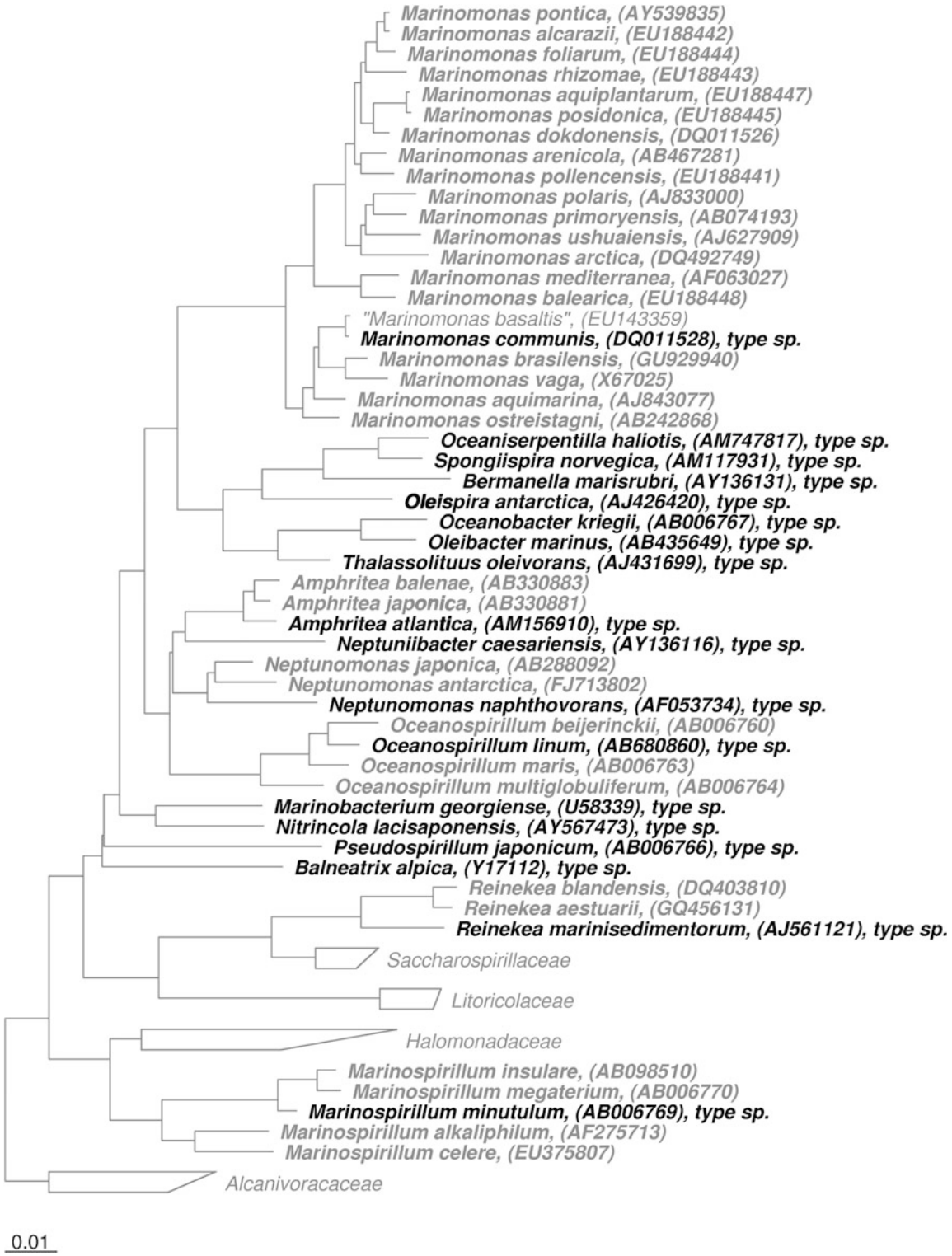

Fig. 24.1

Phylogenetic reconstruction of the family *Oceanospirillaceae* based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes–Cantor correction. The sequence datasets and alignments were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high-quality-type strain sequences proportionally distributed among the different bacterial and archaeal phyla. In addition, a 40 % maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. Scale bar indicates estimated sequence divergence

Table 24.1

Phenotypic characteristics of members of *Oceanospirillaceae*

	Marinomonas	*Marinospirillum*	*Oceanospirillum*	*Pseudospirillum*	*Amphritea*	*Balneatrix*	*Bermanella*	*Neptunibacter*
Morphology	Helical, curved or straight rods	Helical	Helical	Helical	Rods	Rods	Helical	Rods
Number and arrangement of flagella	1 Polar or bipolar tufts	1 Polar or bipolar tufts	Bipolar tufts	Bipolar tufts	1, Polar or bipolar tufts	1 Polar	1 polar	nd
Optimal temperature (°C)	4–40	15–25	25–32	30–32	20–34	20–46	25–30	15–37
Growth at 4 °C	d	+	d	–	+	–	–	–
Growth at 45 °C	–	–	–	–	–	–	–	–
Optimal NaCl (%) for growth	nd	2–3	0.5–8	nd	3	<1	nd	nd
Maximal NaCl (%) for growth	nd	10	8	8	6	1	12	6
Nitrate reducted to nitrite	d	d	–	–	nd	+	–	–
Nitrite reduced to N_2	nd	–	–	–	nd	–	–	nd
Oxidase	d	+	+	+	+	+	+	+
Catalase	d	– or W	d	– or W	+	nd	+	+
Gelatin liquefaction	d	–	d	– or W	d	nd	–	–
Starch hydrolysis	d	–	–	–	nd	–	–	–
Urease	d	–	–	–	d	–	nd	nd
Utilization of								
D-Glucose	+	–	–	–	d	+	–	–
D-Fructose	d	–	–	–	d	+	–	–
D-Mannose	d	–	–	–	nd	+	–	–
Sucrose	d	–	–	–	nd	nd	–	–
Cellobiose	d	nd	–	nd	nd	–	–	–
D-Mannitol	d	nd	–	nd	nd	+	–	–
Glycerol	d	–	–	–	d	+	–	–
Gluconate	d	nd	nd	nd	nd	+	–	–
Succinate	d	+	d	+	nd	+	+	+
L-Glutamate	d	+	d	+	nd	+	W	+
L-Aspartate	d	–	–	–	nd	+	–	+
DL-Alanine	d	–	–	+	+	+	–	+
L-Arginine	d	d	–	–	nd	nd	–	+
L-Serine	d	–	–	–	nd	+	–	–
Pyruvate	d	+	d	+	nd	nd	–	+
Acetate	d	+	d	+	nd	nd	–	+
Hexadecane	d	nd	nd	nd	nd	nd	nd	nd
p-Hydroxybenzoate	d	nd	d	–	nd	nd	nd	nd
PHB accumulation	–	+	+	+	+	nd	+	+
Mol% G + C in DNA	41–50	42–45	45–50	44–46	47–52	54	44	47
Major ubiquinone	Q-8	Q-8	Q-8	Q-8	Q-8	nd	nd	Q-8
Type species	*M. communis*	*M. minutulum*	*O. linum*	*P. japonicum*	*A. atlantica*	*B. alpica*	*B. marisrubri*	*N. caesariensis*
References	Espinosa et al. (2010)	Watanabe (1959)	Hylemon et al. (1973)	Watanabe (1959)	Gärtner et al. (2008)	Dauga et al. (1993)	Pinhassi et al. (2009)	Arahal et al. (2007)
		Hylemon et al. (1973)		Hylemon et al. (1973)	Miyazaki et al. (2008)			
		Satomi et al. (2002)		Satomi et al. (2002)				

Symbols and abbreviations: + present in all strains, – lacking in all strains, *d* differs among strains, *nd* not determined, *W* weak reaction, *PHB* poly-β-hydroxybutyrate, *M* molar

Neptunomonas	*Nitrincola*	*Oceaniserpentilla*	*Oceanobacter*	*Oleibacter*	*Oleispira*	*Reinekea*	*Spongiispira*	*Thalassolituus*	*Marinobacterium georgiense*
Rods	Rods	Helical	Rods	Rods	Helical	Rods	Helical	Helical	Rods
1 Polar	1 Polar	nd	1 Polar	1 Polar	1 Polar	1 Polar	1 Polar	1 Polar	1, polar
nd	37	2–8	20–30	25–30	2–4	25	20–30	20–25	37
+	–	+	–	–	+	d	–	+	+
–	nd	–	–	–	–	–	–	–	–
1.75–7	5	nd	nd	1–7	3–5	2–5	2–3	2.7	0.1–0.5 M
7	8	nd	nd	7	10.7	12	7	5.7	2 M
–	nd	nd	–	+	+	d	–	nd	–
nd	nd	nd	nd	nd	nd	–	nd	nd	–
+	+	+	+	+	+	+	+	+	+
+	+	–	nd	+	+	+	–	+	+
–	–	nd	–	(+)	–	– or W	nd	–	nd
–	–	nd	–	nd	–	d	nd	–	nd
nd	–	nd	nd	(+)	nd	nd	nd	nd	nd
+	–	nd	+	–	–	+	–	–	+
+	–	nd	+	–	–	d	–	–	+
–	–	nd	–	–	–	d	–	nd	+
–	–	nd	–	–	nd	+	–	nd	–
nd	–	nd	–	nd	–	nd	–	nd	+
+	–	nd	+	–	–	+	–	–	+
+	–	nd	–	nd	–	nd	–	nd	+
–	nd	nd	–	nd	–	nd	–	nd	nd
+	+	nd	nd	nd	–	nd	–	nd	+
+	nd	nd	d	nd	nd	nd	–	nd	+
nd	nd	nd	–	–	–	nd	–	nd	+
+	–	+	nd	nd	–	nd	–	–	+
nd	–	nd	d	nd	–	nd	–	nd	–
+	–	nd	–	nd	–	nd	–	nd	–
+	+	nd	nd	+	–	nd	–	nd	+
+	+	nd	nd	nd	nd	nd	–	nd	+
nd	nd	nd	nd	nd	+	nd	–	nd	nd
nd	nd	nd	+	nd	–	nd	–	–	+
+	nd	nd	+	nd	–	nd	nd	–	–
46	47.4	nd	54–56	53.0–53.1	41–42	51–52	62.6	53.2	54.9
Q-8	nd	nd	Q-8	Q-9	nd	Q-8	nd	Q-9	Q-8
N. naphthovorans	*N. lacisaponensis*	*O. haliotis*	*O. kriegii*	*O. marinus*	*O. antarctica*	*R. marinisedimentorum*	*S. norvegica*	*T. oleivorans*	*M. georgiense*
Hedlund et al. (1999)	Dimitriu et al. (2005)	Schlösser et al. (2008)	Bowditch et al. (1984)	Teramoto et al. (2011)	Yakimov et al. (2003)	Romanenko et al. (2004)	Kaesler et al. (2008)	Yakimov et al. (2004)	González et al. (1997)
Miyazaki M et al. (2008)			Sakane and Yokota (1994)					Teramoto et al. (2011)	Satomi et al. (2002)
Zhang et al. (2010)									

multiglobuliferum (Terasaki 1973, 1979), and together these nine species (including one subspecies) were subsequently described in *Bergey's Manual of Systematic Bacteriology* (Krieg 1984). In 1984, Bowditch et al. described two new species, *Oceanospirillum jannaschii* and *O. kriegii*, as well as transferred two species, *Alteromonas communis* (currently *Marinomonas communis*) and *A. vaga* (currently *M. vaga*), to *Oceanospirillum* based on the immunological similarity analysis in marine bacteria, though van Landschoot and De Ley (1983) have proposed the establishment of a new genus for the two based on DNA–rRNA hybridization data. As a result, the genus definition of *Oceanospirillum* needed to be changed drastically, with the unfortunate loss of most of the readily determinable phenotypic features from the genus definition (Krieg 1984) and the extension of the upper mol% G+C limit for the genus from 51 to 57. By this extension, a considerable overlap of mol% G+C range was introduced between the genera *Aquaspirillum* (49–65 mol% G+C) and *Oceanospirillum* (42–51 mol% G+C), confounding one of the most reliable genotypic features discriminating between the genera (Pot et al. 1989, 1992). Although many of these species have since been reclassified into other genera or recognized as subjective synonyms of other species, some of taxonomic discrepancies remained within the genus solely based on their phenotypic characteristics (McElroy and Krieg 1972; Terasaki 1972, 1973; Hylemon et al. 1973; Carney et al. 1975; Krieg and Hylemon 1976). To resolve the problem, chemotaxonomic and genetic analyses were performed for the genus, including fatty acid composition analysis (Sakane and Yokota 1994), isoprenoid quinone profiling (Sakane and Yokota 1994), polyamine composition (Hamana et al. 1994), DNA–DNA hybridization (Pot et al. 1989), DNA–rRNA hybridization (Pot et al. 1989), and 16S rRNA sequence analyses (Woese et al. 1982, 1985). The resulting DNA–rRNA hybridization data (Pot et al. 1989) have indicated that *O. communis* (currently *Marinomonas communis*) and *O. vaga* (currently *M. vaga*) cannot be regarded as members of the genus *Oceanospirillum* and that the *Oceanospirillum sensu stricto* or the "core group" consisted of only five species, including the type species *O. linum, O. maris, O. beijerinckii, O. multiglobuliferum*, and, more distantly, *O. japonicum. O. pelagicum* and *O. hiroshimense* were unified as subspecies into *O. beijerinckii* and *O. maris*, respectively, and *O. kriegii, O. jannaschii, O. minutulum*, and *O. pusillum* were eliminated from the genus *Oceanospirillum* altogether (taxonomic positions for these species have yet to be determined). The analysis of the 16S rRNA oligonucleotide data catalogs of the *Oceanospirillum* species *O. linum, O. maris, O. japonicum* (currently *Pseudospirillum japonicum*), and *O. minutulum* (currently *Marinospirillum minutulum*) indicated that *O. japonicum* and *O. minutulum* were phylogenetically distinct from *O. linum* and *O. maris*, respectively (Woese et al. 1982, 1985). Subsequent chemotaxonomic studies (Sakane and Yokota 1994; Hamana et al. 1994) confirmed that *O. pusillum* (currently *Terasakiella pusilla*) had different profiles of 3-hydroxy fatty acids and quinine composition (comprised primarily of Q-10) from that of the *Oceanospirillum sensu stricto* and other pending *Oceanospirilla* species, whereas phylogenetic analysis indicated it should be assigned in alphaproteobacteria (Kawasaki et al. 1997). More recently, Satomi et al. (1998, 2002) conducted polyphasic taxonomic analyses targeting 16S rRNA and gyrB genes to examine the phylogeny of numerous *Oceanospirillum* strains. It was demonstrated that *O. minutulum* clustered on a separate branch together with new isolates from kusaya gravy (Satomi et al. 1998); thusly, a new genus, *Marinospirillum*, was proposed (Satomi et al. 1998) containing the two species, *M. minutulum* and *M. megaterium* (details given in the *Marinospirillum* description below). As a result, it was suggested that the *Oceanospirillum* core group consisted of four species: *Oceanospirillum linum*, *O. maris, O. beijerinckii*, and *O. multiglobuliferum*. Four other *Oceanospirillum* species were demonstrated to occupy taxonomic positions separate from the *Oceanospirillum* core group: *O. jannaschii, O. japonicum*, and *O. kriegii* in the gammaproteobacteria and *O. pusillum* in the alphaproteobacteria. Subsequently, *O. jannaschii* was transferred to the genus *Marinobacterium* as it was demonstrated to cluster with *Marinobacterium georgiense* (González et al. 1997), *Pseudomonas iners* (Iizuka and Komagata 1964), and *P. stanieri* (Baumann et al. 1983). Given that the other three species did not cluster with known genera, Satomi et al. (2002) proposed the creation of three new genera: *Pseudospirillum* gen. nov. for *Oceanospirillum japonicum* (*Pseudospirillum japonicum* comb. nov.), *Oceanobacter* gen. nov. for *Oceanospirillum kriegii* (*Oceanobacter kriegii* comb. nov.), and *Terasakiella* gen. nov. for *Oceanospirillum pusillum* (*Terasakiella pusilla* comb. nov). These reclassifications were further supported by phenotypic characteristics. For instance, *O. japonicum* differs from other *Oceanospirillum* species as it grows best at 35–37 °C, it does not form coccoid bodies, and its flagella appear to be crescent shaped with less than one helical turn (*Oceanospirillum* sp. typically have one or more helical turns). In addition, *O. jannaschii* and *O. kriegii* both have a higher mol% G+C (54.8–54.9) as well as other phenotypic characteristics that discriminate these species from the genus *Oceanospirillum* (Table 24.1). Moreover, as was discussed previously, *O. pusillum* possessed completely different chemotaxonomic features from the genus core group. Utilizing DNA–DNA hybridization, gyrB sequence analysis, and phenotypic characteristics, Satomi et al. (2002) further demonstrated a lack of significant diversity among the subspecies of *O. maris* and *O. beijerinckii*, suggesting that they should not be regarded as such. However, as González and Whitman (2006) pointed out, in the absence of a formal ruling by the International Committee on Systematic Bacteriology (ICSB), the subspecies designations are still valid, and these taxa continue to possess standing in the nomenclature. It should be noted that surveys of large collections of strains have never been performed, and thus, 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 current taxonomic status of species that have been assigned at one time or another to *Oceanospirillum* is summarized in Table 24.2.

Table 24.2

List of strains previously included in the genus *Oceanospirillum* and their current classification

Species names	Current classification	Type strain	Accession number[a]	Source, place and year of isolation	References
O. beijerinckii subsp. *beijerinckii* (Williams and Rittenberg 1957); Hylemon et al. (1973), subsp. nov.[b]	*Oceanospirillum beijerinckii*	ATCC 12754	AB006760	Coastal water, United States, 1957	Williams and Rittenberg (1957); Hylemon et al. (1973); Pot et al. (1989)
O. beijerinckii subsp. *pelagicum* (Terasaki 1973) Pot et al. (1989), comb. nov.	*Oceanospirillum beijerinckii*	IFO 13612	AB006761	Marine shellfish, 1961	Terasaki (1973, 1979); Pot et al. (1989)
Basonym: *O. pelagicum* (Terasaki 1973) Terasaki (1979)					
Other synonym: *Spirillum pelagicum* Terasaki (1973)					
O. commune (Baumann et al. 1972) van Landschoot and De Ley (1983), Bowditch et al. (1984) comb. nov.	*Marinomonas communis*	ATCC 27118	Ribosomal Database Project	Coastal surface water, Oahu, Hawaii, 1972	Baumann et al. (1972); van Landschoot and De Ley (1983); Bowditch et al. (1984); Pot et al. (1989)
Basonym: *Alteromonas communis* (Baumann et al. 1972)					
O. kriegii (Bowditch et al. 1984) Satomi et al. (2002) comb. nov.	*Oceanobacter kriegii*	ATCC 27133	AB006767	Coastal surface water, Oahu, Hawaii, 1972	Baumann et al. (1972); Bowditch et al. (1984); Satomi et al. (2002)
O. jannaschii (Bowditch et al. 1984) Satomi et al. (2002) comb. nov.	*Marinobacterium jannaschii*	ATCC 27135	AB006765	Coastal surface water, Oahu, Hawaii, 1972	Baumann et al. (1972); Bowditch et al. (1984); Satomi et al. (2002)
O. japonicum (Watanabe 1959; Hylemon et al. 1973); Satomi et al. (2002) comb. nov.	*Pseudospirillum japonicum*			Marine shellfish, 1959	Watanabe (1959); Hylemon et al. (1973); Satomi et al. (2002)
Synonym: *Spirillum japonicum* Watanabe –1959					
O. linum (Williams and Rittenberg 1957) Hylemon et al. (1973)	*Oceanospirillum linum*	ATCC11336	M22365	Coastal water, United States, 1957	Williams and Rittenberg (1957); Hylemon et al. (1973)
Synonym: *Spirillum linum* Williams and Rittenberg (1957)					
O. maris subsp. *maris* Hylemon et al. (1973) subsp. nov.[c]	*Oceanospirillum maris*	ATCC 27509	AB006771	Sea water 1973	Hylemon et al. (1973)
O. maris subsp. *williamsae* Linn and Krieg –1978	*Oceanospirillum maris*	ATCC 29547	AB006763	From mixed culture, 1978	Linn and Krieg (1978)
O. maris subsp. *hiroshimense* (Terasaki 1973) Pot et al. (1989) comb. nov.	*Oceanospirillum maris*	IFO 13616	AB006762	Marine shellfish, 1963	Terasaki (1973, 1979); Pot et al. (1989)
Basonym: *O. hiroshimense* (Terasaki 1973) Terasaki (1979)					
Other synonym: *Spirillum hiroshimense* Terasaki (1973)					
O. minutulum (Watanabe 1959) Hylemon et al. (1973) comb. nov.	*Marinospirillum minutulum*	ATCC 19193	AB006769	Marine shellfish, 1959	Watanabe (1959); Hylemon et al. (1973); Satomi et al. (1998)
Synonym: *Spirillum minutulum* Watanabe –1959					
O. multiglobuliferum (Terasaki 1973) Terasaki –1979	*Oceanospirillum multiglobuliferum*	IFO 13614	AB006764	Marine shellfish, 1960	Terasaki (1973, 1979)

Table 24.2 (continued)

Species names	Current classification	Type strain	Accession number[a]	Source, place and year of isolation	References
Synonym: *Spirillum multiglobuliferum* Terasaki (1973)					
O. pusillum (Terasaki 1973; Terasaki 1979) Satomi et al. (2002) comb. nov.	*Terasakella pusilla*	IFO13613	AB006768	Marine shellfish, 1961	Terasaki (1973, 1979); Satomi et al. (2002)
Synonym: *Spirillum pusillum* Terasaki (1973)					
O. vagum (Baumann et al. 1972) van Landschoot and De Ley (1983), Bowditch et al. (1984) comb. nov.	*Marinomonas vaga*	ATCC 27119	X67025	Coastal surface water, Oahu, Hawaii, 1972	Baumann et al. (1972); van Landschoot and De Ley (1983); Bowditch et al. (1984); Pot et al. (1989)
Basonym: *Alteromonas vaga* Baumann et al. (1972)					

[a]16S rRNA accession number of the type strain

[b]The subspecies name *O. beijerinckii* subsp. *beijerinckii* (Williams and Rittenberg 1957); Hylemon et al. (1973) was automatically created by the valid publication of *O. beijerinckii* subsp. *pelagicum* (Terasaki 1973); Pot et al. (1989; Rule 40d [formerly Rule 46])

[c]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)

The genus *Marinomonas* was established in 1984 by van Landschoot and De Ley and represents the second oldest and largest genus in the *Oceanospirillaceae*, currently encompassing 20 species. As mentioned previously the original two species, *M. communis* (type species; type strain ATCC 27118) and *M. vaga*, were initially described as belonging to *Alteromonas* (Baumann et al. 1972), a genus created to accommodate Gram-negative heterotrophs with a single polar flagellum that, with a lower DNA mol% G+C of 38–50, were distinct from the *Pseudomonas* (DNA mol% G+C of 55 to 64; Baumann et al. 1972). In 1983, van Landschoot and De Ley demonstrated that *A. vaga* and *A. communis* belonged to a different DNA–rRNA hybridization group than other species of *Alteromonas* and proposed the genus *Marinomonas*. A year later, Bowditch et al. (1984) independently proposed that these species instead be classified within *Oceanospirillum* on the basis of immunological studies of the iron-containing superoxide dismutases and glutamine synthetases. They found that these enzymes cross-reacted most strongly with antisera prepared to the enzymes from *Oceanospirillum beijerinckii* and *O. jannaschii*. They also further characterized species in the genus *Oceanospirillum*, in addition to 33 and 17 strains of *M. communis* and *M. vaga*, respectively. However, Pot et al. (1989) again demonstrated with DNA–rRNA hybridization that these strains were not closely related to the type species of *Oceanospirillum*, further supporting their currently classification within *Marinomonas*, a conclusion later validated by 16S rRNA sequencing (Satomi et al. 1998, 2002). A third species, the melanin-producing *Marinomonas mediterranea*, was later described by Solano and Sanchez-Amat (1999), and the genus has subsequently expanded to include 20 total species. Almost all species were isolated from the marine environment, including habitats such as seawater, sediment, and seaglass. It should be noted that the genus *Marinomonas* cannot be clearly differentiated from other groups of marine, Gram-negative bacteria solely on the basis of phenotypic characteristics as numerous major phenotypic characteristics are shared with the genera *Alteromonas* and *Pseudoalteromonas*, as well as others (Akagawa-Matsushita et al. 1992; Baumann et al. 1972; González and Whitman 2006), although the use of 16S rRNA sequence analysis or DNA–DNA hybridization readily resolves these taxa.

Oceanobacter was created by Satomi et al. in 2002 and to date consists solely of one species, *Oceanobacter kriegii*. Originally isolated as strain H-1 (Baumann et al. 1972), this rod-shaped species was classified as *Oceanospirillum kriegii* on the basis of immunological analysis of their superoxide dismutases and glutamine synthetases (Bowditch et al. 1984). Subsequent DNA–rRNA hybridizations between this species and representatives of closely related organisms, along with 16S rRNA gene sequence analysis of the 16S rRNA gene, supported reclassification and the establishment of this genus (Pot et al. 1989; Satomi et al. 2002).

The genus *Pseudospirillum* was created by Satomi et al. (2002) for *Oceanospirillum japonicum* (former name), which was isolated from putrid infusions of shellfish and initially described as *Spirillum japonicum* (Watanabe 1959). Terasaki (1963, 1972) additionally described three similar strains, namely, IF4 (IFO 15447), IF8, and UF3, that based on phenotype and cellular morphology may belong to this or a closely related species. In 1973 Hylemon et al. reclassified *S. japonicum*, along with the other marine strains of *Spirillum*, into a new genus, *Oceanospirillum*. However, numerous independent lines of investigation indicated that this species had low phenotypic similarity to the *Oceanospirillum sensu stricto*; for example, older cultures did not form cocci or microcysts (Terasaki 1972; Carney et al. 1975), while the fatty acid composition of *P. japonicum* (current name) significantly differed from that of other members of the *Oceanospirillum* (Sakane and Yokota 1994). Based on

DNA–rRNA hybridizations and 16S rRNA gene sequence analysis, it was ultimately reclassified as the type species of the new genus, *Pseudospirillum* (Pot et al. 1989; Satomi et al. 1998, 2002).

The genus *Marinospirillum* currently encompasses five species and was created to accommodate *Oceanospirillum minutulum*, originally classified as *Spirillum minutulum* (Watanabe 1959), and a new isolate *Marinospirillum megaterium* (Satomi et al. 1998). Currently, this genus is classified as a member of *Oceanospirillaceae*, although the phylogenetic position of this genus has been shown to be significantly closer to the family *Halomonadaceae*, suggesting that it should be reclassified at the family level. However, in accordance with the current taxonomic classification, this genus will be briefly described here. Originally, Watanabe (1959) isolated two strains, ATCC 19192 and ATCC 19193, and described them as *Spirillum halophilum* and *S. minutulum*, respectively. When the genus *Spirillum* was divided into freshwater and marine species, these strains were moved to *Oceanospirillum* along with the other marine species. Due to their shared similarities, Hylemon et al. (1973) proposed that the two strains did not warrant classification as two different species and they were thus reclassified together as *Oceanospirillum minutulum*. Subsequently, DNA–rRNA hybridization studies (Pot et al. 1989) and fatty acid composition analysis (Sakane and Yokota 1994) indicated that *O. minutulum* was significantly different from *O. linum* and members of the *Oceanospirillum sensu stricto*, respectively. Finally, based on 16S rRNA gene sequence analysis, *Marinospirillum minutulum* (current name) was reclassified as the type species of a new genus, *Marinospirillum*, along with the new isolate *M. megaterium* (Satomi et al. 1998), as described previously. Since then, three alkaliphilic species, *M. alkaliphilum* (Zhang et al. 2002), *M. insulare* (Satomi et al. 2004), and *M. celere* (Namsaraev et al. 2009), have additionally been added to the genus.

The genus *Marinobacterium* was created by Gonzalez et al. (1997) and, in accordance with current classification, is encompassed by the family *Alteromonadaceae*. However, its affiliation is unclear as based on 16S rRNA gene sequence analysis, the genus clusters with *Nitrincola* and occupies a position along the *Oceanospirillaceae* branch. Reclassification at the family level should therefore be considered.

The years of establishment and features for genera belonging to the *Oceanospirillaceae* are shown in ❯ *Table 24.3*.

Phylogenetic Structure of the Family and Its Genera

According to the phylogenetic branching of the type strains of *Oceanospirillales* in the 16S rRNA gene tree of the Living Tree Project (Yarza et al. 2008, 2010), *Oceanospirillaceae* is moderately related to the families *Saccharospirillaceae*, *Litoricolaceae*, *Halomonadaceae*, and several genera for which taxonomic affiliation at the family level remains unclear (❯ *Fig. 24.1*). Although the genus *Marinospirillum* and *Reinekea* are affiliated with the family *Oceanospirillaceae*, phylogenetically both genera are more closely related to *Halomonadaceae* and *Saccharospirillaceae*, respectively. Conversely, based on 16S rRNA sequence analysis, the genera *Amphritea, Bermanella, Marinomonas, Neptuniibacter, Neptunomonas, Oceaniserpentilla, Oceanobacter, Oceanospirillum, Oleibacter, Oleispira, Spongiispira, and Thalassolituus* comprise one defined *Oceanospirillaceae* familial cluster. The robustness of this clustering is supported by both neighbor-joining and maximum likelihood algorithms, although the clustering patterns vary for the remaining genera (*Balneatrix, Nitrincola*, and *Pseudospirillum*) depending on the sequence analysis method utilized. In the case of NJ method, these three genera cluster with and form loose groups to the *Oceanospirillaceae* core genera. However, when the ML method is utilized (data not shown), *Balneatrix* and *Pseudospirillum* are positioned outside of the *Oceanospirillaceae* cluster and apart from other described families, while *Nitrincola* groups within the *Neptunomonas* cluster along with Marinobacterium (which itself belongs to the family *Alteromonadaceae*). Thus, more study and consideration is required to adequately describe the family affiliation of *Oceanospirillaceae*. Of the genera with consistently defined phylogenetic positions, the *Marinomonas* form a robust cluster with sufficient phylogenetic distance among its 20 species. Neighboring branches to *Marinomonas* consist of *Bermanella, Oceaniserpentilla, Oceanobacter, Oleibacter, Oleispira, Spongiispira*, and *Thalassolituus*, which form robust clusters based on NJ analysis. These bacterial genera each consists of a single species and is physiologically associated with petroleum degradation. The genera *Amphritea, Neptuniibacter, Neptunomonas*, and *Oceanospirillum* also form a distinct cluster, although the clustering partners of *Oceanospirillum* are varied when using ML analysis. On the basis of phylogenetic analysis, it is clear that the genus *Marinobacterium* should be transferred from *Alteromonadaceae* to *Oceanospirillaceae*, while the genera *Marinospirillum* and *Reinekea* should be reclassified out of *Oceanospirillaceae*.

Molecular Analyses

Phylogeny

The widespread adoption of molecular tools such as the polymerase chain reaction (PCR) and DNA sequencing and subsequent phylogenetic studies based on 16S rDNA gene sequences have resulted in a major taxonomic reclassification of bacteria, including the establishment of the family *Oceanospirillaceae*. Phylogenetic analysis of 16S rRNA sequences provides relatively accurate information for *Oceanospirillaceae* taxonomy, although it occasionally lacks the required specificity for the differentiation of close relatives (Fox et al. 1992; Stackebrandt and Goebel 1994; Yamamoto and Harayama 1995, 1996, 1998), and thus, higher-resolution molecular identification markers have been required to distinguish between some species. Studies targeting the rapidly evolving gyrB gene, encoding the B subunit of DNA gyrase, have been utilized to elucidate the phylogeny of several taxonomically ambiguous

Table 24.3
List of genera established in the *Oceanospirillaceae*

Genus names	Species		Strain no. as received	Source, place and year of isolation	References
Amphritea	*atlantica*	Type species	LMG 24143	Warm sediment samples at 3,000 m depth, Mid-Atlantic Ridge, 1993	Gärtner et al. (2008)
Balneatrix	*alpica*	Type species	CIP 103589	Cerebrospinal fluid of patient recovered from pneumonia and meningitis, 1987	Dauga et al. (1993)
Bermanella	*marisrubri*	Type species	CECT 7074 CCUG 52064	Surface water from the Red Sea, 2000	Pinhassi et al. (2009)
Marinomonas	*communis*	Type species	ATCC 27118	Coastal surface water, Oahu, Hawaii, 1972	Baumann et al. (1972); van Landschoot and De Ley (1983); Bowditch et al. (1984); Pot et al. (1989)
Marinobacterium	*georgiense*	Type species	ATCC 700074	Salt marsh, Georgia, U.S.A, 1997	González et al. (1997)
Marinospirillum	*minitulum*	Type species	ATCC 19193	Marine shellfish, Japan, 1959	Watanabe (1959); Hylemon et al. (1973); Satomi et al. (1998)
Neptuniibacter	*caesariensis*	Type species	CECT 7075 CCUG 52065	Surface water from the eastern Mediterranean Sea, 2007	Arahal et al. (2007)
Neptunomonas	*naphthovorans*	Type species	ATCC 700637	Eagle Harbor, a creosote-contaminated EPA superfund site, Puget Sound, Washington, 1993	Hedlund et al. (1999)
Nitrincola	*lacisaponensis*	Type species	ATCC BAA-920 DSM 16316	Decayed wood collected at Soap Lake, 2005	Dimitriu et al. (2005)
Oceaniserpentilla	*haliotis*	Type species	DSM 19503 LMG 24225	Haemolymph serum of the blacklip abalone Haliotis rubra, 2006	Schlösser et al. (2008)
Oceanobacter	*kriegii*	type species	ATCC 27133	Coastal surface water, Oahu, Hawaii, 1972	Baumann et al. (1972); Bowditch et al. (1984); Satomi et al. (2002)
Basonym: *Oceanospirillum kriegii* Bowditch et al. (1984) Satomi et al. (2002) comb. nov.					
Oceanospirillum	*linum*	Type species	ATCC11336	Coastal water, United States, 1957	Williams and Rittenberg (1957); Hylemon et al. (1973); Satomi et al. (2002)
Oleibacter	*marinus*	Type species	NBRC 105760 BTCC B-675	Indonesian seawater, Pari Island, 2006	Teramoto et al. (2011)
Oleispira	*antarctica*	Type species	DSM 14852 LMG 21398	Superficial sea-water samples, Rod Bay (Ross Sea, Antarctica), 1999	Yakimov et al. (2003)
Pseudospirillum	*japonicum*	Type species	ATCC 19191	Visceral organs of marine shell-fishes, 1959	Watanabe (1959); Hylemon et al. (1973); Satomi et al. (2002)
Basonym: *Oceanospirillum japonicum* Watanabe (1959); Hylemon et al. (1973) Satomi et al. (2002) comb. nov.					
Other synonym: *Spirillum japonicum* Watanabe (1959)					
Reinekea	*marinisedimentorum*	Type species	DSM 15388	Marine coastal sediments, Reineke Island, 2002	Kaesler et al. (2008)

Table 24.3 (continued)

Genus names	Species		Strain no. as received	Source, place and year of isolation	References
Spongiispira	*norvegica*	Type species	DSM 17749 NCIMB 14401	Marine sponge, *Isops phlegraei*, 2008	Kaesler et al. (2008)
Thalassolituus	*oleivorans*	Type species	DSM 14913 LMG 21420	Sea water/sediment samples, harbor of Milazzo, 2004	Yakimov et al. (2004)

bacterial species (Venkateswaran et al. 1999; Satomi et al. 2003). Satomi et al. (2002) previously demonstrated that gyrB sequence analysis demonstrated good correlation with DNA–DNA hybridization values and provided higher resolution for describing the taxonomy of *Oceanospirillum* species. Recently, a more precise method for phylogenetic evaluation has been reported. Multilocus sequence typing (MLST) has been shown proficient at analyzing phylogenetic relationships between individual species or strains and in cases has been utilized to verify the validity of subspecies; however, to date this method has not been applied to *Oceanospirillum* strains.

rRNA–DNA Hybridization

Before widespread adoption of molecular tools, such as PCR and DNA sequencing, and subsequent phylogenetic studies based on 16S rDNA gene sequences, rRNA–DNA hybridization was the powerful tool to prove the phylogenetic relationships between bacterial species based on molecular base study. In fact, rRNA–DNA hybridization resulted in a major reclassification of bacterial taxonomy including the marine bacteria (van Landschoot and De Ley 1983; Pot et al. 1989; De Vos et al. 1989). As was mentioned above, this technique was applied to study for intra-/intergeneric relationships of the genus *Oceanospirillum* and demonstrated that *Oceanospirillum sensu stricto* or the "core group" belongs to rRNA superfamily II. *O. japonicum* also positioned in this group but not making clusters with *Oceanospirillum sensu stricto*. *O. pusillum* was also misnamed as it belongs in rRNA superfamily IV (Pot et al. 1989). This study also proves *Oceanospirillum vagum* and *O. communis* should be relegated to their real generic positions, *Marinomonas vaga* and *Marinomonas communis*, respectively (Pot et al. 1989). However, the methods for rRNA sequence analysis are shifted to the recent PCR-based DNA–RNA sequence methods.

DNA–DNA Hybridization

In accordance with the consensus molecular definition of a species (Wayne et al. 1987), phylogenetic definitions generally include strains with "approximately 70 % or greater DNA–DNA relatedness and with 5 °C or less ΔTm." Furthermore, "phenotypic characteristics should agree with this definition and are allowed to override the phylogenetic concept of species only in a few exceptional cases." Thus, DNA–DNA reassociation values derived from DNA–DNA hybridization methods with labeled DNA (Ezaki et al. 1989) or thermal renaturation methods (De ley et al. 1970) should be respected before 16S rRNA sequence analysis in bacterial taxonomy. As mentioned previously, because 16S rRNA gene analysis occasionally lacks the specificity to differentiate close relatives, DNA–DNA relatedness values among closely related microbes are essential information for the determination of species' taxonomic affiliation. With respect to *Oceanospirillaceae*, DNA–DNA hybridization is generally performed to describe new species in the case of the genus consisting of multiple species.

Historically, such techniques have been used for the taxonomic study of *Oceanospirillum* species as it often has been difficult to distinguish each species based solely on morphological and biochemical characteristics. In fact, Krieg (1984) suggested that "Species distinctions are less firmly based within a particular morphological group of strains, especially if they have a similar DNA base composition. Therefore, it is possible that some presently recognized species may not deserve separate species status (for example, *O. maris* vs. *O. beijerinckii*). It is likely that DNA/ DNA hybridization experiments could resolve many such questions." Pot et al. (1989) demonstrated that only five species, including the type species, constituted the genus *Oceanospirillum* (*O. linum, O. maris, O. beijerinckii, O. multiglobuliferum*, and, more distantly, *O. japonicum*) and that the former species *O. hiroshimense* and *O. pelagicum* should be assigned as the subspecies, *O. maris* subsp. hiroshimense and *O. beijerinckii* subsp. pelagicum, respectively. Although *O. japonicum* was transferred to the new genus *Pseudospirillum* based on 16S rRNA gene sequence analysis (Satomi et al. 2002), the proposal by Pot et al. (1989) that the *Oceanospirillum sensu stricto* or the "core group" constituted *O. linum, O. maris, O. beijerinckii*, and *O. multiglobuliferum* was strongly supported. Additionally, as both reports indicated, the DNA–DNA reassociated values were sufficiently high within subspecies of *O. maris* (three subspecies) and *O. beijerinckii* (two subspecies) to label them as the same species, respectively. This, however, has yet to be demonstrated with a large-scale phenotypic investigation.

In the cases of the other genera, DNA–DNA hybridization experiments were performed to propose the new species or to study taxonomy within the genus. The phylogenetic study based on 16S rRNA gene sequence analysis within the genus *Marinomonas* species showed occasionally close and unclear to determine as different species due to lacking of resolution as the molecular identification markers. Thus, DNA–DNA hybridization is necessary to evaluate new taxa. In most of the cases, the DNA–DNA hybridization values showed less than 40 % among *Marinomonas* species, even though they have close relationships (>99 %) with each other on the 16S rRNA sequence analysis. As well as *Marinomonas*, DNA–DNA hybridization is available to distinguish species in *Marinospirillum*, *Marinobacterium*, and *Reinekea*, which constituted by multiple species, though their appropriate taxonomic positions are pending.

GC Content

DNA base composition values for *Oceanospirillaceae* vary from 41 to 63 mol%, and the ranges for individual genus are shown in *Table 24.1*. DNA base compositions are analyzed using high-performance liquid chromatography methods (Tamaoka and Komagata 1984), buoyant density method described as Bd (Schildcraut et al. 1962), and thermal denaturation methods described as Tm (Marmur and Doty 1962). These methods can determine GC base composition, although the resulting values may be slightly different. Recent whole-genome sequence analysis is more precise for the determination of GC content. DNA base composition values are good chemotaxonomic marker to distinguish genus, especially for marine bacteria, which have similar phenotypic characteristics. Most *Oceanospirillaceae* genus show 44–54 GC mol%; hence, *Spongiispira norvegica*, which is the only species of this genus, have high GC content, 62.6 %. Since the genus *Spongiispira* have been established based on single strain, further study is necessary based on multiple strains.

Whole-Cell Protein Profile

Whole-cell protein profile is also a common strain or species typing method and has been applied to distinguish some *Oceanospirillum* strains, including formerly *Oceanospirillum* (Pot et al. 1989). Protein electropherogram analysis for *Oceanospirillum* core group demonstrated that the genus distinguished the following three groups of strains: (i) *O. maris* subsp. maris ATCC 27509^T, ATCC 27648, and ATCC 27649; (ii) *O. maris* subsp. williamsae ATCC 29547^T and *O. hiroshimense* IFO13616^T; and (iii) *O. linum* ATCC 11336^T and ATCC 12753. The rest of the species, *O. japonicum* ATCC 19191^T, *O. pelagicum* IFO 13612^T, *O. multiglobuliferum* IFO13614^T, and *O. beijerinckii* NCMB 52^T, occupied a separate position on the dendrogram based on whole-cell protein profiling. It was supported by rRNA–DNA hybridization and DNA–DNA hybridization experiment performed at same time. Thus, the method is available for separating species–subspecies level; it is likely rather than strain typing. As a recent protein-based bacterial identification method, the whole-cell protein fingerprinting technique using matrix-assisted laser desorption–ionization time-of-flight mass spectrometry has been developed (Bohme et al. 2010). However, it has not performed for *Oceanospirillaceae* strains yet.

Other Molecular Technique-Based Analysis

Nucleotide- or protein-based techniques have been developed to detect specific bacteria from various samples, including food, clinical, and environmental samples. PCR and quantitative PCR are useful and sensitive detection methods for specific genes from bacteria including *Oceanospirillaceae*. Since most interests for *Oceanospirillaceae* bacteria is related to diversity and their habitation including main role in ocean environment, most researches were performed to monitor the microbial dynamics in ocean environment using PCR-clone library methods, in which microbial DNA was directly extracted from samples and bacterial species existing in the sample were identified using 16S rRNA gene sequence. The method can give useful information about microbial diversity and assumed bacterial flora in samples without cultural bias, though DNA extraction, PCR condition, and copy number of target gene for identification are effected to quantitative results. For example, Giovannoni and Rappé (2000) studied microbial diversity in the Sargasso Sea by using PCR-clone library sequence method and indicated that rRNA genes closely related to *Oceanospirillum* species have not been encountered in libraries prepared from marine environmental rDNA, suggesting that the abundance of *Oceanospirillum* in the marine environment is low. The other biochemical identification methods, immunobased methods, are additional common methods for the detection of specific bacteria. DeLong et al. (1984) tried to apply bacterial identification–classification based on immunological reaction using the antiserum prepared by the iron-containing superoxide dismutases and glutamine synthetases. The method is suitable to detect the specific bacteria; however, it is difficult to presume genetic relationships among irrelativeness bacteria.

Genome Analysis

Whole-genome sequences for some *Oceanospirillaceae* species have been determined and reported in public databases: *Bermanella marisrubri, Marinomonas mediterranea, M. pontica*, and *Neptuniibacter caesariensis*. The genome size of the species sequenced ranges from 3.53 to 4.68 Mb, and 7–8 copies of the ribosomal RNA operon are present in each genome and no plasmids in sequenced strains. The GC content of each species agrees with data obtained using high-performance liquid chromatography or the Tm method. Whole-genome sequences are expected to provide useful information with which to elucidate metabolic pathways for particular characteristics of *Oceanospirillaceae* strains, e.g., petroleum degradation, melanin

production, and bactericidal compounds secretion. Thus, Marinomonas *mediterranea* MMB-1^T is of interest, and its whole genome is sequenced because it can synthesize melanin pigments, which are mediated by the activity of a tyrosinase and also express other oxidases with biotechnological interest, such as a multicopper oxidase with laccase activity and a novel L-lysine-epsilon-oxidase. As the detailed data of whole-genome sequence in *M. mediterranea* showed that 4,684,316 bp long genome harbors 4,228 protein-coding genes and 98 RNA genes (Lucas-Elío et al. 2012).

Plasmid and Page

There is less information about phages and plasmids harbored in *Oceanospirillaceae* species. Full genome sequence of bacteriophage P12026 that can lytically infect bacterial strain IMCC12026, a member of the genus *Marinomonas*, was reported (Kang et al. 2012). Bacteriophage P12026 was isolated by using enrichment culture followed by plaque assay from a seawater sample collected from the same station. On the basis of transmission electron microscopy observation, the phage P12026 was regarded as a member of the *Siphoviridae*, since it has isometric heads and long noncontractile tails and double-stranded DNA. The genome sequence of phage P12026 was 31,766 bp in length with G+C content of 46.0 mol% and had 54 open reading frames (ORFs) predicted. The genome of phage P12026 seemed to have modular structure, as shown in many other phages (Krupovick et al. 2011).

Phenotypic Analyses

General Description

O.ce.an.o.spi.ril.la'les. M.L.neut.n.*Oceanospirillum* type genus of the family;-aceae ending to denote family; M.L.fem pl. n. *Oceanospirillaceae* the *Oceanospirillum* family. The main features of members of *Oceanospirillaceae* are listed in *Table 24.1*. Most genera are halotolerant or halophilic and motile. Almost all genera are aerobic or microaerophilic chemoorganotroph. As mentioned above, most genera of this family are consist of single species; moreover, in some cases, the genus established only single strain. Thus, genus characteristics are lesser known.

Type genus: *Oceanospirillum* Hylemon, Wells, Krieg and Jannasch 1973, 361AL.

Differentiation of the *Oceanospirillaceae* from Other Families

Major phenotypic characteristics among *Oceanospirillaceae* are similar to other members of *Oceanospirillales* and major marine bacteria, mainly *Alteromonadales*, indicating that based only upon phenotypic characteristics, it is difficult to classify in relation to other Gram-negative marine bacteria. Owing to the diversity of phenotypic characteristics within the family, only a few properties are shared by all of members of this family, thus indicating that some of strains may be misidentified. Moreover, the problem is also 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 (Pot et al. 1989; Satomi et al. 2002), 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, 2002), and chemotaxonomy of polyamines, fatty acids, and isoprenoid quinones (Hamana et al. 1994; Sakane and Yokota 1994). Based upon these analyses, the classification of *Oceanospirillaceae* is accomplished.

Morphology

Oceanospirillaceae species shows that various cell shapes depend on genus or species and most species are curved or rod-shaped form. In the case of *Oceanospirillum*, which is type genus of this family, all species consist of rigid helical cells, although variants having less curvature may arise after prolonged transfer. *Marinospirillum* also have rigid helical shape. The cells have a constant and characteristic type of clockwise (right-handed) helix in both genera. On the other hand, *Terasakiella pusilla* belonging to the alphaproteobacteria, which has been classified as member of *Oceanospirillum*, has a counterclockwise (left-handed) helix (Terasaki 1972), indicating that helix characteristics also give taxonomic information. *Bermanella, Oceaniserpentilla, Oleispira, Pseudospirillum, Spongiispira*, and *Thalassolituus* have thin helical shape. The type strain of *Pseudospirillum japonicum* consisted initially of long, helical cells with several turns (Watanabe 1959), but now consists of slightly curved or S-shaped cells. An unusual elaboration of the plasma membrane, the "polar membrane," occurs in all of the *Oceanospirillum* species so far examined (Krieg 1984). It is attached to the inside of the plasma membrane by bar-like links and is located, most commonly, in the region surrounding the polar flagella (Krieg 1984). Such a membrane has been found mainly in genera of helical bacteria, such as *Spirillum, Campylobacter, Aquaspirillum, Ectothiorhodospira*, and *Rhodospirillum*. However, further study related to membrane structure and flagella formation has not done since it has been reported. As a recent study based on transmission electron microscope observation for cells with ultrathin section, *Thalassolituus* and some thin spiral-shaped species show one to four polar flagella and present a Gram-negative cell wall architecture with an outer membrane (Yakimov et al. 2003, 2004).

As the unique characteristics, *Oceanospirillum* and other rigid helical bacteria can form coccoid bodies (sometimes termed "microcysts") in old culture. These bodies have thin walls and resemble spheroplasts; however, they are resistant to lysis in distilled water (Krieg 1984). Whether coccoid bodies are resistant to desiccation is not known. There is still less

information about these characteristics due to lacking of study in this field past 20 years. Three main modes of formation of coccoid bodies were described by Williams and Rittenberg (1957) as follows: (a) two cells may entwine and apparently fuse. The cells become shorter and thicker and a protuberance develops at the point of fusion. This gradually enlarges and absorbs the organisms to form the coccoid body. More than one coccoid body may develop from a pair of entwined spirilla; (b) a *Spirillum* may become shorter and thicker and a protuberance arises from the center of the cell or from each end of the cell. The protuberances enlarge and eventually merge into a single coccoid body as the helical cell is absorbed; (c) a *Spirillum* may undergo a gradual shortening and rounding to form a coccoid body. The majority of coccoid bodies present in old cultures appear to be viable and can "germinate" when placed into a fresh medium (Williams and Rittenberg 1957). Germination is by unipolar or bipolar growth of a helical cell from the coccoid body, with the latter being absorbed into the developing helical cell. To elucidate details of this character, further studies including molecular sequence and gene expression analysis are needed.

Genus Description

Oceanospirillum

O.ce.an.o.spi.ril'lum. Gr. n. okeanos the ocean; N.L. dim. neut. n. *spirillum* a small spiral from Gr. n. spira spiral; N.L. *Oceanospirillum* a small spiral organism from the ocean (seawater). Rigid, helical cells with clockwise helix. Cells 0.4–1.2 μ-m in diameter; length of the helix, 2.0–4.0 μ-m. A polar membrane underlies the cytoplasmic membrane at the cell poles in all species examined so far by electron microscopy. Intracellular poly-b-hydroxybutyrate (PHB) is formed. All species form thin-walled coccoid bodies, which predominate in old cultures. Gram negative. Motile by bipolar tufts of flagella. Chemoorganotrophic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Nitrate respiration does not occur; nitrate is not reduced to nitrite or beyond the nitrite stage. Optimum temperature, 25–32 °C. Oxidase positive. Indole negative. Casein, starch, hippurate, and esculin are not hydrolyzed. Seawater required for growth. Carbohydrates are neither fermented nor oxidized. Amino acids or the salts of organic acids serve as carbon sources. Growth factors are not usually required. Isolated from coastal seawater, from decaying seaweed, and from putrid infusions of marine mussels. The G+C content of the genomic DNA ranges from 45 to 50 mol% (as determined by the thermal denaturation method). Type species is Oceanospirillum linum (Williams and Rittenberg 1957) Hylemon et al. 1973 (Approved Lists 1980). The genus is currently encompassing four species. Phenotypic features of this genus are shown in ❯ *Table 24.4*.

Marinomonas

Ma.ri.no.mo.nas.L. adj. *marinus* pertaining to the sea; Gr. *N. monas* a unit, monad; M.L. *Marinomonas*. Gram negative, straight, or curbed rods. Motile by means of polar flagella at one or both poles. Aerobic, having a strictly respiratory type of metabolism. Oxidase positive or oxidase negative. Na+ is required for growth. Do not accumulate poly-β-hydroxybutyrate. Do not require organic growth factors. Do not produce extracellular amylase. Utilize acetate but not butyrate or valerate. Utilize glutamate, sorbitol, and malate. Commonly isolated from seawater. The mol% G+C of the DNA is 45–50. The genus is currently encompassing 20 species. Type species: Marinomonas communis (Baumann et al. 1972) van Landschoot and De Ley 1983. Phenotypic features of *Marinomonas* species are shown in ❯ *Table 24.5*.

Amphritea

Am.phri'tea. N.L. fem. n. *Amphritea* from Gr. fem. n. Amphrite, a nymph of the ocean in Greek mythology, referring to the habitat of the bacteria. Cells are Gram-negative rods, motile by monopolar flagella. Coccoid bodies may be formed in old cultures. Catalase and oxidase positive and accumulate PHB. Growth range is from 4 °C to 40 °C, from 0.3 % to 9 % salinity, and from pH 4.6 to 9.5. Various sugars and carboxylic acids are oxidized. Predominant fatty acids are C18: 1ω7c, C16: 1ω7c, and C16: 0. 16S rRNA gene sequence analysis positions the genus in close proximity to the genera *Oceanospirillum* and *Neptunomonas* within the family *Oceanospirillaceae*. The type species is *Amphritea atlantica*. The genus constitute with three species, *A. atlantica, A. balenae*, and *A. japonica*. *A. atlantica* was isolated from a *Bathymodiolus* sp. specimen collected from the Logatchev hydrothermal vent field at the Mid-Atlantic Ridge at a depth of about 3,000 m (Gärtner et al. 2008). It is not unclear whether barophilic or not. *A. balenae* and *A. japonica* were isolated from the sediment adjacent to sperm whale carcasses off Kagoshima, Japan, at a depth of about 230 m (Miyazaki et al. 2008b). The genus *Amphritea* was regarded as the relatives to a symbiotic bacteria of Osedax (Goffredi et al. 2007), because its phylogenetic position was nearby that of a symbiotic bacterial clone when the first species of the genus isolated. The genus *Osedax* (*Polychaeta*: *Siboglinidae*) has recently been discovered in *Osedax* specimens host symbiotic bacteria in their ovisac and root systems. In fact, the latter two species, *A. balenae* and *A. japonica*, were isolated from the sediment adjacent to sperm whale carcasses, which are closely related to *Osedax* sp. habitats on the deep-sea floor. It is indicated that *Amphritea* is associated with *Bathymodiolus* sp. and *Osedax* sp. as symbiotic bacteria; however, there is less information to prove it. Phenotypic features are shown in ❯ *Table 24.6*.

Table 24.4

Phenotypic characteristics of members of the *Oceanospirillum*

Feature	*O. linum*	*O. maris*	*O. beijerinckii*	*O. multiglobuliferum*
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
Flagellar arrangement	BT	BT	BT	BT
Intracellular PHB formed	+	+	+	+
Coccoid bodies predominant after 3–4 weeks	+	+	+	+
Coccoid 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_2S	d	+	d	W
Phosphatase	+	d	+	+
Production of indole	–	–	–	–
Hydrolysis of casein, starch, esculin and hippurate	–	–	–	–
Urease	–	–	–	nd
Growth in presence of 1 % oxgall	+	+	+	nd
Growth in presence of 1 % glycine	+	d	–	nd
Deoxyribonulease	–	d	+	nd
Ribonuclease	d	d	+	nd
Auxotrophic growth requirement	+[a]	d	–	–
Pigmentation				
Ultilization 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	+	+

Table 24.4 (continued)

Feature	*O. linum*	*O. maris*	*O. beijerinckii*	*O. multiglobuliferum*
Tartrate	–	–	–	–
Pyruvate	–	d	+	+
Methanol	–	–	–	–
Ethanol	–	d	d	–
n-Propanol	–	d	d	–
Glycerol	–	–	–	–
L-Alanine	–	–	–	nd
L-Glutamate	–	+	–	nd
L-Proline	–	d	–	nd
Nonpolar fatty acids (%)				
C16 : 1[b]	47	48	48	44
C16 : 0	16	29	27	28
C18 : 1	30	13	16	20
3-Hydroxy fatty acid (%)				
C10 : 0[b]	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)[c]	0.7	0.9	0.6	0.4
Putrescine content (μmol/g of wet wt)[c]	0.02	0.03	0.03	0.08
Mol% G + C in DNA	48–50[d]	45–47[d]	47–49[d]	46[e]
Reference	Williams and Rittenberg (1957)	Hylemon et al. (1973)	Williams and Rittenberg (1957)	Terasaki et al. (1973)
	Pot et al. (1989)	Pot et al. (1989)	Hylemon et al. (1973)	Pot et al. (1989)
			Pot et al. (1989)	Terasaki et al. (1979)

Data from Pot et al. (1992) and also Terasaki (1972)

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

[a]*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

[b]From Sakane and Yokota (1994). Mean values are reported

[c]From Hamana et al. (1994). Mean values are reported

[d]By the thermal denaturation and HPLC methods

[e]By the HPLC method only

Balneatrix

Bal'ne.a.trix,L. fem. n., ba ther. Gram-negative, straight or curved rods, motile by a single polar flagellum. Strictly aerobic. Growth occurs at a wide range of temperatures (20–46 °C) on nutrient agar. Growth occurs in media containing 0–0.5 % (w/v) NaCl and not in media with more than 1 % NaCI. No growth factors required. Nitrate reduced to nitrite. Carbohydrates utilized with acid production. G+C content of the DNA (one strain determined) is around 54 mol%. The type species is *Balneatrix alpica*. The genus *Balneatrix* is consist of a single species, *B. alpaca* (Dauga et al. 1993), which is the only species are reported as clinical related bacteria in *Oceanospirillaceae*. *B. alpaca* have occurred the outbreak of pneumonia and meningitis in a spa therapy center, southern France, in 1987 (Hubert et al. 1991). However, no more outbreaks caused by *B. alpaca* have been reported. Details are described in pathogenicity and clinical relevance section. Phenotypic features of this genus are shown in *Table 24.1*.

Bermanella

Ber.ma.nel'la. N.L. fem. dim. n. *Bermanella* named after the aquatic microbial ecologist Dr. Tom Berman. Gram-negative, strictly aerobic, chemoorganotrophic bacteria. Oxidase and catalase positive. Cells are motile, thin spirilla. Gas vesicles are not observed. Poly-b-hydroxybutyrate granules are produced. Slightly halophilic; no growth without seawater or the addition of combined marine salts to the medium. Mesophilic. Do not

ferment carbohydrates, as determined on anaerobic Hugh and Leifson O/F medium (Difco) with half-strength artificial seawater (Baumann et al. 1972). Stenotrophic; the only carbon sources that serve as single carbon sources are organic acids. The type species is *Bermanella marisrubri*. The genus *Bermanella* is consist of a single species, *B. marisrubri* (Pinhassi et al. 2009), isolated from a surface seawater sample collected from the Gulf of Eilat in the northern Red Sea. The DNA G+C content of the type strain is 44.0 mol%. As the genus was established based on the single strain, the true habitat and biological features of genus are less known. Phenotypic features of this genus are shown in ❯ *Table 24.1*.

Marinospirillum

Ma. ri. no. spi. ril'lum. L. adj . mar inus of the sea; Gr. n. spira a spiral; M.L. dim. neut. n. *spirillum* a small spiral; *Marinospirillum* a small spiral from the sea. Gram-negative, rigidly helical, nonspore-producing, coccoid body-forming, halophilic, aerobic or microaerobic, chemoheterotrophic, and PHB-accumulating bacteria. Motile by means of flagella. Oxidase positive. Catalase reaction is negative or positive. Carbohydrates are not catabolized. Genomic DNA G+C content of 42–45 mol% (as determined by HPLC). The isoprenoid quinone type is Q-8. The type species is *Marinospirillum minutulum*. The genus *Marinospirillum* currently encompasses five species and was created to accommodate *Oceanospirillum minutulum*, originally classified as *Spirillum minutulum* (Watanabe 1959). Currently, this genus is classified as a member of *Oceanospirillaceae*, although the phylogenetic position of this genus has been shown to be significantly closer to the family *Halomonadaceae*, suggesting that it should be reclassified at the family level. Phenotypic features of this genus are shown in ❯ *Table 24.7*.

Neptuniibacter

Nep.tu.ni.i.bac'ter. L. adj. Neptunius Neptunian, pertaining to Neptune, Roman god of the sea; N.L. masc. n. bacter a rod; N.L. masc. n. *Neptuniibacter* a Neptunian rod, referring to the habitat of the bacteria.

Gram-negative, strictly aerobic, chemoorganotrophic bacteria. Oxidase and catalase positive. Cells are rod shaped and motile. Gas vesicles not observed. Produce poly-b-hydroxybutyrate granules. Slightly halophilic; no growth can be obtained without seawater or the addition of combined marine salts to the medium. Mesophilic. Do not ferment carbohydrates. Preferred carbon sources are organic acids and amino acids. Possess ubiquinone Q-8 as a respiratory quinone. DNA G+C content is around 47 mol%. The type species is *Neptuniibacter caesariensis* isolated from a surface seawater sample collected from the eastern Mediterranean Sea, offshore from the historic location of Caesarea. The genus constitutes with a single species and is established based on the single strain; the true habitat and biological features of genus are less known. Phenotypic features of this genus are shown in ❯ *Table 24.1*.

Neptunomonas

Nep-.tu.no.mo'nas. Rom. myth. Neptune, the Roman god of the sea; Gr. n. monas, unit; M.L. n. *Neptunomonas*, Neptune's bacterium. Gram-negative rod-shaped bacteria. Cells of the type species are approximately 0.7–0.9 by 2.0–3.0 mm and are motile by means of a single polar flagellum. Facultatively aerobic. Oxidase and catalase positive. May utilize amino acids, carbohydrates, organic acids, sugar alcohols, and some polycyclic aromatic hydrocarbons (PAHs) as sole carbon and energy sources. Cells can degrade PHAs and require sodium ions for growth. The DNA G+C content is 46 mol%. The type species of the genus is *Neptunomonas naphthovorans*, isolated from Eagle Harbor, a creosote-contaminated Environmental Protection Agency superfund site in Puget Sound, Washington. The genus is currently encompassing three species, which is related to petroleum and PAHs degrading. Phenotypic features of this genus are shown in ❯ *Table 24.8*.

Nitrincola

Nit.rin'co.la. L. neut. n. nitrum soda; L. masc. n.incola inhabitant, dweller; N.L. masc. n. *Nitrincola* an inhabitant of a soda environment. Alkaliphilic, halotolerant and heterotrophic. Cells are non-pigmented, asporogenous, motile, Gram-negative rods. NO_2 and O_2 can be used as electron acceptors. Fermentable carbon sources do not support growth. Chemoorganotrophic. Requires sodium for growth. Oxidase and catalase positive. Optimal pH for growth is 9.0. The genus is isolated from a saline, alkaline lake. The DNA G+C content is 47.4 mol% (Tm). The type species is *Nitrincola lacisaponensis* isolated from decomposing wood taken from the shore of the Soap Lake, a saline, alkaline lake in Grant County, WA, USA. Soap Lake is a closed, evaporative system with neither a significant surface inlet nor outlet. This results in the concentration of salts, mainly sodium carbonate and sodium sulfate (Anderson 1958). As the genus is established based on the single strain, the true habitat and biological features of genus are less known. Phenotypic features of this genus are shown in ❯ *Table 24.1*.

Oceaniserpentilla

O.ce.a.ni.ser.pen.til'la. L. masc. n. oceanus the ocean; L. fem. n. serpens -tis a snake; N.L. fem. n. serpentilla a small snake; N.L. fem. n. *Oceaniserpentilla* small snake of the ocean, indicating shape and origin. Gram-negative, motile, obligately aerobic,

Table 24.5

Phenotypic characteristics of the species of *Marinomonas*

Feature	*M. alcarazii*	*M. aquimarina*	*M. aquiplantarum*	*M. arctica*	*M. arenicola*	*M. balearica*	*M. brasilensis*	*M. communis*	*M. dokdonensis*
Morphology	Helical	Straight rod	Helical	Curved rods	Rod	Straight rod	Straight rod	Curved rods	Straight rod
Number and arrangement of flagella	1, polar	1, polar	1, polar or bipolar tufts	nd	nd	1, polar	nd	Single bipolar	1, polar
Cell width (μm)	nd	0.5–1	nd	0.3–0.4	nd	nd	1	0.7–0.9	0.4–0.6
Cell length (μm)	nd	1–4	nd	1.2–2.6	2	nd	1.5–3	1.8–2.5	1.0–2.5
Temperature range (°C)	5–37	13–40	5–37	0–37	4–37	15–25	20–35		4–37
Growth at 4 °C	nd	–	nd	+	+	–	–	–	+
Growth at 35 °C	+	+	+	+	+	–	+	+	+
Growth at 40 °C	–	+	nd	–	–	–	–	+	–
Growth at 45 °C	–	nd	nd	–	–	–	–	–	–
Nitrate reduced to nitrite	nd	–	nd	–	–	nd	–	–	–
Nitrite reduced to N_2	nd	nd	nd	–	nd	nd	–	–	nd
Oxidase	–	+	–	+	–	–	+	+	+
Catalase									
Gelatin liquefaction	–	–	–	–	–	+	–	–	–
Alginase	nd	nd	nd	nd	nd	nd	nd	–	nd
Lipase	–	–	d	–	–	+	–	–	+
Starch hydrolysis	–	–	–	–	–	–	nd	–	–
Chitinase	nd	nd	nd	–	–	nd	nd	–	nd
Oxygen requirement	Strictly aerobic	Strictly aerobic	Strictly aerobic	Aerobic	Aerobic	Strictly aerobic	Aerobic		Aerobic
Glucose oxidized	+	+	+	+	–	+	+	–	+
Glucose fermented									
Without nitrate	–	–	–	–	–	–	–	–	–
With nitrate				–			–		
Utilization of									
D-Glucose	+	+	+	+	–	+	+	+	+
D-Fructose	+	+	+	+	–	+	+	+	+
D-Mannose	nd	W	nd	+	–	nd	+	d	+
Citrate	nd	+	nd	+	–	nd	+	+	+
Aconitate	nd	W	nd	nd	nd	nd	nd	+	nd
Sucrose	+	–	+	+	–	+	+	–	d
Cellobiose	nd	–	nd	+	–	nd	W	–	+
Malate	+	+	+	+	–	+	–	+	+
Sorbitol	+	–	+	+	–	–	–	+	nd
α-Ketoglutarate	+	+	+	nd	nd	+	nd	+	nd
D-Mannitol	nd	–	nd	+	–	nd	–	+	nd
Erythritol	nd	nd	nd	+	–	nd	–	–	nd
Glycerol	nd	–	nd	+	–	nd	W	+	nd
γ-Aminobutyrate	nd	+	nd	nd	nd	nd	nd	+	nd
Gluconate	nd	+	nd	+	–	nd	–	+	nd

M. foliarum	*M. mediterranea*	*M. ostreistagni*	*M. polaris*	*M. pollencensis*	*M. pontica*	*M. posidonica*	*M. primoryensis*	*M. rhizomae*	*M. ushuaiensis*	*M. vaga*
Rods	Straight rods	Straight rods	Rod	Curved rods	Helical*	Helical	Straight rods*	Rods	Straight rods	Straight rods
1, polar	1, polar	1, polar	nd	1, polar	Subpolar/ polar	1, polar	1, polar	1, polar	nd	Single bipolar
nd	nd	0.4–0.6	0.6–0.8	nd	0.4–0.6	nd	0.4–0.6	nd	0.5–0.7	0.8–1.1
nd	nd	0.6–3.6	2–3.2	nd	0.8–1.6	nd	1.6–1.8	nd	2–3	2–2.8
5–25		16–40	4–37	5–37	4–33	5–25	4–30	5–25	4–25	
–	–	–	+	nd	+	–	+	–	+	–
–	–	+	+	+	–	–	–	–	–	+
–	–	+	–	nd	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
nd	+	–	–	nd	–	nd	–	nd	–	–
nd	–	nd	–	nd	nd	nd	nd	nd	–	–
–	–	+	W	–	+	–	+	–	–	–
–	+	–	–	–	–	–	–	–	–	–
nd	nd	nd		nd	nd	nd	nd	–	nd	–
–	+	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	nd	–	+	–
nd	nd	nd	nd	nd	–	nd	nd	nd	nd	–
Strictly aerobic		Strictly aerobic	Aerobic	Strictly aerobic	Aerobic	Strictly aerobic	Strictly aerobic	Strictly aerobic	Aerobic	
+	+	+		+	+	+	+	+	+	+
	–	–	–	–	–		–		–	–
	–									–
+	+	+	+	+	+	+	W	+	+	+
+	–	nd	nd	+	+	+	nd	+	nd	+
nd	+	nd	+	nd	–	nd	nd	nd	+	+
nd	+	+	–	nd	+	nd	+	nd	–	+
nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	+
+	nd	+	+	–	+	+	nd	+	nd	–
nd	nd	nd	+	nd	+	nd	–	nd	–	d
+	+	–	–	+	+	+	+	+	–	+
+	+	–	+	+	–	+	nd	+	–	+
+	–	nd	nd	+	+	+	nd	+	nd	+
nd	nd	+	nd	nd	+	nd	+	nd	–	+
nd	nd	nd	+	nd	nd	nd	–	nd	–	+
nd	+	–	W	nd	–	nd	+	nd	–	+
nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	+
nd	nd	nd	–	nd	nd	nd	nd	nd	nd	+

Table 24.5 (continued)

Feature	*M. alcarazii*	*M. aquimarina*	*M. aquiplantarum*	*M. arctica*	*M. arenicola*	*M. balearica*	*M. brasilensis*	*M. communis*	*M. dokdonensis*
Succinate	nd	+	nd	nd	nd	nd	+	+	+
Fumarate	nd	+	nd	nd	nd	nd	nd	+	nd
DL-Lactate	nd	+	nd	nd	nd	nd	nd	+	nd
meso-Inositol	nd	–	nd	nd	–	nd	*nd*	+	nd
L-Glutamate	+	d	+	nd	nd	+	nd	+	–
L-Aspartate	nd	+	nd	nd	nd	nd	nd	d	nd
DL-Alanine	nd	+	nd	+	–	nd	–	+	nd
L-Ornithine	nd	+	nd	nd	nd	nd	–	+	nd
L-Arginine	nd	nd	nd	nd	–	nd	nd	+	nd
L-Serine	nd	+	nd	nd	–	nd	–	+	nd
Pyruvate	nd	+	nd	nd	nd	nd	–	+	+
Acetate	+	+	+	nd	–	–	nd	+	d
n-Hexadecane	nd	nd	nd	nd	nd	nd	nd	–	nd
N-Acetylglucosamine	nd	–	nd	nd	–	nd	–	–	nd
m-Hydroxybenzoate	–	nd	–	nd	–	–	nd	+	nd
p-Hydroxybenzoate	nd	+	nd	nd	nd	nd	nd	+	nd
Sarcosine	nd	+	nd	nd	nd	nd	nd	+	nd
Putrescine	nd	–	nd	nd	nd	nd	–	+	nd
Betaine	nd	nd	nd	nd	nd	nd	nd	+	nd
Pigmentation	–	–	–	–	–	–		–	–
PHB accumulation	nd	–	nd	nd	nd	–	nd	–	nd
Mol% G + C in DNA	46.2	nd	47.8	45.2	50	43.4 ± 1.4	46.5	46–48	45.3–45.7
Ubiquinone type	nd	nd	nd	nd	nd	nd	nd	Q-8	Q-8
Reference	Lucas-Elío et al. (2011)	Macian et al. (2005)	Lucas-Elío et al. (2011)	Zhang et al. (2008)	Romanenko et al. (2009)	Espinosa et al. (2010)	Chimetto et al. (2011)	Baumann et al. (1972)	Yoon et al. (2005)
								Akagawa-Matsushita et al. (1992)	

Symbols: see footnote in Table 24.1

M. foliarum	*M. mediterranea*	*M. ostreistagni*	*M. polaris*	*M. pollencensis*	*M. pontica*	*M. posidonica*	*M. primoryensis*	*M. rhizomae*	*M. ushuaiensis*	*M. vaga*
nd	+	–	–	nd	+	nd	nd	nd	–	+
nd	nd	nd	nd	nd	+	nd	nd	nd	nd	+
nd	nd	+	nd	nd	+	nd	nd	nd	nd	+
nd	nd	nd	+	nd	–	nd	nd	nd	nd	+
+	nd	nd	nd	+	+	+	nd	+	nd	+
nd	nd	nd	nd	nd		nd	nd	nd	nd	d
nd	nd	+	–	nd	+	nd	nd	nd	nd	+
nd	nd	nd	nd	nd	+	nd	nd	nd	nd	+
nd	nd	–	+	nd	+	nd	–	nd	–	d
nd	nd	+	–	nd	+	nd	nd	nd	nd	d
nd	nd	+	nd	nd	+	nd	nd	nd	nd	+
+	nd	+	+	+	+	+	+	+	+	+
nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	–
nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	d
–	–	nd	–	+	+	–	+	–	+	+
nd	nd	nd	nd	nd	nd	nd	–	nd	–	+
nd	nd	nd	nd	nd	+	nd	nd	nd	nd	+
nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	d
nd	nd	nd	nd	nd	+	nd	nd	nd	nd	–
–	Melanin-like	–	–	–	–	–	Yellowish	–	–	–
nd	–	–	–	nd	–	nd	nd	nd	–	–
46.2	46	49.8 ± 0.5	41.2	47.8 ± 0.4	46.5	44.7	43.6	45.6	43.6	46–48
nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	Q-8
Lucas-Elío et al. (2011)	Solano and Sanchez-Amat (1999)	Lau et al. (2006)	Gupta et al. (2006)	Espinosa et al. (2010)	Ivanova et al. (2005)	Lucas-Elío et al. (2011)	Romanenko et al. (2003)	Lucas-Elío et al. (2011)	Prabagaran et al. (2005)	Baumann et al. (1972)
					Espinosa et al. (2010)		Prabagaran et al. (2005)			Akagawa-Matsushita et al. (1992)

Table 24.6
Phenotypic characteristic of the three species of *Amphritea*

	Amphritea atlantica	*Amphritea balenae*	*Amphritea japonica*
Morphology	Rods	Rods	Rods
Number and arrangement of flagella	1, Polar	1, Polar or bipolar tufts	1, Polar
Optimal temperature (°C)	31–34	20–22	22–24
Growth at 4 °C	+	+	+
Growth at 45 °C	–	–	–
Optimal NaCl (%) for growth	3	3	3
Maximal NaCl (%) for growth	6	4	5
Nitrate reducted to nitrite	nd	+	+
Nitrite reduced to N_2	nd	–	–
Oxidase	+	+	+
Catalase	+	W	+
Gelatin liquefaction	–	+	–
Starch hydrolysis	nd	–	–
Urease	+	–	–
Utilization of			
D-Glucose	+	–	–
D-Fructose	+	–	–
D-Mannose	nd	–	–
Sucrose	nd	–	–
Cellobiose	nd	–	–
D-Mannitol	nd	nd	–
Glycerol	nd	+	+
Gluconate	nd	nd	nd
Succinate	nd	nd	nd
L-Glutamate	nd	nd	nd
L-Aspartate	nd	nd	nd
DL-Alanine	+	+	+
L-Arginine	nd	nd	nd
L-Serine	nd	nd	nd
Pyruvate	nd	nd	nd
Acetate	nd	nd	nd
Hexadecane	nd	nd	nd
p-Hydroxybenzoate	nd	nd	nd
PHB accumulation	+	+	+
Mol% G + C in DNA	52.2	46.6–47	46.7
Major ubiquinone	nd	Q-8	Q-8
Reference	Gärtner et al. (2008)	Miyazaki et al. (2008)	Miyazaki et al. (2008)

vibroid to spiral, nonspore-forming cells. Oxidase activity is present, whereas catalase activity is absent. The type species is *Oceaniserpentilla haliotis* isolated from after filtration of abalone hemolymph serum through a filter with a pore size of 0.2 mm. The hemolymph serum was isolated from the blacklip abalone Haliotis rubra harvested near Hobart, Tasmania. Its habitat can be associated with black abalone; however, details are not known. As the genus is established based on the single strain, the true habitat and biological features of genus are less known. Phenotypic features of this genus are shown in *Table 24.1*.

Oceanobacter

Gram-negative, straight rod, nonspore-forming, halophilic, aerobic, chemoheterotrophic, PHB-accumulating and

Table 24.7

Phenotypic characteristics of the species of *Marinospirillum*

Feature	*M. alkaliphilum*	*M. celere*	*M. insulare*	*M. minutulum*	*M. megaterium*
Morphology	Helical	Helical	Helical	Helical	Helical
Cell width (μm)	0.2–0.3	0.2–0.4	0.1–0.2	0.2–0.4	0.8–1.2
Cell length (μm)	2.7–4.0	1.4–1.5	2.5–7.5	2–6	5–15
Number and arrangement of flagella	Bipolar	Bipolar	Bipolar tufts	1, Polar or bipolar tufts	Bipolar tufts
Forms coccoid bodies	+	+	+	+	+
Temperature range (°C)	8–49	13–55	4–37	4–30	4–25
Optimal temperature (°C)	38	35–45	25–30	15–22	20–25
Oxygen requirement	Aerophilic	Aerophilic	Aerophilic	Aerophilic	Microaerophilic
Range of NaCl for growth (%)	0.2–5.0	0.5–2.0	0.5–10.0	0.2–10.0	0.5–9.0
Optimal NaCl (%)	2	2	2–3	2–3	3
Range of pH for growth	7.0–11.0	8.0–10.5	6.5–10.0	7.0–10.5	7.5–9.0
Optimal pH	9.5	9.5	8.0	6.8–7.4	8.0
Nitrate reduced to nitrite	+	−	+	+	−
Nitrite reduced to N_2	nd	−	nd	−	−
Oxidase	+	+	+	+	+
Catalase	+	+	+	+	− or W
Gelatin liquefaction	−	−	−	−	−
Growth with 1 % glycine	nd	+	+	+	−
Growth with 1 % ox gall	nd	+	+	+	−
Production of H_2S	−	−	nd	−	nd
Production of indole	−	−	nd	−	nd
Starch hydrolysis	−	−	−	−	−
Urease	+	−	−	−	−
Phosphatase	nd	−	−	−	−
DNase	nd	−	−	−	−
RNase	nd	−	−	−	−
Utilization of					
D-Glucose	−	−	nd	−	nd
D-Fructose	−	nd	−	−	nd
Sucrose	−	−	−	−	nd
Xylose	−	nd	−	−	nd
Formate	nd	nd	nd	−	nd
Acetate	nd	nd	nd	+	nd
Propionate	nd	nd	nd	+	nd
Butyrate	nd	nd	nd	+	nd
Succinate	nd	nd	nd	+	nd
Fumarate	nd	nd	−	+	nd
Malonate	nd	nd	nd	−	nd
Lactate	nd	+	nd	+	nd
Citrate	nd	−	nd	−	nd
Malate	nd	nd	−	+	nd
Tartrate	nd	nd	nd	−	nd
Pyruvate	nd	+	−	+	nd
Methanol	nd	nd	nd	−	nd
Ethanol	nd	nd	nd	−	nd

Table 24.7 (continued)

Feature	*M. alkaliphilum*	*M. celere*	*M. insulare*	*M. minutulum*	*M. megaterium*
n-Propanol	nd	nd	nd	–	nd
Glycerol	nd	–	–	–	nd
Ubiquinone type	Q-8	Q-8	Q-8	Q-8	Q-8
Mol% G + C in DNA	46.8	52.3	42–43	42.5,[a] 42–44.1[b]	44–45[a]
Reference	Zhang et al. (2002)	Namsaraev et al. (2009)	Satomi et al. (2004)	Watanabe (1959)	Satomi et al. (1998)
				Satomi et al. (1998)	

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

Symbols: see footnote in *Table 24.1*

[a]By the HPLC method

[b]By the thermal denaturation method

Table 24.8

Phenotypic characteristics of the three species of *Neptunomonas*

	Neptunomonas japonica	*Neptunomonas antarctica*	*Neptunomonas naphthovorans*
Morphology	Rods	Rods	Rods
Number and arrangement of flagella	1, polar or bipolar tufts	1, polar	1, polar
Cell width (μm)	0.8–1.0	0.6–1.0	0.7–0.9
Cell length (μm)	1.6–1.8	1.2–1.8	2–3
Temperature range (°C)	5–25	4–25	4–24
Optimal temperature (°C)	20	15	nd
Growth at 4 °C	–	+	+
Growth at 45 °C	–	–	–
Range of pH for growth	7.0–8.5	6.0–8.0	6.5–8.5
Optimal pH	7.5	6.5–7.0	7.5
Optimal NaCl (%) for growth	2	2–3	nd
Maximal NaCl (%) for growth	6	5	7
Nitrate reducted to nitrite	+	+	–
Nitrite reduced to N_2	–	nd	nd
Oxidase	+	+	+
Catalase	+	+	+
Gelatin liquefaction	+	–	–
Starch hydrolysis	–	–	–
Urease	–	–	nd
Production of H_2S	–	–	–
Production of Indole	–	–	+
Utilization of			
D-Glucose	nd	–	d
D-Fructose	nd	+	+
D-Mannose	nd	nd	–
Sucrose	nd	–	–
Cellobiose	nd	nd	nd
D-Mannitol	nd	+	+
Glycerol	nd	nd	+

Table 24.8 (continued)

	Neptunomonas japonica	*Neptunomonas antarctica*	*Neptunomonas naphthovorans*
Gluconate	nd	nd	–
Succinate	nd	nd	+
L-Glutamate	nd	nd	+
L-Aspartate	nd	nd	nd
DL-Alanine	nd	nd	nd
L-Arginine	nd	nd	nd
L-Serine	nd	nd	nd
Pyruvate	nd	nd	+
Acetate	nd	nd	+
Hexadecane	nd	nd	nd
p-Hydroxybenzoate	nd	nd	nd
Naphthalene	nd	nd	+
Water-soluble brown pigment	+	nd	+
PHB accumulation	nd	nd	+
Mol% G + C in DNA	43.6–43.8	45.6	46
Major ubiquinone	Q-8	Q-8	Q-8
Reference	Miyazaki et al. (2008)	Zhang et al. (2010)	Hedlund et al. (1999)

Symbols: see footnote in Table 24.1

oxidase-positive bacteria. 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 µmol/g of wet cells) and putrescine (0.03 µmol/g of wet cells) were the only detectable polyamines (Hamana et al. 1994). The type and only species of this genus is *Oceanobacter kriegii* isolated from seawater. Phenotypic features of this genus are shown in Table 24.1.

Oleibacter

O.le'i.bac'ter. L. n. oleum oil; N.L. masc. n. bacter rod; N.L. masc. n. *Oleibacter* an oil (−degrading) rod. According to 16S rRNA gene sequence analysis, belongs to the gammaproteobacteria. Cells are Gram-negative, motile, aerobic rods. *n*-Alkane-degrading activity is observed. Predominant cellular fatty acids are C16: 0, C16: 1ω7, and C18: 1ω9, and hydroxy fatty acids are C12: 0 3-OH and C10: 0 3-OH. The major isoprenoid quinone is Q-9 and minor amounts of Q-8 are present. Polar lipids are phosphatidylglycerol, a ninhydrin-positive phospholipid(s) and glycolipids. The DNA G+C content of known strains of the type species is 53.0–53.1 mol%. The type and only species of this genus is *Oleibacter* marinus isolated from Indonesian seawater after enrichment with crude oil and a continuous supply of supplemented seawater. The strains exhibited high *n*-alkane-degrading activity, which indicated that the strains were important degraders of petroleum aliphatic hydrocarbons in tropical marine environments. Phenotypic features of this genus are shown in Table 24.1.

Oleispira

O.le.i'spi.ra. L. n. oleum oil; Gr. fem. n. spira a spire; N.L. fem. n. *Oleispira* an oil-degrading, spiral-shaped organism. Gram-negative, vibroid to spiral cells, 2.0–5.0 mm long by 0.4–0.8 mm wide, motile by a single polarly inserted, long (>5 mm) flagellum. Chemoheterotroph with strong preference for aliphatic carbon substrates. Aerobic. Able to grow under anaerobic conditions by nitrate reduction. Oxidase and catalase are present. Ammonia and nitrate may serve as nitrogen sources. The narrow range of growth-supporting substrates is restricted to aliphatic hydrocarbons, Tweens, and volatile fatty acids. Uptake of common carbohydrates or amino acids as sole carbon sources for growth is detected in a very narrow spectrum. Stenohaline requires Na+ ions, exhibiting optimal growth in the presence of 3–5 % (w/v) NaCl. Psychrophilic growth, with optimal growth temperature of 2–4 °C. The major cellular fatty acids are monounsaturated fatty acids. The DNA G+C content of known strains of the type species is 41–42 mol%. The type and only species of the genus is *Oleispira antarctica* isolated from hydrocarbon-degrading enrichment cultures obtained from Antarctic coastal marine environments (Rod Bay, Ross Sea). Phenotypic features of this genus are shown in Table 24.1.

Table 24.9

Phenotypic characteristics of the three species of *Reinekea*

	Reinekea marinisedimentorum	*Reinekea aestuarii*	*Reinekea blandensis*
Morphology	Straight rods	Curved rods	Curved rods
Number and arrangement of flagella	1, polar		
Cell width (μm)	0.4–0.5	0.4–0.5	0.3–0.7
Cell length (μm)	1.5–1.7	1.2–2.5	1.2–2.8
Temperature range (°C)	4–37	15–42	15–42
Optimal temperature (°C)	nd	25	
Growth at 4 °C	+	–	–
Growth at 45 °C	–		–
Range of pH for growth	nd	5.0–12.0	
Optimal pH	nd	6.0–8.0	
Optimal NaCl (%) for growth	0.5–5	2.0–5.0	
Maximal NaCl (%) for growth	5	10.0	12
Nitrate reducted to nitrite	+	–	–
Nitrite reduced to N_2	nd		–
Oxidase	+	+	+
Catalase	+	+	+
Gelatin liquefaction	–	W	–
Starch hydrolysis	–	–	+
Urease	–	–	nd
Production of H_2S	nd	–	nd
Production of Indole	–	–	–
Utilization of			
D-Glucose	+	+	+
D-Fructose	nd	+	+
D-Mannose	–	+	+
Sucrose	W	+	+
Cellobiose	nd	+	+
D-Mannitol	+	+	+
Glycerol	nd	+	+
Gluconate	–	nd	+
Succinate	nd	nd	+
L-Glutamate	nd	nd	W
L-Aspartate	nd	nd	–
DL-Alanine	nd	+	–
L-Arginine	nd	nd	+
L-Serine	nd	nd	–
Pyruvate	nd	nd	+
Acetate	nd	nd	+
Hexadecane	nd	nd	nd
p-Hydroxybenzoate	nd	nd	nd
PHB accumulation	nd	nd	+
Mol% G + C in DNA	51.1	51.3	52.4
Major ubiquinone	Q-8	Q-8	Q-8
Reference	Romanenko et al. (2004)	Choi and Cho (2010)	Pinhassi et al. (2007)

Symbols: see footnote in *Table 24.1*

Pseudospirillum

Pseudospirillum (Pseu.do.spi.ril«lum. Gr. adj. pseudes false ; N.L. n. *Spirillum* genus of spiral-shaped bacteria ; N.L. n. *Pseudospirillum* false *Spirillum*). Gram-negative, curved, straight or S-shaped, nonspore-forming, halophilic, aerobic, chemoheterotrophic, and PHB-accumulating bacteria. Motile by means of flagella. Oxidase positive. Catalase-negative or catalase-positive reaction. Carbohydrates are not catabolized. Coccoid body is not forming. Genomic DNA G+C content of 45 mol% (as determined by HPLC). Major fatty acids are C16:0, C16:1ω7c, and C18:1ω7c. The isoprenoid quinone type is Q-8. The type and only species of this genus is *Pseudospirillum japonicum* (basonym *Oceanospirillum japonicum*). Phenotypic features of this genus are shown in Table 24.1.

Reinekea

Rei.ne.ke'a. N.L. fem. n. *Reinekea* derived from Reineke, geographical name of Reineke Island, Peter the Great Bay, Sea of Japan, Russia, the place where the bacterium was first isolated. Gram negative, heterotrophic, oxidase and catalase positive, rod shaped, and motile. Sodium ions are essential for growth. Growth occurs in 0.5–5 % NaCl and between 4 °C and 37 °C. No growth is observed in >5 % NaCl or at 40 °C. Facultatively anaerobic; acid is produced from some carbohydrates under anaerobic and aerobic conditions. Predominant isoprenoid quinone is Q-8. Polar lipids include phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, and an unknown phospholipid. Major fatty acids are C16:0, C16:1ω7c, and C18:1ω7c. The type species is *Reinekea marinisedimentorum*. The genus is currently encompassing three species. Currently, this genus is classified as a member of *Oceanospirillaceae*, although the phylogenetic position of this genus has been shown to be significantly closer to the family *Saccharospirillaceae*, suggesting that it should be reclassified at the family level. Phenotypic features of this genus are shown in Table 24.9.

Spongiispira

Spon.gi.i.spi9ra. L. fem. n. spongia sponge; L. fem. n. spira curvature, spiral; N.L. fem. n. *Spongiispira* spiral-shaped bacterium from a sponge. Gram-negative, spiral-shaped cells, motile by a single polar flagellum. Aerobic, chemoheterotrophic, positive for lipase activity, relatively restricted nutritional profile, not able to reduce nitrate, oxidase positive, catalase negative, and mesophilic. Salt is essential for growth. The major cellular fatty acids are C16:1ω7 and C16:0. The type and only species of this genus is *Spongiispira* norvegica isolated from the cold-water sponge *Isops phlegraei* (class *Demospongiae*) collected from a depth of approximately 320 m in the Sula Ridge off the coast of mid-Norway. As the genus is established based on the single strain, the true habitat and biological features of genus are less known. Phenotypic features of this genus are shown in Table 24.1.

Thalassolituus

Tha.las.so.li.tu9us. Gr. fem. n. thalassa the sea; L. masc. n. lituus a curved rod, crook; N.L. masc. n. *Thalassolituus* a marine, curve-shaped organism. Gram-negative, vibroid to spiral, motile cells, 1.2–3.5 mm long by 0.6 mm wide. Strictly halophilic: Na+ ions are required for growth. Chemoorganoheterotrophic; strictly aerobic; unable to grow under anaerobic conditions by fermentation, nitrate reduction, or phototrophically. Oxidase positive. Ammonia and nitrate may serve as nitrogen sources. Indole, arginine dihydrolase, and gelatinase negative. Acetate, C7–C20 aliphatic hydrocarbons, and their oxidized derivatives are the only carbon sources that are used for growth. Principal cellular fatty acids are laurate, palmitate, and octadecenoate. Predominant isoprenoid quinone is Q-9 (Teramoto et al. 2011). The type and only species (to date) of the genus is *Thalassolituus* oleivorans isolated from extinction dilution from an *n*-tetradecane enrichment culture that was established from seawater–sediment samples collected in the harbor of Milazzo, Italy. As the genus is established based on the single strain, the true habitat and biological features of genus are less known. Phenotypic features of this genus are shown in Table 24.1.

Marinobacterium

Ma.ri.no.bac.te'ri.um. L. adj. marinus, of the sea; Gr. neut. n. bakterion, a small rod; L. neut. n. *Marinobacterium*, marine rod. Cells are rod shaped and Gram negative and have numerous vesicles on their surfaces. Strict aerobe. Oxidase and catalase positive. Grows on sugars, fatty acids, aromatic compounds, and amino acids. Requires sea salt-based medium for growth. The type species is *Marinobacterium georgiense*. The genus *Marinobacterium* was created by Gonzalez et al. (1997) and, in accordance with current classification, is encompassed by the family *Alteromonadaceae*. However, its affiliation is unclear as based on 16S rRNA gene sequence analysis, the genus clusters with *Nitrincola* and occupies a position along the *Oceanospirillaceae* branch. Reclassification at the family level should therefore be considered. For reference, here, genus description is shown. Details of this genus are described in section *Alteromonadaceae*.

Chemotaxonomic Characteristics

Fatty Acid Profiles

Fatty acid profile in *Oceanospirillaceae* is reported for almost all species belonging to the family with description of the new genus or species. *Oleispira antarctica* can synthesize EPA (C20:5ω3). The most abundant nonpolar fatty acids are C16: 1, C16: 0, and C18: 1 in most species of this family. The fatty

acid profiles can be different depending on the analytical method used in each study—for instance, results obtained using a microbial identification system differ from those obtained with traditional methods that analyze extracted cytoplasmic lipids using gas chromatography and gas chromatography–mass spectrometry. In fact, results of fatty acid profile are different among the papers describing the new species of *Marinomonas*, even though they analyzed the same strain at the same condition, for instance, major fatty acid in *M. aquimarina* was reported as C16:0 (Espinosa et al. 2010); hence, Gupta et al. (2006) determined isoC16:0 as predominant fatty acid. However, it is obvious that fatty acid profile is a distinctive feature on the bacterial taxonomy. For instance, the thirteen strains of formerly *Oceanospirillum* that have been investigated for their fatty acid composition by Sakane and Yokota (1994) were divided into three groups. Group I included the ten strains belonging to *O. linum, O. maris* subsp. hiroshimense, *O. maris* subsp. williamsae, *O. beijerinckii* subsp. *beijerinckii*, *O. beijerinckii* subsp. *pelagicum*, *O. multiglobuliferum*, and *O. japonicum* (currently *Pseudospirillum japonicum*), all of which have a low mol% G+C (42.5–48.4). Group II included the two type strains of *O. jannaschii* (currently *Marinobacterium jannaschii*) and *O. kriegii* (currently *Oceanobacter kriegii*) and had a high mol% G+C content (54.8–54.9). Group III included only *O. pusillum* (currently *Terasakiella pusilla*) and could be clearly distinguished from other marine spirilla in having C14:0 3OH as the major 3-hydroxy fatty acid, which is a useful chemical indicator for separating bacterial species (Oyaizu and Komagata 1983), besides Q-10. Bertone et al. (1996) confirmed the separate position of *O. japonicum, O. jannaschii*, and *O. kriegii*. Yakimov et al. (2004) analyzed detail fatty acid profile in *Thalassolituus oleivorans*. The principal fatty acids in total major cellular fatty acids (TLFA), phospholipid fatty acids (PLFA), and glycolipid fatty acids profiles are C12: 0, C16: 0, and C18: 1. The TLFA and PLFA profiles are characterized by an almost equal presence of saturated and monounsaturated fatty acids, with a strong predominance of C14: 0, C16: 1, C16: 0, and C18: 1. They also analyzed the fatty acid profile at the position of the two fatty acids in the glycerol moiety, PE and PG, using CID-MS spectra analysis. They demonstrated that all lipids were possessed with an unsaturated fatty acid at sn-2 of the glycerol moiety, whereas the sn-1 position was mainly occupied by saturated fatty acids, as well as general feature of bacterial phospholipids.

Quinones

The quinones found in *Oceanospirillaceae* are mainly ubiquinone-8. Menaquinone-6 is found in some species (Zhang et al. 2002), but its quantities is low. Exceptionally *Oleibacter marinus* and *Thalassolituus oleivorans* have Q-9 as major isoprenoid quinone with minor amounts of Q-8 (Teramoto et al. 2011). Details of quinine profile in *Oceanospirillum* including formerly members of this genus were described by Sakane and Yokota (1994); all species, except *T. pusilla*, contained ubiquinone-8 as major respiratory quinone (more than 80 %). Like other spirilla from the alphaproteobacteria (see the genus *Aquaspirillum* in this book), *T. pusilla* contained over 90 % Q-10.

Lipids

The polar lipids of *Oceanospirillaceae* species consist almost entirely of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and diphosphatidylglycerol (DPG) with variable proportions of their lyso derivatives. The predominant phospholipids of *Oceanospirillum linum* are PE, PG, and trace amount of DPG (Wilkinson 1988). This pattern is typical of Gram-negative bacteria (Wilkinson 1988). As the other reports, *Bermanella marisrubri* have PG, PE, DPG, amino phospholipid, and glycolipid (Pinhassi et al. 2007). In the case of *Marinomonas polaris*, PE and PG are predominant (Gupta et al. 2006). *Neptuniibacter caesariensis* have PE and PG as major components and also have a moderate amount of unknown aminophospholipid and a minor amount of DPG (Arahal et al. 2007). Polar lipids of *Oleibacter marinus* are PG, a ninhydrin-positive phospholipid(s), and glycolipids (Teramoto et al. 2011). Phospholipids in *Thalassolituus oleivorans* are also represented by the PE and PG types (Yakimov et al. 2004).

The structure of the lipid A from the LPS of *Marinomonas vaga* (formerly *Alteromonas vaga*) ATCC 27119^T has been described (Krasikova et al. 2004). Its lipid A shows stoichiometric lack of the phosphate ester group at C4'. Moreover, also in this case, the main form is represented by a penta-acyl species, with a (3 + 2) distribution of fatty acids, and acylation is principally performed by the short-chained 10:0 (3-OH). It has been reported for the first time in this occasion that the 3-hydroxy moiety is also present as the secondary substituent at the amide-linked fatty acid of GlcN II (Krasikova et al. 2004). However, there is less information on the details of structure of LPS in other species of this family. Thus, further studies are necessary to elucidate the structure of LPS in *Oceanospirillaceae*.

Polyamines

There is little information on the polyamine composition in *Oceanospirillaceae* species. However, Hamana et al. (1994) demonstrated analysis of polyamine components in *Oceanospirilla* (formerly members of this genus) and reported that all *Oceanospirillum* species including *O. jannaschii* and *O. kriegii* contain both putrescine and spermidine. The relative content of putrescine is very small when compared with the level found in members of the alphaproteobacteria, e.g., *Terasakiella pusilla* (formerly *O. pusillum*). The absence of 2-hydroxy putrescine and homospermidine is a unifying character for the gammaproteobacteria.

Isolation, Enrichment, and Maintenance Procedures

General Isolation Methods

Most *Oceanospirillaceae* species can be isolated by direct plating of seawater samples on a complex medium such as marine agar (Zobell 1941) without enrichment procedures, though incubation temperature should be carefully considered as many marine species fail to grow at mesophilic temperatures (e.g., the psychrophilic genera *Oceaniserpentilla* and *Oleispira* require cultivation temperatures of 2–10 °C). Nevertheless, *Marinomonas* species, as well as species belonging to neighboring genera, are routinely isolated from seawater by direct plating methods onto marine agar and form colonies at 20–30 °C within 1 week. Suzuki et al. (1997) demonstrated that organisms related to *Oceanospirillum* could be isolated without enrichment using a modified version of R2A** medium incubated at 15 °C in the dark (Reasoner and Geldreicht 1985).

Enrichment

Generally, most species of *Oceanospirillaceae* grow readily in artificial medium and can be isolated using simple procedures, such as direct plating method onto agar plates. However, the isolation of species of *Oceanospirillum* often requires enrichment techniques utilizing seawater and infusions of marine shellfish (Williams and Rittenberg 1957; Hylemon et al. 1973; Terasaki 1970, 1973, 1979) due to the low predominance of marine *spirilla* in environmental samples. Briefly, the following is the method used by Williams and Rittenberg (1957) to enrich and isolate *O. linum* and *O. beijerinckii*: Seawater was mixed with an equal volume of Giesberger's medium (Williams and Rittenberg 1957) and incubated at 30–32 °C until the appearance of *spirilla* in microscopic observation. At this time, a portion of the culture was autoclaved and then mixed with an equal volume of Giesberger's medium lacking NH_4Cl. This medium was then inoculated with the unsterilized portion of the initial culture and again incubated at 30–32 °C. Spirilla were shown to predominate after one to three subcultures in this medium. For isolation, the enrichment was decimally diluted 1: 100 to 1: 100, 000 in sterile seawater. After mixing, the flasks were allowed to stand for 20 min to allow the *spirilla* to migrate to the surface. Plates with the appropriate medium were then inoculated with surface water. The method of Terasaki (1970) has yielded excellent results for the isolation of *Oceanospirilla* from putrid infusions. In a typical enrichment, the bodies of one to three pulverized shellfish were submerged in a Petri dish containing 2.5 % NaCl and the suspension incubated for up to 3 days at 20–30 °C. Microscopic examination revealed that spirilla were generally apparent early in the putrefaction. When *spirilla* became abundant, a loopful of the suspension was removed and touched successively to a sterile glass slide to produce small droplets. The smallest of these droplets was then streaked onto agar medium containing peptone and 2.75 % NaCl. This methodology was utilized to isolate *O. multiglobuliferum, O. beijerinckii*, and *O. maris* (Terasaki 1973, 1979). Another enrichment method using a horizontal glass tube for the isolation of spirilla has also been reported (Fujii et al. 1990). The principal of this method was based on high motility of *spirilla*, which allowed them to migrate rapidly through medium in a horizontal glass tube. Subsequent to repeated enrichments in this manner, pure cultures were isolated via streaking on Petri plates. This methodology was particularly effective in the isolation of *Marinospirillum megaterium* due to its requirement for microaerobic, reductive, and viscous environments, although the ability to form colonies on agar plates was lost upon subsequent transfers (Fujii et al. 1990). Lastly, isolation of the genus *Balneatrix*, the only species reported as a causative agent of human infection, can be achieved using TSA or other clinical media as, despite being a member of the *Oceanospirillaceae*, it is non-halophilic with a low tolerance to salt (lower than 1 % NaCl for growth).

Enrichment for Unique Characteristic Bacteria

For the isolation of species having specific characteristics other than the abovementioned bacteria, several enrichment methods have additionally been reported.

m-Hydroxybenzoate Degrading Bacteria

Some *Marinomonas* strains degrade *m*-hydroxybenzoate and 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 NH_4Cl, 38 mg of $K_2HPO_4 \cdot 7\ H_2O$, 14 mg of $FeSO_4 \cdot 7\ H_2O$, and 0.5 g of *m*-hydroxybenzoate. Cultures are incubated at 20–25 °C for up to 10 days, and isolates are obtained on Basal Medium Agar (BMA) plates containing 0.1 % *m*-hydroxybenzoate (Baumann et al. 1984). Using a complex medium, Eilers et al. (2000) reported 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 μm 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 Ansede et al. (2001) using media containing 1 mM of the osmolyte dimethylsulfoniopropionate (DMSP). Either basal salts or f/2 media was used with the pH adjusted to 7.2 by adding potassium phosphate to a final concentration of 10 mM. Serial dilutions of seawater or sediment slurries were 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.

Petroleum-Degrading Bacteria

Some *Oceanospirillaceae* species have been reported to degrade petroleum compounds (Teramoto et al. 2009). Briefly, methods

for enrichment of these bacteria are described here: One liter of non-sterilized seawater was supplemented with 1 g NH_4NO_3, 0.2 g K_2HPO_4, and 12 mg $FeCl_3$ (SW medium) and incubated in a glass basin with gentle shaking at room temperature (around 25 °C). Three grams of chocolate-mousse crude oil (comprised of 0.5 g crude oil and 2.5 g seawater) was applied to one side of a sterile pumice stone and floated on the SW medium with the coated side down. SW medium was continuously supplied to the glass basin at a rate of 200 ml per day, while the same amount of the medium was pumped out from the glass basin to maintain the culture volume at 1 L. Continuous flow cultures were conducted in two different modes to isolate hydrocarbon-degrading bacteria of wider diversity. In the first type (culture 1), non-sterilized SW medium prepared with fresh seawater was supplied for the first 10 days, and sterilized SW medium, prepared by autoclaving, was supplied for the next 6 days. In the second type (culture 2), non-sterilized SW medium prepared with fresh seawater was supplied for the first 3 days, and sterilized SW medium was supplied for the next 13 days. On the 16th day after starting the cultivation, the surface of the chocolate-mousse oil and the aqueous phase of the culture were spread onto an SW medium plate (1.5 % (w/v) agar; 9 cm in diameter) covered with 30 ml crude oil. The plates were incubated at 18 °C for up to 6 weeks. Bacterial colonies that appeared on the crude oil-covered SW medium plates were purified at room temperature (around 25 °C) on dMB plates containing 0.5 % (w/v) pyruvate. The dMB plate medium comprised (per liter) 15 g agar, 0.9 L seawater, 0.1 L distilled water, and 3.74 g marine broth 2216 (Difco).

*Ingredients of Giesberger's medium (per liter): 1 g of NH_4Cl, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7\ H_2O$, with 1 % calcium lactate (Williams and Rittenberg 1957).

**Ingredients of R2A medium in g/l 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.

***Preparation of crude oil and of its chocolate mousse. Arabian light crude oil was treated at 214 °C for 10 h to remove the volatile fraction (30 % in volume) and used in this study. Chocolate-mousse crude oil was prepared by mixing the crude oil and fresh seawater collected at Pari Island in a ratio of 1 : 5 (w/w), followed by vigorous and continuous shaking for 1 day. The resultant chocolate mousse was stable for several weeks.

Phenotypic Test

In general, common and conventional methods can be used to characterize for most *Oceanospirillaceae* strains, although media containing natural or artificial seawater must be used for all characterization tests. For some of the species, the genus *Oceaniserpentilla* and *Oleispira*, low cultural temperature should be employed, e.g., 2-10 °C, due to their psychrophilic property. The capability to ferment carbohydrates is tested most effectively in using oxidation–fermentation medium of Leifson (1963) to which carbohydrates have been added at a concentration of 0.5 % (w/v). This medium is more sensitive than the usual Hugh and Leifson medium because it contains phenol red instead of bromothymol blue. Other biochemical and nutritional traits can be tested using standard procedures carried out under suitable growth conditions, e.g., appropriate incubation temperature and sodium and iron concentration. Commercial identification kits (API, Biolog, and others) are also available for routine biochemical testing. Baumann's minimal medium (Baumann et al. 1972) is a common basal medium for carbohydrate or amino acid utilization tests. For *Oceanospirilla* characterization methods have been described in detail by Terasaki (1972, 1979) and Hylemon et al. (1973). The comments given in the Prokaryotes for the genus *Aquaspirillum* also apply to the genus *Oceanospirillum*, except that media containing natural or artificial seawater must be used for all characterization tests. For alkaliphilic species, such as *Marinospirillum alkaliphilum* and *M. celere*, growth medium should be adjusted to high pH unit (around 9.0 pH unit) with appropriate buffer, for instance, $NaHCO_3$ and Na_2CO_3 buffer (Namsaraev et al. 2009).

Maintenance Procedures

In general, common and conventional methods can be used to preserve and maintain most of *Oceanospirillaceae* strains in a carbohydrate-free medium, such as nutrient or marine agar in which they grow well, because these species are relatively robust. Long-term preservation of *Marinomonas* species and most of *Oceanospirillaceae* strains can be achieved by lyophilization using 20 % skim milk as a cryoprotectant. A suitable protocol for the preparation of the cells has been described by Gauthier and Breittmayer (1992). The lyophilized cultures are reconstituted by adding 0.5 ml of marine broth. A few drops are streaked onto marine agar, and the remaining is transferred to a tube containing 4 mol of marine broth. It is advisable to avoid high aeration of the culture during the first hours of incubation. Growth is observed after 2–3 days. An alternative method of preservation is freezing. Strains can sustain viability for over 6 months when frozen in liquid nitrogen or cryopreserved at −80 °C in broth containing 20–30 % glycerol or dimethyl sulfoxide. Using this protocol, frozen *Marinomonas communis* and *M. vaga* cells have remained viable for more than 5 years. Strains can be maintained by serial transfer on marine agar for routine work in the lab. After 2–3 days of growth at 25 °C, the plates can be preserved for 3–4 weeks at 15 °C. It is not recommended to keep the cultures at 4 °C, because viability is lost much faster than when the cultures are stored at 15 °C.

Also in the case of *Oceanospirilla*, preservation can be accomplished by suspending a dense suspension of cells in seawater nutrient broth or marine broth containing 10 % (v/v) dimethyl sulfoxide or 20 % (v/v) glycerol, with subsequent freezing in liquid nitrogen (Krieg 1984) or cryopreserved at −80 °C. Freeze-drying can be performed with cells grown on the appropriate medium (Terasaki 1975). Cells may be

maintained in semisolid PSS seawater medium (containing 0.15 % agar to give a jelly-like consistency) at 30 °C with weekly transfer (Hylemon et al. 1973). Cultures may also maintain as stabs in seawater nutrient agar at room temperature with monthly transfer (Terasaki 1972).

Peptone–Succinate–Salt (PSS) Medium

Peptone 10 g, succinic acid 1 g, $(NH_4)_2SO_4$ 1 g, $MgSO_4 \cdot 7\ H_2O$ 1 g, $FeCl_3 \cdot 6\ H_2O$ 0.002 g, $MnSO_4 \cdot H_2O$ 0.002 g, agar 1.5 g, and synthetic seawater 1 l; the pH is adjusted to 7.8.

Synthetic Seawater

NaCl 27.5 g, $MgCl_2$ 5 g, $MgSO_4 \cdot 7\ H_2O$ 2 g, $CaCl_2$ 0.5 g, KCl 1 g, $FeSO_4$ 0.01 g, and distilled water 1,000 ml.

Energy Metabolism

Oceanospirillaceae is basically aerobic having a strictly respiratory type of metabolism with oxygen as the terminal electron accepter. *Neptunomonas* is exceptionally facultative aerobic. *Oceanospirillaceae* strains are able to utilize various carbohydrate and amino acid compounds, as a sole carbon and energy source (◉ *Tables 24.1* and ◉ *24.4*–*24.9*). In the case of *Marinomonas communis* and *M. vaga*, it was proved that they 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 γ-proteobacteria (Baumann and Baumann 1973; Sawyer et al. 1977). Some petroleum-degrading strains can utilize unusual carbohydrates, e.g., aliphatic, aromatic, and branched-alkane compounds, as a sole carbon and energy source.

Ecology (Habitation)

Oceanospirillaceae species distribute basically marine environment and require sodium ion for growth. Almost all species have been isolated from marine environments, seawater, seawater sediments, putrid shell fish, seaglass surface, and others, strongly indicating that their main habitats are marine or similar environment to marine. The only exceptional case is *Balneatrix* isolated from freshwater spa, and moreover, the bacterium caused pneumonia and meningitis in human. Here we discuss habitation of each genus separately.

Oceanospirillum

Oceanospirilla have been isolated from coastal seawater (Williams and Rittenberg 1957), decaying seaweed (Jannasch 1963), and putrid infusions of marine mussels (Terasaki 1963, 1970, 1979). Isolation sources of each *Oceanospirillum* species are shown in ◉ *Table 24.2*. Because the main isolation source of *Oceanospirillum* spp. is coastal marine environments, *O. linum, O. maris*, and *O. beijerinckii*, or putrid shellfish infusions, *O. maris* (formerly *O. hiroshimense*), *O. beijerinckii* (formerly *O. pelagicum*), and *O. multiglobuliferum*, interesting question arose as to whether it lives as epibiotic strains in the tissues of various marine animals or as a free-living organism in seawater. By direct microscopic counts of the bacteria present in clear and turbid seawaters near Port Aransas, Texas, Oppenheimer and Jannasch (1962) found that spirilla comprised only 0.1–2.5 % of the total bacteria present. Also, rRNA genes closely related to *Oceanospirillum* species have not been encountered in libraries prepared from marine environmental rDNA (Giovannoni and Rappé 2000), which suggests that the abundance of *Oceanospirillum* in the marine environment is low. In chemostat experiments, Jannasch (1963) suggested that the growth of *Oceanospirilla* might be restricted to environments of higher nutrient concentration than is found in ordinary seawater, such as in zones surrounding decaying particulate matter. Hence, multiple strains have been isolated from putrid infusions of marine mussels, suggesting that this trait appears to be associated with the capability of these species to survive as epibiotic strains in the tissues of various marine animals. However, it can be simply indicated that the source is most likely marine mud adherent to the mussels (Terasaki 1970). The true habitat is still unclear.

Marinomonas

All *Marinomonas* species appear to be strictly marine in origin, although *M. polaris* has no requirement of sodium ion for growth (Gupta et al. 2006). At the time of writing, the genus *Marinomonas* comprise 20 species, mainly originating from seawater from different geographical locations, e.g., *Marinomonas communis* and *M. vaga* (Baumann et al. 1972; van Landschoot and De Ley 1983) were isolated from the Pacific Ocean, *M. polaris* (Gupta et al. 2006) and *M. ushuaiensis* (Prabagaran et al. 2005) were isolated from subantarctic regions, while *M. primoryensis* (Romanenko et al. 2003) and *M. arctica* (Zhang et al. 2008) were isolated from sea ice. The next major isolation source is the seaglass, *P. oceanica*, which plays an important role in Mediterranean Sea ecosystems. Totally seven *Marinomonas* species have been isolated from this sea plant, as part of a study to characterize the microbiota associated with *P. oceanica* (Espinosa et al. 2010; Lucas-Elío et al. 2011). The coral *Mussismilia hispida* and oyster are also reported as isolation source for *Marinomonas* species, *M. brasiliensis* (Chimetto et al. 2011), and *M. aquimarina* (Macian et al. 2005), respectively. Also in this genus, interesting question arose as to whether it lives as epibiotic strains on the surface of various sea plants and organisms or as a free-living organism in seawater. A high number of *M. communis* and *M. vaga* strains have been isolated using enrichment methods with different compounds as carbon

sources (Baumann et al. 1972). These isolations led to the view that *Marinomonas* is a usual component of the bacterial flora in marine waters; however, there are few data about the ecological distribution of each *Marinomonas* species owing to the scarcity of strains in each species. The true habitat is also unclear, however.

Marinospirillum and *Nitrincola*

As eluted above, the phylogenetic position of the genus *Marinospirillum* is clearly far from *Oceanospirillaceae*, indicating it belongs to other bacterial family. Since *Marinospirillum* is included in *Oceanospirillaceae* at the time of writing, its habitation is also shown here. The genus *Marinospirillum* is encompassing five species and isolated from putrid shellfish infusions, fermented sea food, and in an alkali lakes called a sada Lake. Three species of *Marinospirillum, M. minutulum, M. megaterium*, and *M. insulare*, 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. *M. megaterium* and *M. insulare* were isolated from kusaya gravy, which is rich in nutrients; contains 3 % NaCl, volatile nitrogen compounds, and low oxygen concentrations; and is used to make Japanese dried fish (Fujii 1977, 1978; Fujii et al. 1985, 1990, 1993). Satomi et al. (1997) investigated predominant microbial species using PCR-clone library sequence in kusaya gravy. Although helical or S-shaped cells were obviously recognized on microscopic observation in this gravy sauce, *Marinospirillum* spp. or putative helical bacteria were not determined, indicating that the abundance of *Marinospirillum* and its relatives in the gravy sauce can be low. As was mentioned in the section enrichment, the strains should be enriched to isolate on the basis of their high motility, which allowed them to migrate rapidly through medium in a horizontal glass tube. *M. alkaliphilum* was isolated from Haoji Soda Lake (pH 9.5) in Inner Mongolia Autonomous Region of China as an alkaliphilic helical bacterium (Zhang et al. 2002). Subsequently, *M. celere* was isolated from combined water – a novel sediment slurry sample taken from a hot spring (40 °C, pH 9.3, salinity 25 g l^{-1}) on Paoha Island on Mono Lake (CA, USA) as a haloalkaliphilic, helical bacterium in *Marinospirillum*. Both species prefer alkaline environment and require sodium ion for growth, suggesting that their habitats can be ocean or ocean-like environments other than alkali Soda Lakes. As well as two *Marinospirillum*, *M. alkaliphilum* and *M. celere*, a novel alkaliphilic bacterium, *Nitrincola* lacisaponensis, strain was also isolated from decomposing wood taken from the shore of Soap Lake, a saline, alkaline lake in Grant County, WA, USA (Dimitriu et al. 2005). Although all *Marinospirillum* species are halophilic alkaliphilic bacteria as a common characteristic, there are few data about the ecological distribution of *Marinospirillum*. The true habitat in nature is also unclear.

Other *Oceanospirillaceae* Species

Other *Oceanospirillaceae* species also appear to be strictly marine in origin (see *Table 24.3*). As describe later, petroleum-degrading bacteria are widely distributed in ocean with no limit to seawater temperature from tropical to polar region. Population of these bacteria in environment are likely to vary always depending on nutritional condition or competition against other microorganisms. These bacteria could dominate in the natural marine environment after an accidental oil spill and would continue to dominate in the environment after biostimulation (Teramoto et al. 2009), despite low occupation under usual environmental condition. *Amphritea balenae, A. japonica*, and *Neptunomonas japonica* were isolated from sediment adjacent to sperm whale carcasses. These species are regarded as the most closely related to a symbiotic bacterial clone of the genus *Osedax* (Hedlund et al. 1999; Arahal et al. 2007; Goffredi et al. 2007). The genus *Osedax*, composed of siboglinid polychaete, has recently been discovered in whale carcasses on the deep-sea floor (Rouse et al. 2004; Glover et al. 2005; Fujikura et al. 2006; Braby et al. 2007). Members of the genus *Osedax* host symbiotic bacteria in the ovisac and root systems. These bacterial species may have related to the symbiont-like bacteria clone of *Osedax japonicus*. As well as two *Marinospirillum, M. alkaliphilum* and *M. celere*, a novel alkaliphilic bacterium, *Nitrincola lacisaponensis*, was also isolated from decomposing wood taken from the shore of Soap Lake, a saline, alkaline lake in Grant County, WA, USA (Dimitriu et al. 2005).

Pathogenicity and Clinical Relevance

Balneatrix alpaca is the only species that is reported as clinical related bacteria in *Oceanospirillaceae*. The genus *Balneatrix* is consist of single species and established in 1993 by Dauga et al. for an unknown bacterium occurred during the outbreak of pneumonia and meningitis in a spa therapy center, southern France, in 1987 (Hubert et al. 1991). Thirty-five cases of pneumonia and two cases of meningitis occurred. Isolates from eight patients were recovered from blood, cerebrospinal fluid, and sputum and one from water. Morphology and conventional biochemical characteristics have been described (Casalta et al. 1989). This organism, previously referred to as a "new non-fermentative unknown Gram-negative bacterium," shared some phenotypic properties with the genus *Flavobacterium*, although the G+C content of the DNA was 54 mol%. Further studies indicated that nine isolates of this pathogen constituted a tight DNA hybridization group and belonged to the gamma subclass of the proteobacteria with close relationships to *Oceanospirillum* based on the rRNA–DNA hybridization and 16S rRNA sequencing. The new bacterium differed from the genus *Oceanospirillum* by lacking the NaCI requirement and by reducing nitrate into nitrite, producing indole from tryptophan and producing acid from carbohydrates. It is obvious that *Balneatrix alpaca* is a remarkable causative agent of human

infection; however, the fact is that also it is a rare case that *Oceanospirillaceae* strains become an infectious agent. *Balneatrix* strains are susceptible in vitro to a variety of antimicrobial agents, including ß-lactam, macrolides, and aminoglycoside antibiotics; sulfamethoxazole–trimethoprim; chloramphenicol; doxycycline; minocycline; ofloxacin; and nalidixic acid. They are resistant to clindamycin and vancomycin. To data, *Balneatrix* strains have been isolated only from thermal water and clinical specimens at a spa therapy center in southern France. According to epidemiological data, the bacteria were present in the hot water spring spa, and favorable growing conditions were found only in vapor baths. After disinfection of water pipes by chlorination, no further cases of infection were observed.

Applications

Applications for *Oceanospirillaceae* strains as current-generating devices include degradation of chemical pollutants including petroleum-related compounds, production of bactericidal elements, and extraction of useful enzymes, such as unique oxidases related to melanin pigment production.

Degradation of Petroleum-Related Compounds

Unique features of some *Oceanospirillaceae* strains are known. Some species have been isolated from hydrocarbon-rich environments, crude oil-contaminating temperate seawater, seawater and sediment after oil spill, adjacent area of sperm whale carcasses, and others. They are able to degrade petroleum hydrocarbons, such as aliphatic, aromatic, and branched-alkane compounds (Hedlund et al. 1999; Yakimov et al. 2003, 2004; Teramoto et al. 2009). Yakimov et al. (2003) isolated hydrocarbon-degrading strains from Antarctic coastal marine environments (Rod Bay, Ross Sea) using enrichment method and established new genus *Oleispira*, which is psychrophilic, halophilic, aerobic, and Gram negative with polar flagella. As unique characteristics, the strains were able to synthesize the polyunsaturated fatty acid eicosapentaenoic acid (20: 5w3) at low temperatures, as well as many psychrophilic marine bacteria, and exhibited a restricted substrate profile, with a preference for aliphatic hydrocarbons, that is typical of marine hydrocarbonoclastic microorganisms such as *Alcanivorax, Marinobacter*, and *Oleiphilus*. Also Teramoto et al. (2009) reported that *Oleibacter* isolated from Indonesian seawater accidentally contaminated by crude oil can degrade *n*-alkane-hydrocarbons. Although the bacteria did not show degrading activity for branched-alkane degradation, they have high degrading activity for *n*-alkane-hydrocarbons and become the most dominant in microcosms that simulated a crude oil spill event with Indonesian seawater, thus could be key bacteria for biodegradation in tropical seas. As well as *Oleibacter*, *Thalassolituus oleivorans* also has been reported to degrade aliphatic hydrocarbons (Yakimov et al. 2004), and *Thalassolituus* strains have recently been shown to dominate in *n*-alkane-containing temperate seawater microcosms (Yakimov et al. 2005; McKew et al. 2007) and in crude oil-containing temperate estuarine seawater microcosms (Harayama et al. 1999; Kasai et al. 2001; Coulon et al. 2007; McKew et al. 2007). On the other hand, *Neptunomonas naphthovorans* is isolated from creosote-contaminated Puget Sound sediment based on their ability to utilize naphthalene as a sole carbon and energy source and able to degrade polycyclic aromatic hydrocarbon (PAH) compounds, including 2-methylnaphthalene, 1-methylnaphthalene, 2, 6-dimethylnaphthalene, and phenanthrene. Details for mechanisms for degradation of PAHs including related genes were investigated (Hedlund et al. 1999) in *N. naphthovorans*. The bacteria were not able to degrade acenaphthene as a sole carbon source but degrade with a mixture of seven other PAHs. A naphthalene dioxygenase iron–sulfur protein (ISP) gene was determined in the bacteria, and PAH dioxygenase ISP-deduced amino acid sequences showed close relationships between the genes encoding naphthalene dioxygenases of Pseudomonas and Burkholderia strains. *N. japonica* was isolated from sediments adjacent to sperm whale carcasses, which may contain rich and unique lipids, suggesting that the bacteria can be related to lipid derogation. However, there is less information that all of *Neptunomonas* can degrade PAH compounds. Also *Neptuniibacter* sp. strain CAR-SF can utilize carbazole as its sole carbon and nitrogen sources. The genes related to carbazole degradation pathway, consisting with carAa, carBa, carBb, and carC, were investigated (Nagashima et al. 2010). As mentioned above, some *Oceanospirillaceae* strains can degrade petroleum and related compounds and are expected to apply their ability to improve environment, such as oil spill and artificial pollution.

Marinomonas mediterranea is melanogenic (Solano et al. 1997; Solano and Sanchez-Amat 1999; Solano et al. 2000; Sanchez-Amat et al. 2001, 2010) and produces polyphenol oxidase, an enzyme involved in melanin synthesis. Melanin pigments are made from L-tyrosine as precursor and by the involvement of the enzyme tyrosinase (EC 1.14.18.1) (Solano et al. 1997), which is a copper protein belonging to the group of polyphenol oxidases (PPOs). The other important copper enzyme in this group is laccase (EC 1.10.3.2). M. mediterranea also shows this activity, due to a multipotent enzyme showing both tyrosinase and laccase activities (Solano et al. 1997). 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. *Marinomonas mediterranea* synthesizes a novel antimicrobial protein (LodA) with lysine-epsilon-oxidase activity (EC 1.4.3.20). As mentioned above, production of useful enzymes have been investigated in *M. mediterranea* deeply. Since main habitation of most *Marinomonas* species is similar to that of *M. mediterranea*, they also may have unique and useful properties as well as *M. mediterranea*. Further investigation including whole-genome analysis in these bacteria may lead to developing new technology.

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25 The Family *Oleiphilaceae*

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Abstract

During cultivation on *n*-alkanes as the sole source of carbon and energy, a bacterial isolate, ME102^T, was obtained from a seawater/sediment sample collected in the harbor of Messina (Italy) from the depth of 8 m. Possessing a very narrow spectrum of consumable organic substrates almost exclusively restricted to long-chain aliphatic hydrocarbons, alkanoates and alkanoles, this Gram-negative, aerobic, motile, rod-shaped bacterium appeared to belong to the group of the so-called obligate marine hydrocarbonoclastic bacteria. The ME102^T cells formed a biofilm on the surface of the alkane droplets and were likely adapted to the lifestyle on organic aggregates. Large-scale intracellular accumulation of $C_{16:0}$ and $C_{18:0}$ alkanoates (more than 50 % of the total cell mass) was observed at stationary phase of growth. 16S rRNA gene sequence analysis showed that ME102^T isolate represents a distinct lineage in the order *Oceanospirillales* and had <92 % sequence identity to the closest members from families *Hahellaceae* and *Oceanospirillaceae*. On the basis of physiological, phenotypic, metabolic data and distant phylogenetic position, the hydrocarbonoclastic isolate ME102^T was described as a novel species within a new genus, *Oleiphilus messinensis* gen. nov., sp. nov., in a new family, *Oleiphilaceae* fam. nov. Although many years have passed since the first isolation and valid publication, the family *Oleiphilaceae* and genus *Oleiphilus* are still represented by single species *O. messinensis* and the type strain ME102^T remains as unique isolated bacterium.

Taxonomy, Historical and Current

Since the first description in 2002 (Golyshin et al. 2002), the family *Oleiphilaceae* did not change the structure and content. The type and only genus is *Oleiphilus* (O.le.i'phi.lus. L. n. *oleum* oil; Gr. adj. *philos* loving; N.L. n. *Oleiphilus* oil-loving organism). Gram-negative, motile, aerobic thick rods are generally 0.7–1.4 μm wide and 1.5–2.0 μm long when growing in medium supplemented with *n*-hexadecane. Only aliphatic hydrocarbons and their derivatives are used as carbon sources for growth. The type and only species is *Oleiphilus messinensis* ME102^T.

16S rRNA gene sequence analysis showed that *Oleiphilus messinensis* represents a distinct lineage in the order *Oceanospirillales* and has less than 92 % sequence identity to members of *Hahellaceae* and *Oceanospirillaceae*, the closest families (Fig. 25.1). As we mentioned above, since its first description, ME102^T isolate remains the unique cultivated bacterium, representing the family *Oleiphilaceae*, genus *Oleiphilus*, and species *Oleiphilus messinensis*. Survey for similar 16S rRNA genes in available nucleotide databases was failed to find any of sequences with more than 93.5 % of identity (Fig. 25.2). Although retrieved from different marine habitats, all these *Oleiphilus*-related uncultivated organisms were associated with either sediments or biofilms and microbial mats. Coincidently with both historical isolation and nutritional preferences, some *Oleiphilus*-like sequences (FM242233, JQ579692, and JQ580103) were recovered from oil-polluted subtidal shallow sediments (Paisse et al. 2010; Acosta-Gonzalez et al. 2013).

Molecular Analyses

Genome sequence of *O. messinensis* ME102^T has been determined, although not yet presented in a published form. Unlike other obligate marine hydrocarbonoclastic bacteria, such as *Alcanivorax borkumensis* SK2^T (3.12 Mbp; Golyshin et al. 2003; Schneiker et al. 2006), *Cycloclasticus pugetii* (2.66 Mbp; Lai et al. 2012), *Oleispira antarctica* (4.4 Mbp; Kube et al. 2013), and *Thalassolituus oleivorans* (3.92 Mbp; Golyshin et al. 2013), *O. messinensis* ME102^T has a much larger genome. Preliminary molecular studies indicate that the size of genome is about 6.38 Mbp with 47.8 % of G+C content. Five rRNA operons were identified within the genome. No plasmids were detected.

The putative gene encoding the key enzyme of alkane catabolism, alkane hydroxylase or monooxygenase (AlkB), has been subjected to phylogenetic analysis (Golyshin et al. 2002). The deduced amino acid sequence of ME102^T AlkB was related to the putative alkane monooxygenases of *Salinisphaeraceae* bacterium PC39 (ADE05603) and of uncultured bacterium from Antarctic marine sediments (Kuhn et al. 2009),

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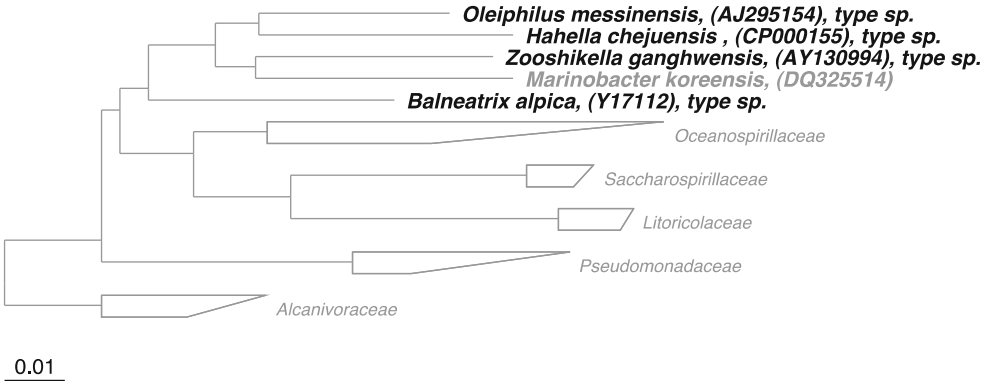

Fig. 25.1

Phylogenetic tree showing the position of *Oleiphilaceae, Hahellaceae*, and related families of the class *Gammaproteobacteria*. The type species are shown in *black* color. Accession numbers of the nucleotide sequences used for the tree construction are shown in parenthesis as follows. Scale bar, 0.01 nucleotide substitutions per position

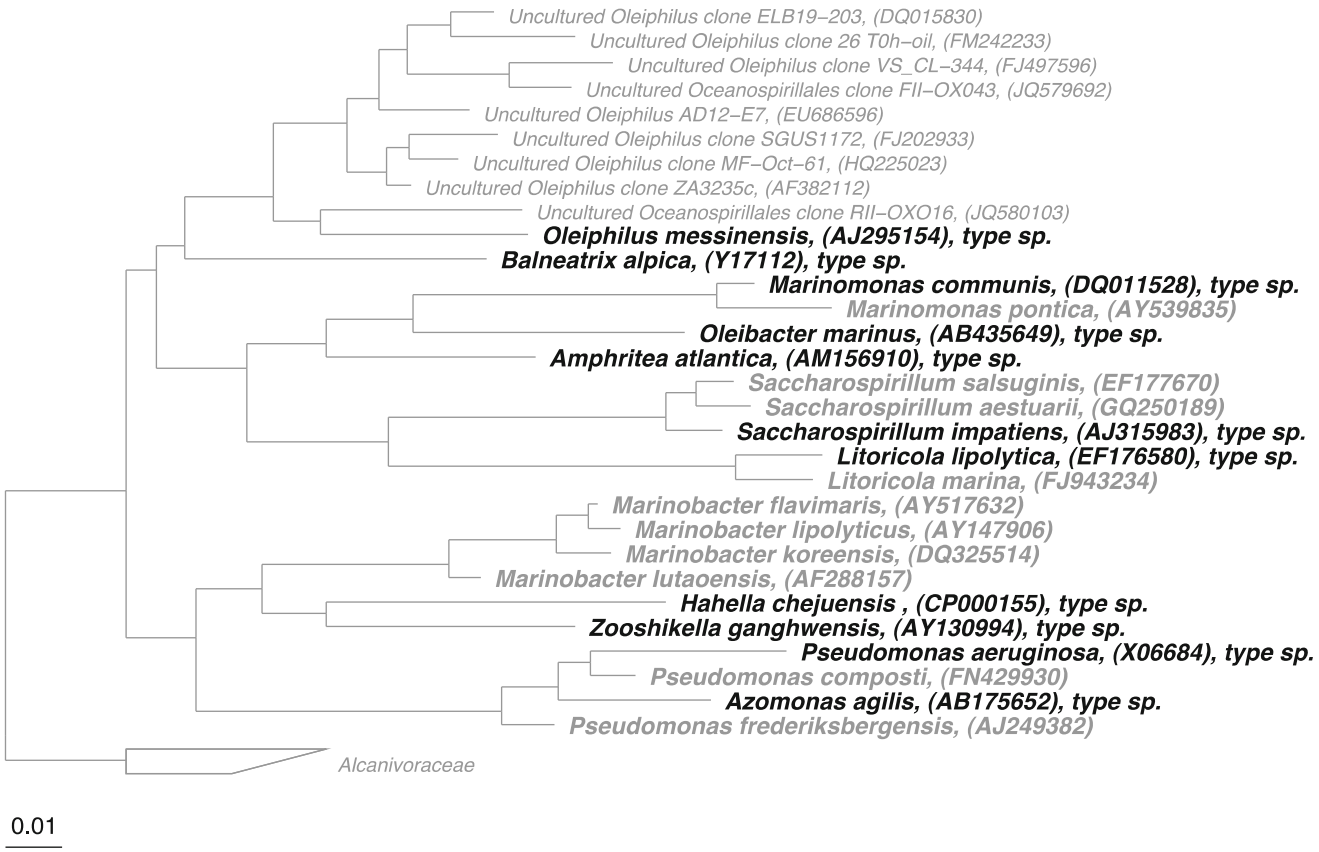

Fig. 25.2

Phylogenetic affiliations of *Oleiphilus messinensis* ME102^T and other related to *Oleiphilaceae* uncultured marine bacteria. The type species are shown in *black* color. Accession numbers of the nucleotide sequences used for the tree construction are shown in parenthesis. Scale bar, 0.01 nucleotide substitutions per position

respectively, exhibiting 65 % and 60 % sequence identity. Among cultivated organisms, AlkB of *Pseudomonas oleovorans* and *Alcanivorax borkumensis* were most similar to alkane hydroxylase of ME102^T.

Phenotypic Analyses

Oleiphilus messinensis is a Gram-negative, strictly aerobic, chemoorganotrophic, oxidase-positive and catalase-negative,

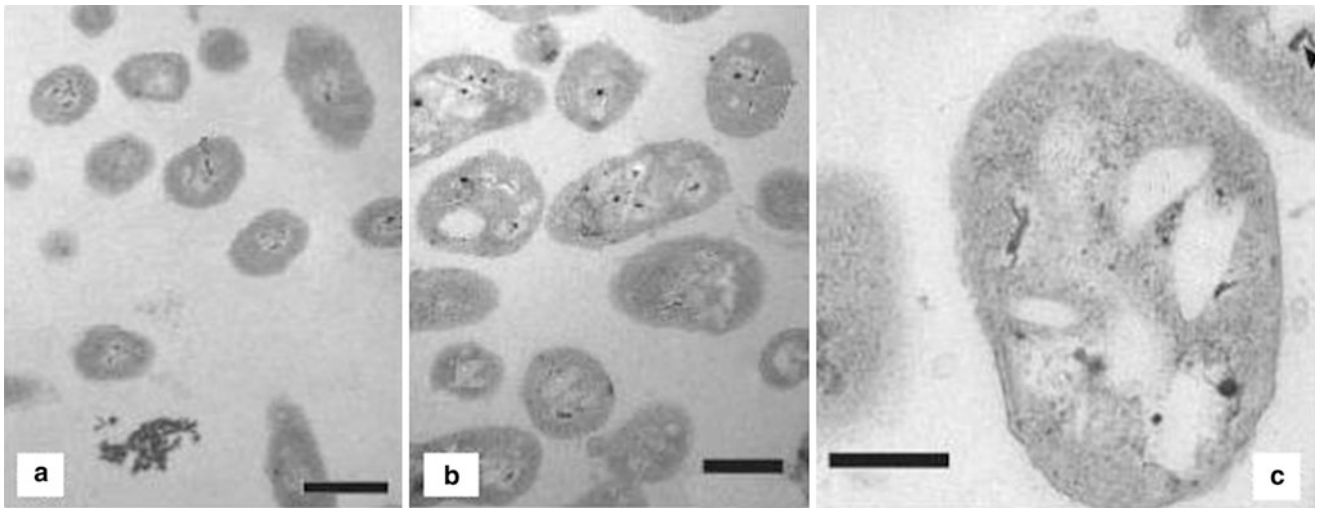

Fig. 25.3
Ultrastructural analysis of mid-exponentially growing cells of *O. messinensis* ME102^T. Representative views of ultrathin-sectioned, rodlike cells from the water phase (a) and the oil phase (b) (bars, 1 μm). "Oil" cells are shown in detail (c) (bar, 300 nm)

nonspore-forming straight rod, 0.7–1.4 μm in diameter and 1.5–2.0 μm long, motile by single unsheathed polar flagellum, not luminescent, and nonpigmented. Strains grow at 10–37 °C, with optimal growth between 20 °C and 25 °C. Growth occurs at NaCl concentrations of 0.06–10.5 % (w/v), with an optimum between 2.5 % and 5 %. Strains exhibit tweenase activity, but not agarase, amylase, arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase, gelatinase, or aesculinase activities. It has capability to grow anaerobically reducing nitrate to nitrite. Polar lipid fatty acid analysis has allowed the detection of four different types of polar lipid: phosphatidyl glycerol, phosphatidyl ethylamine, phosphatidyl dimethylethylamine, and lipids belonging to an unknown type of phospholipid. However, the saturated fatty acid C_{14}–C_{18} represented more than 57 % of the total extracted PLFA, and palmitate and palmitoleate were principal fatty acids in the PLFA profile.

The growth with different sources of carbon revealed that *O. messinensis* uses a very narrow spectrum of organic substrates: aliphatic hydrocarbons with alkyl chain lengths between C_{11} and C_{20} as principal carbon and energy sources. In liquid media, cells colonize the surface of alkane droplets and grow in a dense film on this hydrophobic substrate, although some cells are detectable in an aqueous phase. The oxidized derivatives of these *n*-alkanes, i.e., corresponding fatty acids and alcohols, are also used by the isolate ME102^T, as well as some other compounds possessing hydrocarbon chains, e.g., Tween 40 and Tween 80.

When *O. messinensis* grows in an aqueous emulsion of oil/water, two physiologically diverse cell fractions are obtained, those in the water phase and those in contact with the oil droplets. Ultrastructural analysis of cells from the water phase shows the general rod bacterial morphology (mean diameter 0.77 ± 0.11 μm) with a central chromosome surrounded by a homogeneous cytoplasm (*Fig. 25.3*). The cells collected from oily phase revealed three distinct morphological differences. In general, the diameter of "oil" cells (mean diameter 1.07 ± 0.19 μm) is larger if compared to the "water" cells. Oil-attached cells contain a large number of irregularly shaped, electron-translucent inclusions, which are often outlined by sharp edges and edge-angle characteristic of crystalline inclusions, that can make up to 50 % or more of the total cell volume. Mass-spectrometry analysis of intracellular inclusions isolated from "oil" cells of *O. messinensis* revealed the presence of palmitic and stearic acids as principal storage compounds (35 % and 55 % of total amount, respectively). Dihydroxybutyl-dicarboxylic acid was found in amounts a bit less than 10 %, while myristic ($C_{14:0}$) and pentadecylic ($C_{15:0}$) fatty acids were detected in trace quantities.

Isolation, Enrichment, and Maintenance Procedures

O. messinensis ME102^T was obtained from seawater/sediment samples collected in the harbor of Messina, 38.1909 N, 15.5677 E (Sicily, Italy) from a depth of about 8 m, using enrichment culture with *n-hexadecane* (Fluka) as sole carbon source in synthetic seawater medium ONR7a (Dyksterhouse et al. 1995). One liter of seawater/sediment sample was filtered through a sterile nylon membrane filter (0.2 μm pore diameter) covered with a glass-wool prefilter. Both prefilter and filter were sectioned in four pieces and one section was placed into a 50 ml Falcon polypropylene tube containing 20 ml ONR7a medium, supplemented with 1.5 % (vol/vol) *n*-hexadecane, and incubated at 20 °C for 1 week. The resulting mixed culture was diluted and 5–50 μl of culture were plated onto solid ONR7a medium supplemented with the same carbon source. All single colonies possessing different morphologies were isolated and sequenced. Strain ME102^T was selected from a number of Gram-negative isolates for further studies. *O. messinensis* ME102^T can be routinely cultivated aerobically in either

ONR7a or SM1 (Yakimov et al. 1998) liquid media supplemented with 1.5 % (vol/vol) *n*-hexadecane. Bacto agar (Difco) (15 g l^{-1}) was added for the preparation of solid medium. Optimal temperature for growth is 20–25 °C.

Ecology

During the past few years, efforts to isolate indigenous obligate marine microbes from extinction dilution on artificial seawater medium supplemented only with petroleum hydrocarbons have yielded the group of taxonomically and physiologically new hydrocarbon-degrading bacteria from different sites all over the world (Head et al. 2006; Yakimov et al. 2007). Such bacteria exhibit highly restricted substrate profiles, essentially being able to use only petroleum hydrocarbons as carbon and energy sources. Significantly, these hydrocarbonoclastic bacteria are found in small numbers in unpolluted waters but in high abundance in oil-polluted waters (Harayama et al. 2004). In accordance with this observation, the isolation of *O. messinensis* ME102^T was occurred from seawater/sediments collected in the harbor of Messina (Italy, 38°11′22″N; 15°33'55″E). This area is significantly impacted by intensive maritime traffic and constantly polluted by fuel hydrocarbons (Golyshin). At present, this site is only known habitat of cultivated *Oleiphilaceae*. Culture-independent studies indicated the presence of *Oleiphilaceae*-related organisms in subtidal sediments of North Atlantic cost of Spain (Cies Islands, Galicia, 42°13′56.15″N; 8°53′50.18″W) impacted by tanker *Prestige* oil spill and chronically contaminated coastal sediments of Etang de Berre lagoon (France, 43°28′00″N; 5°10′00″E).

On the basis of the phenotypic and physiological analyses, the marine environment as a source of isolation, the restricted nutritional profile, and the absence of fermentative metabolism, *O. messinensis* ME102^T shares many traits with OMHCB of the order *Oceanospirillales* belonging to the genera *Alcanivorax*, *Oleispira*, and *Thalassolituus*. The above genera, however, have greater nutritional versatility. In contrast, *O. messinensis* exhibits an extremely narrow spectrum of substrates that support growth. Isolate ME102^T utilizes only *n*-alkanes, alkane alcohols, and alkanoates with a chain length between C_{11} and C_{20}. Another characteristic feature of isolate ME102T is the large-scale accumulation of crystals of $C_{16:0}$ and $C_{18:0}$ alkanoates when cells are grown under nitrogen-limiting conditions or in direct contact with alkane droplets.

In liquid media, *O. messinensis* ME102^T cells colonized the surface of alkane droplets and formed a dense biofilm on this hydrophobic substrate. Biofilm formation at the alkane-water interface seems an efficient strategy employed by this organism to overcome the low bioavailability of aliphatic hydrocarbons and to colonize hydrophobic interfaces. It could also be related with the particle-associated lifestyle because an adhesion and biofilm formation could be a win-win strategy to acquire carbon and energy from hydrophobic compounds contained in marine aggregates.

Application

Along with other representatives of the order *Oceanospirillales* from the genera *Alcanivorax*, *Oleispira*, and *Thalassolituus*, *Oleiphilus messinensis* comprises a specific group of so-called obligate marine hydrocarbonoclastic bacteria (OMHCB) whose metabolism is restricted to the linear and branched aliphatic, saturated, and non-saturated hydrocarbons and their derivatives: fatty acids or alcohols. Being usually present at low or undetectable levels in pristine marine environments, the accidental load of oil or oil constituents into seawater leads to their successive blooms. Over the past few years, the OMHCB have been shown to play a significant role in the biological removal of petroleum hydrocarbons from polluted marine waters and sediments. As an example, the recent blowout of Deepwater Horizon platform in the Gulf of Mexico was followed by one of the largest offshore oil spills with ~four million barrels crude oil released into the deep sea. This accident resulted in the formation of a continuous and stable 35-km-long plume at the depth of 1,100 m (Camilli et al. 2010). As reported elsewhere (Hazen et al. 2010; Mason et al. 2012), a number of autochthonous marine hydrocarbon-degrading bacteria from the order *Oceanospirillales* have been identified to be actively involved in the oil degradation. The bloom of these organisms resulted in the rapid degradation of many oil constituents, hence highlighting the importance of OMHCB in bioremediation of marine environment and a necessity of comprehensive studies to unveil the genomic and physiological backgrounds of hydrocarbonoclastic lifestyle of OMHCB. Initial genome analyses and genome-based functional studies (Golyshin et al. 2003, 2013; Schneiker et al. 2006; Kube et al. 2013) have already revealed unique insights into OMHCB capability of alkane degradation, siderophore production, micronutrients scavenging, coping with various habitat-specific stress factors at the levels of protein folding, structural adaptation of hydrolytic enzymes, and chemical composition of fatty acid residues in bacterial lipids. The physiological studies coupled with genome and proteome analyses have also provided the understanding of the efficiency and versatility of OMHCB hydrocarbon utilization (Lemak et al. 2012; Alcaide et al. 2013), the metabolic routes underlying their special hydrocarbon diet, and their ecological success. These and other studies have revealed the potential of OMHCB for multiple biotechnological applications that include not only oil pollution mitigation but also biopolymer production and biocatalysis. Moreover, these studies provided essential knowledge to establish rational strategies aiming at the mitigation of environmental damage caused by oil spills in the process of oil exploration, production, and transportation in marine environments.

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26 The Family *Pasteurellaceae*

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Abstract
This chapter describes the systematics and evolution of *Pasteurellaceae* with emphasis on new information generated since the 3rd edition of The Prokaryotes which only included chapters dealing with *Haemophilus*, *Actinobacillus*, and *Pasteurella*. A major source of new information for the current chapter has been provided by whole genome sequences now available for many taxa of the family. Some 100 species and species-like taxa have been documented and 18 genera of *Pasteurellaceae* reported so far. Members of the family include specialized commensals, potential pathogens, or pathogens of vertebrates and mainly survive poorly in other habitats including the external environment. The pathogenic members are of major importance to animal production and human health. Members of *Pasteurellaceae* have relatively small genomes, probably as a result of adaption to a special habitat. The most important species in veterinary microbiology include *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, [*Haemophilus*] *parasuis*, *Mannheimia haemolytica*, *Bibersteinia trehalosi*, and *Avibacterium paragallinarum*, while *Haemophilus influenzae* and *Aggregatibacter actinomycetemcomitans* represent the most important species as to human disease. Traditional isolation techniques are still used in both human and veterinary clinical diagnostic laboratories although genetically based diagnostic methods have replaced traditional biochemical/physiological methods for characterization and identification. For all species, MALDI-TOF can now be used as a diagnostic tool. As control and if MALDI-TOF equipment is not at hand, PCR-based specific detection is possible for *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, [*Haemophilus*] *parasuis*, *Mannheimia haemolytica*, *Avibacterium paragallinarum*, *Gallibacterium anatis*, *Haemophilus influenzae*, and *Aggregatibacter actinomycetemcomitans*. A lot of work has been directed towards identification of virulence factors and understanding host microbe interactions involved in disease.

Introduction

Members of the family *Pasteurellaceae* represent specialized commensals or parasites of vertebrates and survive poorly outside their natural host. Members of the *Pasteurellaceae* have been found to have relatively small genomes probably as a result of adaption to their special habitat. While the family includes some of the most common and devastating pathogens of animals (*Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, [*Haemophilus*] *parasuis*, *Mannheimia haemolytica*, *Bibersteinia trehalosi*, *Avibacterium paragallinarum*), the family, apart from *Haemophilus influenzae* and *Aggregatibacter actinomycetemcomitans*, seems only to play a minor role in humans. Compared to the 3rd edition of The Prokaryotes which only included chapters dealing with *Haemophilus* (Fink and St. Geme 2006), *Actinobacillus* (MacInnes and Lally 2006), and *Pasteurella* (Christensen and Bisgaard 2006), this chapter has been significantly extended as to taxa reported. As a consequence, the current presentation is less detailed. For more information readers are recommended to consult reviews and monographs cited. Compared to the 3rd edition of The Prokaryotes, important new information has been gained from analysis of the whole genome sequences now available for many taxa of the family. Although a lot of work has been directed towards identification of virulence factors and understanding host – parasite interactions involved in disease, surprisingly little is still known on these topics and for the same reasons, these organisms are still of major importance in animal production and to human health.

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Taxonomy, Historical and Current

The family *Pasteurellaceae*, initially proposed by Pohl (1979) to include the genera *Pasteurella* (Trevisan 1887), *Actinobacillus* (Brumpt 1910), and *Haemophilus* (Winslow et al. 1917), is the single member of the order *Pasteurellales* – one of the 15 orders of the class *Gammaproteobacteria*. None of the other members of the class *Gammaproteobacteria* are close relatives to the *Pasteurellaceae*. However, on single gene level, comparisons have shown homology of some genes of *Pasteurellaceae* to families of *Gammaproteobacteria* and *Betaproteobacteria* (Maughan et al. 2008).

The properties of the family described by Pohl (1979) were based on characterization of 59 strains representing 38 species by phenotypic characterization and DNA-DNA hybridization experiments. The strains investigated were connected by a single linkage dendrogram constructed from the comparison of DNA reassociation values. This was the starting point for a natural classification of this group of bacteria. Genetic relationships later investigated by rRNA-DNA hybridizations showed the existence of at least seven evolutionary clades and also recognized heterogenicity of taxa within *Pasteurella*, *Haemophilus*, and *Actinobacillus* (De Ley et al. 1990). 16S rRNA gene sequence-based phylogenetic analysis confirmed a common ancestry for members of the family and documented monophyly of genera-like groups as well as polyphyly within *Pasteurella*, *Haemophilus*, and *Actinobacillus* (Dewhirst et al. 1992). As a consequence of this polyphyly, there have been numerous reclassifications of species within the three original genera *Pasteurella*, *Actinobacillus*, and *Haemophilus*, and reclassification of species that are not true *sensu stricto* members of the three genera is still ongoing. With *sensu stricto* we refer to a well-supported monophyletic group of taxa including the type species of the genus, all of which possess key phenotypic characteristics for the genus. The genera *Pasteurella*, *Actinobacillus*, and *Haemophilus* now include five, ten, and three species considered *sensu stricto* members with standing in nomenclature. The remaining validly named species of the three genera, however, probably need reclassification with other genera. For the same reason, the genus name of these species is enclosed in brackets. The most recent reclassification of this sort included [*Actinobacillus*] *actinomycetemcomitans*, [*Haemophilus*] *aphrophilus*, [*Haemophilus*] *paraphrophilus*, and [*Haemophilus*] *segnis* all reclassified with the new genus *Aggregatibacter* and [*Haemophilus*] *paraphrophilus* included with *Aggregatibacter aphrophilus* (Norskov-Lauritsen and Kilian 2006). After the original description of the family, 15 new genera have been validly named, and the family now includes 18 genera (*Table 26.1*). Five genera, *Mannheimia* (Angen et al. 1999), *Gallibacterium* (Bisgaard et al. 2009), *Avibacterium* (Blackall et al. 2005), *Aggregatibacter* (Norskov-Lauritsen and Kilian 2006), and *Volucribacter* (Christensen et al. 2004b), include six, four, five, three, and two species, respectively, whereas the remaining nine genera are monotypic: *Lonepinella koalarum* (Osawa et al. 1995), *Phocoenobacter uteri* (Foster et al. 2000), *Histophilus somni* (Angen et al. 2003), *Nicoletella semolina* (Kuhnert et al. 2004), *Bibersteinia trehalosi* (Blackall et al. 2007), *Chelonobacter oris* (Gregersen et al. 2009), *Basfia succiniciproducens* (Kuhnert et al. 2010), *Necropsobacter rosorum* (Christensen et al. 2011a), *Bisgaardia hudsonensis* (Foster et al. 2011) and *Otariodibacter oris* (Hansen et al. 2012) (*Table 26.1*). In total, the family *Pasteurellaceae* includes 73 validly named species in current use (LPSN, ICSP) and some 30 unnamed taxa (*Tables 26.2* and *26.3*). Out of the named 73 species, only 49 are considered to have been classified properly at genus level while 24 species are labeled with genus name in brackets. Two validly named species (*Haemophilus piscium*, *Pasteurella lymphangitidis*) should not be classified with *Pasteurellaceae* (Christensen and Bisgaard 2008).

Molecular Analyses

Phylogeny of type strains of species. *Figure 26.1* shows a neighbor-joining phylogeny based on 16S rRNA gene sequence comparison of type strains of validly named species in current use. The type species of the 18 genera of the family are distributed all over the tree. It is difficult to recognize a root for the family, and it has been suggested that the deeper branches are following a star-like topology (Christensen et al. 2004c). All genera except of *Pasteurella*, *Actinobacillus*, and *Haemophilus* are monophyletic. For these, the monophyletic *sensu stricto* groups can be recognized when comparison is made to *Tables 26.2* and *26.3*. *Actinobacillus capsulatus* is not monophyletic with the type species of the genus, *Actinobacillus lignieresii*, based on 16S rRNA comparison in agreement with the conclusion of Kuhnert et al. (2007) since 16S rRNA probably has undergone horizontal gene transfer in this taxon recently, whereas other conserved protein coding genes were monophyletic with *Actinobacillus lignieresii*. Similarly, *Haemophilus pittmaniae* and *Haemophilus sputorum* are not monophyletic with the type species of *Haemophilus* (*Haemophilus influenzae*) by 16S rRNA gene sequence comparison (Norskov-Lauritsen et al. 2005, 2012).

DNA reassociation. DNA reassociation data mainly generated by the spectrophotometric method provided the background for classification of most species within the family (Mutters et al. 1989; Pohl 1979). Contrary to most prokaryotic species, that are separated by more than 70 % DNA reassociation (Tindall et al. 2010), some members of *Pasteurellaceae* are separated by 80 or even 85 % DNA reassociation (Christensen et al. 2007; Mutters et al. 1989). In addition to the spectrophotometric method, attempts have been made to simplify DNA-DNA hybridization procedures (Christensen et al. 2000). The use of the *recN* sequences to estimate DNA reassociation was also found rather efficient (Kuhnert and Korczak 2006), and more recently, the use of whole genomic sequences to estimate DNA reassociation by use of average nucleotide identity (ANI) seems very promising (Bisgaard et al. 2012).

Population structural analysis. MLSA (multilocus sequence analysis) has been published for many taxa of *Pasteurellaceae*

Table 26.1

Genera of *Pasteurellaceae* and their phenotypic separation

Character	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Catalase	d	d*	–	+*	+	–	d	d	–	d	+	d	d	–	+	+	+	+
Oxidase	+*	d*	–	d	d	+	d	d	+	+	+	d	–	+	+	+	+	+
X factor requirement[a]	+*	–*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
V factor requirement[a]	+*	d*	–	–	–	–	–	–	–	d	–	–*	d	–	–	–	–	–
Methyl red	nd	–	nd	nd	–	nd	+	+	nd	–	nd	–	nd	+	nd	+	nd	nd
Voges Proskauer	–*	–*	+*	–	–*	+	–	–	–	–	nd	–	nd	–	nd	–*	–	+
Urease	d*	+	–	–	d	–	d*	–	–	–	+	–	–	–	–	–	–	d*
Ornithine decarboxylase	d*	–*	–*	d	d	–	–	–	–	d	–	–	–	–	–	–	–	–*
Indole	d	–	+ [b]	–	+*	–	d*	–	+	–	–	–	–	–	d	–	–	+
Acid from																		
L-arabinose	nd	d*	+*	d	d*	nd	d	d	nd	d	–	–	–	–*	+	+	nd	+
Dulcitol	–*	–	nd	–*	d*	–*	d*	–	nd	–	–	–	nd	–*	+	+	nd	–
D-fucose	nd	d*	nd	–*	d*	nd	–	–	nd	–	nd	–	nd	nd	nd	–*	nd	nd
L-fucose	nd	d*	nd	–*	d*	nd	d*	d	nd	d	nd	–	nd	nd	nd	+*	nd	+
D-mannitol	–*	+*	–*	+	d	–*	+	–	nd	d	–	+	d	+	+	–	+	–
D-mannose	–*	d*	nd	–	+*	–*	+	+	nd	+	–	+	d	+	+	+	+	–
Maltose	d	+*	nd	d*	d*	–*	d*	d	–	d	–	+	+	–	+	+	+*	+
D-sucrose	d	+*	d*	+*	+*	–*	+	+	–	+	–	+	d	+	+	+*	+	–
Trehalose	–*	d	nd	–	d	–*	d	–	–	d	–	+	d	+	+	+	+	+
MacConkey	nd	+	–*	nd	d	–	d	–	nd	–	–	nd	nd	nd	nd	nd	–	–*
o-nitrophenyl-beta-D-galactopyranoside (ONPG)	nd	+	+*	nd	nd	+*	d*	d	nd	d	nd	–	nd	+*	+	+*	–	+*
Alpha-glucosidase (PNPG)	nd	nd	nd	nd	nd	nd	d*	–	nd	d	nd	d	nd	–	–	nd	–	nd
GC mol %	39 *	35.5–43.7*	37.5*	39.2	37.7–43.9	41.5	39.9–42.3	40.8	nd	44.2–47	nd	42.6	42–44	42.5	47.2	52.5	39.5	36.2

1, *Haemophilus sensu stricto* (Kilian 2005; Norskov-Lauritsen et al. 2005; Winslow et al. 1917; Zinnemann and Biberstein 1974); 2, *Actinobacillus sensu stricto* (Brumpt 1910; Christensen and Bisgaard 2004); 3, *Lonepinella* (Osawa et al. 1995); 4, *Mannheimia* (Angen et al. 1999); 5, *Pasteurella sensu stricto* (Trevisan 1887) (Christensen and Bisgaard 2006); 6, *Phocoenobacter* (Foster et al. 2000); 7, *Gallibacterium* (Bisgaard et al. 2009); 8, *Volucribacter* (Christensen et al. 2004b); 9, *Histophilus* (Angen et al. 2003); 10, *Avibacterium* (Blackall et al. 2005); 11, *Nicoletella* (Kuhnert et al. 2004); 12, *Bibersteinia* (Blackall et al. 2007); 13, *Aggregatibacter* (Norskov-Lauritsen and Kilian 2006); 14, *Basfia* (Kuhnert et al. 2010); 15, *Chelonobacter* (Gregersen et al. 2009); 16, *Necropsobacter* (Christensen et al. 2011a); 17, *Bisgaardia* (Foster et al. 2011); 18, *Otariodibacter* (Hansen et al. 2012 in press).

Abbreviations: + only positive reactions occur, – only negative reactions occur, d positive or negative, nd no data available. * not part of formal genus description. All tests performed at 37 °C

[a]X factor, referring to the dependence on hemin for growth in vitro and V factor related to dependence on NAD (or related substances) for growth in vitro

[b]Negative reaction listed in Table of Osawa et al. (1995)

Table 26.2

Members of *Pasteurellaceae* with four or more records in PubMed, pathogenic potential, examples of important diseases reported, hosts, and X and V factor requirements (Alphabetic order)

Taxon[a]	Genus or genus-like taxon	Pathogenic potential	Main disease(s)	Hosts	X factor[b] requirement	V factor[b] requirement	References
Actinobacillus capsulatus	*Actinobacillus*	Opportunistic	Arthritis, septicaemia	Rabbits, hares	–	–	Kuhnert et al. (2007), Pohl (1979)
Actinobacillus equuli subsp. *equuli*	*Actinobacillus*	Opportunistic	Septicaemia "sleepy foal disease"	Horses, pigs	–	–	Christensen et al. (2002a)
Actinobacillus equuli subsp. *haemolyticus*	*Actinobacillus*	Opportunistic	NI [c]	Horses	–	–	Christensen et al. (2002a)
Actinobacillus lignieresii	*Actinobacillus*	Opportunistic	Actinobacillosis, pyogranulomatous lesions, "wooden tongue"	Ruminants	–	–	Christensen and Bisgaard (2004)
Actinobacillus pleuropneumoniae	*Actinobacillus*	Primary pathogen	Fibrinous-necrotizing pleuropneumonia	Pigs	–	d	Christensen and Bisgaard (2004)
[*Actinobacillus*] *seminis*	Aerogenes	Opportunistic	Urogenital tract infection, epididymitis	Sheep	–	–	Christensen et al. (2005b), Sneath and Stevens (1990)
[*Actinobacillus*] *succinogenes*	Succinogenes	Commensal	NI	Ruminants	–	–	Guettler et al. (1999)
Actinobacillus suis	*Actinobacillus*	Opportunistic	Pneumonia, septicaemia	Pigs	–	–	Christensen and Bisgaard (2004)
Actinobacillus hominis, *Actinobacillus ureae*	*Actinobacillus*	Opportunistic	NI	Human beings	–	–	Christensen and Bisgaard (2004), Friis-Moller et al. (2001)
[*Actinobacillus*] *minor*	Minor	Commensal	NI	Pigs	–	+	Moller et al. (1996)
[*Actinobacillus*] *muris*	Muribacter	Commensal	NI	Rodents	–	–	Bisgaard (1986)
[*Actinobacillus*] *rossii*	Rossii	Opportunistic	Reproductive tract infections	Pigs	–	–	Christensen et al. (2005b)
"*Actinobacillus porcitonsillarum*"	Minor	Commensal	NI	Pig	–	+	Gottschalk et al. (2003)
Aggregatibacter actinomycetemcomitans	*Aggregatibacter*	Opportunistic	Juvenile periodontitis (localized aggressive)	Primates	–	–	Norskov-Lauritsen and Kilian (2006)
Aggregatibacter segnis	*Aggregatibacter*	Opportunistic	Endocarditis	Primates	–	+	Norskov-Lauritsen and Kilian (2006)
Aggregatibacter aphrophilus (includes *Haemophilus paraphrophilus*)	*Aggregatibacter*	Opportunistic	Endocarditis, brain abscess	Primates	–	d	Norskov-Lauritsen and Kilian (2006)
Avibacterium gallinarum	*Avibacterium*	Opportunistic	Chronic fowl cholera-like lesions	Galliforme birds	–	–	Blackall et al. (2005)

Avibacterium paragallinarum	*Avibacterium*	Primary pathogen	Infectious coryza	Galliforme birds	–	d	Blackall et al. (2005)
Avibacterium endocarditidis	*Avibacterium*	Opportunistic	Valvular endocarditidis	Galliforme birds	–	–	Bisgaard et al. (2007)
Bibersteinia trehalosi	*Bibersteinia*	Opportunistic	Pneumonia, septicemia, mastitis	Ruminants	–	–	Blackall et al. (2007)
Gallibacterium anatis	*Gallibacterium*	Opportunistic	Salpingitis, peritonitis septicemia	Birds	–	–	Bisgaard et al. (2009)
Gallibacterium genomospecies 1 and 2	*Gallibacterium*	Opportunistic	Salpingitis, peritonitis, septicemia	Galliforme birds	–	–	Bisgaard et al. (2009)
[*Haemophilus*] *ducreyi*	Ducreyi	Opportunistic	Cancroid	Human beings	+	–	Bergey et al. (1923)
Haemophilus influenza (including *Haemophilus aegyptius* [Koch-Weeks bacillus])	*Haemophilus sensu stricto*	Opportunistic	Meningitidis, pneumonia, otitis media	Human beings	+	+	Fink and St. Geme (2006)
[*Haemophilus*] *haemoglobinophilus*	Haemoglobinophilus	Opportunistic	Urogenital tract infection	Dogs	+	–	Murray (1939)
Haemophilus haemolyticus	*Haemophilus sensu stricto*	Opportunistic	NI	Humans	+	+	Pittman (1953)
Haemophilus pittmaniae	Pittmania	Opportunistic	NI	Humans	–	+	Norskov-Lauritsen et al. (2005)
[*Haemophilus*] *parahaemolyticus*	Parahaemolyticus	Opportunistic	NI	Humans, Porcine isolates are *Actinobacillus pleuropneumoniae*	–	+	Kilian (2005), Pittman (1953)
[*Haemophilus*] *parainfluenzae*	Parainfluenzae	Opportunistic	Many types of infections	Humans	–	+	Fink and St. Geme (2006)
[*Haemophilus*] *paraphrohaemolyticus*	Parahaemolyticus	NI	NI		–	+	Zinnemann et al. (1971)
[*Haemophilus*] *parasuis*	Parasuis	Opportunistic	''Glässer's disease'' (polyserositis, meningitis, polyarthritis), bronchopneumonia	Pigs	–	+	Rapp-Gabrielson et al. (2006)
Histophilus somni	*Histophilus*	Opportunistic	Pneumonia, myocarditis, thrombotic meningoencephalitis, urogenital disease	Ruminants	–	–	Angen et al. (2003), Siddaramappa et al. (2011)
Mannheimia haemolytica	*Mannheimia*	Opportunistic	Pneumonia, septicemia, mastitis	Ruminants	–	–	Angen et al. (1999)
Mannheimia granulomatis	*Mannheimia*	Opportunistic	Panniculitis, bronchopneumonia	Ruminants, hares	–	–	Angen et al. (1999)
Mannheimia glucosida	*Mannheimia*	Opportunistic	Various infections	Ruminants	–	–	Angen et al. (1999)
Mannheimia varigena	*Mannheimia*	Opportunistic	*Pneumonia, septicemia*	Ruminants, pigs	–	–	Angen et al. (1999)
Mannheimia ruminalis	*Mannheimia*	Commensal		Ruminants	–	–	Angen et al. (1999)
Pasteurella canis, Pasteurella dagmatis, Pasteurella stomatis	*Pasteurella sensu stricto*	Opportunistic	Bite wounds	Cats, dogs, human beings	–	–	Mutters et al. (1985)

Table 26.2 (continued)

Taxon[a]	Genus or genus-like taxon	Pathogenic potential	Main disease(s)	Hosts	X factor[b] requirement	V factor[b] requirement	References
Pasteurella multocida subsp. *multocida*	*Pasteurella sensu stricto*	Primary or opportunistic	Hemorrhagic septicemia, fowl cholera, pneumonia, atrophic rhinitis, snuffles	Birds, mammals	–	–	Mutters et al. (1985)
Pasteurella multocida subsp. *gallicida*	*Pasteurella sensu stricto*	Opportunistic	Fowl cholera, pneumonia	Birds, mammals	–	–	Mutters et al. (1985)
Pasteurella multocida subsp. *septica*	*Pasteurella sensu stricto*	Opportunistic	Septicaemia, bite wounds, fowl cholera	Birds, felidae	–	–	Mutters et al. (1985)
[*Pasteurella*] *caballi*, Bisgaard taxon 42	Caballi	Opportunistic	Bite wounds	Horses, pigs, humans bitten by pigs and horses	–	–	Christensen et al. (2006), Schlater et al. (1989)
[*Pasteurella*] *aerogenes*, [*Pasteurella*] *mairii*	Aerogenes	Opportunistic	Abortion, septicemia	Pigs	–	–	Christensen et al. (2005b)
[*Pasteurella*] *bettyae*	Bettyae	Opportunistic	Tropism for Bartholin's glands of vagina	Human beings	–	–	Sneath and Stevens (1990)
[*Pasteurella*] *pneumotropica*	Pneumotropica	Opportunistic	Pneumonia, abscesses	Rodents	–	–	Jawetz and Baker (1950)
[*Pasteurella*] *testudinis*	Testudinis	Opportunistic	Pneumonia	Tortoise	–	–	Snipes and Biberstein (1982)
Volucribacter psittacicida, *Volucribacter amazonae*	*Volucribacter*	Opportunistic	Pneumonia	Psittacine birds	–	–	Christensen et al. (2004), Gregersen et al. (2010)
Bisgaard taxon 14	Taxon 14	Opportunistic	Septicaemia	Birds	–	–	Bisgaard and Mutters (1986b)
Bisgaard taxon 16	Taxon 16	Opportunistic	Bite wounds	Vertebrates	–	–	Bisgaard and Mutters (1986a)
Bisgaard taxon 45	*Pasteurella sensu stricto*	Opportunistic	Bite wounds	Large cats, human beings	–	–	Christensen et al. (2005a)

[a]References to publication of names are provided at List of Prokaryotic names with Standing in Nomenclature (http://www.bacterio.cict.fr/) and at the ICSP Subcommittee on the Taxonomy of *Pasteurellaceae* homepage (http://www.the-icsp.org/taxa/Pasteurellaceaelist.htm

[b]X factor, referring to the dependence on hemin for growth in vitro and V factor related to dependence on NAD (or related substances) for growth in vitro

[c]NI, no or limited record

Table 26.3

Rarely reported (less than four records in PubMed), new or poorly known members of *Pasteurellaceae* (Alphabetic order)

Taxon[a]	Genus or genus-like group	Hosts of isolation	X factor requirement[b]	V factor requirement[b]	References
Actinobacillus anseriformium	*Actinobacillus*	Ducks	–	–	Bisgaard and Christensen (2012)
Actinobacillus arthritidis	*Actinobacillus*	Horses	–	–	Christensen et al. (2002b)
[*Actinobacillus*] *indolicus*	Parasuis	Pigs	–	+	Moller et al. (1996)
[*Actinobacillus*] *porcinus*	Rossii	Pigs	–	+	Christensen et al. (2005b), Sneath and Stevens (1990)
[*Actinobacillus*] *delphinicola*, [*Actinobacillus*] *scotia*	Scotia	Cetacea	–	–	Foster et al. (1996), Foster et al. (1998)
Avibacterium avium, *Avibacterium volantium*	*Avibacterium*	Galliforme birds	–	+	Blackall et al. (2005)
Basfia succiniproducens	*Basfia*	Ruminants	–	–	Kuhnert et al. (2010)
Bisgaardia hudsonensis *Bisgaardia* genomo sp. 1	*Bisgaardia*	Seals	–	–	Foster et al. (2011)
Chelonobacter oris	*Chelonobacter*	Tortoise, turtles	–	–	Gregersen et al. (2009)
Gallibacterium trehalosifermentans, *Gallibacterium melopsittaci*	*Gallibacterium*	Budgerigars and psittacine birds	–	–	Bisgaard et al. (2009)
Gallibacterium genomospecies 3, *Gallibacterium salpingitidis*	*Gallibacterium*	Birds	–	–	Bisgaard et al. (2009)
Haemophilus sputorum	Parainfluenzae	Human	–	+	Norskov-Lauritsen et al. (2012)
[*Haemophilus*] *felis*	Felis	Cat	–	–	Inzana et al. (1992)
[*Haemophilus*] *paracuniculus*	Paracuniculus	Rabbit	–	+	Targowski and Targowski (1984)
Lonepinella koalarum	*Lonepinella*	Koala	–	–	Osawa et al. (1995)
Mannheimia caviae	*Mannheimia*	Guinea pig	–	–	Christensen et al. 2011b)
Necropsobacter rosorum	*Necropsobacter*	Several hosts	–	–	Christensen et al. (2011d)
Nicoletella semolina	*Nicoletella*	Horses	–	–	Kuhnert et al. (2004)
Otariodibacter oris (Bisgaard taxon 56)	*Otariodibacter*	Pinnipeds	–	–	Hansen et al. (2012) in press
[*Pasteurella*] *langaaensis*	Langaa	Galliforme birds	–	–	Mutters et al. (1985)
Pasteurella oralis	*Pasteurella sensu stricto*	Hedgehog, cat and dog bite infections of humans, mongoose, lizard	–	–	Christensen et al. (2012)
Phoconobacter uteri	*Phoconobacter*	Harbour porpoise	–	–	Foster et al. (2000)
Kangaroo I	Kangaroo	Kangaroos	–	d	Christensen et al. (2011a)
Kangaroo IV	Kangaroo	Kangaroos	–	d	Christensen et al. (2011a)
Kangaroo II	Kangaroo	Kangaroos	–	d	Christensen et al. (2011a)
Kangaroo V	Kangaroo	Kangaroos	–	d	Christensen et al. (2011a)
Petra (Tonsil1Pastlikesmaa)	Petra	Tiger, cheetah	–	–	Olesen et al. (2011)
Red panda	Red panda	Red panda	–	–	Christensen et al. (2011a)
Cheetah (22721_4_1gingiva)	Cheetah	Cheetah	–	–	Olesen et al. (2011)

Table 26.3 (continued)

Taxon[a]	Genus or genus-like group	Hosts of isolation	X factor requirement[b]	V factor requirement[b]	References
Bisgaard taxon 5	Taxon 5	Guinea pig	–	–	Bisgaard (1993)
Bisgaard taxon 7	Taxon 7	Guinea pig	–	–	Bisgaard (1993)
Bisgaard taxon 8	*Actinobacillus*	Guinea pig	–	–	Bisgaard (1993)
Bisgaard taxon 6 and 10	Taxon 6/10	Guinea pig, horse	–	–	Bisgaard (1993)
Bisgaard taxon 17	Taxon 17	Mouse	–	–	Christensen et al. (2003c)
Bisgaard taxon 22	Taxon 22	Mice, chicken	–	–	Christensen et al. (2003c)
Bisgaard taxon 29 and 30	Taxon 29/30	Rabbit	–	–	Christensen et al. (2011c)
Bisgaard taxon 32	Taxon 14	Falconiformes	–	–	Christensen et al. (2003)
Bisgaard taxon 44	Taxon 44	Psittacine birds	–	–	Gregersen et al. (2010)
Bisgaard taxon 46	*Pasteurella sensu stricto*	Large cats	–	–	Christensen et al. (2005a)

[a]References to publication of names are provided at List of Prokaryotic names with Standing in Nomenclature (http://www.bacterio.cict.fr/) and at the ICSP Subcommittee on the Taxonomy of *Pasteurellaceae* homepage (http://www.the-icsp.org/taxa/Pasteurellaceaelist.htm)
[b]X factor, referring to the dependence on hemin for growth in vitro and V factor related to the dependence on NAD (or related substances) for growth in vitro

(Christensen and Bisgaard 2010; Korczak and Kuhnert 2008). The purpose has mostly been to improve separation of species-like taxa compared to 16S rRNA, aiming at classification and identification. In addition, MLSA has been used to confirm monophyly of taxa by sequence comparisons of more genes than just 16S rRNA.

MLST (multilocus sequence typing) with a permanent web service and database has been established for *Haemophilus influenzae* (http://haemophilus.mlst.net/) (Meats et al. 2003), [*Haemophilus*] *parasuis* (http://pubmlst.org/hparasuis/) (Olvera et al. 2006), *Mannheimia haemolytica* (http://pubmlst.org/mhaemolytica/ *M. haemolytica*) (Petersen et al. 2009), *Pasteurella multocida* (http://pubmlst.org/pmultocida/) (Subaaharan et al. 2010), and most recently *Gallibacterium anatis* (http://pubmlst.org/gallibacterium/). MLST analysis has been used to establish population structures for molecular epidemiology and to investigate specific host associations. So far, unique ST68 and ST69 of *Pasteurella multocida* have been isolated from chimpanzees (Kondgen et al. 2011) while ST143 and ST144 have been isolated from red-necked wallabies (Bertelsen et al. 2012). The hope is to use MLST and similar-based methods to identify populations involved in specific disease manifestations linked to the presence of virulence factors, persistence traits, and host associations (Christensen and Bisgaard 2010). It has already been shown that ST122 is involved in hemorrhagic septicemia (HS) in bovines (Christensen et al. 2011b).

Phylogenies based on single genes may yield erroneous historical signal due to horizontal gene transfer, recombination, selection, or duplication events. In addition, the amount of information in a single gene sequence may not be sufficient to robustly resolve the relationships between close relatives. Several groups have proposed that phylogenies based on multiple genes may help to ameliorate the problems inherent in single gene phylogenetics (Christensen et al. 2004c; Gioia et al. 2006; Redfield et al. 2006). Whole genome sequencing has provided a powerful new data source for understanding the relationship among members of the *Pasteurellaceae* family. Bonaventura et al. (2010) presented the first whole genome phylogeny for the family, which included 3,130 concatenated genes and over one million amino acid characters. The resulting tree supported extremely well the phylogeny with 100 % bootstrap values at every node despite a high level of phylogenetic disagreement among the individual genes in the analysis. The results were also robust as to the inclusion of genes that did not have a representative in every taxon in the tree. ❯ *Figure 26.2* shows a subsequent analysis based on 54 *Pasteurellaceae* genomes and a concatenated dataset of 4,110 genes (1,421,165 amino acids). This tree is also very well supported with more than 83 % of branches having an 80 % bootstrap value or greater. Nearly all of the branches with weaker support fall within species, but there are many strongly supported intraspecies relationships as well. These findings suggest that whole genome sequencing is particularly effective in resolving relationships among species and genera.

Whole genome analyses have underscored the separation of two major groups within the *Pasteurellaceae* family (❯ *Fig. 26.2*). The first group includes *Haemophilus*, *Pasteurella*, and *Aggregatibacter* as predominant. The second group included the genera *Actinobacillus* and *Mannheimia* in addition to [*Actinobacillus*] *minor* and [*Haemophilus*] *ducreyi*. This pattern is roughly consistent with the 16S rRNA gene tree for the species included, but it is important to note that there are multiple instances of species in both trees with genus names which are

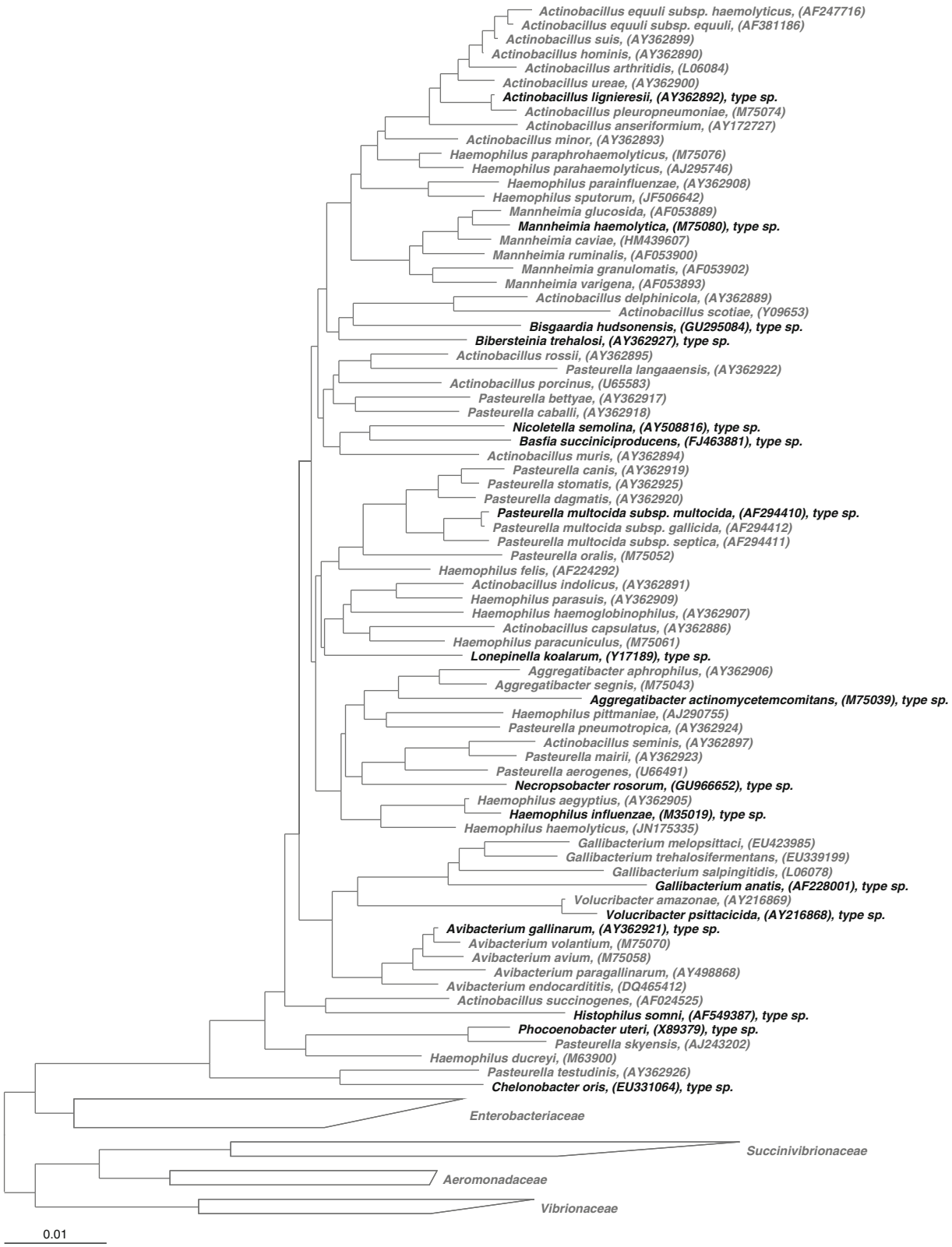

Fig. 26.1

Phylogeny of validly named species of *Pasteurellaceae* based on 16S rRNA gene sequences of the type strains compared by neighbor-joining analysis. The scale bar indicates 1 % of nucleotide substitution with respect to branch length given the substitution matrix. Genera names of species not included in *sensu stricto* groups have not been enclosed in brackets for typological reasons

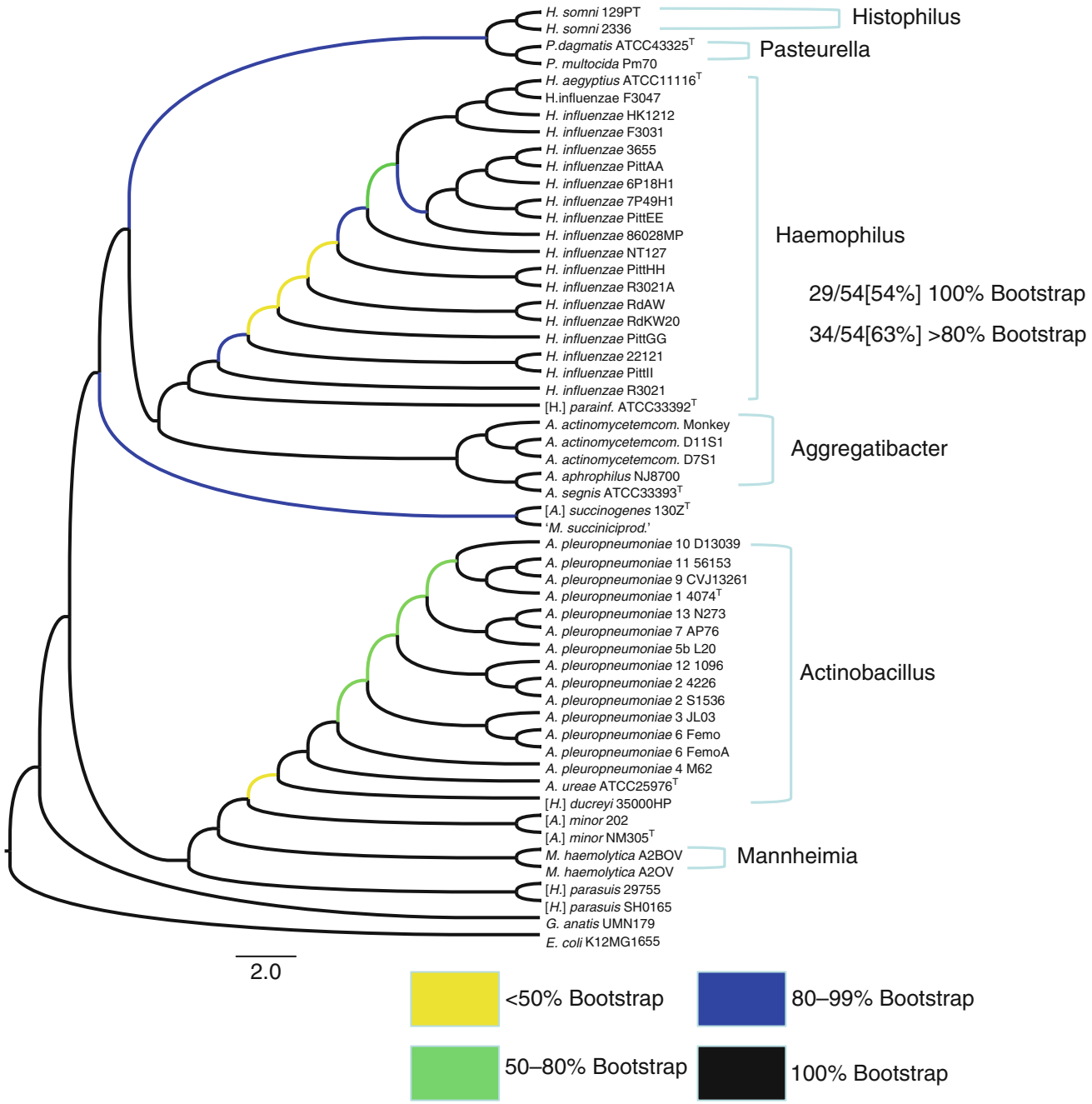

Fig. 26.2

Cladogram based on the concatenated matrix of 4110 genes 1,421,165 amino acids. The tree was constructed using maximum parsimony (MP) criterion and a heuristic search with 1000 iterations of random addition followed by tree bisection and reconnection. The final single MP tree had 1,626,219 steps (CI = 0.769, RI = 0.852, HI = 0.231)

in opposition to this general classification (e.g., [*Actinobacillus*] *minor* and [*Haemophilus*] *ducreyi*). This signals that major revisions of these taxa are needed.

Protein signatures and DNA motifs. Protein signatures identified based on comparison of published genome sequences separated the taxa within *Pasteurellaceae* into two groups. One group consisted of *Pasteurella*, *Aggregatibacter*, *Basfia*, *Haemophilus*, *Histophilus*, and [*Actinobacillus*] *succinogenes*, while the other included *Actinobacillus*, *Mannheimia*, [*Haemophilus*] *ducreyi*, and [*Haemophilus*] *parasuis* (Naushad and Gupta 2012). These signatures might be relevant for the classification at genus level in the future. Such investigations

should always include type strains of species compared. This separation is in accordance with the whole genome phylogeny mentioned above and with one obtained based on motifs of single-stranded DNA uptake sequences (Redfield et al. 2006). One type of DNA uptake sequence separates members of [*Haemophilus*] *ducreyi*, *Actinobacillus pleuropneumoniae*, and *Mannheimia haemolytica* from another group with *Aggregatibacter actinomycetemcomitans*, *Pasteurella multocida*, *Histophilus somni*, *Basfia succiniciproducens*, and *Haemophilus influenzae* (Redfield et al. 2006).

Genome structure and *rrn* operons. Only a single chromosome has been found in strains investigated. Five *rrn* operons were reported in *Aggregatibacter aphrophilus* (Di Bonaventura et al. 2009), and six *rrn* operons have been found in all other species investigated (Chen et al. 2010, 2012; Fleischmann et al. 1995; May et al. 2001), with the gene order (synteny) 16S rRNA-23SrRNA-5SrRNA (Fleischmann et al. 1995; May et al. 2001).

Plasmids. Beta-lactam resistance among members of *Pasteurellaceae* is often associated with small plasmids (4.1–10.5 kb). A 15 kb plasmid carrying multiresistance was found in *Actinobacillus pleuropneumoniae* (Kang et al. 2009). Larger conjugative plasmids of 40 kb were found to mediate resistance to tetracycline, chloramphenicol, or kanamycin (Schwarz 2008). The blaTEM-1 gene is usually located on large (40-kb), integrative, and conjugative elements (ICEs) when present in *Haemophilus influenzae* and less commonly on small (<10-kb) nonconjugative plasmids (Leaves et al. 2000). Plasmids were only found in one of the strains of *Histophilus somni* subjected to whole genome sequencing (Siddaramappa et al. 2011), whereas members of *Gallibacterium* were reported with from none to four plasmids (Christensen et al. 2003b). In conclusion, members of *Pasteurellaceae* have been reported with none or up to four plasmids of variable size, often associated with antibiotic resistance and with plasmid content variable within the species.

Bacteriophages. With the advent of whole genome sequencing, the genetic background of bacteriophages has been investigated in detail. A Mu-like prophage was reported in *Haemophilus influenzae* strain Rd (Fleischmann et al. 1995; Morgan et al. 2002). The first phage sequenced was HP1 (Esposito et al. 1996) and later in a non-typeable strain of *Haemophilus influenzae*, prophage HP2 was investigated (P2 family) (Williams et al. 2002). In *Pasteurella multocida* a lambda-like prophage was found associated with the *toxA* gene (Pullinger et al. 2004). A temperate bacteriophage was sequenced from *Aggregatibacter actinomycetemcomitans* and found closely related to lambdoid bacteriophages (Resch et al. 2004). From *Mannheimia haemolytica* a lysogenic bacteriophage of the P2 family was found by Highlander et al. (2006), while prophage regions were further reported in the genome of *Histophilus somni* (Siddaramappa et al. 2011). In the genome of *Avibacterium paragallinarum* a complete lysogenic temperate prophage similar *to Haemophilus influenzae* HP2, a Mu-like prophage, as well as lambdoid genes was found (Roodt et al. 2011). In conclusion, bacteriophages have been reported in members of *Haemophilus*, *Actinobacillus*, *Pasteurella*, and *Mannheimia*, and prophages related to Mu the P2 family and lambdoid phages are known from members of *Pasteurellaceae*.

Genomic islands. Genomic islands have been found in genomes of *Haemophilus influenzae*, *Histophilus somni* (Siddaramappa et al. 2011), and *Aggregatibacter actinomycetemcomitans* (Chen et al. 2010, 2012). Phage-related genes and genes of unknown function have been predicted on the genomic islands (Chang et al. 2000; McGillivary et al. 2005). Most remarkable is the finding of a syntenic type IV secretion system that facilitates conjugation and replication of the genomic island between members of *Haemophilus influenzae* and [*Haemophilus*] *parainfluenzae* (Juhas et al. 2007a, b).

Population genetic mechanisms. In addition to members of *Haemophilus*, *Aggregatibacter*, and *Actinobacillus* which are well known for their natural competence, *Gallibacterium anatis* was recently found natural transformable (Kristensen et al. 2012). This species as well as some other members of *Pasteurellaceae* have been found to prefer own DNA over foreign (Kristensen et al. 2012). Most of the DNA taken up by natural competence from the environment is probably degraded; however, homologous recombination of some DNA can take place and lead to change in the genotype of the recipient cell. For genetic manipulation, transformation is preferred with *Haemophilus* (Fink and St. Geme 2006), and it can be hypothesized that this natural competence is also the predominant mechanism for horizontal transfer of genetic material in the natural habitat for members of *Pasteurellaceae*. *Haemophilus influenzae* is a model organism for investigation of competence. When a large number of strains were compared with respect to the genetic traits linked to competence, it was difficult to trace the evolution of traits linked to competence, and it was concluded that competence has changed frequently during the evolution of this species (Maughan and Redfield 2009). In *Actinobacillus pleuropneumoniae*, only one strain of serotype 15 was highly transformable while other strains, representing all serotypes, were poorly transformable or non-transformable (Bosse et al. 2009). In members of *Pasteurellaceae*, the mechanisms for competence have been linked to short DNA uptake sequences with specific motifs. Based on model experiments and simulations, it was argued that a preference for a certain motif type and neutral homologue recombination were enough to explain uptake and accumulation of short uptake DNA sequences to high densities in the genomes (Maughan et al. 2010; Redfield et al. 2006).

Phenotypic Analyses

Members of *Pasteurellaceae* are facultative anaerobes and have both respiratory and fermentative types of metabolism including alternative terminal reductases that catalyze respiratory chain electron transfer to nitrite, nitrate, and fumarate (Olsen et al. 2005). Acid and sometimes gas are formed from D-glucose. They are nonmotile and nonspore-forming, and they reduce nitrate. Optimal growth is at 37 °C. Biochemical and

Table 26.4

Ubiquinone, menaquinone, and demethylmenaquinone composition of selected genera of *Pasteurellaceae*

	% total						
General	Q-7	Q-8	MK-7	MK-8	DMK-6	DMK-7	DMK-8
1	65		12		22		
2	27, 59.7	9	22.4, 35		21	17.9	
3	4		1.0, 3		87–97	3–93	6.0
4			88	5	6		
5			19.0	2.0		74.0	5.0
6						99.0–100.0	1.0
7	2–6	14.1–95		1–37.4		5	2–60.6
8		58.2		29.0			12.8
9	18.7–56	21.2–63	3.0, 3.6	1–60.1		79.4	10.3–18.7
	% total ubiquinone		% total menaquinone and demethylmenaquinone				
10	1	98		26			74
11	4	96					100
12	14	86					100

Comparison of ubiquinone 6, 7, 8, and 9 (Q-6, Q-7, Q-8, Q-9); menaquinone 7 and 8 (MK-7, MK-8); and demethylmenaquinone 6, 7, and 8 (DMK-6, DMK-7, DMK-8) for members of *Pasteurellaceae*. The color refers to chain lengths of 6, orange; 7, yellow; and 8, red. All data based on analysis with oxygen as the terminal electron acceptor

1, *Bibersteinia* (*Bibersteinia trehalosi*) (Mutters et al. 1993); 2, *Mannheimia* (*Mannheimia haemolytica*) (Kroppenstedt and Mannheim 1989; Mutters et al. 1993); 3, *Aggregatibacter* (*Aggregatibacter aphrophilus*, *Aggregatibacter actinomycetemcomitans*, *Aggregatibacter segnis*) (Kroppenstedt and Mannheim 1989; Mutters et al. 1993); 4, *Histophilus* (*Histophilus somni*) (Mutters et al. 1993); 5, *Actinobacillus sensu stricto* (*Actinobacillus lignieresii*, *Actinobacillus equuli*) (Kroppenstedt and Mannheim 1989); 6, *Haemophilus* (*Haemophilus influenzae*, *Haemophilus parainfluenzae*) (Kroppenstedt and Mannheim 1989); 7, *Pasteurella sensu stricto* (*Pasteurella multocida*, *Pasteurella dagmatis*, *Pasteurella canis*, *Pasteurella stomatis*, *Pasteurella oralis*) (Engelhard et al. 1991; Kainz et al. 2000; Kroppenstedt and Mannheim 1989); 8, *Gallibacterium anatis* (Engelhard et al. 1991); 9, *Avibacterium* (*Avibacterium gallinarum*, *Avibacterium avium*, *Avibacterium volantium*) (Kroppenstedt and Mannheim 1989; Kainz et al. 2000; Engelhart et al. 1991); 10, *Otariodibacter oris* DSM 23800^T (Hansen et al. 2012); 11, *Bisgaardia hudsonensis* M327/99/2^T (Hansen et al. 2012); (Foster et al. 2011); 12, *Phocoenobacter uteri* DSM 15746^T (Foster et al. 2000)

physiological characteristics are of major importance for separation of the genera (*Table 26.1*). At the genus level, genera need to be circumscribed by a unique set of phenotypic characteristics, and in principle only characteristics included in the original description of genera can be used for their phenotypic separation (*Table 26.1*). In practice, this is not possible for all genera. For genera described a long time ago, characteristics for separation will have to be based on their *sensu stricto* interpretation. For monotypic genera it has to be assumed that the characteristics included with the type species are also valid for the genus. Members of *Actinobacillus sensu stricto* are urease positive which separate them from members of *Pasteurella sensu stricto* and several other genera. Members of *Pasteurella sensu stricto* are indole positive which separate them from most other genera except *Histophilus* and *Lonepinella*. Other phenotypic characteristics (see below) vary less between genera making them less useful for practical identification.

Species need to be recognized by unique phenotypic characters. However, for most members of *Pasteurellaceae*, traditional phenotypic identification is very tedious, and phenotypical identification is often problematic. This is underlined by description of an increased number of genomospecies (*Tables 26.2* and *26.3*). In addition, revised genotypic data might compromise previous phenotypic separation if the genotype is not in accordance with the phenotype as demonstrated for subspecies of *Pasteurella multocida* (Christensen et al. 2004a; Kondgen et al. 2011). In case of problems as to phenotypic separation, the use of data on host reservoirs might be useful.

Chemotaxonomic characteristics. For "quinones," genus level differences seem to exist between a chain length of 7 for *Bibersteinia*, *Mannheimia*, *Histophilus*, *Aggregatibacter*, *Actinobacillus sensu stricto*, and *Haemophilus sensu stricto* compared to other genera with chain length of 8 (*Table 26.4*). This separation seems unrelated to the one obtained by protein

signatures and DNA uptake sequences mentioned above (Naushad and Gupta 2012; Redfield et al. 2006).

Polar lipids are conserved within *Pasteurellaceae*, with phosphatidyl ethanolamine, phosphatidyl glycerine and minor amounts of lyso-phosphatidyl ethanolamine. The latter compound was suggested as a marker for the family; however, this has not been further tested (Mutters et al. 1993). Polyamines of the family were extensively investigated by Busse et al. (1997), and the major composition within genera is included in *Table 26.5*. Some genera, like *Aggregatibacter*, *Mannheimia*, and *Bibersteinia*, are conserved in their polyamine contents. For other genera, like *Gallibacterium* and *Pasteurella sensu stricto*, diversity of polyamines between species seems to exist (Busse et al. 1997, Bisgaard et al. 2009).

Fatty acids are rather conserved between members of *Pasteurellaceae* with a dominance of C14:0, C16:0, C16:1 ω7c, and C14:0 3−OH/iso−C16:1 I (Christensen et al. 2011a).

With respect to the genera of *Pasteurellaceae*, the GC mol % varies between 36.2 and 52.5 (*Table 26.1*).

Isolation, Enrichment, and Maintenance Procedures

Members of *Pasteurellaceae* can be isolated from infected tissue, blood, or affected mucosal surfaces of vertebrates including humans. Blood or serum-enriched media, including chocolate agar, are incubated under microaerophilic conditions (sealed plastic bags or candle jar sometimes used in the veterinary clinic) at 37 °C for 24–48 h. Some taxa of *Pasteurellaceae* have a requirement for elevated levels of CO_2 (capnophiles). The species epithet of *Aggregatibacter aphrophilus* has been derived from the Greek "aphros" referring to the CO_2 bubbles produced when wine is fermenting. Most isolates of *Aggregatibacter* and *Histophilus* have been referred to as capnophilic.

Members of *Pasteurellaceae* typically show small regular, grayish, and shiny colonies on solid media. Colony size may vary from pinpoint to 2 mm in diameter. Some taxa may adhere to the media on primary isolation. Cultivation on blood agar allows observation of beta hemolysis. Some taxa have special characteristics and need special conditions for isolation and cultivation. One prominent growth requirement is the X factor, referring to the inability of some members of *Haemophilus* to synthetize porphyrins (*Tables 26.1*, *26.2* and *26.3*). Another growth factor is the V factor, which covers a demand for NAD (*Tables 26.1*, *26.2* and *26.3*). A few taxa (certain strains of *Avibacterium paragallinarum* and some taxa from kangaroos) have even been found to require extra V factor, the so-called double V factor requirement (Blackall et al. 2011). For these taxa, a filtrate of *Staphylococcus epidermidis* needs to be supplied to the medium for growth to take place. The need for special media for growth should always be considered before isolation in a clinical diagnostic laboratory. In chocolate agar, NAD is liberated from the red blood cells. For subcultivation of members of *Haemophilus*, brain-heart infusion broth has been recommended (Fink and St. Geme 2006) – still with an extra supply of X and V factor. [*Haemophilus*] *ducreyi* is recognized to be notoriously difficult to cultivate with special procedures needed such as the use of vancomycin in the medium to suppress the resident microflora and the addition of hemoglobin, fetal calf serum, or chocolarized horse blood as well as chemically defined growth-promoting substances such as IsoVitaleX^{TM} (Blackall and Nørskov-Lauritsen 2008). In - veterinary diagnostic laboratories involved in porcine and avian respiratory diseases, V factor will typically be provided by cross-streaking isolation media with *Staphylococcus aureus* (Blackall and Nørskov-Lauritsen 2008). One should remember that one of the most common members of the family, *Pasteurella multocida*, also might require V factor for growth (Krause et al. 1987). In addition, one should keep in mind that growth requirements might increase with host adaptation. Finally, extended incubation should be carried out in case of chronic infections to ensure growth of small colony variants, so far observed for *Pasteurella multocida* (Christensen et al. 2008) and *Avibacterium endocarditidis* (Pors et al. 2011).

When isolated, members of *Pasteurellaceae* easily die out at ambient temperature. Neither do they survive for long at 4 °C. They should be lyophilized or frozen in special media at −80 °C to survive, and repeated subculturing of the original isolates should be avoided. A special 7.5 % glucose serum medium (Redway and Lapage 1974) is recommended for storage of the more sensitive cultures at −80 °C. The glucose solution is sterilized by filtration and mixed with sterile calf serum and stored at −20 °C until use. Bacteria to be frozen are subcultured from a single colony on blood agar, and following overnight incubation at 37 °C, one ml of the glucose serum medium is used for suspension of the overnight growth. Storage in liquid nitrogen can also be used if facilities for freezing or freeze-drying are not available (Christensen et al. 2007). For transport of *Avibacterium paragallinarum*, it was recommended to use a commercial medium without charcoal and supply it with NAD and horse serum. This extended viability tremendously even at 37 °C (Vazquez 2011).

Clinical Relevance and Pathogenicity

The majority of taxa seem to represent harmless commensals, while some 10 % of all taxa have been associated with disease so far. Only three taxa (*Actinobacillus pleuropneumoniae*, *Avibacterium paragallinarum*, and *Pasteurella multocida*) are considered to include populations that may act as primary pathogens under all conditions; the remaining taxa are being considered as opportunistic pathogens or commensals (*Table 26.2*).

Haemophilus influenzae is the most important species in relation to human disease either involved in acute pyogenic and usually invasive infections mainly related to serotype B or involved in noninvasive infections where primarily non-encapsulated types are important (Kilian 2005). *Haemophilus influenzae* is among the leading causes of otitis media and meningitis in children as well as pneumonia and many other

Table 26.5

Dominant polyamines (more than 5 % of total) in genera or genera-like groups of *Pasteurellaceae*

Genus like group (sensu 16S rRNA)												
Aggregatibacter [a]	*Bibersteinia* [a]	*Mannheimia* [a]	*Actinobacillus sensu stricto* [a]	*Necropsoacter* and SL group [a]	*Avibacterium* [a]	[*Haemophilus*] *ducreyi* [a]	Bisgaard Taxon 14 [a]	*Haemophilus sensu stricto* [a]	*Histophilus*[a]	*Gallibacterium* [a, b]	[*Pasteurella*] *aerogenes* [*Pasteurella*] *mairii* [*Actinobacillus*] *seminis* [a]	*Pasteurella sensu stricto* [a]
DAP	DAP	DAP	DAP	DAP	DAP	DAP		DAP	DAP	DAP	DAP	DAP
			SPD	SPD	SPD	SPD	SPD		SPD	SPD	SPD	SPD
							PUT	PUT	PUT	PUT	PUT	PUT
					SPM	SPM			SPM	SPM	SPM	SPM
								CAD		CAD	CAD	CAD
												NSPD

DAP 1,3-diaminopropane, *PUT* putrescine, *CAD* cadaverine, *TYR* tyramine, *NSPD* sym-norspermidine, *SPD* spermine, *HSPD* sym-nomospermidine, *SPM* spermine.

[a] (Busse et al. 1997).

[b] (Bisgaard et al. 2009).

diseases (Fink and St. Geme 2006). This species was regarded as the cause of influenza until 1920 (Olitsky and Gates 1921). Serotyping has demonstrated six capsular types (Pittman 1933) which has been used to categorize populations of this species. An association between certain lesion types as well as their pathogenesis and epidemiology and serotypes has been reported. However, the majority of isolates cannot be serotyped since they are non-encapsulated (Kilian 2005); such isolates are traditionally referred to as non-typeable (NT) *Haemophilus influenzae*.

A number of taxa or unnamed *Haemophilus* species are difficult to discriminate from *Haemophilus influenzae* (Norskov-Lauritsen et al. 2009). These taxa have been referred to as "nonhemolytic *Haemophilus haemolyticus*" and appear to be of little or no pathogenic significance (Murphy et al. 2007). Such strains may be more prevalent in specimens from the respiratory tract than anticipated so far, comprising 16–21 % of presumptive *Haemophilus influenzae* throat isolates from healthy children and adults (Mukundan et al. 2007; Xie et al. 2006) and almost 40 % of presumptive *Haemophilus influenzae* sputum isolates from patients with chronic obstructive pulmonary disease (Murphy et al. 2007). Misidentification of strains of low pathogenicity as *Haemophilus influenzae* in airway samples could result in unnecessary antimicrobial therapy being prescribed. *Haemophilus influenzae* is invariably nonhemolytic and dependent on X factor. The presence or absence of several genes is significantly associated with *Haemophilus influenzae* in contrast to "nonhemolytic *Haemophilus haemolyticus*." Such marker genes include *sodC* (Fung et al. 2006; Langford et al. 2002), IgA1 (McCrea et al. 2008; Mukundan et al. 2007), *fucK* (Norskov-Lauritsen 2009; Norskov-Lauritsen et al. 2009), and *hpd* (Theodore et al. 2012; Wang et al. 2011). Occasionally strains may give aberrant results in PCRs for the marker genes, and such strains may require extended sequencing for characterization, such as that included in the MLST scheme (Meats et al. 2003).

Aggregatibacter actinomycetemcomitans is a well-known cause of infectious endocarditis, the epidemiological and clinical features of which have recently been reviewed (Paturel et al. 2004). The species has attracted particular attention because of its association with periodontitis (Henderson et al. 2010; Slots and Ting 1999). Especially a single clone of serotype B (designated the JP2 clone) has been associated with the aggressive form of localized periodontitis in adolescents (early-onset periodontitis). The JP2 clone of *Aggregatibacter actinomycetemcomitans* has a 530 bp deletion in the leukotoxin promoter, which results in significant enhancement of leukotoxin production (Brogan et al. 1994). The JP2 clone has predominantly been found in Africa but is absent in non-African populations from Northern Europe (Contreras et al. 2000; Haraszthy et al. 2000; Haubek et al. 1997). The clone was linked with a statistically significant risk of periodontitis during a two-year prospective longitudinal cohort study of Moroccan adolescents who were initially free of periodontitis (Haubek et al. 2008). With the co-occurrence of non-JP2 clones with a reduced risk of development of periodontitis, it was suggested that JP2 and non-JP2 clones of *Aggregatibacter actinomycetemcomitans* competed for the same ecological niche.

Pasteurella multocida is the most important species in relation to disease in animals. It has been reported involved in lesions in almost all types of animals as well as occasionally humans. The pathogenesis of the different manifestations of *Pasteurella multocida* infections is also highly diverse ranging from fatal septicemia as observed with fowl cholera in poultry and HS in cattle and buffalo to chronic or latent infections as observed with progressive atrophic rhinitis (PAR) in pigs (Dziva et al. 2008). *Pasteurella multocida* is an important pathogen in relation to all farm animals. The two serotyping systems of Carter (A, B, D, E, F) (Carter 1955; Rimler and Rhoades 1987) and Heddleston (1–16) (Heddleston et al. 1972) are usually combined (e.g., A:1).

In poultry *Pasteurella multocida* is mainly causing fowl cholera but is also isolated from other lesion types in acutely as well as chronically infected animals (Christensen and Bisgaard 2000; Dziva et al. 2008). In pigs, it is associated with pneumonia and atrophic rhinitis (Jong de 2006; Pijoan 2006). "Snuffles" and other less-frequent lesions are reported in rabbits (DeLong and Manning 1994). *Pasteurella multocida* isolated from lesions of rabbits belongs to serotypes A, D, and lately also F. Members of serotype F isolated from fowl cholera were able to cause disease in rabbits (Jaglic et al. 2011). Chickens infected with rabbit isolates of serotype F resulted in less severe lesions compared to chicken isolates of serotype F (Petersen et al. unpubl.).

HS has been described as the most important disease in certain regions of Asia and Africa (Carter and De Alwis 1989; Shivachandra et al. 2011). Isolates obtained from HS have been found to be included in a specific clonal population of *Pasteurella multocida* (ST122). Isolates of *Pasteurella multocida* from HS have been referred to belong to serotypes B:2 and E:2 (Carter and De Alwis 1989), and a specific PCR has been published for the detection of isolates belonging to serotype B:2 of *Pasteurella multocida* from HS (Townsend et al. 1998). However, other serotypes have also been isolated from HS (Shivachandra et al. 2011).

Other types of *Pasteurella multocida* than those isolated from HS seem to be involved in bovine respiratory disease often affecting calves (enzootic calf pneumonia). When older calves have been affected by bovine respiratory disease complex (shipping fever) during weaning or restocking, *Pasteurella multocida* has also been frequently implicated (Griffin 2010). Certain STs (13, 79, 80) have been identified in association with bovine respiratory disease (Hotchkiss et al. 2011a, b). Recently, a new ST62 of serotype B was isolated from different outbreaks in dairy-rearing farms (McFadden et al. 2011).

Mannheimia haemolytica is the principal bacterium implicated in bovine respiratory disease (shipping fever) (Griffin 2010; Rice et al. 2007). The most common serotypes implicated include A:1 followed by A:6 (Griffin 2010). In sheep, serotypes A:2 dominated followed by A:6 and A:9 (Gilmour and Gilmour 1989).

Bibersteinia trehalosi is a major pathogen in sheep causing serious systemic infections in lambs. It is also associated with pneumonia in sheep (Gilmour and Gilmour 1989).

Table 26.6
RTX (repeat in toxin) in members of *Pasteurellaceae*

Species of Pasteurellaceae with RTX toxin	RTX	Reference accession number	Primary reference
Actinobacillus equuli subsp. *haemolyticus*	Aqx	AF381185	Berthoud et al. (2002)
Actinobacillus pleuropneumoniae, *Actinobacillus suis*	ApxI	X68595	Frey et al. (1994)
Actinobacillus pleuropneumoniae, *Actinobacillus suis*, [*Actinobacillus*] *rossii*, '*Actinobacillus porcitonsillarum*'	ApxII	M30602	Chang et al. (1989)
Actinobacillus pleuropneumoniae, [*Actinobacillus*] *rossii*	ApxIII	L12145	Chang et al. (1993b)
Aggregatibacter actinomycetemcomitans	Ltx	X16829	Kraig et al. (1990)
Mannheimia/Bibersteinia	Lkt/Plkt	M20730	Chang et al. (1993a), Lo et al. (1987)
[*Pasteurella*] *aerogenes*, [*Pasteurella*] *mairii*	Pax	U66588	Kuhnert et al. (2000)
[*Pasteurella*] *pneumotropica*	PnxI-III	AB466276, AB466280	Sasaki et al. (2009, 2011)
Actinobacillus pleuropneumoniae	ApxIV	AF030511	Schaller et al. (1999)
Gallibacterium anatis	Gtx	FJ917356	Kristensen et al. (2010)
Avibacterium paragallinarum	AvxI	JQ289154	Pan et al. (2012), Küng and Frey J (2013)

The two species *Actinobacillus pleuropneumoniae* and [*Haemophilus*] *parasuis* are important pathogens exclusively causing diseases in pigs. Both species are V factor dependent (*Table 26.2*). *Actinobacillus pleuropneumoniae* is a pathogen resulting in pleuropneumonia in the respiratory tract. Sixteen serotypes (5a and 5b are considered separate) (Blackall et al. 2002; Nielsen 1986) have been reported. Serotyping has been important to understand the pathogenesis and epidemiology of this infection for prevention and treatment (Gottschalk and Taylor 2006). In some older articles, this species is reported as [*Haemophilus*] *parahaemolyticus* (*Table 26.2*).

[*Haemophilus*] *parasuis* is commonly found in the respiratory tract of healthy pigs. Unfortunately some strains can migrate to the lungs and cause pneumonia. Polyserositis (Glässer's disease) represents a severe systemic manifestation (Rapp-Gabrielson et al. 2006). A serotyping system is available; however, many isolates are not typeable. As mentioned above, a MLST system is available (Rapp-Gabrielson et al. 2006).

Avibacterium paragallinarum is an important pathogen of chickens in which it causes infectious coryza. Economic losses are mainly due to decreased growth, uneven flocks, and reduction in egg production (Blackall and Soriano 2008). The impact is much greater in developing countries than in developed. The serotypes A, B, and C of Page were recognized based on a plate agglutination test using whole cells and chicken antisera. A hemagglutination inhibition test is now recommended to detect the three serotypes (Blackall and Soriano 2008). Recently, a PCR has been published that should be able to determine the three serotypes of *Avibacterium paragallinarum* (Sakamoto et al. 2012). Serotyping is of importance in relation to selection of strains to be included with killed vaccines (*Table 26.8*).

RTX toxins. The family of RTX toxins, named for their repeats in structural toxin, is a group of mainly pore-forming hemolytic and/or cytotoxic protein toxins. However, they can also have other biological functions (Linhartova et al. 2010). They are found in several Gram-negative bacteria but are especially prominent in members of *Pasteurellaceae*, where they are often associated with disease-causing taxa (Frey and Kuhnert 2002). Classical RTX toxins are encoded on a CABD operon, in which the protein C posttranslationally activates the toxin A by acylation. The toxin is then secreted by a type I secretion system formed mainly by the B and D proteins. This type of RTX toxin has been described in species of the genera *Actinobacillus*, *Aggregatibacter*, *Bibersteinia*, *Mannheimia*, and *Pasteurella* (*Table 26.6*). Besides these classical RTX toxins, larger variants lacking the classical operon structure have been described like the ApxIV and Gtx in *Actinobacillus pleuropneumoniae* and *Gallibacterium anatis*, respectively, and most recently AvxI in *Avibacterium paragallinarum* (*Table 26.6*).

RTX toxins show high inter- as well as intraspecies genetic dynamics, with horizontal gene transfer and genetic reorganization. This genetic plasticity is typically seen with the ApxI, ApxII, and ApxIII toxin operons of *A. pleuropneumoniae* which are found in different combinations in the various serotypes, whereby the combination defines the pathogenic potential of types (Frey 1994). A new variant of *A. pleuropneumoniae* serotype 3 still having the ApxIII but lacking the entire ApxII operon was just recently described, indicating the potential of ongoing genetic reorganization of these toxins (Kuhnert et al. 2011). RTX toxins are not only found in pathogens since a functional ApxII operon has been found in *Actinobacillus porcitonsillarum*, a hemolytic commensal of porcine tonsils (Kuhnert et al. 2005). Moreover, the potential role in pathogenesis of some RTX toxins found in *Pasteurellaceae*, as, e.g., Pax in *Pasteurella aerogenes* or Aqx in hemolytic *Actinobacillus equuli*, is not yet clearly elucidated.

Despite their genetic plasticity, RTX toxins from *Pasteurellaceae* show host-specific presence as well as host-specific cytotoxic activity towards eukaryotic cells (Frey 2011) as observed for the phylogenetically closely related *Actinobacillus suis* harboring ApxI/II and hemolytic *Actinobacillus equuli* harboring Aqx which are host-specifically found in pig and horse, respectively (Kuhnert et al. 2003a). While the ApxI/II, only found in pig-adapted species, are cytotoxic for porcine but not equine lymphocytes, the Aqx, only found in horse-adapted species, likewise kills equine but not porcine lymphocytes (Kuhnert et al. 2003b). The specific cytotoxicity is determined by binding of RTX toxins to the CD18 subunit of β2-integrins on host lymphocytes as shown for the leukotoxins of *Mannheimia* and *Aggregatibacter* as well as ApxIII of *Actinobacillus pleuropneumoniae* (Deshpande et al. 2002; Lally et al. 1999; Vanden Bergh et al. 2009).

Knowledge gained so far points to a determinative role of these toxins in host specificity of *Pasteurellaceae* species. The fact that *Pasteurellaceae* species containing RTX toxins are mostly opportunistic pathogens and that also commensal species contain functional RTX operons might suggest a more general role of RTX in host-bacteria interaction. Therefore, it can be hypothesized that RTX toxins are needed for the commensal lifestyle of *Pasteurellaceae* and that disease is a collateral damage of a more profound symbiosis and the result of an impaired balance between toxic and beneficial action of RTX proteins.

***Pasteurella multocida* toxin**. The *P. multocida* toxin (PMT) has only been found in isolates of *Pasteurella multocida* mainly from pigs. PMT is also referred to as dermonecrotic toxin expressed in a skin test performed in guinea pigs. PMT positive isolates are involved in porcine atrophic rhinitis. PMT is encoded by *toxA*, and the gene is located on a lysogenic prophage of alpha type (Lax and Chanter 1990; Pullinger et al. 2004). The toxin is unique to *Pasteurella multocida* and is probably only present in specific populations of this species.

Iron uptake. Many members of *Pasteurellaceae* are dependent on the uptake of iron from host proteins that sequester iron such as transferrin (Gonzalez et al. 1990). Proteins were identified in *Haemophilus influenzae* that showed specific affinity to transferrin and occasionally lactoferrin binding (Schryvers 1989). Transferrin-binding proteins, TbpA and TbpB, were predicted in genome sequences of *Histophilus somni* (Siddaramappa et al. 2011). There seems to be host-specific binding and uptake mechanisms for iron with some members of *Pasteurellaceae* (Fink and St. Geme 2006; Ogunnariwo and Schryvers 1990; Veken et al. 1994; Yu and Schryvers 1994). Members *of Pasteurellaceae* may face anaerobic conditions in the host. Under these conditions dramatic changes in the expression of sets of genes seem to occur. In *Actinobacillus pleuropneumoniae*, HlyX was found to act as a global regulator under anaerobic conditions and regulation included genes involved in iron acquisition (Buettner et al. 2009).

Adhesins. Adhesins expressed by different members of *Pasteurellaceae* represent potential virulence factors. Overabundance of genes predicted to encode adhesion-like proteins was detected in *Histophilus somni* (Siddaramappa et al. 2011). The adhesion protein, Hap of *Haemophilus influenzae*, has been found to belong to the group of self-associating autotransporters which promote aggregation and microcolony formation (Meng et al. 2011). Further description of adhesions of *Haemophilus influenzae* can be found in Fink and St. Geme (2006).

Capsule. The capsule of members of *Pasteurellaceae* has been found as a potential virulence factor, and its expression and molecular background have been investigated in detail for most of the important pathogenic members of the family. *Haemophilus influenzae* is traditionally separated into six capsular serotypes A–F, of which type B (Hib) is most frequently involved in invasive infections. The biochemical basis for the synthesis of the different capsular types is also known (Fink and St. Geme 2006). The structure of the capsular locus in *Haemophilus influenzae* is typical for other members of *Pasteurellaceae* as well, with regions encoding export, polysaccharide biosynthesis, and proteins involved in post-polymerization modifications, respectively. In *Actinobacillus pleuropneumoniae*, the genes *cpxDCBA* are involved in export of capsular material, and their DNA sequences were conserved in all serotypes, whereas genes of the *cps* cluster showed high variability between serotypes (Xu et al. 2010). Serotypes 1, 9, and 11 were closely related to each other than to the other serotypes based on DNA sequence comparison of capsular genes (Xu et al. 2010). This relationship is also recognized on whole genome level (*Fig. 26.2*). The capsular types of *Haemophilus influenzae* and *Pasteurella multocida* can be determined by PCR (Falla et al. 1994; Lam et al. 2011; Townsend et al. 2001).

LPS and LOS. Lipopolysaccharides (LPS) are glycolipids found on the outer membrane. LPS appear as a ladder of distinct evenly separated bands that span the length of a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) in which each higher-sized band represents an additional O antigen subunit. Lipooligosaccharide (LOS) appears on the SDS-PAGE as a heterogeneous mixture of closely spaced bands of low molecular size (see Fig. 9.1 in Inzana et al. (2008)). On DNA level, LOS are encoded by tandem repeats with a translational switch (High et al. 1996). LOS have been considered as O antigen-deficient LPS (Harper et al. 2011). Most species of *Actinobacillus*, *Aggregatibacter*, and *Mannheimia* produce LPS, whereas most members of *Haemophilus*, *Histophilus*, and *Pasteurella* produce LOS (Inzana et al. 2008; Siddaramappa et al. 2011). The biochemical and genetic background for the Heddleston serotypes of *Pasteurella multocida* has been investigated by Harper et al. (2011). It was found that serotypes 1 and 14 shared the same genetic structure of the LPS locus and that serotypes 2 and 5 also have high similarities on the genetic level. A diagnostic PCR that will substitute the traditional serotyping is being developed.

Extracellular appendages in the *Pasteurellaceae*. Two types of pili have been described in *Pasteurellaceae* species. Flp pili and the genomic locus that encodes their assembly (the *tad* locus) have been most fully studied in *Aggregatibacter actinomycetemcomitans* (Tomich et al. 2007). *Tad* loci are also found in multiple other species including *Pasteurella multocida*, *Actinobacillus*

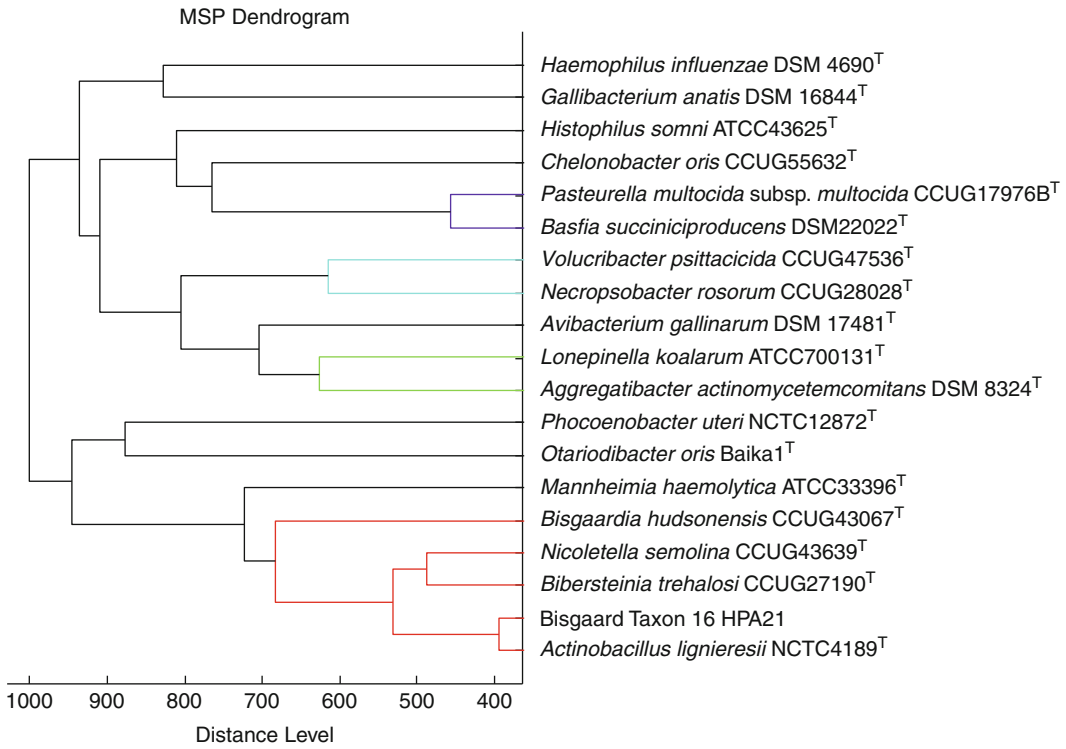

Fig. 26.3

Score-oriented dendrogram based on mass spectra profiles (MSP) of type species of currently validated genera and Bisgaard taxon 16. The dendrogram was generated in Biotyper V.3.0. MSP were taken from the Biotyper database and from Kuhnert et al. (2012)

pleuropneumoniae, [*Haemophilus*] *ducreyi*, *and Aggregatibacter aphrophilus* but are not found in *Haemophilus influenzae*. Indeed, 9 out of the 13 *Pasteurellaceae* species with available whole genome sequences have loci that are highly similar to tad loci. Flp pili and the genes responsible for their assembly have been shown to be important in phenotypes such as adherence, biofilm formation, and colony morphology (Kachlany et al. 2000, 2001a, b; Labrie et al. 2010; Li et al. 2012; Nika et al. 2002; Planet et al. 2003; Spinola et al. 2003). In *Aggregatibacter actinomycetemcomitans* (Schreiner et al. 2003), [*Haemophilus*] *ducreyi* (Janowicz et al. 2011; Nika et al. 2002; Spinola et al. 2003), *Pasteurella multocida* (Guo et al. 2012; Harper et al. 2003; Nika et al. 2002; Spinola et al. 2003), and *Actinobacillus pleuropneumoniae* (Auger et al. 2009), the *tad* locus has been implicated in the disease process.

The second type of pili is referred to as the classical type IV pili (TFP). TFP have been shown to be involved in twitching motility (Bakaletz et al. 2005) biofilm formation and colonization of the upper airway (Jurcisek and Bakaletz 2007; Jurcisek et al. 2007) in *Haemophilus influenzae*. Genes that appear to encode a TFP in *Aggregatibacter actinomycetemcomitans* are required for natural DNA competence (Wang et al. 2003). TFP have also been identified in *Actinobacillus pleuropneumoniae* (Stevenson et al. 2003) where they are regulated in response to host cells (Boekema et al. 2004). TFP are also found in *Pasteurella multocida* (Doughty et al. 2000; Ruffolo et al. 1997).

Detection and Identification of Members of *Pasteurellaceae*

Traditional isolation techniques are still used in diagnostic laboratories to allow subsequent characterization to investigate epidemiology, prognosis, and possible prevention of disease. An alternative to culture-based identification of members of *Pasteurellaceae* is microscopic detection in specimens prepared for histopathology (Blackall and Nørskov-Lauritsen 2008). Fluorescent in situ hybridization (FISH) has been developed for specific detection of *Haemophilus influenzae* (Hogardt et al. 2000), *Pasteurella multocida* (Mbuthia et al. 2001), and *Gallibacterium* (Bojesen et al. 2003). PCR detection methods for specific detection of members of *Pasteurellaceae* have recently been reviewed (Christensen et al. 2003a; Dziva et al. 2008) and are now widely used on DNA from tissue extracts directly.

Procedures for traditional culture-based identification are available from common textbooks. Lists of specialized methods for identification of *Pasteurellaceae* have been provided (Christensen et al. 2007; Kilian and Frederiksen 1981). For species level identification, PCR methods are available for the most important members of *Pasteurellaceae* (Christensen et al. 2003a; Dziva et al. 2008) including [*Haemophilus*] *parasuis* (Frandoloso et al. 2012; Turni et al. 2010). The kmt1-based PCR (Townsend et al. 1998) is specific for *Pasteurella multocida* (Register and DeJong 2006; Sellyei et al. 2008).

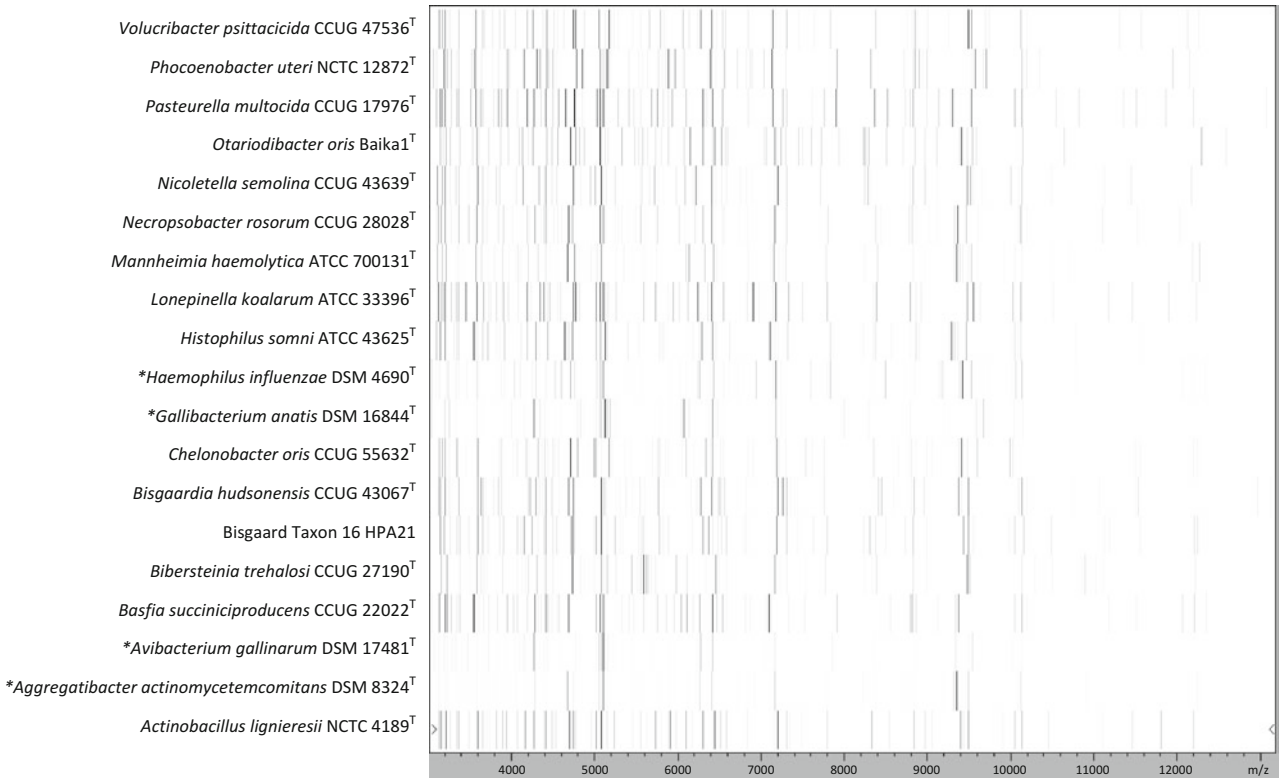

Fig. 26.4

Comparison of mass spectra profiles (MSP) of type species of currently validated genera and Bisgaard taxon 16. Peak locations and their relative intensities as contained in the MSP are indicated. MSP were taken from the Biotyper database (*indicated by an asterisk*) and from Kuhnert et al. (2012)

PCR detection methods have also been highly efficient for further identification of populations of special importance below the species level. More specialized typing methods based on PCR have been used for investigation of virulence-associated genes as well as for serotyping (see above). Prediction of virulence of *Pasteurella multocida* based on PCR-based profiling of virulence-associated genes (VAG) showed relationships to hosts and to some extent also to lesions observed (Ewers et al. 2006). Investigation of pig isolates of *Pasteurella multocida* showed strong clonal groupings of the VAGs (Bethe et al. 2009).

The MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry is a fast, low-cost, and universal method for bacterial identification which has revolutionized diagnostics (Croxatto et al. 2012). The method is based on the detection of (mainly ribosomal) protein masses resulting in a specific profile/fingerprint that is compared against a database for identification. For *Pasteurellaceae* it has so far been used for investigation of the genus *Gallibacterium* (Alispahic et al. 2011, 2012), description of *Haemophilus sputorum* (Norskov-Lauritsen et al. 2012), and recently extended for identification of members of the entire family (Kuhnert et al. 2012). It has been proved to be a very reliable approach with a high discrimination being achieved at the genus and species level. Generally, there was only little influence due to culture variations. Moreover, the simple direct transfer method by spotting single colony material directly to the steel plate template without further purification was applicable. High homogeneity and therefore high reproducibility was observed within species; however, slight discrepancies in the identification scores were seen when comparing reference spectra of a commercial database and own-generated reference spectra (Kuhnert et al. 2012). Even though the method can normally compensate for such differences, it is recommended to generate own reference databases for optimal identification and discrimination. Reference spectra can also be exchanged between laboratories if the corresponding species are not covered by the commercial databases. Only phenotypically and phylogenetically closely related species and subspecies might be problematic to be clearly distinguished by MALDI-TOF, analogous to known classical identification problems. These include, e.g., *Actinobacillus pleuropneumoniae* and *Actinobacillus lignieresii*, and [*Actinobacillus*] *minor*. However, such species can be further identified by conventional tests like RTX-toxin typing. Given the speed, high throughput, and low cost, MALDI-TOF is a straightforward technique for identification of *Pasteurellaceae* or for screening and monitoring purposes. ❯ *Figure 26.3* shows the relationship of genera based on MALDI-TOF analysis.

The *Pasteurellaceae* generally show many mass peaks which is an ideal prerequisite for proper separation of taxa and their identification (❯ *Fig. 26.4*). This is also a promising basis for its application in taxonomic studies. The profiles shown in ❯ *Fig. 26.4* are very diverse and allow identification of specific

Table 26.7
Antibiotic resistance to selected antibiotics

Antibiotic agent	Species	Resistance	References
Amoxicillin/clavulanic acid	*Mannheimia haemolytica*	Few	Cote et al. (1991), Klima et al. (2011)
Ampicillin	*Pasteurella multocida*	Few	McKay et al. (1996), Kehrenberg et al. (2001)
	Mannheimia haemolytica	Some	Kehrenberg et al. (2006)
	Haemophilus influenzae	Few	Leaves et al. (2000)
Penicillin	*Pasteurella multocida*	Some	McKay et al. (1996)
	[*Pasteurella*] *aerogenes*	NI	Kehrenberg et al. (2001)
	Haemophilus influenzae	Few	Leaves et al. (2000)
Ticarcillin	[*Pasteurella*] *aerogenes*	NI	Frederiksen (1989)
	Pasteurella multocida	NI	Blackwood et al. (1996)
Ceftriaxone	*Pasteurella multocida*	NI	Boerlin et al. (2000)
Ceftiofur	*Pasteurella multocida*	Few	Kehrenberg et al. (2001), Salmon et al. (1995)
	Mannheimia haemolytica	Few	Klima et al. (2011)
Cefquinome	*Pasteurella multocida*	Some	Kehrenberg et al. (2001), Michael et al. (2012)
	Mannheimia haemolytica	Some	Michael et al. (2012)
Erythromycin	*Pasteurella multocida*	None	McKay et al. (1996), Kehrenberg et al. (2001), Salmon et al. (1995)
	Mannheimia haemolytica	Some	Kehrenberg et al. (2006)
	Avibacterium paragallinarum	Most	Schwarz (2008)
Tilcomycin	*Pasteurella multocida*	None	McKay et al. (1996), Kehrenberg et al. (2001)
Tylosin	*Pasteurella multocida*	Some	Kehrenberg et al. (2001)
Gentamicin	*Pasteurella multocida*	None	McKay et al. (1996), Snipes et al. (1989)
	Mannheimia haemolytica	None	Klima et al. (2011)
Kanamycin	*Pasteurella multocida*	None	Snipes et al. (1989), Cote et al. (1991)
	[*Pasteurella*] *aerogenes*	None	Kehrenberg et al. (2006)
Spectinomycin	*Pasteurella multocida*	Few	Kehrenberg et al. (2006)
	Mannheimia haemolytica	Few	Kehrenberg et al. (2006)
Streptomycin	[*Pasteurella*] *aerogenes*	All	Kehrenberg et al. (2001)
	Pasteurella multocida	Some	McKay et al. (1996)
	Avibacterium paragallinarum	Most	Schwarz (2008)
Minocycline	*Pasteurella multocida*	None	Chaslus-Dancla et al. (1995)
Tetracycline	[*Pasteurella*] *aerogenes*	All	Kehrenberg and Schwarz (2001)
	Pasteurella multocida	Few	Kehrenberg et al. (2001), McKay et al. (1996)
Oxytetracycline	*Mannheimia haemolytica*	Few	Klima et al. (2011)
	[*Pasteurella*] *aerogenes*	Most	Kehrenberg and Schwarz (2001)
	Pasteurella multocida	Most	Kehrenberg et al. (2001), McKay et al. (1996)
	Mannheimia haemolytica	Few	Klima et al. (2011)
Trimethoprim	[*Pasteurella*] *aerogenes*	Most	Kehrenberg and Schwarz (2001)
	Pasteurella multocida	Few	McKay et al. (1996)
Sulfonamide	*Pasteurella multocida*	Some	Schwarz (2008)
	Mannheimia haemolytica	Some	Kehrenberg et al. (2006)
Sulfamethoxazole/ trimethoprim	*Mannheimia haemolytica*	Few	Klima et al. (2011)
	Haemophilus influenzae	Most	Schwarz (2008)
	Pasteurella multocida	Few	Schwarz (2008)
Chloramphenicol	[*Pasteurella*] *aerogenes*	Some	Kehrenberg et al. (2001)
	Pasteurella multocida	Most	Kehrenberg et al. (2001)

Table 26.7 (continued)

Antibiotic agent	Species	Resistance	References
Enrofloxacin	*Pasteurella multocida*	Few	Kehrenberg et al. (2001), Salmon et al. (1995)
	Mannheimia haemolytica	Few	Klima et al. (2011), Schwarz (2008)
Marbofloxacin	*Pasteurella multocida*	None	Spreng et al. (1995)
Nalidixic acid	*Pasteurella multocida*	Most	Kehrenberg et al. (2001)
Florfenicol	*Mannheimia haemolytica*	Rare	Schwarz (2008)
	Pasteurella multocida	Rare	Schwarz (2008)
	Actinobacillus pleuropneumoniae	Rare	Schwarz (2008)
Fluoroquinolones	*Haemophilus influenzae*	Few	Schwarz (2008)
Neomycin	*Pasteurella multocida*	None	McKay et al. (1996)
	Avibacterium paragallinarum	Most	Schwarz (2008)
Nitrofurantoin	*Pasteurella multocida*	None	McKay et al. (1996)
Novobiocin	*Pasteurella multocida*	NI	Morris et al. (1989), Morris et al. (1989)
Spiramycin	*Pasteurella multocida*	NI	Kehrenberg et al. (2001)
Tilmicosin	*Mannheimia haemolytica*	Few	Schwarz (2008)
	Pasteurella multocida	Few	Schwarz (2008)
Tulathromycin	*Mannheimia haemolytica*	Few	Schwarz (2008)
	Pasteurella multocida	Few	Schwarz (2008)

Abbreviations: none, 0 %; few, 1–33 %; some, 34–66 %; most 67–99 %; all, 100 % of strains; and NI, no information

peaks that discriminate the genera. Especially the peaks between 9,000 m/z and 10,000 m/z could be used for such purposes. However, including more species of genera into such analyses has to be considered to define stable markers.

Given its simplicity and speed of analysis, MALDI-TOF is a suitable screening tool for identification of not only known but also new taxa. The open system of the commercially available platforms allows building of own databases that can be fed with yet uncharacterized isolates. Once in the databases, new groups can easily be recognized in future samples, and data from MALDI-TOF together with other analyses in a polyphasic approach should be used for description of new species.

In conclusion, traditional isolation techniques are still needed in diagnostic laboratories. When isolated, identification, however, is difficult based on traditional biochemical/physiological characterization. Instead, MALDI-TOF should be used. As a control and if MALDI-TOF is not at hand, PCR-based specific detection is possible for *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, [*Haemophilus*] *parasuis*, *Mannheimia haemolytica*, *Avibacterium paragallinarum*, *Haemophilus influenzae*, and *Aggregatibacter actinomycetemcomitans*. Isolates remaining unidentified by these methods should be subjected sequencing of the 16S rRNA gene and eventually selected housekeeping genes such as *rpoB* and *infB* for identification.

Application

Only few members of *Pasteurellaceae* are used as production organisms in biotechnology. Industrial application of *Basfia succiniproducens* and [*Actinobacillus*] *succinogenes* is performed with respect to succinic acid that is produced under anaerobic conditions (Scholten et al. 2009; Van der Werf et al. 1997).

Control

Antibiotic resistance. An overview of antibiotic resistance reported in members in *Pasteurellaceae* is given in *Table 26.7*. Standardized and internationally accepted guidelines for in vitro testing are only available for *Haemophilus influenzae* (Schwarz 2008) (http://mic.eucast.org/Eucast2/SearchController/search.jsp?action=performSearch&BeginIndex=0&Micdif=mic&NumberIndex=50&Antib=-1&Specium=10). For veterinary important members of *Pasteurellaceae*, only recommendations are available. With respect to mechanisms and genetic backgrounds for antibiotic resistance, we refer to the reviews and monographs on the topic (Kehrenberg et al. 2006; Schwarz 2008). In the latter reference (Table 10.1), an overview of genes and mutations in members of *Pasteurellaceae* is provided. Genes providing resistance to chloramphenicol, florfenicol, kanamycin, neomycin, penicillins, streptomycin, spectinomycin, and tetracycline have been identified on small (4–11 kb) plasmids in at least nine species of *Pasteurellaceae* (Kehrenberg et al. 2006).

Vaccines. Commercial vaccines for members of *Pasteurellaceae* are widely used for the protection of animals. In humans only vaccines against *Haemophilus influenzae* type b are used. The classical live vaccines might revert to the virulent wild type or just become pathogenic under conditions such as stress or by immunosuppression (Morris et al. 1989).

Table 26.8

Vaccines for prevention of diseases in relation to members of *Pasteurellaceae*. Either live attenuated bacteria or bacterins are used as vaccines. With live attenuated vaccines, immunity might be obtained against different serotypes

Name	Disease	Species of *Pasteurellaceae*	References
Classical live vaccine			
CU and M-9 strains	Fowl cholera	*Pasteurella multocida*	Kim and Nagaraja (1990)
aroA mutant strains	Fowl cholera	*Pasteurella multocida*	Homchampa et al. (1997), Scott et al. (1999)
Avichol, Orachol, PM one vax, M Nine, Vax C, Choleravac-PM-1, ViClemcol-C	Fowl cholera	*Pasteurella multocida*	
Inactivated (**killed vaccines**)			
Coryza vaccine with strains of serovars A, B, and C	Infectious coryza	*Avibacterium paragallinarum*	Blackall and Soriano (2008)
Volvac Gallibacterium a.	Salpingitis and peritonitis	*Gallibacterium anatis*	
Inacti VACFC3 With strains of Heddleston serovars 1, 3, and 4	Fowl cholera	*Pasteurella multocida*	
PABAC IV with strains of Heddleston serovars 1, 3, 4, and 3x4	Fowl cholera	*Pasteurella multocida*	
Porcilis® Glässer Strain 4800 of serovar 5	Glässer's disease	[*Haemophilus*] *parasuis*	Frandoloso et al. (2011)
Bacterins			
HbOC PRP-OMP PRP-T PRP-D	Pneumonia, meningitis	*Haemophilus influenzae*	Fink and St. Geme (2006)
Purified OMP of *P. multocida*	Fowl cholera	*Pasteurella multocida*	Ruffolo and Adler (1996)

The alternative is to use killed bacteria or cellular components (bacterin). 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. Vaccines are most widely used for protection against *Pasteurella multocida*, *Mannheimia haemolytica*, and *Haemophilus influenzae* (Table 26.8). For the latter, the Hib (*Haemophilus influenzae* serotype B) conjugate vaccine was introduced into national childhood immunization programs in the 1990s. It is based on the polysaccharide polyribosylribitol phosphate (PPP) (Fink and St. Geme 2006). Vaccination with this type, however, seems to have led to an increase in the incidence of non-capsulated *Haemophilus influenzae* (Ladhani et al. 2010). Recently, an edible alfalfa has been constructed with the GC60 outer membrane protein of *Mannheimia haemolytica* (Lee et al. 2008). The proteins extracted from the alfalfa were able to stimulate an immune response in rabbits, and one rabbit responded to the vaccine after having eaten the alfalfa.

Ecology and Host Association

To investigate associations between members of *Pasteurellaceae* and their vertebrate hosts at the level of orders, the family was separated into 52 genera-like groups by 16S rRNA sequence comparisons. The recognized monophyletic genera listed in Table 26.1 were included in addition to 34 mainly monotypic genera-like groups (Tables 26.2, 26.3). Association between a single genus-like group of *Pasteurellaceae* and a single-animal host group has been found for only two out of the 52 genera-like groups of *Pasteurellaceae* (Model A in Fig. 26.5). Red panda enabled isolation of a new genus-like group, and previously, the monotypic genus *Lonepinella koalarum* has been reported isolated from the alimentary tract of koala only (Osawa et al. 1995) (Tables 26.2, 26.3). Model B (Fig. 26.5), in which more genera-like groups of *Pasteurellaceae* are associated with a single host is the most common, found 40 genera-like groups with 65 species or species-like taxa. To name some of these associations, the group of

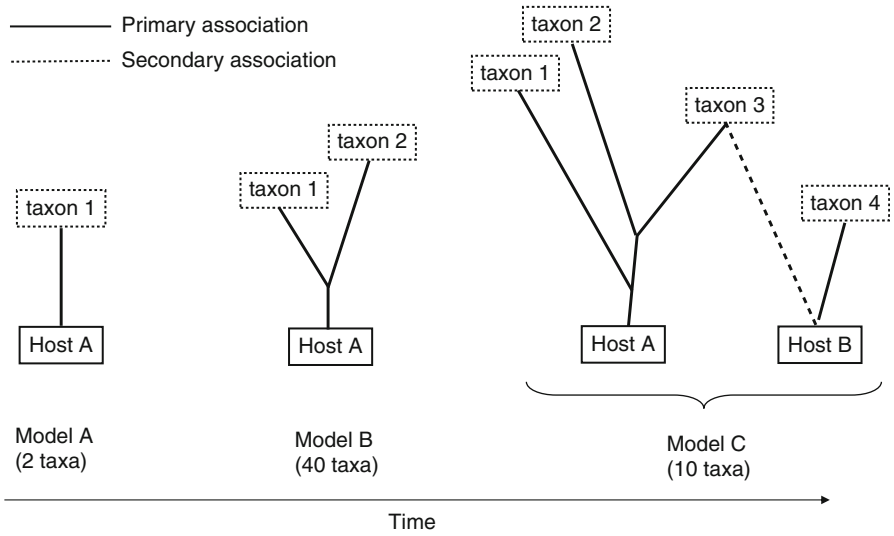

Fig. 26.5

Coevolution of members of *Pasteurellaceae* and their vertebrate hosts (level of order) according to three models on an evolutionary time line. In model A, association between a single genus-like group of *Pasteurellaceae* and a single-animal host group is shown. Most genera-like groups of *Pasteurellaceae* are associated a single host as shown in model B, and the same animal host has more of these groups associated. The third group of genera or genera-like taxa are associated more hosts groups, and this association is assumed to have developed secondary and late in the evolution of the family (Model C)

taxa 29/30 and [*Haemophilus*] *paracuniculus* is associated with the animal order *Lagomorpha* which includes rabbits and hares. A genus-like group including [*Pasteurella*] *pneumotropica* and the three divergent taxa [*Actinobacillus*] *muris* and the taxa 5 and 7 of Bisgaard are associated with the order *Rodentia*, which includes mice and rats. The species [*Pasteurella*] *langaaensis* and members of *Avibacterium* are associated with the order *Galliformes* (chicken and related birds), whereas two other divergent taxa, *Volucribacter* and taxon 44, are associated with the order *Psittaciformes* (parrots and related birds). Two related genera-like groups *Chelonobacter* and [*Pasteurella*] *testudinis* are associated with the order *Testudines* (turtles). Host relationships for the order of *Primates* is observed for the six genera-like groups *Haemophilus sensu stricto*, *Haemophilus pittmaniae*, [*Haemophilus*] *parainfluenzae*, [*Haemophilus*] *ducreyi*, [*Pasteurella*] *bettyae*, and *Aggregatibacter* (Fig. 26.5). With respect to suborder *Selenodontia* (cows and other ruminants), five divergent genera-like groups of *Pasteurellaceae*, *Basfia*, [*Actinobacillus*] *seminis*, *Bibersteinia*, *Histophilus*, and [*Actinobacillus*] *succinogenes* have been found associated. Model C in which a single genus or genus-like group of *Pasteurellaceae* is associated with many hosts (Fig. 26.5) is observed for the ten groups: *Actinobacillus sensu stricto*, *Mannheimia*, Aerogenes, Caballi, *Pasteurella sensu stricto*, taxon 6/10, *Necropsobacter*, taxon 16, taxon 14/32, and *Gallibacterium*. For all species of *Actinobacillus sensu stricto* except *Actinobacillus equuli*, four out of the six species of *Mannheimia*, and some taxa of *Pasteurella sensu stricto*, host associations exist at the species level (Table 26.2). It is assumed that these associations including diversity as to hosts for some species (*Pasteurella multocida*) developed secondary to primary associations of a genus-like group (Model C in Fig. 26.2). Species with more than a single host relationship such as *Pasteurella multocida* and *Actinobacillus equuli* are expected to have evolved this dual association secondary. It might be assumed that *Pasteurellaceae* to some extent have evolved in close contact with vertebrates. Only minor changes in the genotype might change the host reservoir as to disease as observed for *Pasteurella multocida* in which only a single restriction fragment differed between isolates from outbreaks in the wild avifauna and domestic chicken (Eigaard et al. 2006).

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27 The Genus *Piscirickettsia*

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Abstract
The genus *Piscirickettsia* is part of the *Piscirickettsiaceae* family, belonging to the *Gammaproteobacteria* class within the *Thiotrichales* order. The family contains seven phylogenetically related genera (*Cycloclasticus*, *Hydrogenovibrio*, *Sulfurivirga*, *Thioalkalimicrobium*, *Methylophaga*, *Thiomicrospira*, and *Piscirickettsia*), with highly diverse characteristics, making them very different from one another. The genus *Piscirickettsia* comprises a single species called *Piscirickettsia salmonis*, a Gram-negative facultative intracellular fish pathogen that significantly affects the salmon industry. Since its first isolation in Chile in 1989, the bacterium has been reported in Norway, Scotland, Greece, Canada, and the USA, among others. To date, the complete genome sequence of *P. salmonis* has not been reported, and relevant aspects of its metabolism, virulence, and life cycle are still poorly understood.

Historical and Current Taxonomy

Short Description of the Other Organisms of the *Piscirickettsiaceae* Family

The *Piscirickettsiaceae* family is composed of seven genera, *Piscirickettsia*, *Cycloclasticus*, *Hydrogenovibrio*, *Sulfurivirga*, *Thioalkalimicrobium*, *Methylophaga*, and *Thiomicrospira*, which are all phylogenetically related, but with very different phenotypic characteristics. Unlike the *Piscirickettsia* genus, the other members of this family are motile, and morphologically they are rod-, comma-, or spiral-shaped bacteria (Fryer and Lannan 2005). Although the current taxonomy places these seven genera within the *Piscirickettsiaceae* family, it isclear from a phylogenetic tree based on 16S rDNA (◉ *Fig. 27.1*) that these genera do not cluster neatly into a single family and that taxonomic reclassification will be necessary to accurately depict relationships among these bacteria.

The organisms of the *Cycloclasticus* genus have been characterized as Gram-negative rod-shaped bacteria, with a cell size of 0.5 × 1.0–2.0 μm; the cells are motile due to a single polar flagellum (Geiselbrecht 2005). Only one species has been reported in this genus, *Cycloclasticus pugetii* (ATCC 51542), which grows poorly on complex bacteriological media containing no aromatic compounds. Aromatic compounds, including biphenyl, naphthalene, phenanthrene, anthracene, and toluene, are used as sole or principal carbon sources for growth (Dyksterhouse et al. 1995). Bacteria of this genus play a key role in the degradation of low-molecular-weight polycyclic aromatic hydrocarbons (PAHs) in marine environments (Niepceron et al. 2010). *Cycloclasticus* sp. A5 has been suggested as a major degrader of petroleum aromatics spilled in temperate seas, showing the highest degrading activity for petroleum aromatics at 25 °C (Teramoto et al. 2010).

Bacteria of the *Hydrogenovibrio* genus are comma shaped with a cell size of 0.2–0.5 × 1–2 μm and motile by means of a single polar flagellum (Nishihara 2005). The unique species of this genus, *H. marinus*, is an obligately chemolithoautotrophic marine bacterium that uses molecular hydrogen or reduced sulfur compounds, such as elemental sulfur, thiosulfate, and tetrathionate, as electron donors and carbon dioxide as its carbon source (Nishihara et al. 1991). A taxonomic analysis of the amino acid sequences of the two *H. marinus* ribulose-1,5 bisphosphate carboxylase oxygenases (RuBisCO), the enzyme required for carbon fixation, shows that they are similar to those of the sulfur oxidizer *Thiobacillus* and to a purple sulfur bacterium *Chromatium vinosum*, suggesting that an ancestor of

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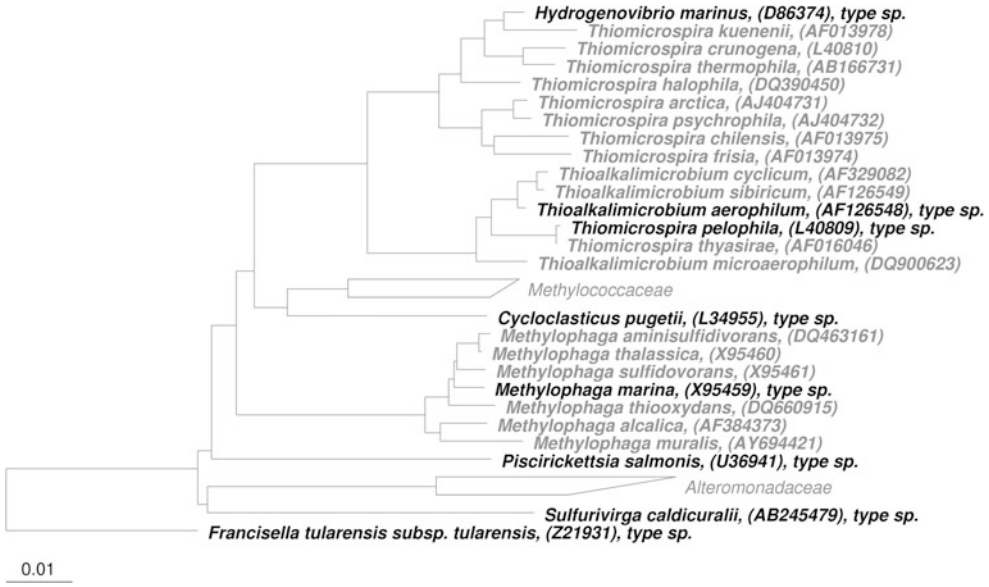

Fig. 27.1

Phylogenetic reconstruction of the family *Piscirickettsiaceae* based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes-Cantor correction. The sequence datasets and alignments were used according to the All-Species Living Tree Project (*LTP*) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high-quality type strain sequences proportionally distributed among the different bacterial and archaeal phyla. In addition, a 40 % maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. Scale bar indicates estimated sequence divergence

purple sulfur bacteria might be a common root of *H. marinus* and related sulfur oxidizers (Nishihara et al. 1998).

The *Methylophaga* genus is composed of rod-shaped bacteria with a cell size of 0.2 × 0.9–1.0 μm, which are motile by means of a single polar flagellum. These organisms are chemoheterotrophic (methylotrophic), having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor (Bowman 2005). This genus contains eight species: *M. alcalica*, *M. aminisulfidivorans*, *M. lonarensis*, *M. marina*, *M. murata*, *M. sulfidovorans*, *M. thalassica*, and *M. thiooxidans*. Members of this genus have been isolated from various marine sediments and soda lakes, and they play an important role in coastal environments by participating in biogeochemical cycling of one-carbon substrates containing nitrogen, sulfur, or halogens (Han et al. 2011). Genome sequences have been reported only for *M. thiooxidans* (strain DMS010T), *M. aminisulfidivorans* (strain MPT), and *Methylophaga* sp. strains JAM1 and JAM7 (Villeneuve et al. 2012).

The genus *Thiomicrospira* is composed of nine Gram-negative species: *T. arctica, T. chilensis, T. crunogena, T. frisia, T. halophila, T. pelophila, T. psychrophila, T. thyasirae*, and *T. thermophila*. The general characteristics of these species are small spiral-, comma-, or rod-shaped cells, with a size of 0.2–0.5 × 0.8–3.0 μm, either nonmotile or motile due to a single polar flagellum. These organisms are aerobic, chemolithoautotrophic, using reduced inorganic sulfur compounds and CO_2 as carbon source (Brinkhoff et al. 2005). This genus appears to be ecologically significant in hydrothermal vent systems, though in this environment the genus is found in lower densities than other sulfur-oxidizing bacteria (Brinkhoff et al. 1998).

The genus *Sulfurivirga* is composed of only one species, *S. caldicuralii* (strains VW1 and MM1^T), collected from a shallow marine hydrothermal system (water depth 22 m) occurring in coral reefs off Taketomi Island, Okinawa, Japan. These organisms are motile rods with a single polar flagellum in the exponential growth phase and with a cell size of 0.3–0.6 × 1–3 μm; they are microaerobic chemolithoautotrophs capable of using thiosulfate or tetrathionate as their sole energy source, O_2 as the sole electron acceptor, and CO_2 as the sole carbon source (Takai et al. 2006).

Thioalkalimicrobium contains four species, *T. aerophilum*, *T. cyclicum*, *T. microaerophilum*, and *T. sibiricum*. These bacteria are motile due to 1–3 polar flagella and their cells are curved rods or spiral shaped with a size of 0.4–0.5 × 0.8–1.5 μm (Sorokin et al. 2001). In general, the genus *Thioalkalimicrobium* includes fast-growing species with high activity for thiosulfate and sulfide oxidation but relatively low salt tolerance; most of the strains were obtained from low-mineralized steppe soda lakes (Sorokin et al. 2002). *Thioalkalimicrobium* species are obligate chemolithoautotrophs and are able to grow only in the presence of thiosulfate or sulfide (Sorokin et al. 2001).

Characteristics of the *Piscirickettsia* Genus

The *Piscirickettsia* genus is composed of one species, *P. salmonis*, which is a fish pathogen. The wild-type strain,

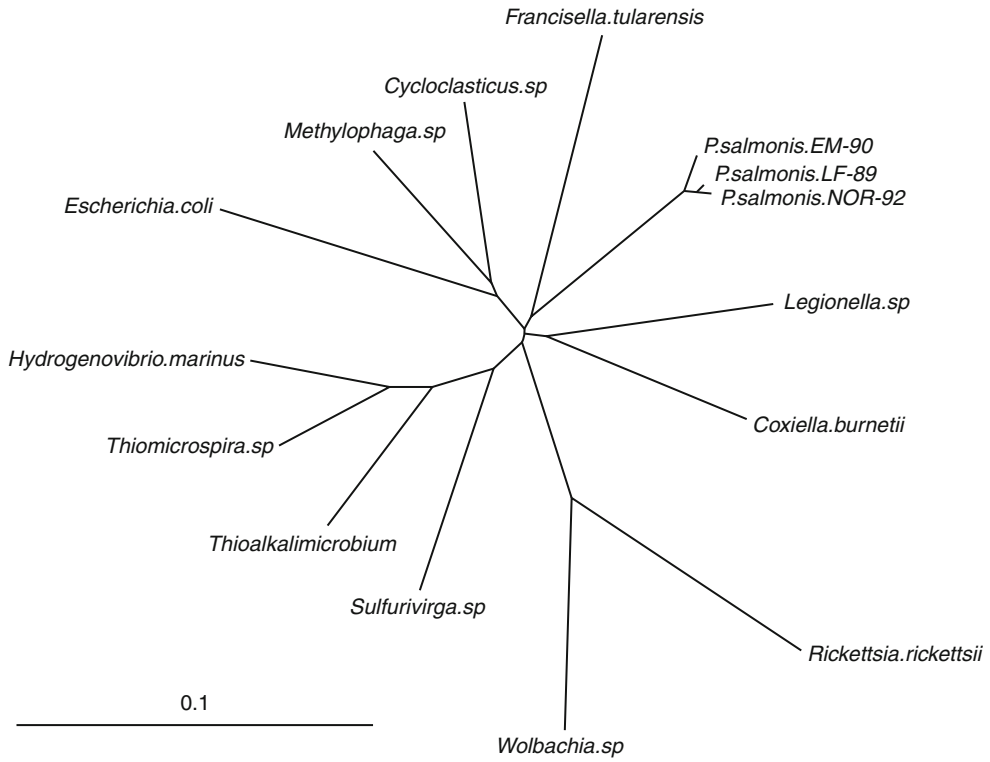

Fig. 27.2

16S cladogram of bacteria from the *Piscirickettsiaceae* family and related organisms from Gamma and Alphaproteobacteria groups. The 16S sequences were aligned with ClustalW software and the cladogram was made using the TreeView software by maximum parsimony methods

LF89 (ATCC VR-1361), was the first Gram-negative intracellular bacterium isolated from fish. The bacterium was initially isolated in 1989 from coho salmon (*Oncorhynchus kisutch*) specimens affected by high mortality (70–90 %) in southern Chile (Bravo and Campos 1989). *P. salmonis* was initially classified via phenotypic characterization as a member of the *Alphaproteobacteria* class in the order *Rickettsiales*, in the *Rickettsiaceae* family, and was phylogenetically related with the genera *Rickettsia*, *Wolbachia*, *Anaplasma*, and *Ehrlichia* (Fryer et al. 1990). Closer analysis of the 16S rRNA led to the reclassification of *P. salmonis* in the *Gammaproteobacteria* class in the *Thiotrichales* order, constituting the new genus and species *Piscirickettsia salmonis* gen. nov., sp. nov. (Fryer et al. 1992) which is phylogenetically related to the genera *Francisella*, *Legionella*, and *Coxiella* (Fig. 27.2).

In addition to the wild-type strain LF89, other strains have been reported at different latitudes. CI-95 was isolated from coho salmon in Canada in 1995 (data unpublished), ATL-491 was isolated form Atlantic salmon in Canada in 1992 (Brocklebank et al. 1992), NOR-92 was isolated from Atlantic salmon in Norway in 1992 (data unpublished), and SLGO-94 was obtained from rainbow trout (*Oncorhynchus mykiss*) in Chile in 1994 (data unpublished). Additionally, EM-90, a putative novel strain isolated in Chile in 1990 from Atlantic salmon (*Salmo salar*), as well as EM-90-like counterparts reported in Norway, is the most phylogenetically distant specimens of the group.

Phylogenetic analysis of the 16S, internal transcribed spacer (ITS), and 23S ribosomal RNA of these strains reveals that they form a tight monophyletic cluster in the Gammaproteobacteria class, where five of the strains (LF-89, CI-95, NOR-92, ATL-491, and SLGO-94) have 16S rRNA similarities of 99.4 %, ITS similarities between 99.1 %and 99.7 %, and 23S similarities ranging from 99.3 % to 99.8 %; only the Chilean strain EM-90 shows low similarities with the other strains (98.5–98.9 % for 16S, 95.2–96.9 % for ITS, and 97.6–98.5 % for 23S) (Mauel et al. 1999) (Fig. 27.3).

Morphology and Phenotypic Characteristics

P. salmonis is a nonmotile, nonencapsulated, pleomorphic but generally coccoid bacterium, with a diameter ranging from 0.1 to 1.5 μm (Cvitanich et al. 1991). The pathogen is able to infect fish cell lines producing strong cytopathic effect (CPE) characterized by the production of clusters of rounded and vacuolated cells (Fig. 27.4A), culminating in detachment of the monolayer (Fryer et al. 1990; Rojas et al. 2008). Five susceptible fish cell lines have been derived from salmonid species: CHSE-214 derived from Chinook salmon (*Oncorhynchus tshawytscha*), CHH-l derived from chum salmon (*Oncorhynchus keta*) heart, CSE-119 derived from coho salmon embryo, RTG-2 derived from rainbow trout gonad, and RTS11 (Fig. 27.4B) derived

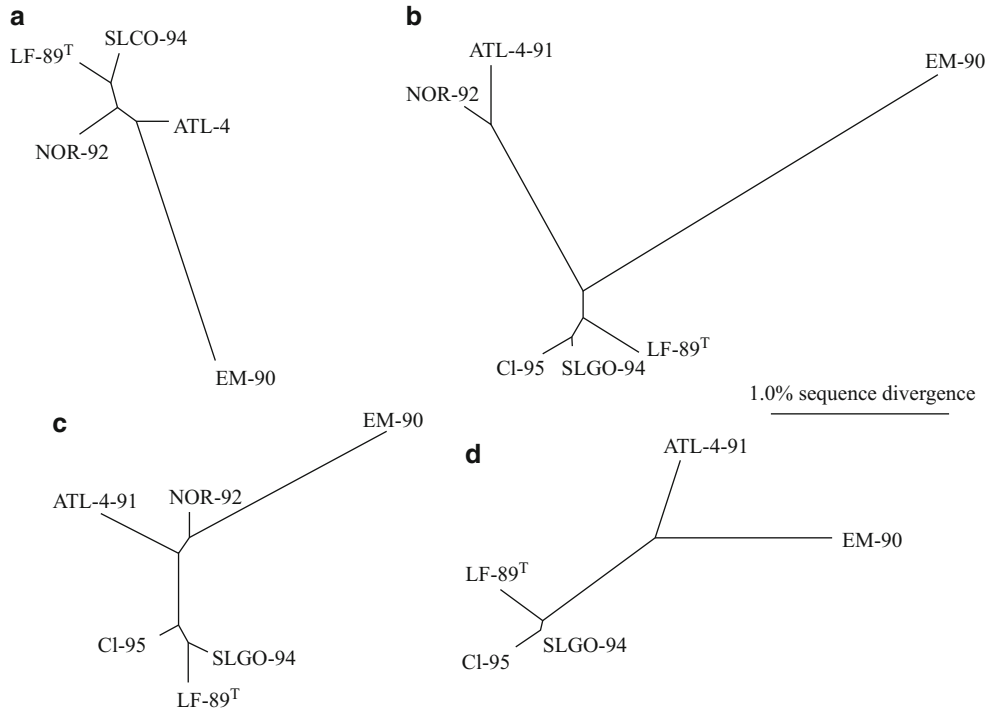

Fig. 27.3

Cladograms of phylogenetic relationships among *P. salmonis* strains. (a) 16S rDNA, (b) ITS (16S–23S internal transcribed spacer), (c) the first 450 bp of 23S rDNA sequences, and (d) 1902 bp of 23S rDNA sequence. Evolutionary distances were calculated by the method of Jukes and Cantor and the trees inferred by distance method of De Soete (Mauel et al. 1999)

from rainbow trout spleen (Fryer et al. 1990; Cvitanich et al. 1991; Ganassin and Bols 1998; Rojas et al. 2009). Other cell lines from warm water fish species are also susceptible to *P. salmonis* such as EPC (epithelioma papulosum cyprini) from *Cyprinus carpio* and FHM (fat head minnow) from *Pimephales promelas*, derived from caudal fin epithelial cells (Almendras et al. 1997). Additionally, an insect cell line (Sf21) has also been reported to be susceptible to *P. salmonis* infection, producing high pathogen titers after 15 days postinfection, considerably superior to the titers produced in fish cell lines (Birkbeck et al. 2004).

A small isoform of the wild-type strain LF-89, named sP.s, has been reported. It has a diameter less than 0.2 μm (*Fig. 27.3d*) and has been isolated in vivo from sick fish and in vitro from 1 month-infected CHSE-214 cell culture. This variant is highly infective and exhibits some genetic differences within the ITS region to the wild-type strain. The authors suggest that sP.s represents a selectable infective variant of the LF-89 strain and not new a strain, likely resulting from a survival strategy of the bacteria in response to limiting growth conditions. As such, sP.s could be responsible for horizontal infection of fish in the field (Rojas et al. 2008).

P. salmonis is considered a facultative intracellular organism due to its ability to grow in cell-free media (*Fig. 27.3d*), although with low growth efficiency in liquid media (Mauel et al. 2008; Mikalsen et al. 2008; Gómez et al. 2009; Yañez et al. 2012). The typical *P. salmonis* colonies in sheep blood agar are 1 mm in diameter, slightly convex, grey-white, shiny, and centrally opaque with translucent, slightly undulating margins (*Fig. 27.4C*) (Mikalsen et al. 2008). In addition, in vitro tests have shown that the wild-type strain is sensitive to a broad range of antibiotics including chloramphenicol, streptomycin, gentamicin, tetracycline, erythromycin, oxytetracycline, clarithromycin, and sarafloxacin (Fryer et al. 1990; Cvitanich et al. 1991), while showing resistance to beta-lactam antibiotics (Almendras et al. 1997). Biochemical tests have shown beta-lactamase production in the LF-89 strain and have provided additional phenotypic features, including H_2S production positive, catalase positive, alkaline phosphatase positive, esterase (C4) and lipase (C8) positive, acid phosphatase positive, and naphthol-AS-BI-phosphohydrolase positive (Mikalsen et al. 2008). Growth in sheep blood agar has revealed that *P. salmonis* requires the amino acid L-cysteine for growth (Mauel et al. 2008; Gómez et al. 2009). Phage particles have been identified in close association with *P. salmonis* in electron micrographs, but to date no *P. salmonis*-specific bacteriophage have been characterized (Yuksel et al. 2001).

An interesting phenotypic characteristic was described during fish ova infection experiments in vitro. Infected ova were examined at sample periods from 30 s to 60 min by scanning electron microscopy. These studies found that 45 s after initial contact *P. salmonis* was attached to the surface of the ova by means of pseudopod-like extensions that apparently reached

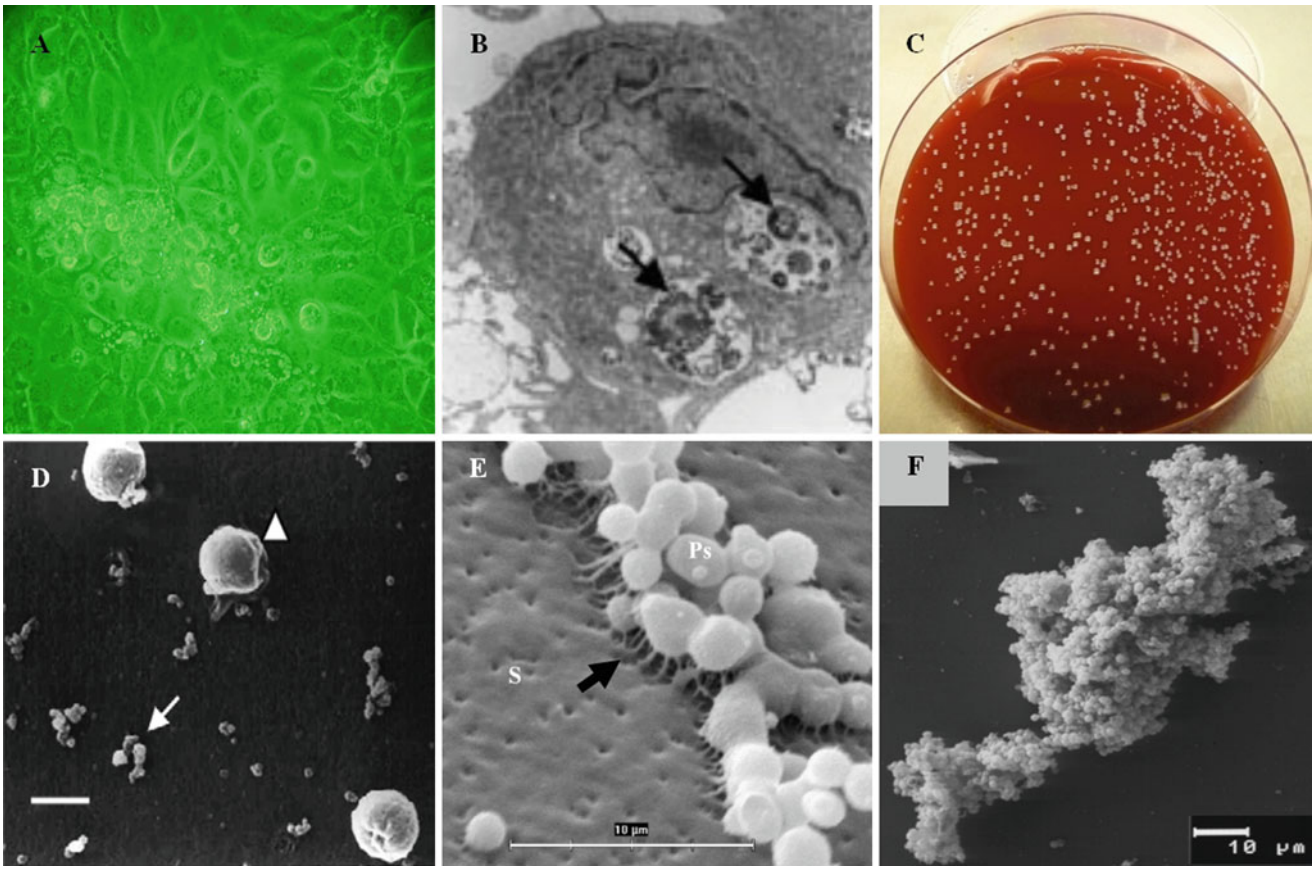

Fig. 27.4

Relevant phenotypic characteristics of *P. salmonis*. (A) *P. salmonis* producing typical CPE in the CHSE-214 cell line at 5 days postinfection. (B) Scanning electron microscopy (*SEM*) image of *P. salmonis* inside RTS11 macrophages (Rojas et al. 2009). (C) *P. salmonis* colonies grown on sheep blood agar plates (Gómez et al. 2009). (D) SEM images of the small isoform of *P. salmonis* (*sP.s*); the *white arrow* shows sP.s and the triangle indicates LF-89 prototype (bar: 1 µm) (Rojas et al. 2008). (E) SEM image of *P. salmonis* attaching on the surface of a salmonid ovum; the *black arrow* shows the PAC complex characterized (Larenas et al. 2001). (F) SEM image of *P. salmonis* biofilm complex produced by nutritional stress in liquid cultures (Marshall et al. 2012)

from the external membrane of the bacteria to the chorion of the ovum (*Fig. 27.4E*). These structures were called "piscirickettsial attachment complex" (PAC), and the authors suggested that they may allow later penetration into the ovum (Larenas et al. 2003).

A recent study demonstrated that *P. salmonis* LF-89 produces biofilm during growth under nutritional stress conditions, resulting in big cell aggregates (*Fig. 27.4F*) linked by an exopolysaccharide matrix (EPS). The *P. salmonis* EPS was characterized by two conventional lectins, concanavalin A (Con A) and wheat germ agglutinin (WGA), indicating that the EPS may be rich in levan polysaccharides due by its high affinity with Con A. Growth kinetics in limiting growth medium showed that biofilm formation is detectable at 3 h' growth and the EPS increases proportionally with time. In addition, during the early stages of *P. salmonis* biofilm formation, overexpression of the *mazEF* toxin-antitoxin (TA) operon was detected; a similar event has been observed in other related organisms (Marshall et al. 2012).

Molecular Analyses

To date, *P. salmonis* remains relatively uncharacterized at the molecular level, and the genome sequence has been published recently (Eppinger et al. 2013). Most of the molecular studies of *P. salmonis* have focused on the search for antigenic molecules for the development of new vaccines. In this area, some molecular chaperones have been characterized by different authors as alternatives to vaccine development: HSP60 (ChaP.s/GroEL), HSP70 (DnaK), HSP10 (GroES), and HSP16 (Marshall et al. 2007; Wilhelm et al. 2005, 2006). The OspA antigen has also been characterized and used as a recombinant vaccine with moderate efficiency (Kuzyk et al. 2001).

Interestingly, the analysis of ITS (internal transcribed spacer) from 11 Chilean isolates of *P. salmonis* has been reported. This analysis shows that PCR amplification revealed a *P. salmonis*-specific ITS electrophoretic pattern that was distinguishable from the ITS of other bacteria, including fish pathogens. Moreover, two separate *P. salmonis* patterns were seen: ITS with higher

(LF-89 strain) or with lower (EM-90 strain) electrophoretic mobility. In addition, in the 11 *P. salmonis* isolates analyzed, two primary amplification products were observed, demonstrating that *P. salmonis* has two ITS regions, ITSA and ITSB. The larger region (ITSA) is the same as the smaller (ITSB) region, but it is interrupted by two tRNA genes, tRNA-Ile and tRNA-Ala (Casanova et al. 2003).

Similar to other bacteria, *P. salmonis* contains insertion sequences (ISs) in its genome. To date, two ISs have been reported, ISPsa1 (GenBank: AF184152) and ISPsa2 (Marshall et al. 2012). ISPsa2 has been detected in the wild-type strain and in different natural isolates and is characterized by the presence of two identical 16/16 bp inverted terminal repeats flanking a 726-bp ORF that encodes a putative transposase (Tnp-Psa). The coding sequence of Tnp-Psa transposase shares similarities to that described in some *Bacillus* species and particularly to those of the IS6 family, such as the presence of DDE motifs (Marshall et al. 2010). The existence of at least two ISs in *P. salmonis* suggests the potential for fluidity in its genome (e.g., via rearrangements).

Two toxin-antitoxin (TA) systems have been described in *P. salmonis*. The first, the *marzEF* (TA) operon, may have implications for biofilm formation, since overexpression of this operon was detected during the early stages of biofilm development in *P. salmonis* (Marshall et al. 2012). The other TA system described is a homologue of VapBC. This TA system has all the characteristics present in the VapBC family, such as the presence of a PIN domain in the VapC toxin; a SpoVT/AbrB domain, related to transcriptional regulation, in the VapB antitoxin; and the ribonuclease activity of VapC (Gómez et al. 2011).

Antigenic characterization of *P. salmonis* has been reported. Using immune sera from rabbits immunized with *P. salmonis* whole-cell extract, four protein antigens were detected with relative molecular weights of 65, 60, 54, and 51 kDa and two carbohydrate antigens with molecular weights of 16 and 11 kDa (Kuzyk et al. 1996).

A study to determine the composition of *P. salmonis* lipid A revealed moderate compositional and structural heterogeneity with respect to the content of phosphate groups and 4-amino-4-deoxy-L-arabinopyranose (Ara4N) residues and with regard to the degree of acylation. At least two molecular species of *P. salmonis* lipid A were detected. The major species represented in the hexaacyl lipid A is the ss-(1– > 6)-linked D-glucosamine (GlcN) disaccharide backbone carrying two phosphate groups: the first at the glycosidic hydroxyl group of the reducing GlcN I and the second at the O-4′ position of the nonreducing GlcN II. The primary fatty acids are three 3-hydroxytetradecanoic [C14:0(3-OH)] acids and one 3-hydroxyhexadecanoic [C16:0(3-OH)] acid. The latter was amide linked to GlcN I and one C14:0(3-OH) was amide linked to GlcN II. Two secondary fatty acids were represented by C14:0(3-OH) and were equally distributed between the O-2′ and O-3′ positions. The phosphate group at O-4′ carried a non-stoichiometric substituent Ara4N. The minor lipid A species contained exclusively C14:0(3-OH) with an asymmetric distribution (4 + 2) at GlcN II and GlcN I, respectively. Finally, the authors suggested that the *P. salmonis* lipid A strongly resembles endotoxic enterobacterial lipid A in terms of structure (Vadovic et al. 2007).

Has been reported the presence of a functional Dot/Icm (Deficient in Organelle Trafficking/Intracellular Multiplication) Type IV Secretion Systems homologoue in *P. salmonis* (Gómez et al. 2013). This secretion system is the major virulence mechanism *L. pneumophila* and *C. burnetii*, being the responsible for their intracellular survival and multiplication and also is also involved in phagocytosis, cytotoxicity, apoptosis and in inhibition of phagosome-lysosome fusion which leads to the formation of a novel ribosome-lined phagosome (Zusman et al. 2003). At least four *P. salmonis* dot/icm homologues (dotA, dotB, icmE and icmK) are expressed both during in vitro tissue culture cells infection and growing in cell-free media, suggestive of their putative constitutive expression. Additionally, as it happens in other referential bacterial systems, temporal acidification of cell-free media results in over expression the *P. salmonis* dot/icm genes, a well-known strategy by which SSTIV-containing bacteria inhibit phagosome-lysosome fusion to survive (Gómez et al. 2013).

Isolation, Enrichment, and Maintenance Procedures

P. salmonis is routinely isolated from infected fish kidneys. Although the growth of *P. salmonis* has been described in at least two different agar media (Mauel et al. 2008; Mikalsen et al. 2008), to date the cell lines CHSE-214 or EPC are widely used for the growth and subsequent isolation of this organism in vivo.

Three liquid media have been described for the successful growth of this organism. The first contains a fish blood lysate, but its efficiency is very low, reaching a maximum OD_{600} of 0.3 after 8 days (Gómez et al. 2009). The second is a rich medium, supplemented with amino acids, vitamins, and fetal bovine serum (FBS), and shows moderate growth efficiency (OD_{600} of 1.7 after 6 days of growth) (Yañez et al. 2012). The last medium contains peptone and yeast extract as the carbon source and is supplemented with 0.1 % L-cysteine and 10 % FBS, obtaining moderate growth efficiency ($OD_{600} \sim 0.7$ after 48 h) (Marshall et al. 2012).

For in vivo isolation from infected fish, the kidney must be aseptically removed and transferred to a sterile container; antibiotics must not be used during this procedure. Tissues must be kept at 4 °C or on ice until processed, but not frozen. The kidney tissues must be homogenized at a dilution of 1/20 in antibiotic-free salt solution or 1X PBS and then diluted further to 1/5 and 1/50 in antibiotic-free salt solution for inoculation in cell cultures (dilutions as inocula are 10^{-2} and 10^{-3}). The infected cell lines must be incubated at 15–18C° for 28 days and observed for the appearance of the cytopathic effect (CPE). If CPE does not occur, the cultures should be incubated for an additional 14 days (OIE 2003).

P. salmonis can be stored at 4 °C for at least 14 days. Additionally, frozen vials in 20 % DMSO can be stored at−80 °C for at least 2 years.

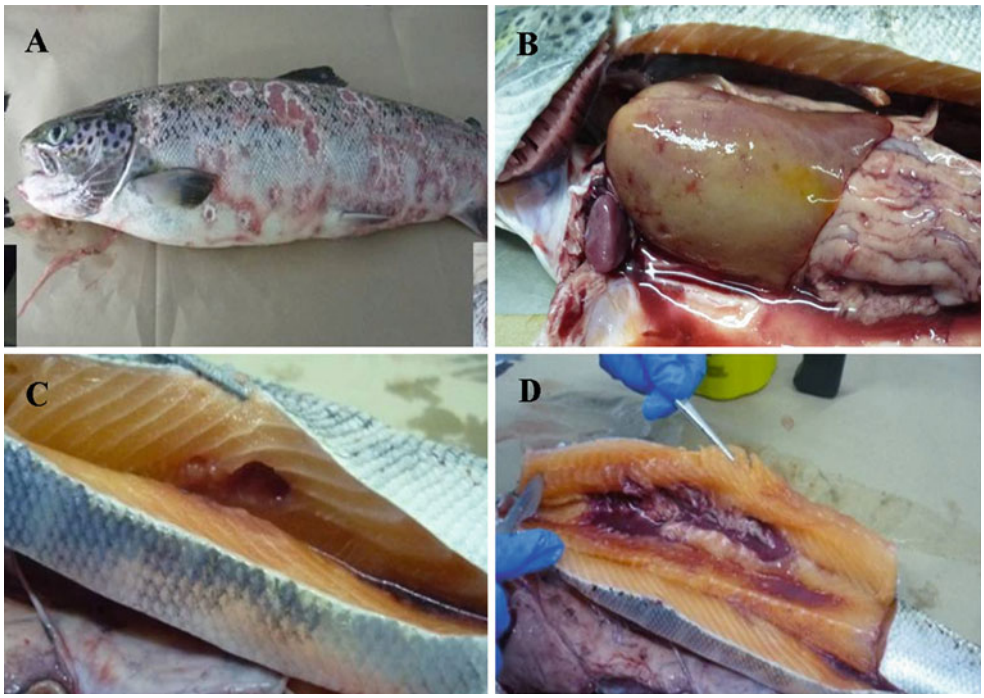

Fig. 27.5
Clinical signs of Piscirickettsiosis. (A) Skin ulcers produced by *P. salmonis*, (B) internal signs, with characteristic ring-shaped lesions and paleness in liver, (C) muscle caverns produced by *P. salmonis* infection (sign of virulent isolates), and (D) muscle caverns with purulent and bloody exudates

Pathogenicity, Clinical Relevance

Due to the ineffectiveness of antimicrobial agents against *P. salmonis* and the resulting high rate of fish mortality, this pathogen has become a major problem for the Chilean salmon industry, accounting for annual losses of over US$100 million (Bravo and Midtlyng 2007).

Clinical Signs and Pathology

A range of external signs of disease can be observed in salmonids affected by *P. salmonis*. Severely affected fish have the following symptoms: dark skin, anorexia, lethargic behavior, and erratic swimming. In addition, infected fish are often found near the surface or at the edges of cages. In some cases there is also branchial paleness, bleeding at the base of the fins, and the appearance of nodules on skin ulcerations due to scale loss (Lannan et al. 1999). Infections of the brain may cause erratic swimming behavior. Fish with milder infections may appear normal.

P. salmonis produces a systemic infection in fish predominantly targeting the kidney, liver, spleen, intestine, brain, ovary, and gills (Bravo and Campos 1989; Kuzyk et al. 1996). The internal signs include swollen and discolored kidneys and an enlarged spleen (*Fig. 27.4*). Ascites in the peritoneal cavity and hemorrhages on the visceral fat, stomach, swim bladder, and body musculature can also occur (Cvitanich et al. 1991; Schafer et al. 1990). Hallmark internal lesions are found in the liver, which may exhibit large, whitish or yellow, multifocal, coalescing, pyogranulomatous nodules. These lesions often rupture, resulting in shallow crater-like cavities in the liver.

In recent years atypical symptoms have been detected in rainbow trout and in Atlantic salmon, including muscle hemorrhagic inflammation associated with muscular lysis, giving rise to muscle caverns, which may or may not be visible externally (*Fig. 27.5*). The content of these caverns is viscous opaque variable-color bleeding with high bacterial content of typical *P. salmonis* morphology (Marshall et al. unpublished).

Epidemiology and Ecology

Existing studies have described the disease primarily in seawater and in estuaries (Fryer et al. 1990; Cvitanich et al. 1991), although it has also been reported in freshwater (Gaggero et al. 1995). The most accepted theory of the mode of spread of the pathogen is via the feces and urine of infected fish, where the agent is able to enter other individuals through the gills and/or intact skin (Larenas et al. 1997). The presence of ectoparasites or lesions on the skin of fish would likely favor pathogen entry and subsequent infection of other individuals (Fryer and Mauel 1997). There may also exist a method of vertical transmission of the disease by ova or sperm, which has been demonstrated in naturally infected coho salmon (Larenas et al. 1996). It has been reported that certain marine vectors may be involved in maintaining the bacteria in the environment. Direct immunofluorescence was used to detect

P. salmonis in the hematophagous parasite *Ceratothoa gaudichaudi* and in the ectoparasite *Caligus sp.*, indicating that these species may be vectors or reservoirs for the pathogen (Garcés et al. 1991; Correal 1995, Venegas 1996). Recently, it was reported that *P. salmonis* is able to stay in seawater at certain population densities for at least 40 days, both at the surface and at a depth of 5 m, suggesting a reservoir for the organisms during interepidemic periods (Olivares and Marshall 2010).

It has been observed that it is not only salmonid species that are affected by this organism. The presence of *P. salmonis* has been detected in specimens of white sea bass (*Atractoscion nobilis*) on the Southern California coast (Arkush et al. 2005), while European sea bass (*Dicentrarchus labrax*) in Greece have been affected by a pathogen similar to *P. salmonis* (Athanassopoulou et al. 2004). In Hawaii, tilapia populations (*Oreochromis mossambicus* and *Sarotherodon melanotheron*), both free-living and farmed fish, have suffered a Piscirickettsiosis-type disease (Mauel et al. 2003), suggesting the expansion of this agent to other fish of commercial importance (Marshall et al. 2007).

At present, several injectable vaccines against *P. salmonis* are commercially available. Although they produce variable long-term results, all of these vaccines are in some way effective in preventing the initial Piscirickettsiosis outbreaks that otherwise typically occur after the transfer of salmonid fish from fresh water to sea water. After the initial outbreak, the fish become susceptible to a second more aggressive Piscirickettsiosis outbreak that correlates with the weakening of the specific immune response elicited by the first immunization event. The second outbreak usually affects large fish and occurs 10–12 months after transfer, resulting in greater financial losses. Protecting these fish by revaccination is an attractive solution, but this is more difficult to accomplish, mainly due to financial, practical, and stress-related issues (Tobar et al. 2011).

Additional information derived from in vitro experiments has revealed that *P. salmonis* induces apoptosis in trout macrophages by activation of caspase-3, which could lead to bacterial survival and evasion of the host immune response and play an important role in the establishment of infection in the host. Morphologic and physiologic changes in the infected macrophages, such as nuclear fragmentation and the increased binding of annexin V to externalized phosphatidylserine, constitute evidence of apoptosis type I activation. Finally, immunofluorescence microscopy has been used to detect the presence of activated caspase-3 in infected cells, an effect that was not observed in noninfected cells or in infected macrophages treated with a caspase inhibitor (Rojas et al. 2010).

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28 The Family *Pseudoalteromonadaceae*

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Abstract
The family *Pseudoalteromonadaceae* (class *Gammaproteobacteria*, order *Alteromonadales*) consists of three genera: the type genus *Pseudoalteromonas*, containing 37 species; the genus *Algicola*, containing two species; and the genus *Psychrosphaera* with one species. All species are Gram-negative rods, with the exception of *Psychrosphaera saromensis* which is a coccus. Historically, many species of *Pseudoalteromonas* were assigned to the genus *Alteromonas*. Similarly, both *Algicola bacteriolytica* and *A. sagamiensis* were reclassified from *Pseudoalteromonas* to form a separate genus. Many species of this family produce a variety of primary and secondary metabolites including hydrolytic enzymes, cyclic peptides, proteins and protein inhibitors, pigments, exopolymers, phenolic and pyrrole-containing alkaloids, and unusual brominated compounds with antibacterial and antiviral properties. Due to their versatile metabolic capacities, members of this family are highly adaptable to dissimilar ecological habitats and play important ecological roles in marine environments.

Taxonomy, Historical and Current

Short Description of the Family

Pseudoalteromonadaceae (Ivanova, Flavier, and Christen (2004))

Pseudoalteromonadaceae (Pseu.do.al.te.ro.mo.na.da'ce.ae. N.L. fem. pl. n. *Pseudoalteromonas* type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. *Pseudoalteromonadaceae* the *Pseudoalteromonas* family).

Species of *Pseudoalteromonadaceae* are Gram-negative, rod-shaped bacteria which are motile by means of one or more flagella. Flagella are usually polar, but some species have lateral or bipolar flagella. Some species produce capsules. They are chemoorganotrophic in metabolism and require Na^+ ions for growth, in some cases tolerating up to 15 % NaCl. Cells are aerobic or facultatively anaerobic and usually do not denitrify. In most species, the major isoprenoid quinone is Q8, and the major fatty acids are 16:0, 16:1ω7, and 18:1ω7. Members of the family have been isolated from coastal, open and deep seawaters, sediments, marine invertebrates, fish, and algae from marine environments. The family is a member of the *γ-proteobacteria* with the following nucleotide sequence characteristics: 733 (A), 744 (T), 833 (C), 852 (T), and 853 (T). The family comprises the type genus *Pseudoalteromonas* (Gauthier et al. 1995) and the genera *Algicola* (Ivanova et al. 2004a) and *Psychrosphaera* (Park et al. 2010).

Phylogenetic Position

The family *Pseudoalteromonadaceae* forms a clade which can be observed to be clearly distinct from the other most closely related families. Six other families are moderately related to *Pseudoalteromonadaceae*, namely, *Shewanellaceae*, *Ferrimonadaceae*, *Moritellaceae*, *Psychromonadaceae*, *Colwelliaceae*, and *Idiomarinaceae*, while *Vibrionaceae* and *Alcanivoracaceae* are more distantly related. Within family *Pseudoalteromonadaceae*, the *Pseudoalteromonas* species cluster quite closely, while the single *Psychrosphaera* and the two *Algicola* species form branches on the fringes of the family.

List of *Pseudoalteromonadaceae*-type strains used for dendogram construction: *Pseudoalteromonas agarivorans* DSM 14585^T, *Pseudoalteromonas aliena* SW19^T, *Pseudoalteromonas antarctica* NF3^T, *Pseudoalteromonas arabiensis* k53^T, *Pseudoalteromonas arctica* A 37-1-2^T, *Pseudoalteromonas atlantica* ATCC 19262^T, *Pseudoalteromonas aurantia* 208^T, *Pseudoalteromonas bacteriolytica* ATCC 700679^T, *Pseudoalteromonas byunsanensis* FR1199^T, *Pseudoalteromonas carrageenovora* ATCC 43555^T, *Pseudoalteromonas citrea* ATCC 29719^T, *Pseudoalteromonas denitrificans* ATCC 43337^T, *Pseudoalteromonas distincta* ATCC 700518^T, *Pseudoalteromonas donghaensis* HJ51^T, *Pseudoalteromonas elyakovii* 40MC^T, *Pseudoalteromonas espejiana* ATCC 29659^T, *Pseudoalteromonas flavipulchra* ATCC BAA-314^T, *Pseudoalteromonas haloplanktis* 215^T, *Pseudoalteromonas issachenkonii* CIP 106858^T, *Pseudoalteromonas lipolytica* LMEB

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39^T, *Pseudoalteromonas luteoviolacea* CH130^T, *Pseudoalteromonas maricaloris* CIP 106859^T, *Pseudoalteromonas marina* mano4^T, *Pseudoalteromonas mariniglutinosa* DSM 15203^T, *Pseudoalteromonas nigrifaciens* 217^T, *Pseudoalteromonas paragorgicola* ATCC BAA-322^T, *Pseudoalteromonas peptidolytica* F12-50-A1^T, *Pseudoalteromonas phenolica* O-BC30^T, *Pseudoalteromonas piscicida* ATCC 15057^T, *Pseudoalteromonas prydzensis* ACAM 620^T, *Pseudoalteromonas rubra* ATCC 29570^T, *Pseudoalteromonas ruthenica* CIP 106857^T, *Pseudoalteromonas sagamiensis* B-10-31^T, *Pseudoalteromonas spongiae* UST010723-006^T, *Pseudoalteromonas tetraodonis* GFC^T, *Pseudoalteromonas translucida* ATCC BAA-315^T, *Pseudoalteromonas tunicata* D2^T, *Pseudoalteromonas ulvae* UL12^T, *Pseudoalteromonas undina* ATCC 29660^T, *Psychrosphaera saromensis* SA4-48^T, *Algicola bacteriolytica* ATCC 700679^T, and *Algicola sagamiensis* B-10-31^T.

Taxonomical History

The taxonomic history of *Pseudoalteromonas*-related bacteria dates back to 1995 as the result of the revision of the genus *Alteromonas* (Gauthier et al. 1995). The phylogenetic analysis of 16S rRNA gene sequences confirmed the heterogeneity of *Alteromonas* genus and defined a few rRNA relatedness groups. This conclusion was in agreement with an earlier rRNA–DNA hybridization study (Van Landschoot and De Ley 1983). Therefore *Alteromonas macleodii* representing rRNA group I was separated from the rRNA group II which comprised the rest of the species and composed the genus *Pseudoalteromonas*. This group included *A. haloplanktis*, "*A. marinopraesens*" (reclassified as *A. haloplanktis* (Reichelt and Baumann 1973), *A. rubra* (Gauthier 1976), *A. citrea* (Gauthier 1977), *A. luteoviolacea* (Gauthier 1982), *A. aurantia* (Gauthier and Breittmayer 1979), *A. espejiana*, *A. undina* (Chan et al. 1978), [*A.*] *putrefaciens* (Lee et al. 1977), "*A. thalassomethanolica*" (Yamamoto et al. 1980) and *A. nigrifaciens* (White 1940; Baumann et al. 1984; Ivanova et al. 1996a), *A. denitrificans* (Enger et al. 1987), *A. atlantica*, *A. carrageenovora* (Akagawa-Matsushita et al. 1992), *A. tetraodonis* (Simidu et al. 1990), "*A. rava*" (Kodama et al. 1993), [*A.*] *fuliginea*, *A. distincta*, and *A. elyakovii* (Romanenko et al. 1994, 1995; Ivanova et al. 1996b). The species *A. tetraodonis* has been reclassified as *A. haloplanktis* subsp. *tetraodonis* (Akagawa-Matsushita et al. 1993) but later on retrieved as *Pseudoalteromonas tetraodonis* (Ivanova et al. 2001). In recent years, a number of novel species of marine *Pseudoalteromonas* have been included in the genus, namely, *Pseudoalteromonas antarctica* (Bozal et al. 1997) and *Pseudoalteromonas prydzensis* (Bowman 1998), which were isolated from Antarctic coastal waters; [*Pseudoalteromonas*] *bacteriolytica* (Sawabe et al. 1998), which was isolated from wounded fronds of *Laminaria japonica* of the Sea of Japan; and *Pseudoalteromonas peptidolytica* (Venkateswaran and Dohmoto 2000), which was isolated from seawater. The highly bioactive species *Pseudoalteromonas tunicata* (Holmström et al. 1998) was isolated from the ascidian *Ciona intestinalis* residing in coastal waters of western Sweden. More recently, several more species were proposed, including *Pseudoalteromonas ulvae* (Egan et al. 2001), *Pseudoalteromonas issachenkonii* (Ivanova et al. 2002a), *Pseudoalteromonas ruthenica* (Ivanova et al. 2002c), *Pseudoalteromonas maricaloris*, *Pseudoalteromonas flavipulchra* (former *A. aurantiaca* NCIMB 2033) (Ivanova et al. 2002d), *Pseudoalteromonas translucida*, *Pseudoalteromonas paragorgicola* (Ivanova et al. 2002b), *Pseudoalteromonas agarivorans* (Romanenko et al. 2003), *Pseudoalteromonas phenolytica* (Isnansetyo and Kamei 2003), and *Pseudoalteromonas arabiensis* (Matsuyama et al. 2013).

The family *Pseudoalteromonadaceae* was proposed a decade later as the results of detailed phylogenetic analysis of the *Pseudoalteromonas* cluster. It was reported that this taxon is rather heterogeneous, with interstrain 16S rRNA gene sequence similarity values ranging from 90 % to 99.9 % (Ivanova et al. 2004a), and consisted of several monophyletic clusters, a large cluster of nonpigmented species (currently includes 15 species, including the type strain of the genus *Pseudoalteromonas haloplanktis*) with high interstrain similarity values of 98–99.9 % and a few clusters comprising pigmented species: (i) *Pseudoalteromonas citrea* and *Pseudoalteromonas aurantiaca*; (ii) *Pseudoalteromonas ruthenica*; (iii) *Pseudoalteromonas rubra*, *Pseudoalteromonas luteoviolacea*, *Pseudoalteromonas peptidolytica*, *Pseudoalteromonas piscicida*, *Pseudoalteromonas flavipulchra*, and *Pseudoalteromonas maricaloris*; (iv) *Pseudoalteromonas tunicata* and *Pseudoalteromonas ulvae*; (v) *Pseudoalteromonas denitrificans*; and (vi) [*Pseudoalteromonas*] *bacteriolytica* (Fig. 28.1). The deep branching (low similarity levels for nucleotides of 16S rRNA down to 90.3 %) of the latter species, the lack of sequence signature, the lack of association with other species of the genus, low DNA–DNA hybridization values (3–5 %), and some characteristic phenotypic traits (bacteriolytic activity, requirement for organic growth factors, different pattern of carbohydrate utilization) allowed to place this species in a separate genus *Algicola*, which contained the one species *Algicola bacteriolytica* as its type species. Detailed taxonomic investigation of [*Pseudoalteromonas*] *sagamiensis* (Kobayashi et al. 2003) revealed that this species is most closely related to *Algicola bacteriolytica* and was therefore reassigned to the genus *Algicola* (Nam et al. 2007). Recently one more genus, *Psychrosphaera*, with a single species, *Psychrosphaera saromensis*, has been described and formally validated (Park et al. 2010; Validation List no. 139 2011).

Molecular Analyses

Genome Sequences

The full genomes of 24 strains of *Pseudoalteromonas* have been reported. Typically, the genomes of analyzed strains are between 4 and 6 Mb in size, with 3,000–5,000 predicted genes (Table 28.1). Only a few have been studied in detail. *P. rubra* was found to possess 13 genes involved in the production of cycloprodigiosin (Xie et al. 2012), an algicidal compound with potential anticancer activity (Montaner and Pérez-Tomás

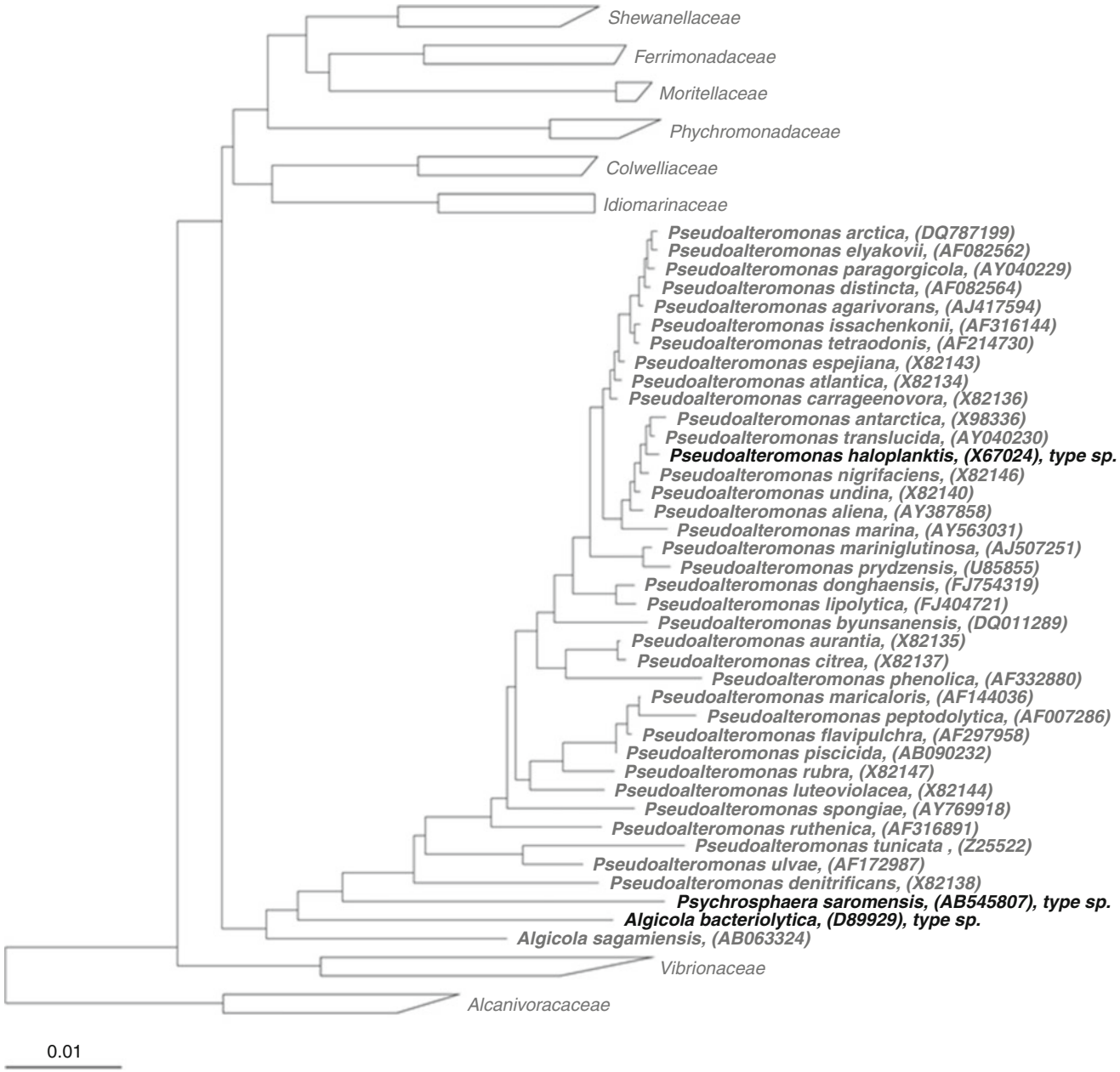

Fig. 28.1

Phylogenetic reconstruction of the family *Pseudoalteromonadaceae* based on 16S rRNA and created using the maximum likelihood algorithm RAxML (Stamatakis 2006). The sequence dataset and alignment were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). Representative sequences from closely related taxa were used as out-groups. In addition, a 40 % maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. Scale bar indicates estimated sequence divergence

2001; Kim et al. 2006). The genome of *P. tunicata* contains a number of artifacts indicative of mobile genetic elements, such as transposons, integrons, clustered regularly interspaced short palindromic repeats (CRISPRs), and a P2-like prophage (Thomas et al. 2008). A significant portion of the *P. tunicata* genome was found to be composed of these elements (2 %).

Protein Profiling

Protein profiling is one of the methods used for strain characterization. In 1997, SDS-PAGE was used to characterize *P. antarctica* NF3^T and its respective isolates to the other *Pseudoalteromonas* species, namely, *P. haloplanktis* CECT 4188^T and *P. atlantica* IAM 14164 (Bozal et al. 1997). Recently, with the

Table 28.1
Genomic characteristics of *Pseudoalteromonas* spp

Species	Strains sequenced	Genome size (Mb)	Predicted CDSs	Predicted rRNA genes	Predicted tRNA genes
P. arctica	A 37-1-2^T	4.63	4,094	3	104
	BSi20429	4.5	4,030	0	62
P. atlantica	T6c	5.19	4,281	15	62
P. citrea	NCIMB 1889^T	5.34	4,438	5	119
P. flavipulchra	JG1	9.93	4,758	1	105
P. haloplanktis	ATCC 14393^T	5.02	0	0	0
	ANT/505	4.49	4,127	5	51
P. issachenkonii	PAMC 22718	4.18	0	0	0
P. luteoviolacea	ATCC 29581	4.05	3,681	7	99
P. marina	mano4^T	4.18	3,711	4	96
	BSi20480	4.15	3,967	0	58
	TW-7	4.10	3,783	14	69
P. piscicida	JCM 20779^T	5.28	4,524	6	94
	NJ631	5.35	0	0	0
P. rubra	ATCC 29570^T	5.97	4,893	3	113
P. ruthenica	CP76	4.01	3,749	3	70
P. spongiae	UST010723-006^T	4.72	4,185	5	96
P. tetraodonis	SM9913	4.04	3,712	25	62
P. tunicata	D2^T	6.46	4,504	31	98
P. undina	NCIMB 2128^T	4.00	3,581	6	94
Unclassified *Pseudoalteromonas*	BSi20311	3.98	3,676	0	67
	BSi20439	3.88	3,612	0	64
	BSi20495	4.83	4,365	0	65
	BSi20652	4.25	4,085	0	53
	BSw20308	4.76	4,172	0	0
	TAC125	3.85	3,485	28	106

development of two-dimensional gel electrophoresis and MALDI-TOF MS, proteome analysis of the species of interest can be determined. *Pseudoalteromonas haloplanktis* strain TAC 125 was one strain belonging to the genus which has its proteome characterized by 2-D gel electrophoresis and MADLI-TOF MS in order to study the fast growth of this psychrophilic bacterium (Wilmes et al. 2011). Two-dimensional gel proteomics was also applied on *Pseudoalteromonas* sp. strain D41 to study bacterial biofilm formation (Ritter et al. 2012). Gel-free approaches have also been developed in the field of proteomics. The development of amine-specific isobaric tagging for relative and absolute quantitation (iTRAQ) method allowed the study of pigments and bioactive compound production of *Pseudoalteromonas tunicata* (Evans et al. 2007; Schweder et al. 2008). Whole cell MALDI-TOF MS is a more rapid and cost-efficient method for protein profiling, where *Pseudoalteromonas* species were successfully characterized and classified from the environmental samples from previous studies (Dieckmann et al. 2005; Emami et al. 2012).

Metabolites

The species within genus *Pseudoalteromonas* can be grouped into two broad clades: the pigmented and the nonpigmented strains. It has been reported that pigmented species generally possess a broad range of biologically active compounds, whereas nonpigmented species tend to have a broad range of enzymatic activities and greater adaptability in terms of nutrient requirement and environmental tolerance (Holmström and Kjelleberg 1999; Bowman 2007).

Antimicrobial Activity

Pseudoalteromonas species have been reported to produce antibiotics that show bactericidal effects. Validly described *Pseudoalteromonas* species that possess antimicrobial activity include *P. aurantia*, *P. luteoviolacea*, *P. maricaloris*, *P. peptidolytica*, *P. phenolica*, *P. piscicida*, *P. rubra*, *P. ruthenica*, and *P. tunicata* (Bowman 2007). Recent biologically active compounds that have been isolated from *Pseudoalteromonas* species include isatin

Table 28.2
Chemotaxonomic and morphological traits of members of the *Granulosicoccus* genus

Phenotypic trait	*Algicola*	*Pseudoalteromonas*	*Psychrosphaera*
Cellular morphology	Rod shaped	Rod shaped	Coccoid
Size	0.6–1.5 μm × 1.9–3.7 μm	0.15–1.5 μm × 0.4–4.0 μm	0.5-0.7 μm × 0.75–1.0 μm
Gram stain	Negative	Negative	Negative
Motility	Motile	Almost always motile[a]	Motile
Pigmentation	Red or none	Variable[b]	–
Oxygen requirements	Aerobic	Almost always aerobic[c]	Aerobic
Metabolic activity	Chemoorganotrophs	Chemoorganotrophs	Chemoorganotrophs
Temperature range	15–35 °C	4–40 °C	4–30 °C
NaCl requirements (%)	1.5–5	up to 15[d]	1–5
pH range	6.0–8.5	5.0–10.0	6.0–9.0
G+C (mol %)	42–46	36.8–48.9	38.7–39.6
Major fatty acids	$C_{16:1}$ $\omega7c$ and/or iso-$C_{15:0}$ 2-OH (38.9 %), $C_{18:1}$ $\omega7c$ (20.4 %), $C_{16:0}$ (13.2 %), and $C_{10:0}$ 3-OH (9.5 %)	$C_{16:0}$, $C_{16:1}$ $\omega7c$, and $C_{18:1}$ $\omega7c$	$C_{18:1}$ $\omega7c$, $C_{16:0}$, and $C_{16:1}$ $\omega7c$
Major respiratory quinones	Ubiquinone 8 (Q-8)	Ubiquinone 8 (Q-8)	Ubiquinone 8 (Q-8)

[a]*P. spongiae* is nonmotile
[b]Pigments include melaninlike, orange, violet, beige/pale yellow-orange, lemon, bright blue/purple/red, brown, red, and dark green
[c]*P. tunicata* is capable of facultatively anaerobic growth
[d]*P. arctica* can grow without NaCl

(indole-2,3-dione) from *P. issachenkonii* KMM 3549^T (Kalinovskaya et al. 2004), bromo-alterochromides A and B from *P. maricaloris* KMM 636^T (Sobolevskaya et al. 2005), isovaleric acid (3-methylbutanoic acid) and 2-methylbutyric acid (2-methylbutanoic acid) from a *P. haloplanktis* strain (Hayashida-Soiza et al. 2008), alkaloid tambjamines from *P. tunicata* (Pinkerton et al. 2010), and thiomarinol from *Pseudoalteromonas* spp. SANK73390 (Murphy et al. 2011).

Enzymes

Cold-adapted enzymes from *Pseudoalteromonadaceae* strains have gained interest for their thermal stability and potential for research as well as biotechnological applications (Georlette et al. 2004). Cold-adapted enzymes that have been characterized include subtilase from *Pseudoalteromonas* sp. SM9913 (Chen et al. 2007), lipase from *Pseudoalteromonas* sp. NJ70 (Venkateswaran and Dohmoto 2000), metalloprotease from *Pseudoalteromonas* sp. SM495 (He et al. 2012), α-amylase from *P. arctica* GS230 (Lu et al. 2010), esterase from *P. arctica* LMG 23753^T (Al Khudary et al. 2010), and glutathione synthetase from *P. haloplanktis* (Albino et al. 2012). Also, *Pseudoalteromonas* species are one group of marine organisms that have been documented to be able to degrade agar. Strains within the genus *Pseudoalteromonas* that possess the agarase activity are *P. atlantica*, *P. carrageenovora* (Akagawa-Matsushita et al. 1992), *P. antarctica* strain N-1 (Vera et al. 1998), and *Pseudoalteromonas* sp. JYBCL1 (Oh et al. 2011).

Extracellular Polysaccharides

The extracellular polymeric substances (EPS) secreted by bacterial cells can aid in surface colonization, increase the survival rate of organisms in specific environment, act as antimicrobial components, protect the cells from antibiotics and protozoa, enhance nutrient uptake, and decrease the diffusion of particular substances in or out of the cells (Holmström and Kjelleberg 1999). Examples of studies on the EPS secreted by *Pseudoalteromonas* species have demonstrated the potential to treat fungal keratitis infection (Chen et al. 2012) to be used as biological response modifiers (Bai et al. 2012) and its ability to adsorb metal ions such as Pb^{2+} and Cu^{2+} (Zhou et al. 2009).

Phenotypic Analyses

Bacteria belonging to family *Pseudoalteromonadaceae* are mostly Gram-negative rods, except for *Psychrosphaera saromensis*, which is a Gram-negative coccus. All cells are typically ~1 μm in diameter, and rod-shaped cells can reach up to 4 μm in length. A variety of pigments are produced by different species, and as a result colonies on agar plates have a variety of colors, including no color/white, yellow, orange, red, beige, violet, bright blue, dark green, and dark brown. Typical phenotypic characteristics for each of the three genera belonging to *Pseudoalteromonadaceae* are given in *Table 28.2*.

Isolation, Enrichment, and Maintenance Procedures

Strains of *Pseudoalteromonadaceae* have been isolated from a number of marine sources, including surface seawater (Kobayashi et al. 2003; Ivanova et al. 2004b), deep seawater (Romanenko et al. 2003), marine sediments (Bozal et al. 1997), tidal flats (Nam et al. 2007), sea ice (Al Khudary et al. 2008), brackish lakes, and marine organisms such as sponges (Lau et al. 2005), seaweeds (Yaphe 1957), and algae (Ivanova et al. 2002a). Most strains within the family were originally isolated via either direct plating or dilution series plating, on marine agar 2216 (BD), Medium B (0.2 % (w/v) peptone, 0.2 % (w/v) casein hydrolysate, 0.2 % (w/v) yeast extract, 0.1 % (w/v) glucose, 0.002 % (w/v) KH_2PO_4, 0.005 % (w/v) $MgSO_4.7H_2O$, 1.5 % (w/v) agar, 50 % (v/v) natural seawater, and 50 %(v/v) distilled water at pH 7.5–7.8) or similar. Isolation of three species of *Pseudoalteromonas* also included an initial enrichment step: *P. arctica* was isolated from a culture that was enriched for psychrophilic bacteria, *P. denitrificans* was recovered from an enrichment for denitrifying bacteria, and *P. issachenkonii* was isolated from an algal degradation enrichment.

Pathogenicity, Clinical Relevance

Some species of *Pseudoalteromonadaceae* have been described to possess characteristics which may have some relevance in clinical settings. *Pseudoalteromonas rubra* is known to produce cycloprodigiosin, which has been shown to possess some anti-cancer activity. *P. denitrificans* also produces a related compound prodigionine, and *P. aliena* has been shown to have a cytotoxic effect against tumor cells.

There is little data to suggest that members of *Pseudoalteromonadaceae* have any pathogenic potential. Genomic analysis of *Pseudoalteromonas tunicata* led to the identification of a number of genes that are likely involved in virulence and pathogenicity. Also, the hemolytic activity of *P. aurantia* and *P. rubra* hints at the possibility of virulent activity.

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29 The Family *Psychromonadaceae*

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Abstract
The family *Psychromonadaceae*, within the order *Alteromonadales*, includes only the genus *Psychromonas*; it is closely related to the three families *Pseudoalteromonadaceae*, *Colwelliaceae*, and *Idiomarinaceae*. The main habitats of the family *Psychromonadaceae* appear to be marine, sea ice, or saline aquatic environments, from which original cultures are isolated. Cells can be Gram-negative rods or oval cells, non-spore, and motile or nonmotile. The flagellum may be single or subpolar, sheathed or unsheathed. The cells grow at temperatures less than 37 °C. They are facultative anaerobes, but some species may grow to be aerotolerant anaerobes or aerobes. They use carbohydrates for energy for growth. Oxidase is positive and catalase is variable. The major isoprenoid quinone is Q-8. The predominant cellular fatty acids are $C_{16:0}$ and $C_{16:1}$. The abundance ratio of unsaturated fatty acids was $\geq$50 %. *P. ingrahamii* 37^T, which can grow at −12 °C, underwent genomic analysis. Some species produced $C_{20:5}\omega3$ and/or $C_{22:6}$. The G+C DNA is 38–43.8 mol%. This chapter is a modified and updated version of previously published family descriptions (Ivanova et al. Int J Syst Evol Microbiol 54:1773–1788, 2004).

Taxonomy, Historical and Current

Short Description of the Families and their Genera

Psychromonadaceae (Psy.chro.mo.na.da'ce.ae. N.L. fem. n. *Psychromonas* type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. *Psychromonadaceae* the *Psychromonas* family Ivanova et al. 2004; Hosoya et al. 2009).

The family *Psychromonadaceae* is phylogenetically a member of the order *Alteromonadales* (Bowman and McMeekin 2005) and class *Gammaproteobacteria*. The family contains only the type genus *Psychromonas* (Mountfort et al. 1998; emended by Nogi et al. 2002). They are Gram-negative, rod- or oval-shaped bacteria and may be motile or nonmotile. They are chemoorganotrophs that do not form endospores or microcysts. They are facultative anaerobes, but some species may grow to be aerotolerant anaerobes or aerobes. Some species do not require Na^+ ions for growth. In most species, the major isoprenoid quinone is Q-8. The major fatty acids are $C_{16:0}$ and $C_{16:1}\omega7$. Members of the family have been isolated from coastal, open, and deep-sea waters, as well as sea ice, sediment, and marine environments. The family has the following nucleotide sequence characteristics: 811 (A), 842 (A), 845 (T), 1336 (T). The G+C contents of DNA range between 38 and 43.8 mol%.

Phylogenetic Structure of the Family and its Genera

The phylogenetic tree in Fig. 29.1 shows the family *Psychromonadaceae*. The family *Psychromonadaceae* includes only the genus *Psychromonas* (Mountfort et al. 1998), and it is closely related to the three families *Pseudoalteromonadaceae*, *Colwelliaceae*, and *Idiomarinaceae*. The species *Psychromonas antarctica* was first reported to be closely related to *Moritella marina* (similarity was 91.0 %), belonging to *Gammaproteobacteria* (Mountfort et al. 1998). This family was created by Ivanova et al. (2004) based on phylogenetic position and the presence of a unique set of 16S rRNA gene sequence signature nucleotides. The phylogenetic dendrogram of *Psychromonas*-type strains indicates the presence of two clades. One clade is composed of *P. antarctica*, *P. boydii*, *P. japonica*, *P. macrocephali*, *P. ossibalaenae*, and *P. aquimarina*, which share 96.5–99.2 % 16S rRNA gene sequence similarity with *P. arctica*. The second clade group of *P. marina*, *P. kaikoae*, *P. profunda*, *P. heitensis*, and *P. hadalis* share a 96.0–98.6 % similarity with *P. agarivorans*. The two clades share ∼96.3 % similarity.

DNA-DNA Hybridization Studies

DNA-DNA hybridization (DDH) studies have been performed on several *Psychromonas*-type strains. The phylogenetic neighbors *P. aquimarina*, *P. macrocephali*, and *P. ossibalaenae*, sharing 98.8–99.2 % 16S rRNA gene sequence similarity, exhibit 36–47 % DDH similarity, whereas *P. antarctica* and *P. ingrahamii*

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Fig. 29.1

Phylogenetic reconstruction of the family *Psychromonadaceae* based on the neighbor-joining algorithm with the Jukes-Cantor correction. Sequence dataset and alignments are according to the All-Species Living Tree Project (LTP) online database (LTPs108, July 2012) (Yarza et al., 2008). The tree topology was stabilized with the use of a representative set of 767 high-quality type strain sequences proportionally distributed among the different bacterial and archaeal phyla. In addition, a 40 % maximum frequency filter was applied to remove hypervariable positions from the alignment. Scale bar indicates estimated sequence divergence

show DDH values less than <20 % (Miyazaki et al. 2008). Additional DDH values are available for the neighboring species *P. boydii* and *P. ingrahamii*, sharing 98.9 % 16S rRNA gene sequence similarity (<63 % DDH similarity; Auman et al. 2010), as well as for *P. marina*, *P. kaikoae* and *P. profunda*, which share 98.3–98.6 % 16S rRNA gene sequence similarity (<38 % DDH similarity) (Xu et al. 2003).

Genome Analyses

Psychromonas ingrahamii 37^T is the only strain of the family for which the full genome sequence has been generated (INSDC ID CP000510) (Riley et al. 2008). The single replicon genome is 4,559,598 bp long, with 40.1 % of extrachromosomal elements absent. There are 10 ribosomal RNA clusters containing 5S, 16S, 23S RNAs. Altogether 3,708 genes were identified, 3,545 of which were proteins of 83 residues or longer. Correspondence analysis of the composition of all *P. ingrahamii* proteins showed the following: (1) there are six classes of proteins, at least one more than other bacteria; (2) integral inner membrane proteins are not sharply separated from bulk proteins, suggesting that, overall, they may have a lower hydrophobic character; (3) there is strong opposition between asparagine and the oxygen-sensitive amino acids methionine, arginine, cysteine, and histidine; and (4) one of the previously unseen clusters of proteins has a high proportion of "orphan" hypothetical proteins, raising the possibility that these are cold-specific proteins. Based on annotation of proteins by sequence similarity, the following can be concluded: (1) *P. ingrahamii* has a large number (61) of regulators of cyclic GDP, suggesting that this bacterium produces an extracellular polysaccharide that may help sequester water or lower the freezing point in the vicinity of the cell; (2) *P. ingrahamii* has genes for production of the osmolyte betaine choline, which may balance the osmotic pressure as sea ice freezes; (3) *P. ingrahamii* has a large number (11) of three-subunit TRAP systems, which may play an important role in the transport of nutrients into the cell at low temperatures; (4) chaperones and stress proteins may play a critical role in transforming nascent polypeptides into three-dimensional configurations that permit low temperature growth; and (5) the metabolic properties of *P. ingrahamii* were deduced. Finally, a few small sets of proteins of unknown function, which may play a role in psychrophily, have been singled out as worthy of future study.

Phenotypic Analyses

Psychromonas (Mountfort et al. 1998, emended by Nogi et al. 2002) Psy.chro.mo'nas. Gr. adj. psychros cold; Gr. fem. n. monas a unit; N.L. n. *Psychromonas* a cold monad.

Psychromonas cells may be Gram-negative rods or oval cells, nonspore, and motile or nonmotile. The flagellum is single or subpolar, sheathed or unsheathed. The cells grow at temperatures less than 37 °C. They are facultative anaerobes, but some species may grow to be aerotolerant anaerobes or aerobes. They use carbohydrates for energy for growth. Oxidase is positive and catalase is variable. The major isoprenoid quinone is Q-8. The predominant cellular fatty acids are $C_{16:0}$ and $C_{16:1}$. The G+C DNA is 38–43.8 mol%.

The type species is *Psychromonas antarctica*, and the type strain is star-1^T. Type strains of the other species are indicated in *Table 29.1*.

Table 29.1
Differential characteristics of *Psychromonas* species

Characteristic	*P. antarctica*[a]	*P. agarivorans*[b]	*P. aquamarina*[c]	*P. arctica*[d]	*P. boydii*[e]	*P. hadalis*[f]	*P. heitensis*[g]
Morphology							
Cells form	Ovoid rods	Rods	Rods	Rods	Large rods, chains, single cells	Rods	Rods
Size (length)	1.3 × 2.5–6	0.5–0.8 × 0.8–1.2	0.9–1.1 × 1.6–3.2	0.7–1.7 × 1.3–2.6	8–18	0.8–1.0 × 1.5–2	0.5–1.0 × 1.0–1.5
Flagellum	Single polar	Single subpolar	Single polar sheathed	ND	No flagella	Single subpolar	Single subpolar
Production of gas vesicles	–	ND	–	–	+	–	–
Colony color	White	ND	Cream	White	White	ND	ND
Motility	+	+	+	+	–	+	+
Growth(°C)							
Optimum	12	20–25	20–25	20	ND	6, 60 MPa	20–25
Maximum	17	30	27	25	10	12	30
Minimum	2	2	5	0	0	2	2
NaCl(%)							
Optimum	3	ND	3	2	3.5	3	ND
Maximum	4	4	5	7	18	ND	4
Minimum	0	1	2	1	2	>0	3
pH							
Optimum	6.5	ND	8.5	8.5–8.8	ND	ND	ND
Maximum	ND	9.0	9.0	9.8	7.4	ND	9.0
Minimum	ND	6.0	6.0	6.5	6.5	ND	6.0
O_2 requirement for growth[l]	ATAN	FAN	FAN	A	FAN	FAN	FAN
Growth at atmospheric pressure	+	+	+	+	+	–	+
Enzyme							
Catalase	+	–	–	+	+	+	+
Oxidase	+	+	+	+	+	+	+
Lipase (tri-n-butyrin)	ND	ND	–	ND	ND	ND	ND
Hydrolysis of							
Gelatin	+	–	+	–	–	–	+
Casein	ND	+	w	ND	ND	ND	ND
Starch	+	v	+	+	–	–	+
DNA	ND	+	+	ND	ND	ND	+
Tween 20	ND	–	+	ND	–	ND	+
Tween 40	ND	–	+	ND	–	ND	+
Tween 60	ND	–	ND	ND	–	ND	+
Tween 80	ND	–	+	ND	–	ND	+
Agar	–	+	–	–	ND	–	–
Production of							
H_2S	–	–	–	ND	–	–	–
Indole	–	–	–	ND	–	–	–

Table 29.1 (continued)

Characteristic	*P. antarctica*[a]	*P. agarivorans*[b]	*P. aquamarina*[c]	*P. arctica*[d]	*P. boydii*[e]	*P. hadalis*[f]	*P. heitensis*[g]
Reduction of							
Nitrate	−	−	+	−	+	+	−
Nitrite	−	ND	−	ND	ND	+	ND
Utilizes or acid production from							
L-Arabinose	−	−	−	ND	−	−	−
Cellobiose	+	+	−	ND	+	−	+
D-Fructose	+	−	+	+	+	+	+
D-Galactose	+	+	+	ND	+	+	−
D-Glucose	+	+	+	+	+	+	+
Glycerol	−	−	+	+	−	+	−
myo-inositol	−	−	−	ND	+	+	−
D-Lactose[m]	−	ND	ONPG+	+	−	−	ONPG+
Maltose	+	+	+	+	+	+	+
D-Mannitol	+	−	+	+	+	+	+
D-Mannose	w	−	+	+	+	+	−
D-Raffinose	−	−	−	ND	ND	−	−
L-Rhamnose	−	−	−	ND	ND	−	−
D-Sorbitol	−	−	−	ND	−	−	ND
Sucrose	+	−	+	+	+	−	+
D-Trehalose	+	−	+	ND	+	+	+
D-Xylose	−	−	−	−	+	−	−
Chemotaxonomic properties							
Predominant fatty acids	$C_{16:1}\omega7c$ and $C_{16:0}$	$C_{16:1}\omega7c$ and $C_{16:0}$	$C_{16:1}$ and $C_{16:0}$	$C_{16:0}$, $C_{16:1}\omega7c$, $C_{16:1}\omega7t$ and$C_{18:1}\omega7$	$C_{16:1}\omega7c$ and $C_{16:0}$	$C_{16:1}$, $C_{16:0}$ and $C_{14:1}$	$C_{16:1}\omega7c$ and $C_{16:0}$
GC (mol%)	42.8	41–42	42.2	40.1	40	39.1	38
Characteristic	***P. ingrahamii***[h]	***P. japonicus***[c]	***P. kaikoae***[i]	***P. macrocephalus***[c]	***P. marina***[j]	***P. ossibalaenae***[c]	***P. profunda***[k]
Morphology							
Cells form	Large rods	Rods	Rods	Rods	Rods	Rods	Rods
Size (length)	1.25–1.5 × 6–14	0.5–0.8 × 1.6–2.2	0.8–1.0 × 2.0–4.0	0.6–0.8 × 2.2–2.9	0.8–1.2 × 1.5–2.0	0.7–1.1 × 1.9–3.7	0.9–1.2 × 2.0–5.5
Flagellum	ND	Single polar	Single polar	Single polar sheathed	Single polar	Single polar	Single polar
Production of gas vesicles	+	−	−	−	−	−	−
Colony color	White	Translucence cream	ND	Cream	Colorless	Cream	Colorless
Motility	−	+	+	+	+	+	+
Growth (°C)							
Optimum	ND	20–22	10, 50 MPa	20	ND	20	ND
Maximum	10	25	15	25	25	25	14
Minimum	−12	5	4	0	0	0	2
NaCl (%)							
Optimum	ND	3	3	3	3–5	3	ND
Maximum	12	5	ND	6	7	5	ND
Minimum	1	2	>0	2	>0	2	>0

Table 29.1 (continued)

Characteristic	*P. ingrahamii*[h]	*P. japonicus*[c]	*P. kaikoae*[i]	*P. macrocephalus*[c]	*P. marina*[j]	*P. ossibalaenae*[c]	*P. profunda*[k]
pH							
Optimum	ND	9.0	ND	8.5	ND	9.0	ND
Maximum	7.4	9.5	ND	9.0	ND	9.5	ND
Minimum	6.5	6.5	ND	6.0	ND	6.5	ND
O_2 requirement for growth[l]	FAN	FAN	FAN	FAN	FAN	FAN	FAN
Growth at atmospheric pressure	+	+	–	+	+	+	+
Enzyme							
Catalase	+	+	+	–	+	–	+
Oxidase	+	+	+	+	+	+	+
Lipase (tri-n-butyrin)	ND	–	ND	–	+	+	–
Hydrolysis of							
Gelatin	–	w	+	+	–	–	–
Casein	ND	–	ND	+	–	–	ND
Starch	–	–	–	–	+	–	w
DNA	ND	+	ND	+	+	+	+
Tween 20	ND	+	ND	+	+	–	ND
Tween 40	ND	–	ND	+	+	–	ND
Tween 60	ND	ND	ND	ND	+	ND	ND
Tween 80	ND	–	ND	+	+	–	ND
Agar	–	–	–	–	–	–	–
Production of							
H_2S	ND	–	–	–	+	–	+
Indole	–	–	–	–	–	–	+
Reduction of							
Nitrate	+	+	+	+	+	+	+
Nitrite	ND	–	–	–	ND	–	ND
Utilizes or acid production from							
L-Arabinose	–	–	–	–	–	–	–
Cellobiose	+	+	+	–	+	+	+
D-Fructose	+	+	+	+	+	+	+
D-Galactose	+	–	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+
Glycerol	+	–	–	+	+	+	+
myo-inositol	–	–	–	–	–	–	+
D-Lactose[m]	–	ONPG–	–	ONPG+	+	ONPG+	+
Maltose	ND	–	+	+	+	+	+
D-Mannitol	+	–	+	–	+	–	+
D-Mannose	–	+	+	+	–	+	+
D-Raffinose	ND	–	–	–	–	w	–
L-Rhamnose	ND	–	–	–	–	–	+
D-Sorbitol	–	–	–	–	–	–	–
Sucrose	+	–	+	–	+	+	+

Table 29.1 (continued)

Characteristic	*P. ingrahamii*[h]	*P. japonicus*[c]	*P. kaikoae*[i]	*P. macrocephalus*[c]	*P. marina*[j]	*P. ossibalaenae*[c]	*P. profunda*[k]
D-Trehalose	+	−	+	+	−	+	+
D-Xylose	−	−	−	−	+	+	+
Chemotaxonomic properties							
Predominant fatty acids	$C_{16:1}\omega7c$ and $C_{16:0}$	$C_{16:1}$ and $C_{16:0}$	$C_{16:1}\omega7c$, $C_{16:0}$ and $C_{14:1}\omega7t$	$C_{16:1}$ and $C_{16:0}$	$C_{16:1}$ and $C_{16:0}$	$C_{16:1}$ and $C_{16:0}$	$C_{16:1}$, $C_{16:0}$ and $C_{14:1}$
GC(mol%)	40	38.8	43.8	41.5	43.5	41.3	38.1

All strains showed identical biochemical characteristics, except those indicated here. + Positive, −, negative, *v* varaiable reaction, *w* week positive, *ND* no data
Data from
[a]Mountfort et al. 1998
[b]Hosoya et al. 2009
[c]Miyazaki et al. 2008
[d]Groudieva et al. 2003
[e]Auman et al. 2010
[f]Nogi et al. 2007
[g]Hosoya et al. 2008
[h]Auman et al. 2006
[i]Nogi et al. 2002
[j]Kawasaki et al. 2002
[k]Xu et al. 2003
[l]*A* Aerobe, *ATAN* aerotolerant anaerobe, *FAN* facultative anaerobe
[m]Results prefixed with ONPG indicate that strains were not tested for lactose utilization directly but, rather, for ONPG hydrolysis/b-galactosidase activity

Some species produced $C_{20:5}\omega3$ and/or $C_{22:6}$. In addition, *P. ingrahamii* (Auman et al. 2006) and *P. boydii* (Auman et al. 2010) have the following properties. They contain two gas vesicle morphologies. Both strains use the following as sole carbon sources: DL-aspartate, DL-lactate, L-aspartate, L-cysteine, L-glutamate, acetate, pyruvate, and *N*-acetyl-glucosamine; they do not use D-gluconate, D-glucuronate, DL-malate, L-leucine, L-proline, α-ketoglutarate, benzoate, caproate, glycolate, or methanol. *P. ingrahamii* uses D-glucuronate, fumarate, propionate, salicin, succinate, and glucosamine as sole carbon sources. *P. boydii* uses only citrate as a sole carbon source. *P. agarivorans* (Hosoya et al. 2009) and *P. heitensis* (Hosoya et al. 2008) use API 20NE, API 50CH, and API ZYM (bioMérieux). The major fatty acids are $C_{16:0}$ and $C_{16:1}$. The abundance ratio of unsaturated fatty acids was ≥50 %, with the exception of *P. marina*.

Isolation, Enrichment and Maintenance Procedures

Isolation and Enrichment

P. agarivorans J42-3A^T (Hosoya et al. 2009) was isolated from marine sediments collected at Kori, off Nomozaki, Nagasaki in Japan, on solidified 1/10 strength marine broth [900 ml filtered seawater and 100 ml marine broth 2216 (Difco)] with 1.5 % (w/v) agar and supplemented with 0.5 % Rose bengal.

P. antarcticus star-1^T (Mountfort et al. 1998) was isolated from the sediment of Salt Pond near Bratina Island on the McMurdo Ice Shelf. A complex medium was prepared as the basal medium, except that trypticase and yeast extract were each present at 0.2 % (w/v). The sporulation medium was according to that described by Duncan and Strong (1968). The solid basal or complex medium for slants contained 2 % agar; the sterile reduced medium was dispensed in 3-ml amounts into sterile 13-mm × 100-mm culture tubes fitted with no. 00 black rubber stoppers. The composition of liquid aerobic complex medium was the same as for the anaerobic medium except that the medium was not boiled during its preparation, a reducing agent was not added prior to use, and air replaced N_2–CO_2 as the gas phase. The solid complex medium for plates contained 2 % agar.

P. aquimarina JAMM 0404^T (Miyazaki et al. 2008) was isolated from the sediment on the seabed adjacent to a sperm whale carcass off Kagoshima, Japan, after a 1-week incubation at 15 °C on marine agar 2216.

P. arctica Pull 5.3^T (Groudieva et al. 2003) was isolated from a seawater sample taken near Svalbard, Spitzbergen. The complex medium contained (L^{-1}): NaCl, 28.13 g; KCl, 0.77 g; $CaCl_2.2H_2O$, 0.02 g; $MgSO_4.7H_2O$, 0.5 g; NH_4Cl, 1.0 g; iron ammonium citrate, 0.02 g; yeast extract, 0.5 g; 10-fold concentrated trace element solution (DSM 141), 1 ml; 10-fold concentrated vitamin solution (DSM 141), 1 ml; KH_2PO_4, 2.3 g; $Na_2HPO_4.2H_2O$, 2.9 g; starch, 5 g. The pH was adjusted with NaOH to 7.2.

P. boydii 174^T (Auman et al. 2010) was isolated from an open water site at Point Barrow, Alaska, USA, about 40–60 cm from the ice/water interface from a 1.8-m ice core grown on Ordal's seawater cytophaga medium (SWCm) using full-strength artificial seawater (Irgens et al. 1989).

P. hadalis K41G^T (Nogi et al. 2007) was isolated from sediment collected from the bottom of the Japan Trench on marine agar 2216 (Difco) and was grown at 6 °C and 60 MPa. High-pressure cultivation was achieved using a liquid hydraulic

system. Piezophilic bacteria were cultivated in a plastic bag containing liquid medium in a pressure vessel made of stainless steel (SUS304). If necessary, oxygen-saturated fluorinert (FC-72; Sumitomo-3 M) was added to supply oxygen to the cultures (20 % of total volume). This was performed according to the procedure reported previously (Kato et al. 1994; Yanagibayashi et al. 1999).

P. heitensis AK15-027^T (Hosoya et al. 2008) was isolated from seawater taken from Heita Bay off Kamaishi in Japan. Strain AK15-027^T was isolated from seawater taken from Heita Bay in July 2003, using full-strength seawater agar that contained 100 % (v/v) filtered seawater and 1.5 % (w/v) agar, supplemented with 5 % (v/v) vitamin solution of the DSM141 medium (DSMZ 1993). The isolates were cultured and maintained on 1/5-strength marine agar [800 ml filtered seawater, 200 ml distilled water, 7.48 g marine broth 2216 (Difco) and 15 g agar].

P. ingrahamii 37^T (Auman et al. 2006) was isolated from a sea ice core collected from Point Barrow, Alaska, USA. Ordal's seawater cytophaga medium (SWCm), prepared in full-strength artificial seawater, was used for the isolation and routine growth of strain 37^T (Irgens et al. 1989).

P. japonica JAMM 0394^T (Miyazaki et al. 2008) was isolated from the sediment on the seabed adjacent to a sperm whale carcass off Kagoshima, Japan, after a 1-week incubation at 15 °C on marine agar 2216.

P. kaikoae AK15-027^T (Nogi et al. 2002) was isolated from sediment collected from the deepest cold-seep environment with chemosynthesis-based animal communities within the Japan Trench, at a depth of 7,434 m. The isolated piezophilic strains were grown in marine broth 2216 (Difco) at 10 °C and 50 MPa. High-pressure cultivation was achieved using a liquid hydraulic system. Piezophilic bacteria were cultivated in a plastic bag containing liquid medium in a pressure vessel made of stainless steel (SUS304). If necessary, oxygen-saturated Fluorinert (FC-72; Sumitomo-3 M) was added to supply oxygen to the cultures (20 % total volume). This method was performed according to the procedure reported previously (Kato et al. 1994; Yanagibayashi et al. 1999).

P. macrocephali JAMM 0415^T (Miyazaki et al. 2008) was isolated from the sediment on the seabed adjacent to a sperm whale carcass off Kagoshima, Japan, after a 1-week incubation at 15 °C on marine agar 2216.

P. marina 4-22^T (Kawasaki et al. 2002) was isolated from a cold current off the Monbetsu coast of the Okhotsk Sea in Hokkaido, Japan, after a 4-week incubation at 0 °C on marine agar 2216.

P. ossibalaenae JAMM 0738^T (Miyazaki et al. 2008) was isolated from the sediment on the seabed adjacent to a sperm whale carcass off Kagoshima, Japan, after a 1-week incubation at 15 °C on marine agar 2216.

P. profunda 2825^T (Xu et al. 2003) was isolated from deep Atlantic sediments at a depth of 2,770 m and a temperature of 2 °C. Sediment from the upper 2-cm layer was suspended in cold 75 % sterile seawater and spread onto chilled seawater agar plates prepared with a medium containing 1.5 g peptone, 0.5 g yeast extract, 0.01 g $FePO_4.4H_2O$, 750 ml seawater, and 250 ml distilled water. Sampling and isolation methods were described in detail by Ruger and Tan (1992).

Maintenance

Serial transfers of each medium on agar or in liquid medium at 4-week intervals followed by maintenance at 4 °C are recommended for medium-term storage, as is maintenance of cells as 15–20 % (w/v) glycerol suspensions in an appropriate medium at −80 °C. Long-term preservation methods include freeze-drying in skim milk or maintenance in liquid nitrogen at −196 °C.

Ecology

The main habitat of family *Psychromonadaceae* appears to be marine, sea ice, or the saline aquatic environments from which the original cultures were isolated. This applies also to *Psychromonas* species described or reclassified recently: *P. antarcticus* was isolated from Antarctic saline pond (Mountfort et al. 1998); *P. agarivorans* (Hosoya et al. 2009), *P. heitensis* (Hosoya et al. 2008) and *P. marina* (Kawasaki et al. 2002) were isolated from the coast of Japan; and *P. ingrahamii* (Auman et al. 2006) and *P. boydii* (Auman et al. 2010) were isolated from the ice core at Point Barrow, Alaska, USA. *P. ingrahamii* and *P. boydii* grow above 10 % (w/v) NaCl, and *P. ingrahamii* grows at −12 °C (Breezee et al. 2004). *P. aquimarina*, *P. japonica*, *P. macrocephali*, and *P. ossibalaenae* (Miyazaki et al. 2008) were isolated from the sediment on the seabed adjacent to a sperm whale carcass off Kagoshima, Japan. This environment has very abundant organic matter (Fujiwara et al. 2007). *P. hadalis* (Nogi et al. 2007), *P. kaikoae* (Nogi et al. 2002), and *P. profunda* (Xu et al. 2003) were isolated from deep sea sediment. Increased pressure enhances the growth of *P. profunda* (Xu et al. 2003) and is required for the growth of *P. hadalisi* (Nogi et al. 2007) and *P. kaikoae* (Nogi et al. 2002). The barophilic strain CNPT3 (Delong et al. 1997) proved to be closely related to *P. kaikoae* (Margesin and Nogi 2004). *P. arctica* (Groudieva et al. 2003) was from a seawater sample taken near Svalbard, Spitzbergen. In this way, all species were isolates from a natural environment that was rich in aqueous and mineral salts. Most of the species were grown at minimum temperatures of 0–4 °C and exhibit both fermentative and respiratory metabolism.

Pathogenicity, Clinical Relevance

The information on antibiotic sensitivity and resistance is limited to the family *Psychromonadaceae*. *Psychromonas profanda* cells are susceptible to penicillin G, tetracycline, chloramphenicol, furazolidone, and polymyxin B. No information on antibiotic sensitivity and resistance is available for the other species.

Application

Serine hydroxymethyltransferase from *Psychromonas ingrahamii* was characterized with respect to its spectroscopic, catalytic, and thermodynamic properties (Angelaccio et al. 2012). The properties of the psychrophilic enzyme have been contrasted with the characteristics of its homologous counterpart from *Escherichia coli*, which has been structurally and functionally characterized in depth and with which it shares 75 % of its sequence identity. Spectroscopic measures confirmed that the psychrophilic enzyme displays structural properties that are almost identical to those of its mesophilic counterpart. At variance, the *P. ingrahamii* enzyme showed decreased thermostability and high specific activity at low temperatures, both of which are typical features of cold-adapted enzymes. Furthermore, the enzyme was a more efficient biocatalyst compared to *E. coli* serine hydroxymethyltransferase (SHMT), particularly for side reactions. Many β-hydroxy-α-amino acids are SHMT substrates and represent important compounds in the synthesis of pharmaceuticals, agrochemicals, and food additives.

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30 The Family *Salinisphaeraceae*

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Abstract
The family *Salinisphaeraceae* (Class *Gammaproteobacteria*, Order *Salinisphaerales*) comprises a single genus, *Salinisphaera*, and six species: *S. shabanensis*, *S. hydrothermalis*, *S. dokdonensis*, *S. orenii*, *S. halophila*, and *S. japonica*. All members of the family *Salinisphaeraceae* were isolated from marine/oceanic and high-salinity environments. These bacteria have coccoid or short rod morphologies and are halophilic or halotolerant. All known members of the family *Salinisphaeraceae* are heterotrophic, mesophilic aerobes, although *S. hydrothermalis* was shown to be a facultative chemolithoautotroph. Isolation and characterization of new members of the *Salinisphaeraceae*, as well as in-depth studies of the currently known species, will allow for a better understanding of this family.

Taxonomy: Historical and Current

Sa.li.ni.shpae'ra'ce.ae, M. L. fem. n. *Salinisphaera* type genus of the family (L. adj. *salinus*, saline; M. L. fem. n. *sphaera*, a sphere; *Salinisphaera*, coccoid microorganism, capable of growth at high salt); -aceae ending to denote a family; M. L. fem. pl. n. *Salinisphaeraceae* the *Salinisphaera* family.

The first representative of the family *Salinisphaeraceae*, *S. shabanensis*, was isolated in 2003 from the brine-seawater interface of the Shaban Deep, Red Sea, at a depth of about 1,325 m (Antunes et al. 2003). At the time of its description, the 16S rRNA gene of *S. shabanensis* was 88 % similar to that of its closest relative. Based on the phylogenetic and phenotypic characteristic of this isolate, the new genus *Salinisphaera* was created, with *S. shabanensis* as the type species. In 2009, *S. hydrothermalis* was isolated from hydrothermal fluids collected from a diffuse-flow deep-sea hydrothermal vent located on the East Pacific Rise at 9° 50′ North, at a depth of 2,500 m (Crespo-Medina et al. 2009). *S. hydrothermalis* was able to grow both heterotrophically and autotrophically, and to oxidize n-alkanes. Hence, the description of the genus *Salinisphaera* was emended to reflect these observations (Crespo-Medina et al. 2009). *S. dokdonensis* was isolated from surface seawater of the East Sea in Korea (Bae et al. 2010), while *S. orenii* and *S. halophila* were isolated from a solar saltern in Korea and from the brine of a salt well in China, respectively (Park et al. 2012; Zhang et al. 2012). The most recent species, *S. japonica*, was isolated from the body surface of the deep-sea fish, *Malacottus gibber*, which was captured at a depth of 600–800 m in Toyama Bay, Japan (Shimane et al. 2013). Recently, the major cellular fatty acids and the predominant quinone were identified in several *Salinisphaera* spp., and the description of the genus was further emended (Shimane et al. 2013).

Phylogeny

Phylogenetic reconstruction based on 16S rRNA and created using the maximum likelihood algorithm showed that the family *Salinisphaeraceae* forms a unique branch in the Class *Gammaproteobacteria* (◉ *Fig. 30.1*). The closest relatives to the *Salinisphaeraceae* are the Families *Sinobacteraceae* and *Halothiobacillaceae*. All the *Salinisphaera* spp. clustered together in a unique group (◉ *Fig. 30.1*). Within this group, *S. shabanensis*, *S. dokdonensis*, *S. orenii*, and *S. halophila* clustered together, with the two latter isolates, which originated from hypersaline environments, closely related (◉ *Fig. 30.1*). In contrast, *S. hydrothermalis* and *S. japonica* formed a distinct sub-cluster (◉ *Fig. 30.1*). The sequence similarity of the 16S rRNA gene among the *Salinisphaera* spp. ranged between 93.8 % and 96.7 %.

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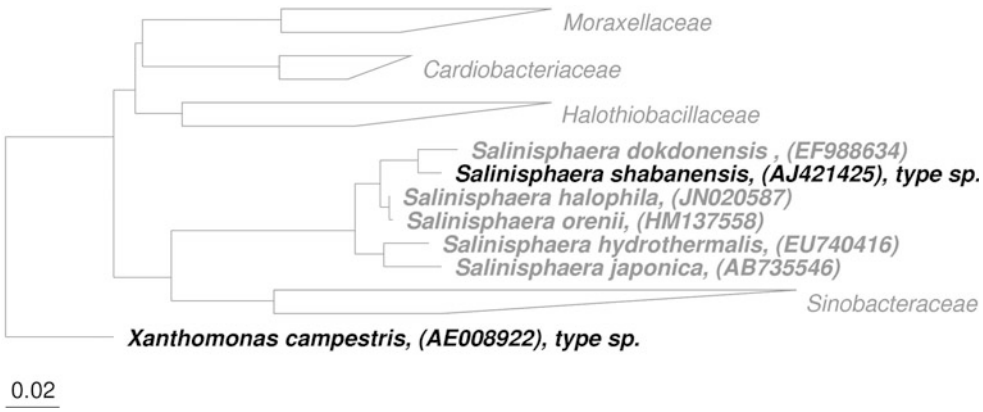

Fig. 30.1

Phylogenetic reconstruction of the family *Salinisphaeraceae* based on 16S rRNA and created using the maximum likelihood algorithm RAxML (Stamatakis 2006). The sequence datasets and alignments were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). Representative sequences from closely related taxa were used as outgroups. In addition, a 40 % maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. Scale bar indicates estimated sequence divergence

Autotrophy and Detection of the *rbcL*/*cbbL* Gene

S. hydrothermalis and *S. shabanensis* were capable of autotrophic growth and carried the *rbcL*/*cbbL* gene (encoding for form I RubisCO), suggesting that the Calvin-Benson-Bassam (CBB) cycle for autotrophic CO_2 fixation is operational in these bacteria (Crespo-Medina et al. 2009). The *cbbM* gene, encoding for form II RubisCO, was not detected in either strain. However, none of the other type strains were tested for autotrophic growth, and further studies are necessary to assess if autotrophy is widespread across all known *Salinisphaera* spp.

Oxidation of *n*-Alkanes and Detection of the *alkB* Gene

S. hydrothermalis and *S. shabanensis* grew on dodecane as their sole carbon and energy source and the *alkB* gene, encoding for the enzyme alkane hydroxylase, which is involved in the first step of the alkane oxidation pathway, was detected in both strains (Crespo-Medina et al. 2009). Currently, it is not known if the ability to oxidize *n*-alkanes is a trait conserved in the other type strains of the family *Salinisphaeraceae*.

Genome

Within the family *Salinisphaeraceae*, only the draft genome sequence of *Salinisphaera shabanensis* is currently available (Antunes et al. 2011). The approach used in this study provided annotation for 98 % of all 4,110 predicted genes. The distribution of genes into COGs functional categories indicated that the majority of genes are involved in energy production and conversion (227; 7.1 %), amino acid transport and metabolism (232; 7.2 %), inorganic ion transport and metabolism (191; 5.9 %), and cell wall/membrane biogenesis (183; 5.7 %). The draft genome has a G+C content of 61.0 mol%, a value only slightly lower than the range determined for the species by the HPLC method (61.8–63.8 mol%; *Table 30.1*). Several of the genes identified were involved in osmoadaptation (*e.g.*, *ectABC* gene cluster), heavy metal detoxification, resistance and efflux (e.g., RND permeases, mercuric and arsenate reductases), and uptake, storage, and degradation of limiting nutrients (e.g., PHB and siderophore production).

Evidence from the draft genome of *S. shabanensis* indicates that, besides the *rbcL*/*cbbL* gene, almost all the other genes coding for enzymes in CBB cycle are present. These findings further attest that the CBB cycle for autotrophic CO_2 fixation is operational in this bacterium. The detection of the *CYP153* and *alkB* genes in the genome of *S. shabanensis*, encoding for the P450 and AlkB alkane hydroxylases, respectively, supports the experimental evidence that this strain can oxidize *n*-alkanes. Furthermore, an almost complete set of genes for one of the branches of the benzoate degradation pathway – a model compound for the degradation of aromatic compounds – was identified in the genome of *S. shabanensis* (Alam et al. 2013).

Phenotypic Analyses

The physiological and phenotypic characteristics of the type strains of the family *Salinisphaeraceae*, including the range in composition of the cellular fatty acids, are shown in *Tables 30.1*–*30.3*. When grown on solid medium, colonies of the *Salinisphaera* type strains were brownish, white or translucent in color and cells were either cocci of 0.7–1.2 μm in

diameter or rods ranging between 0.3 and 1.1 in width by 0.5–2.0 μm in length (Table 30.1). With the exception of *S. japonica*, all the type strains were motile by means of single or more flagella. All the described *Salinisphaera* spp. are mesophilic (the optimum temperature for growth ranged between 25 °C and 37 °C), heterotrophic aerobes which grow over a fairly broad pH range and require at least 1 % w/v NaCl (Table 30.1). Currently, *S. orenii* and *S. halophila* are the most halophilic representatives of the family *Salinisphaeraceae*, with optimal NaCl concentrations for growth of 15–17 % and 14–19 % w/v, respectively (Table 30.1). All the type strains can grow on marine complex medium, and *S. hydrothermalis* and *S. shabanensis* were also shown to grow on acetate and *n*-alkanes as sole carbon and energy sources and chemolithoautotrophically in the presence of thiosulfate and bicarbonate (Crespo-Medina et al. 2009). The G+C content in the *Salinisphaera* type strains ranged between 61.8 % and 69.5 % (Table 30.1). With the exception of *S. japonica*, all the type strains were catalase positive (Table 30.2). The major polar lipids in the *Salinisphaera* type strains were diphosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylglycerol, while *S. japonica* was the only strain to synthesize phosphatidylserine (Shimane et al. 2013; Zhang et al. 2012). Data on the polar lipids of *S. orenii* are not available.

Isolation, Enrichment, and Maintenance Procedures

Members of the family *Salinisphaeraceae* can be enriched and isolated using complex liquid or solid marine media of neutral pH containing, depending on the strain, at least 1–6 % w/v NaCl. The cultures can be incubated at temperature of 20–35 °C. For long-term storage, stocks can be prepared by adding 150 μl of sterile glycerol to 850 μl of a fresh culture. The stocks can then be stored at −80 °C.

Ecology

All known members of the *Salinisphaeraceae* have been isolated from marine (deep-sea and surface water) and hypersaline environments. *S. shabanensis* thrives at the interface between brine and seawater where cells are exposed to drastic increases in salinity, heavy metals, and fluctuating nutrient availability. The adaptation of this bacterium to such harsh conditions is reflected in its genome, which carries genes involved in osmoadaptation, heavy metal detoxification, and nutrient storage (Antunes et al. 2011). *S. hydrothermalis* and its close relatives, *Salinisphaera* spp. EPR71 and EPR72, were isolated from the fluids collected from a diffuse-flow deep-sea hydrothermal

Table 30.1
Physiological characteristics of type strains of the genus *Salinisphaera*[a]

	S. shabanensis E1L3A^T	*S. hydrothermalis* EPR70^T	*S. dokdonensis* CL-ES53^T	*S. orenii* MK-B5^T	*S. halophila* YIM95161^T	*S. japonica* YTM-1^T
Morphology	Cocci	Short rods	Short rods	Short rods	Short rods	Short rods or cocci
Cell size (μm)	0.7–1.2	0.3–0.5 × 0.8–1.0	0.3–0.6 × 0.5–0.9	0.5–0.7 × 1.5–2.0	0.4–0.6 × 0.9–1.4	0.9–1.1 × 1.0–1.8
Motility	+	+	+	+	+	−
Colony color	Brown-yellow	Brown-orange	Brown-yellow	White	Translucent	Brown-pink
Oxygen requirement	Facultative aerobe	Strict aerobe	Strict aerobe	Strict aerobe	Strict aerobe	Strict aerobe
NaCl range (optimum) (w/v %)	1–28 (10)	1–25 (2.5)	4–21 (10)	3–25 (15–17)	6–29 (14–19)	1–30 (7.5–10)
pH range (optimum)	4.0–8.0 (6.5–7.5)	5.0–7.5 (5.5)	5.2–8.8 (6.3–7.2)	5.0–8.5 (7.0–7.5)	5–8 (7.0)	3.8–9.5 (5.0–5.5)
Temperature range (optimum) (°C)	5–42 (30–37)	20–40 (30–35)	5–40 (25)	10–40 (30–37)	15–40 (20–25)	4–30 (20–25)
Nitrate reduction	−	+/−	−	ND	−	−
G+C (mol%)	61.8	64	64.9	63.4	69.5	67.3
Isolation	Deep-sea brine	Deep-sea hydrothermal vent	Seawater	Solar saltern	Salt well brine	Deep-sea fish

Antunes et al. (2003), Crespo-Medina et al. (2009), Bae et al. (2010), Park et al. (2012), Zhang et al. (2012), Shimane et al. (2013)
+ positive, − negative, *ND* no data, +/− variable results
[a]All strains were negative to Gram stain and for the production of H_2S and possess ubiquinone Q-8

Table 30.2

Comparative phenotypic characteristics of type strains of the genus *Salinisphaera*[a]

	S. shabanensis E1L3A^T	*S. hydrothermalis* EPR70^T	*S. dokdonensis* CL-ES53^T	*S. orenii* MK-B5^T	*S. halophila* YIM95161^T	*S. japonica* YTM-1^T
Methyl red test	–	–	–	–	+	+
Enzyme activity for						
Catalase	+	+	+	+	+	–
Oxidase	+	–	+	–	+	–
Acid phosphatase	+/–	+	+	–	–	+
α-chymotrypsin	–	+/–	–	–	+	+
Esterase C4	+	+	+	+	–	+
Esterase lipase C8	+	+	+	+	–	+
Leucine arylamidase	+	+	+	+	–	+
Lipase C14	–	+	+	+	–	+
Naphthol-AS-Bi-phophohydrolase	+	+/–	+	+	–	+
Valine arylamidase	–	+	+	+	–	+
Utilization of carbon sources						
L-Arabinose	–	+	+	+	+	–
D-Fructose	+	–	+	+	–	–
D-Galactose	+	–	+	+	–	–
D-Glucose	+	+/–	+	+	–	–
D-Lactose	–	–	+	–	–	–
Acid Production from						
D-Galactose	–	+/–	–	–	–	+
D-Glucose	–	+/–	–	–	–	+
D-Fructose	–	+/–	–	–	–	+
Salicin	–	–	–	–	+	–
L-Fructose	–	+	–	–	+	–
D-Arabitol	–	+	–	–	–	–
L-Arabitol	–	+	–	–	–	–
Xylitol	+/–	+/–	–	–	+	–
Antibiotic resistance						
Chloramphenicol	–	–	–	–	+	–
Erythromycin	–	–	–	–	+	–

+ positive, – negative, +/– variable results

[a]All strains are negative for utilization of α-frucosidase, α-galactosidase, α-glucosidase, α-mannosidase, B-galactosidase, B-glucosidase, B-glucoronidase, cysteine arylamidase, and *N*-acetyl-B-glucosaminadase, trypsin, and for hydrolysis of casein and gelatin. All strains were positive for utilization of glycerol

vent (Crespo-Medina et al. 2009). The temperature of the fluids at the time of sampling ranged between 2.5 °C and 13 °C. Interestingly, *S. hydrothermalis* has the lowest NaCl optimum (2.5 % w/v) of all the *Salinisphaera* type strains, in line with the lower salinity of hydrothermal fluids as compared to seawater. In contrast, *S. orenii* and *S. halophila* have the highest lowest NaCl optima (between 14 % and 19 % w/v), consistent with the hypersaline environments they originate from (Park et al. 2012; Zhang et al. 2012). The ability of *S. hydrothermalis* to grow chemolithoautotrophically and to conserve energy via the oxidation of reduced inorganic sulfur species suggests that this bacterium is well adapted to the vent environment, where reduced sulfur species and carbon dioxide are readily available. Furthermore, the ability of *S. hydrothermalis* and *S. shabanensis* to oxidize *n*-alkanes relates well to the identification of a variety of hydrocarbons in the original biotopes of these organisms (Brault et al. 1988; Michaelis et al. 1990). Finally, evidence from the genome sequence of *S. shabanensis* suggests that this bacterium may be capable to degrade aromatic hydrocarbons (see Genome section in this chapter).

Table 30.3

Range in the composition of major cellular fatty acids of type strains of the genus *Salinisphaera. Values are percentages of total fatty acids**

	S. shabanensis E1L3A^T	*S. hydrothermalis* EPR70^T	*S. dokdonensis* CL-ES53^T	*S. orenii* MK-B5^T	*S. halophila* YIM95161^T	*S. japonica* YTM-1^T
Straight-chain						
14:0	6.7^d–25.8^a	2.0^c–6.6^a	8.3^b–14.6^a	22.1^b	16.1^a	3.2^c
16:0	1.2c–6.8^b	3.0^c–27.1^b	1.7^c–14.9^b	16.1^b	5.2^a	5.4^c
18:0	2.9^a–15.6^b	5.5^c–21.4^a	3.5^a–10.2^b	8.9^b	6.9^a	3.2^c
Branched						
19:0ω8c cyclo	2.8^a	ND^a–55.6^d	0.8^a–7.6^b	25.8^b	2.2^a	30.7^c
Summed feature 8 (C18:1ω6c and/or 18:1ω7c)	23.5^b–70.2^d	2.7^b–20.4^b	21.5^b–54.8^c	1.4^b	47.1^a	30.2^c

[a]Data from Zhang et al. (2012)
[b]Data from Park et al. (2012)
[c]Shimane (2013)
[d]Bae et al. (2010)
*Shown are fatty acids that represent >10 % of the cellular fatty acids from at least two species, *ND*: no data

Application

No current application for any members of this family has been reported. However, there is significant potential for a wide range of applications, as suggested by several studies.

Bioremediation and Bioleaching: Members of the *Salinisphaeraceae* contain an interesting set of enzymes and genes with potential application in bioremediation and bioleaching and possible interest for the oil and mining industry.

Oil and heavy metal bioremediation. Crespo-Medina et al. (2009) reported that *S. shabanensis* and *S. hydrothermalis* were able to grow with *n*-alkanes as the sole carbon and energy source, and detected the *alkB* gene, which encodes for the alkane hydroxylase, an enzyme that catalyzes the first step in the *n*-alkane oxidation pathway. More recent studies on oil-degrading marine microbial communities resulted in the isolation of several additional *Salinisphaera* strains capable of using a wide variety of alkanes (C5-C38 *n*-alkanes, pristane, paraffinic wax, and diesel fuel) and encoding for three enzymes involved in hydrocarbon oxidation: AlmA, CYP153A, and AlkB (Wang et al. 2010; Wang and Shao 2012). Taken together, these data suggest that *Salinisphaera* spp. might have an application in the bioremediation of oil spills in marine environments.

Multiple genes associated with iron uptake, heavy metal resistance, detoxification, and efflux transporters were reported in the genome of *S. shabanensis* and included siderophore production, mercuric and arsenate reductases, and genes for transporters and resistance proteins involved in resistance to copper, zinc, cobalt, cadmium, magnesium, mercury, and arsenate (Antunes et al. 2011).

Stress-protective agents. Compatible solutes have attracted considerable biotechnological interest as biomolecules (enzymes, proteins, DNA, membranes) and whole cells stabilizers, salt antagonists, or stress-protective agents (da Costa et al. 1998; Margesin and Schinner 2001). Osmoadaptation by accumulation of compatible solutes, such as ectoine and betaine, has been demonstrated experimentally in *S. shabanensis*, in which they reached intracellular concentrations above 4 M (Antunes et al. 2003). In line with these experimental observations, several genes involved in compatible solute synthesis and transport were identified in the genome of *S. shabanensis* (Antunes et al. 2011).

Other applications. The observation of poly-β-hydroxybutyrate (PHB) granules in *S. dokdonensis* (Bae et al. 2010), and detection of PHB synthesis and degradation genes in *S. shabanensis* (Antunes et al. 2011), is also biotechnologically relevant. Microbial PHB-production is seen as an eco-friendly, viable alternative to oil-derived plastics in several fields (Margesin and Schinner 2001).

Finally, Kim et al. (2011) isolated a new *Salinisphaera* strain from the intestinal tract of a fish and reported on the identification of a cold-adapted new esterase that could find applications in biocatalysis under low-water activities for the pharmaceutical and chemical industries.

In conclusion, members of the family *Salinisphaeraceae* are potentially relevant for a range of biotechnological applications.

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31 The Family *Shewanellaceae*

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Abstract
Shewanellaceae, a family within the order *Alteromonadales*, consist of a sole genus *Shewanella*. Almost all species of this genus are Gram-negative, motile rod with positive oxidase and catalase reaction. Facultatively anaerobic. Oxygen is used as the electron acceptor during aerobic growth. The mol% G+C of the DNA is 38–54. The genome size of *Shewanella* is approximately 5 Mb by whole-genome sequences analysis based on some species reported. The organisms are widely distributed in nature; marine environment, marine organisms, deep sea, iced fish, proteinaceous foods, and occasionally clinical samples. Roughly, three major groups are delineated within the genus based on the 16S rRNA gene sequence analysis: high GC content group, psychrotolerant and non-halophilic group, and psychrotolerant and psychrophilic sodium ion-requiring group. Most species related to marine environment are psychrophile and halophile, and can grow at 4 °C, with production of polyunsaturated fatty acid in phospholipids consisting of their cytoplasmic membrane. Psychrotolerant species representing *S. putrefaciens* which is the type of *Shewanella* species generally do not synthesize polyunsaturated fatty acid. Due to the ability to grow at 4 °C and to produce a variety of volatile sulfides, including H_2S, and in marine fish, they reduce trimethylamine oxide (TMAO) to trimethylamine (TMA) which has a fishy smell; some *Shewanella* species are important in the food industry. Additionally, *S. alga*, formerly identified as *S. putrefaciens*, has been implicated in human disease (bacteremia and sepsis). Some *Shewanella* species are known to have unique metabolic characteristics including dissimilatory reduction of manganese, iron oxide, and other metal compounds. Therefore, these bacteria are implicated to apply as the microbial fuel cell based on their ability to make electricity.

Taxonomy, Historical, and Current

Short Description of *Shewanellaceae*

Shewanellaceae (She.wa.nel.la'ce.ae. N.L. fem. n. *Shewanella* type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. *Shewanellaceae* the *Shewanella* family) was established by

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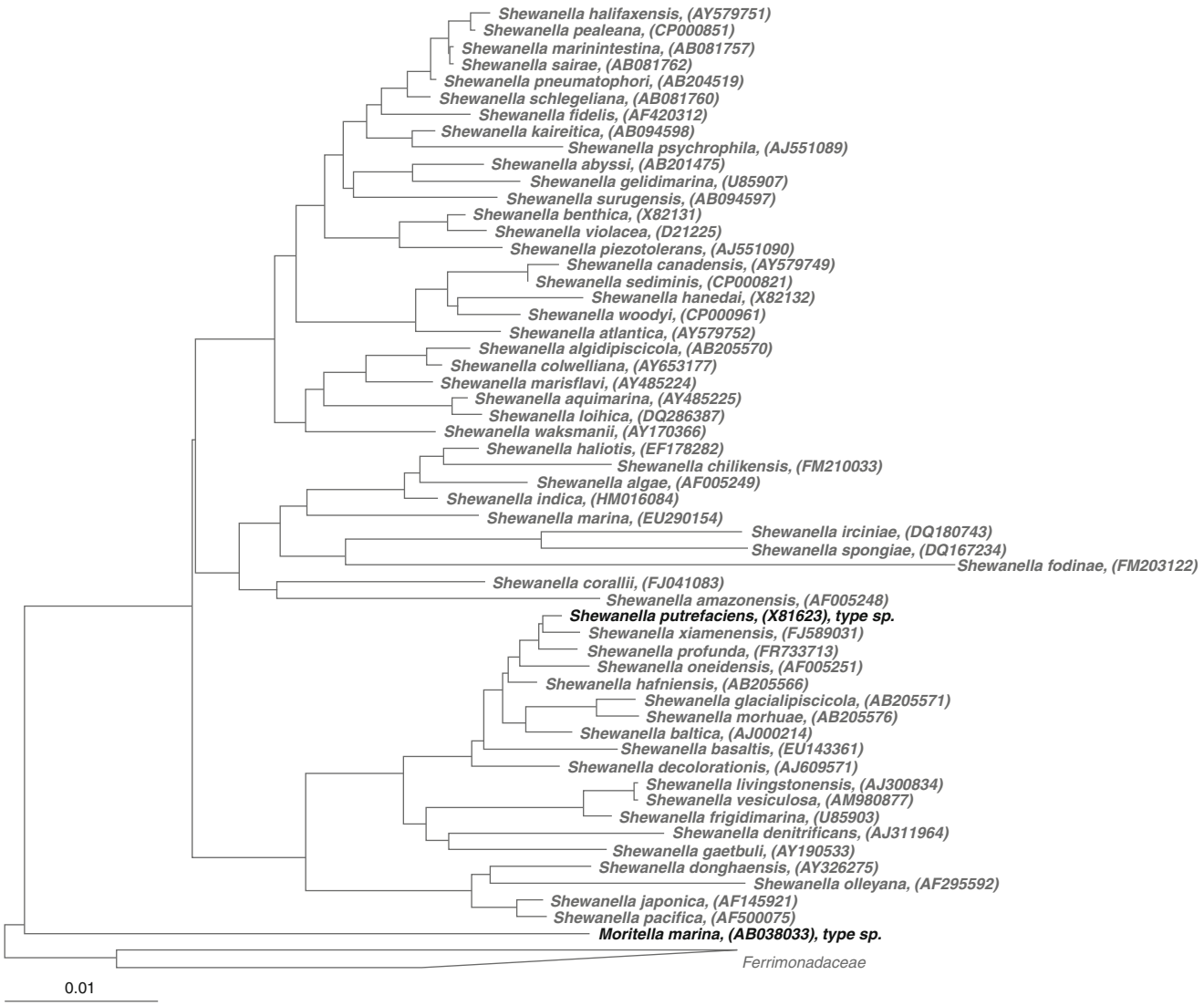

Fig. 31.1

Phylogenetic reconstruction of the family *Shewanellaceae* based on 16S rRNA and created using the neighbor-joining algorithm withthe Jukes–Cantor correction. The sequence datasets and alignments were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high-quality-type strain sequences proportionally distributed among the different bacterial and archaeal phyla. In addition, a 40 % maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. Scale bar indicates estimated sequence divergence.

Ivanova et al. in 1994. The type genus is *Shewanella* (MacDonell and Colwell 1985). The family *Shewanellaceae* is members of the order *Alteromonadales* belonging to the class *Gammaproteobacteria* (Fig. 31.1). *Shewanellaceae* is consisted of sole genera, *Shewanella*, which was named in honor of late Dr J. Shewan for his work in fisheries microbiology. They are Gram-negative, rod-shaped bacteria. Motile by a single unsheathed polar flagellum. Do not form endospores or microcysts. Chemoorganotrophs. Aerobic or facultatively anaerobic. Able to reduce nitrate to nitrite and grow anaerobically by reducing trimethylamine *N*-oxide and ferric compounds. Some species do not require Na^+ ions for growth. In most species, the major isoprenoid quinones are Q7, Q8, and MK7. The major fatty acids are 14:0, 16:1ω7, 16:0, and 17:1ω6. Most species produce polyunsaturated fatty acid (PUFA). To date, the genus encompasses more than 50 recognized species (Fig. 31.1) with broad environmental distribution including (but not limited to) freshwater lakes, coastal, open- and deep-sea waters, ocean sediments, marine organisms, oil fields, iced fish, and proteinaceous foods. Occasionally, some strains have also been isolated from clinical samples. The mol% G+C of the DNA is: 38–54.

Taxonomic History

Shewanella species as a food spoilage organism was first reported by Derby and Hammar (1931). The primary species of interest to the food industry, *Shewanella putrefaciens*, has been known since then, although under changing names and taxonomic positions. As it is regarded as a representative species of fish spoilage bacteria, much research has focused on *Shewanella putrefaciens*. Even so, the taxonomic position of *S. putrefaciens* remains confusing and is still unresolved for some strains. Initially, *S. putrefaciens* was identified as *Achromobacter* (Derby and Hammar 1931), a group comprising various Gram-negative, nonfermentative, oxidase-positive, rod-shaped bacteria. The species was transferred to *Pseudomonas* by Long and Hammer in 1941 and placed in the *Pseudomonas* group III/IV using a classification scheme proposed by Shewan et al. (1960). (Shewan et al. 1960). However, due to the difference in guanine + cytosine (G+C) content between *Pseudomonas putrefaciens* (typically 43–53 %) and other pseudomonads (typically 58–72 %), the species was transferred to the genus *Alteromonas* (Baumann et al. 1972; Lee et al. 1977). In 1985, MacDonell and Colwell demonstrated that *Alteromonas putrefaciens*, along with two other marine species, be transferred to a completely new genus, *Shewanella*. The study was based on comparison of 5S rDNA gene sequences (Demong et al. 1984; Coyne et al. 1989) and suggested that the genus *Shewanella*, at the time comprised of *S. putrefaciens*, *S. benthica* (Nogi et al. 1998), and *S. hanedai* (Jensen et al. 1980), be included in the *Vibrionaceae* family (Farmer 1992). Although the genus *Shewanella* was sharing a number of phenotypic characteristics with other genera in formerly *Vibrionaceae* (e.g., association with the marine environment, the ability to use various electron acceptors, and the production of hydrolytic enzymes), *Shewanella* was easily distinguished from others as a strict aerobic bacteria (*Table 31.1*). The advent of 16S rRNA gene sequence analysis has emended the definition of *Vibrionaceae* (Kita-Tsukamoto et al. 1993; Gauthier et al. 1995) and resulted in the creation of the *Alteromonadaceae* including the genus *Shewanella* and other marine-related genera. Then after the huge bacterial family, *Alteromonadaceae* was divided into eight families by Ivanova et al. in 1994, and at that time *Shewanellaceae* was established.

As eluted previously, *Shewanella putrefaciens* has been known since the early 1930s as a representative species of fish spoilage bacteria with much research. Since its importance stems from its role in spoilage of low-temperature stored protein-rich foods (e.g., marine fish, chicken), *Shewanella* can produce a variety of volatile sulfides, including H_2S, and in marine fish they reduce trimethylamine oxide (TMAO) to trimethylamine (TMA), resulting in a characteristic fishy smell (Herbert et al. 1971; Shewan, 1971; Lee et al. 1977; Shewan, 1977; Parker and Levin, 1983; Jorgensen et al. 1988; Dainty et al. 1989; Stenstrom and Molin, 1990; Russell et al. 1995; Gram and Huss, 1996; Borch et al. 1996; Chiniwasagam et al. 1998; Leroi et al. 1998; Vogel et al. 2004). However, *Shewanella putrefaciens* has been known as very heterogeneous species (Owen et al. 1978; Semple et al. 1989; Vogel et al. 1997; Ziemke et. al. 1998; Venkateswaran et al. 1999). For example, while *S. putrefaciens* were originally typified as being Gram-negative, rod-shaped, motile, positive for oxidative acid production, non-halophilic, and aerobic bacteria, several researchers identified a mesophilic, halotolerant group often associated with warm-blooded animals and, occasionally, with disease in humans (Shewan 1977; Owen et al. 1978). Owen et al (1978) demonstrated that *S. putrefaciens* known as the heterogeneous group was divided into four bacterial groups, characterized as Owen's groups I–IV. Based on genomic DNA relatedness, it was demonstrated that clinical isolates were clearly distinguished from the other food-related strains comprising Owen's groups I and II. Recent research utilizing DNA sequence methods subsequently demonstrated that the vast majority of these mesophilic isolates were members of a different species, *S. alga*, corresponding to Owen's group IV (Gilardi 1972; Riley et al. 1972a; Richard et al. 1985; Nozue et al. 1992; Vogel et al. 1997; Khashe and Janda 1998, Ziemke et al. 1998, Vogel et al. 2000). The use of classical phenotypic characterization to distinguish between *S. algae* and other species (e.g., high G+C% (52–56 %), tolerance to 42 °C, and10 % NaCl are characteristics of *S. alga* that supported this affiliation with group IV. Subsequently, Owen's group I and II were reclassified as *S. putrefaciens* and *S. baltica*, respectively, based on 16S rDNA sequence analysis (Ziemke et al. 1998; Venkateswaran et al. 1999). However, taxonomic positions for Owen's group III strains have not been resolved, primarily because this group was heterogeneous from the first.

After developing 16S rRNA sequence analysis technique, reclassification of bacterial species was significantly accelerated due to clear and simple identification system (Stackebrandt and Goebel, 1994). Also member of the genus *Shewanella* was renewed and species numbers were expanded. Moreover, numerical new isolates derived from unexpected samples where the isolation source for formerly *Shewanella* fall into the genus *Shewanella* based on phylogenetic analysis prior to classical phenotypic identification. In addition to the new isolates being similar phenotype to known *Shewanella*, several new species that have been found in cold ocean environment, Antarctic sea and Arctic sea, or deep-sea environment, were also placed in this genus (Deming et al. 1984; Bowman et al. 1997; Yano et al. 1997; Nogi et al. 1998; Toffin et al. 2004; Miyazaki et al. 2006; Gao et al. 2006; Bozal et al. 2007; Yang et al. 2007; Xiao et al. 2007; Bozal et al. 2009). As the result, the genus encompasses more than 50 recognized species with a wide range of physiological diversity reflecting their habitation.

Phylogenetic Structure of the Family and Its Genera

According to the phylogenetic branching of the type strains of *Alteromonadales* in the RaxML 16S rRNA gene tree of the Living Tree Project (Yarza et al. 2008, 2010), the family is moderately related to the families *Ferrimonadaceae*, *Moritellaceae*, and some genus that taxonomic affiliation on the family level remains unclear. A phylogenetically broad group containing

Table 31.1
Phenotypic characteristics of *Shewanellaceae* and some closely related Gram-negative families

Reaction	*Shewanellaceae*	*Alteromonadaceae*	*Colwelliana*	*Pseudoalteromonadaceae*	*Pseudomonas* spp.	*Vibrionaceae*	*Enterobacteriaceae*
Gram reaction	−	−	−	−	−	−	−
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+	(+)	(+)
Requires Na + for growth	(−)	+	+	+	−	(+)	−
Cytochrome oxidase	+	+	+	+	+	+	−
Catalase	+	+	+	+	+	+	+
Acid from D-Glucose	−/O	−	F	−	O	F	F
TMAO reduction	+	nd	nd	nd	−	+	+
H_2S production	+	(−)	(−)	(−)	−	(+)	(+)
Ornithine decarboxylase	+	−	−	−	−	(−)	+/−
Reduce nitrate to nitrite	+	−	+	(−)	(+)	(+)	(+)
G + C%[a]	38−54	44−47	35−46	36−48	58–70	38–63	38–60

+ positive; (+) a few negative; +/− some positive, some negative; (−) a few positive; − negative, *TMAO* trimethylamine oxide; *nd* no data
[a]May vary slightly depending on method of determination, e.g., high-performance liquid chromatography or Tm

Alteromonadaceae, *Pseudoalteromonadaceae*, *Idiomarinaceae*, *Colwelliaceae*, *Psychromonadaceae*, and several other families appear as a sister clade of these families/groups with *Shewanellaceae* (Fig. 31.1). The species belonging to the genus *Shewanella* fall into one phylogenetical group with clear distinguishable phylogenetical distance, indicating that it is reasonable to be categorized as one bacterial genus. Roughly, three major groups reflecting phenotypic characteristics are delineated within the genus based on the 16S rRNA gene sequence analysis: high GC content group, psychrotolerant and non-halophilic group, and psychrotolerant and psychrophilic sodium ion-requiring group.

Molecular Analyses

Phylogeny

The widespread adoption of molecular tools, such as PCR and DNA sequencing, and subsequent phylogenetic studies based on 16S rDNA gene sequences resulted in a major reclassification of bacterial taxonomy including the establishment of the family *Shewanellaceae* to encompass the genus *Shewanella*. Phylogenetic trees based on 16S rRNA sequences of *Shewanella* strains are shown in Fig. 31.1. Overall, three major groups are delineated within the genus, reflecting their habitat and phenotype with some exception. One large group includes the psychrotolerant non-halophilic species, *S. putrefaciens*, *S. baltica*, *S. frigidimarina*, *S. oneidensis*, and others and some psychrophilic and halophilic species, *S. japonica*, *S. pacifica*, and others. The second group includes the psychrotolerant and psychrophilic Na+ -requiring species, *S. benthica*, *S. gelidimarina*, *S. hanedai*, *S. pealeana*, *S. woodyi*, *S. violacea*, and others. The third group includes the mesophilic and high GC content species, *S. algae*, *S. amazonensis*, and others, with some exceptional species. Although in the previous studies, EPA production feature and growth characteristics, Na+ requirement, and temperature were corresponded with phylogenetic positions in *Shewanella* species (Russell and Nichols, 1999; Bowman, 2005); this classification is to be collapsing with drastic expanding of the genus. The taxonomy of the most species is clear-cut, and phenotypic differences can readily distinguish these species (Table 31.2). However, 16S rRNA gene analysis occasionally lacks the specificity for differentiation of close relatives (Fox et al. 1994; Stackebrandt and Goebel, 1994; Yamamoto and Harayama, 1995, 1996, 1998). Thus, higher-resolution molecular identification markers are required to distinguish the ever-expanding pool of *Shewanella* species. To resolve this limitation, more recent studies have targeted the rapidly evolving gene, *gyrB* (encoding the β subunit of DNA gyrase), to examine the phylogeny of *Shewanella* (Venkateswaran et al. 1999; Satomi et al. 2003). For instance, 16S rRNA gene sequences of *S. marinintestina* and *S. sairae* are completely the same with each other, indicating that it is not distinguishable based on this gene sequence. On the other hand, the sequence analysis of gyrB shows both species were clearly distinguished with approximately 10 % sequence diversity, result which was in strong agreement with those from DNA-DNA hybridization analysis. Moreover, since gyrB sequences of *Shewanella* species deposited in public database are enriched, it facilitates to employ this gene sequence for phylogenetic analysis.

DNA-DNA Hybridization

In accordance with the consensus molecular definition of the species (Wayne et al. 1987), the phylogenetic definition of a species would generally include strains with "approximately 70 % or greater DNA-DNA relatedness and with 5 °C or less ΔTm," and "phenotypic characteristics should agree with this definition and would be allowed to override the phylogenetic concept of species only in a few exceptional cases." Thus, DNA-DNA reassociation values derived from DNA-DNA hybridization methods with labeled DNA (Ezaki et al. 1989) or thermal renaturation methods (De ley et al. 1970) should be respected prior to 16S rRNA sequence analysis in bacterial taxonomy. As mentioned above, since 16S rRNA gene analysis occasionally lacks the specificity for differentiation of close relatives, DNA-DNA relatedness values among close related species in taxonomic study are essential information to decide species affiliation. In the taxonomic study for *Shewanella*, DNA-DNA hybridization is generally performed when describing the new species. Historically DNA-DNA hybridization technique was employed to study for taxonomy in *Shewanella* species, mainly formerly *S. putrefaciens*. Owen et al. (1978) have demonstrated that *S. putrefaciens* isolated various samples including food, clinical specimens, and deep-sea sediment, comprising four DNA hybridization groups, characterized as Owen's groups I–IV. Based on genomic DNA relatedness, it was demonstrated that clinical isolates were clearly distinguished from Owen's groups I and II. Phenotypic characteristics and DNA sequence methods demonstrated that the Owen's group IV was corresponding to *S. alga*, which were coincident with the classical phenotypic characterization to distinguish between *S. algae* and other species (e.g., high G+C% (52–56 %), tolerance to 42 °C and 10 % NaCl are characteristics of *S. alga*). Additional data in the form of ribotypes and protein electrophoretic patterns indicate that strains belonging to DNA groups *S. putrefaciens* can be clearly distinguished from *S. algae* (Vogel et al. 1997). Recent research utilizing DNA sequence methods subsequently demonstrated that Owen's groups I and II were reclassified as *S. putrefaciens* and *S. baltica*, respectively, based on 16S rDNA sequence analysis. Overall, *S. baltica* represents a large proportion of isolates conforming to Gilardi biovars 1 and 3 (Gilardi, 1973). Phenotypic analyses of *S. putrefaciens* by Semple and Westlake (1987) and Ziemke et al. (1998) suggest that phenotypic resolution of Owen's groups I–IV may be possible, although Owen's group III consists heterogeneous strains. However, taxonomic positions for Owen's group III strains have not been resolved, from the first (Owen et al. 1978; Venkateswaran et al. 1999; Vogel et al. 1997, 2000, 2005; Bowman, 2005).

Table 31.2

Phenotypic characteristics which differentiate the species of the genus *Shewanella*

Characteristics	*S. abyssi*	*S. affinis*	S. algae	*S. algidipiscicola*	*S. amazonensis*	*S. aquimarina*	*S. arctica*	*S. atlantica*
Bioluminescence	–		–	–	–			–
Violet pigment	–	–	–	–	–	–	–	–
Growth at 4 °C		–	–	+	+	–	+	+
Growth at 25 °C	+	+	+	+	+	+	+	+
Growth at 37 °C	–	–	+	+	+	+	–	–
Barophilic			–		–			–
Requires Na^+ for growth	+	+	+		d	+	+	+
Tolerates 6 % NaCl	–	+	+	+	–	+	–	–
Fermentation of								
D-Glucose		+	–	–	–			–
D-Glucose (with gas)			–		–			
N-Acetylglucosamine				–				+
Chitinase	–	–	–		–			+
Amylase		–	–		–	+		–
Lipase	+		+			+		+
Hemolysis		+	+		+	+		
Nitrite reduced	+	+	+	+	+	+	+	+
Ornithine decarboxylase		+	+	V	–			+
EPA synthesis	+	+	–		–	–	–	+
H_2S production	+	+	+	+	+	+		+
Iron reduction			+		+		+	-
Acid from D-glucose	–		V			–	+	–
Utilization of								
D-Glucose		+	–	–			+	d
Cellobiose			–				+	
Maltose			+	–	–	+	+	–
Sucrose		d	+	–	–			
N-Acetylglucosamine			+	+			–	+
D-Gluconate			+	–			–	
Mol% G+C	44	45	54	47	52	54	40	46
Quinone type	Q-7, 8	Q-7, 8				MK-7	MK-7	MK, MMK-7
						Q-7, 8	Q-7, 8	Q-7, 8
References	Miyazaki et al. (2006)	Ivanova et al. (2004a)	Simidu et al. (1990)	Satomi et al. (2007)	Venkateswaran et al. (1998)	Yoon et al. (2004a)		Zhao et al. (2007)
		Vogel et al. (2005)	Venkateswaran et al. (1998)	Vogel et al. (2005)	Bowman, 2005			
			Nozue et al. (1992)					
			Bowman, 2005					

	S. baltica	*S. basaltis*	*S. benthica*	*S. canadensis*	*S. chilikensis*	*S. colwelliana*	*S. corallii*	*S. decolorationis*
Bioluminescence	–		–	–		–		
Violet pigment	–	–	–	–	–	–	–	–
Growth at 4 °C	+	+	+	+	–	–	–	+
Growth at 25 °C	+	+	–	+	+	+	+	+
Growth at 37 °C	–	+	–	–	+	–	+	+
Barophilic	–		+	–		–		
Requires Na^+ for growth	–		+	+	–	+	+	–
Tolerates 6 % NaCl	–	–	–	–	+	–	+	–

Table 31.2 (continued)

	S. baltica	*S. basaltis*	*S. benthica*	*S. canadensis*	*S. chilikensis*	*S. colwelliana*	*S. corallii*	*S. decolorationis*
Fermentation of								
D-Glucose	–		+	–	–	–	+	+
D-Glucose (with gas)	–		–		–	–		
N-Acetylglucosamine	–	–	+	+	+	–	+	+
Chitinase	–		+	+		–		
Amylase	–		–	–	–	+		–
Lipase	+		+	–	–	–		
Hemolysis	–		–		+	–	d	
Nitrite reduced	–	+	+	+	–	+	+	+
Ornithine decarboxylase	+		–	+	+	–		
EPA synthesis	–	–	+	+	–		–	–
H_2S production	+		+	d	+	–		+
Iron reduction	+		d	–				+
Acid from D-glucose	+		+	–	–	–		
Utilization of								
D-Glucose	+	+	+		–	–	–	+
Cellobiose	+		–			–		+
Maltose	+	+	–			–	+	
Sucrose	+	–	–			–		+
N-Acetylglucosamine	–		+		–	–		
D-Gluconate	+	–	–			+	–	
Mol% G+C	46-47		47	46	55	46	49	49
Quinone type			MK-7	MK, MMK-7				MK7
			Q-7, 8	Q-7, 8				Q-7, 8
References	Ziemke et al. (1998)	Chang et al. (2008)	Macdonell and Clwell (1985)	Zhao et al. (2007)	Sucharita et al. (2009)	Weiner et al. (1988)	Shnit-Orland et al. (2010)	Xu et al. (2005)
	Bowman, 2005		Deming et al. (1984)			Bowman, 2005		
			Nogi et al. (1998)					
			Bowman, 2005					

	S. denitrificans	*S. donghaensis*	*S. fidelis*	*S. fodinae*	*S. frigidimarina*	*S. gaetbuli*	*S. gelidimarina*	*S. glacialipiscicola*
Bioluminescence					–		–	–
Violet pigment	–	–	–	–	–	–	–	–
Growth at 4 °C	+	–	+	–	+	+	+	+
Growth at 25 °C	+	–	+	+	–	+	–	+
Growth at 37 °C	–	–	–	+	–	+	–	–
Barophilic		+			–		–	
Requires Na^+ for growth	– S	+	–	–	S	+	+	
Tolerates 6 % NaCl	+	–	+	+	+	+	+	–
Fermentation of								
D-Glucose			+	–	+		–	–
D-Glucose (with gas)					–		–	
N-Acetylglucosamine					–		+	
Chitinase	+		–		–		+	
Amylase	+	–	–	–	–	+	–	
Lipase	–	+	V	–	+	+	+	
Hemolysis	–		+	+	–	+	–	
Nitrite reduced	+	+	+	–	+*	–	+	+

Table 31.2 (continued)

	S. denitrificans	*S. donghaensis*	*S. fidelis*	*S. fodinae*	*S. frigidimarina*	*S. gaetbuli*	*S. gelidimarina*	*S. glacialipiscicola*
Ornithine decarboxylase				–	d		–	+
EPA synthesis	–	+	–	–	+	–	+	+
H_2S production	+			+	d	–	+	+
Iron reduction	–				+		+	
Acid from D-glucose	–		+	–	+	–	–	
Utilization of								
D-Glucose	+	–	+		+		–	–
Cellobiose			–		+		–	
Maltose	+	–		–	+	–	–	
Sucrose	–		–		+	–	–	–
N-Acetylglucosamine	+	–	–		–		+	+
D-Gluconate		–			d		–	+
Mol% G+C	47-48	39	45	54	40-43	42	48	44
Quinone type						MK7		
						Q-7, 8		
References	Bretter et al. (2002)	Yang et al. (2007)	Ivanova et al. (2003a)	Kumar et al. (2010)	Bowman et al. (1997)	Yoon et al. (2004b)	Bowman et al. (1997)	Satomi et al. (2007)
					Bowman, 2005		Bowman, 2005	
	S. hafniensis	*S. halifaxensis*	*S. haliotis*	*S. hanedai*	*S. indica*	*S. irciniae*	*S. japonica*	*S. kaireitica*
Bioluminescence	–	–		+				
Violet pigment	–	–	–	–	–	–	–	–
Growth at 4 °C	+	+	–	+	–	–	–	
Growth at 25 °C	+	+	+	d	+	+	+	+
Growth at 37 °C	–	–	+	–	+	–	+	–
Barophilic				–				
Requires Na^+ for growth	+	+	–	+	–	+	–	+
Tolerates 6 % NaCl	+	–	+	–	+	+	–	V
Fermentation of								
D-Glucose	–	–	–	–	–		+	
D-Glucose (with gas)		–		–				
N-Acetylglucosamine		–		–				
Chitinase		+		+	+	–	–	+
Amylase		–		–		–	+	
Lipase				+		–	+	+
Hemolysis				–	+	+	+	
Nitrite reduced	+	+	+	d	+	–	+	+
Ornithine decarboxylase	+	+	+	–	–	–		
EPA synthesis		+	–	+	–	–	+	+
H_2S production	+	+	+	+	+	–		+
Iron reduction		–		–			–	
Acid from D-glucose			–	+	–	–		+
Utilization of								
D-Glucose	+	p	–	d	+	+	+	
Cellobiose		–		–		–	+	
Maltose	+	–	–	–		–	+	

Table 31.2 (continued)

	S. hafniensis	*S. halifaxensis*	*S. haliotis*	*S. hanedai*	*S. indica*	*S. irciniae*	*S. japonica*	*S. kaireitica*
Sucrose	–	–	–	–		+	–	
N-Acetylglucosamine	+	d	+	+			+	
D-Gluconate	+			d			–	
Mol% G+C	47	45	54	45	51	40	43-44	43
Quinone type		MK, MMK-7				MK-7		Q-7, 8
		Q-7, 8				Q-7, 8		
References	Satomi et al. (2006)	Zhao et al. (2006)	Kim et al. (2007)	Jensen et al. (1980)	Verma et al. (2011)	Lee et al. (2006)	Ivanova et al. (2001)	Miyazaki et al. (2006)
				Bowman, 2005				

	S. livingstonensis	*S. loihica*	*S. marina*	*S. marinintestina*	*S. marisflavi*	*S. morhuae*	*S. olleyana*	*S. oneidensis*
Bioluminescence				–		–		–
Violet pigment	–	–	–	–	–	–	–	–
Growth at 4 °C	+	+	+	+	+	+	+	d
Growth at 25 °C	–	+	+	+	+	+	+	+
Growth at 37 °C	–	+	+	–	+	–	–	+
Barophilic								–
Requires Na^+ for growth	–	+	+	+	–		+	–
Tolerates 6 % NaCl	–	–	+	–	+	–	+	d
Fermentation of								
D-Glucose	+			–	+	–	–	–
D-Glucose (with gas)								–
N-Acetylglucosamine	+						–	
Chitinase	–			–			–	
Amylase	–		–	–	–		+	–
Lipase	–			+	+			
Hemolysis	nd			–	+			–
Nitrite reduced	+	–		+	+	+	d	+
Ornithine decarboxylase	–	+	–	–		+	–	–
EPA synthesis	–	–	–	+	–		+	–
H_2S production	+	–	+	+	+	+	+	+
Iron reduction	+	+						+
Acid from D-glucose	+	–	d	–	–			
Utilization of								
D-Glucose	+	+	–	+		–	–	
Cellobiose	+							
Maltose	+	+			+	–	–	–
Sucrose	+					–	+	–
N-Acetylglucosamine	+	+		+		+	–	
D-Gluconate	–					+	+	
Mol% G+C	41	54	41	43	51	44	44	45
Quinone type	MK, MMK-7	MK7		Q-7, 8	MK7			
	Q-7, 8	Q-7, 8			Q-7, 8			
References	Bozal et al. (2002)	Gao et al. (2006)	Park et al. (2009)	Satomi et al. (2003)	Yoon et al. (2004a)	Satomi et al. (2006)	Skerratt et al. (2002)	Venkateswaran et al. (1999)
								Bowman, 2005

Table 31.2 (continued)

	S. pacifica	*S. pealeana*	*S. piezotolerans*	*S. pneumatophori*	*S. profunda*	*S. psychrophila*	*S. putrefaciens*	*S. sairae*
Bioluminescence		–					–	–
Violet pigment	–	–	–	–	–	–	–	–
Growth at 4 °C	+	+	+	+	+	+	d	+
Growth at 25 °C	+	+	+	+	+	–	+	+
Growth at 37 °C	–	–	–	–	+	–	+	–
Barophilic			+		+	+	–	
Requires Na^+ for growth	+	+	+	+	–	+	–	+
Tolerates 6 % NaCl	+	–	+	+	+	+	–	–
Fermentation of								
D-Glucose	+	–		+			–	–
D-Glucose (with gas)							–	
N-Acetylglucosamine		–					–	
Chitinase	–	–	–	–		–	–	–
Amylase	+	–	+	–	+	–	–	–
Lipase	+	+	+			–	+	+
Hemolysis	+	–					–	–
Nitrite reduced	+	+	+	–	+	+	d	+
Ornithine decarboxylase					+		+	–
EPA synthesis	+	–	+	+	–	+	–	+
H_2S production	+	+	+	+	–	–	+	+
Iron reduction		+					+	
Acid from D-glucose		+		+			d	–
Utilization of								
D-Glucose	+	+	+	+	–	–	d	+
Cellobiose	+		–	–	–	–	–	
Maltose	+		+	–	d	+	d	
Sucrose			–	–	–	+	d	
N-Acetylglucosamine	–		+			+	d	+
D-Gluconate	–						-	
Mol% G+C	40-41	45	49	43	45	51	43-47	43
Quinone type	Q-7, 8		MK, MMK-7	Q-7, 8	MK, MMK-7	MK, MMK-7		Q-7, 8
			Q-7, 8		Q-7, 8	Q-7, 8		
References	Ivanova et al. (2004b)	Leonardo et al. (1999)	Xiao et al. (2007)	Hirota et al. (2005)	Toffin et al. (2004)	Xiao et al. (2007)	Lee et al. (1977)	Satomi et al. (2003)
		Zhao et al. (2006)					Bowman, 2005	

Characteristics	*S. schlegeliana*	*S. sediminis*	*S. spongiae*	*S. surugensis*	*S. vesiculosa*	*S. violacea*	*S. waksmanii*	*S. woodyi*	*S. xiamenensis*
Bioluminescence	–	–				–		+	
Violet pigment	–	–	–	–	–	+	–	–	–
Growth at 4 °C	+	+	+		+	+	+	+	+
Growth at 25 °C	+	+	+	–	+	–	+	+	+
Growth at 37 °C	–	–	–	–	–	–	–	–	+
Barophilic						+		–	
Requires Na^+ for growth	+	+	–	+	–	+	+	+	–
Tolerates 6 % NaCl	–	–	+	–	+		+	+	–
Fermentation of									
D-Glucose	–	–				+	+	–	+
D-Glucose (with gas)						+		–	
N-Acetylglucosamine		+						–	

Table 31.2 (continued)

Characteristics	*S. schlegeliana*	*S. sediminis*	*S. spongiae*	*S. surugensis*	*S. vesiculosa*	*S. violacea*	*S. waksmanii*	*S. woodyi*	*S. xiamenensis*
Chitinase	–	+		–			–	–	
Amylase	–	–	–	–	–	–	–	V	–
Lipase	–	+	–	+	+		+	–	
Hemolysis	–						+	–	
Nitrite reduced	+	+	–	–	+	–		+	+
Ornithine decarboxylase	–	+				–			
EPA synthesis	+	+	–	–	–	+	+	–	–
H_2S production	+	+	–	–	+	–	+		+
Iron reduction		–							+
Acid from D-glucose	–	–		+		+			
Utilization of									
D-Glucose	+	–	+		+		+	+	+
Cellobiose					+		–	+	–
Maltose					+			–	+
Sucrose		–	+		+		–	–	+
N-Acetylglucosamine	+	+			+		–	–	d
D-Gluconate									
Mol% G+C	45	45	53	40	42	47	43	46	46
Quinone type	Q-7, 8	MK, MMK-7	Q-8	Q-7, 8	MK, MMK-7	Q-7, 8			MK7
		Q-7, 8			Q-7, 8				Q-7, 8
References	Satomi et al. (2003)	Zhao et al. (2005)	Yang et al. (2006)	Miyazaki et al. (2006)	Bozal et al. (2009)	Nogi et al. (1998)	Ivanova et al. (2003b)	Makemson et al. (1997)	Huang et al. (2010)
						Bowman, 2005		Bowman, 2005	

S growth is stimulated by the presence of Na + ions, *d* weak, *V* variable reaction depending on strain, *p* poorly utilized

GC Contents

DNA base composition values for *Shewanella* vary from 38 to 54 mol%, and the ranges for individual species are shown in ❯ *Table 31.2*. DNA base compositions are analyzed using HPLC methods (Tamaoka and Komagata, 1984) and thermal renatulation methods, describing as Tm (Marmur and Doty, 1962). Both methods are available to determine GC base composition, although values can be slight different. Recent whole-genome sequence analysis is more precise for determination of GC content.

Strain Typing

In food industry and medical field, it demands that development of strain typing technique for food and medical related *Shewanella*, because little is known about clonal differences within the species, e.g., if particular clones are selected for during chill storage of foods or occurrence of infection (Vogel et al. 1997, 2000). For the purpose of clonal analysis in bacteria, randomly amplified polymorphic DNA (RAPD) analysis has been used to assess the genetic diversity of environmental isolates of *S. putrefaciens* identifying several distinct genotypes (Vogel et al. 2000); however, the species appeared to be stable over time. Preliminary experiments with RAPD typing of isolates from fish show that, while the strains isolated from fresh fish are almost all genotypically different, some selection is seen during iced storage. However, a large variation was seen from fish to fish (Vogel and Gram, 1994). Whole cell protein profile is also a common strain typing method and applied to distinguish some Shewanella strains. However, it is suitable for separating species level rather than strain typing; - e.g., separating *S. putrefaciens* and *S. algae* (Vogel et al. 1997), and *S. frigidimarina* and *S. livingstonensis* (Bozal et al. 2002). As a more precise and rapid fingerprinting method for *Shewanella* strains related to a biofuel application, ITS (intergenic transcribed spacer)-DGGE was developed using sequence of 16S-23S ITS regions which result in distinct ITS-DGGE profiles (Kan et al. 2011). Whole-genome sequence with high throughput computer analysis techniques demonstrated to provide useful information related to the clonal differentiation among *S. baltica* strains in recent work. A comparative study of 5 *S. baltica* genomes recovered from the

same sample and those taken 12 years apart from the same sampling station revealed that two strains of *S. baltica* shared 93% of the 3,985 single-nucleotide polymorphisms detected between the two genomes within six syntenic regions (Caro-Quintero et al. 2012). These two strains have apparently recombined with more divergent members of the *S. baltica* population consistent with earlier findings of high intrapopulation recombination within the natural *S. baltica* population (Caro-Quintero et al. 2012). Although further study with more genomic sequences is required, whole-genome sequences are powerful tools in this research field.

Other Molecular Base Analyses

In order to determine the specific bacteria from various samples, such as food, clinical specimens, and environmental samples, nucleotides or protein-based techniques have been developed (DiChristine and Delong, 1993; Venkateswaran et al. 1999; Wang et al. 2004; Coleman et al. 2007; Bohme et al. 2011; Li et al. 2012). The polymerase chain reaction (PCR) and quantitative PCR are useful and sensitive methods to detect specific genes from bacteria including *Shewanella*; for example, temperature and pressure-regulated genes have been studied in a number of barotolerant and barophilic *S. benthica* strains (Li et al. 1997; Chilukuri and Bartlett, 1997; Chilukuri et al. 2002), and then a set of PCR-amplified, pressure-regulated gene products have been found to be universal in *S. benthica* strains and have been proposed as a rapid means to identify this particular barophilic species. A series of cytochrome proteins, including a cytochrome c oxidase and metal-reducing proteins, also have been studied based on PCR-amplified gene sequences (DiChristine and Delong, 1993; Venkateswaran et al. 1999). As the most common cases, the primer sets have been applied to the specific bacterial detection or identification targeting 16S rDNA, 23S rDNA, internal transcribed spacer (ITS) region, and *gyr*β. It is significantly higher (detection limits of 10^2–10^3 cfu g^{-1}) than probe-based methods (10^6–10^7 cfu g^{-1}).

DNA/RNA probe and PCR primer set has been designed and successfully applied in a study of Fe(III) reduction by *Shewanella* species. Recently a molecular-based specific detection system for *S. putrefaciens* was constructed applying a reverse transcription loop-mediated isothermal amplification technique (Li et al. 2012). It must be noted, however, that DNA/RNA extraction procedures on food samples need to be carefully examined, as the existence of PCR inhibitor compounds and overestimation by amplifying DNA from dead cells may bias results. As a rapid identification tool for food spoilage *Shewanella*, whole-cell protein fingerprinting technique by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOFMS) has been developed (Bohme et al. 2010). This method can be identified rapidly and precisely, e.g., it could distinguish among three *Shewanella* species, *Shewanella algae*, *Shewanella baltica*, and *S. putrefaciens*. Immuno-based methods known as the common methods for specific detection of bacteria are also applied. Specific poly- and monoclonal antibodies have been produced against *S. putrefaciens* and employed in various enzyme-linked immunosorbent (ELISA) assays. However, a very high number (approximately 10^7 bacteria per gram) are required to detect the organism in these assays. Some methodological improvement is necessary, e.g., attempts to concentrate the bacteria by immunomagnetic separation. Ribotyping and protein electrophoretic pattern analysis (Vogel et al. 1997; Bozal et al. 2002) also have demonstrated to evaluate species or strain separation in some *Shewanella* species. These methods are also useful to distinguish species based on matrix analysis of gel image; however, in the case of separation for strains among Owen's groups I to IV, the resolution using both procedures was quite poor (Vogel et al. 1997). It suggests that the resolution in each method depends on the bacterial species, though the methods described above are generally useful for separating species/strain and detection for specific species in most cases.

Genome Analysis

Whole-genome sequences for some *Shewanella* species were performed and reported in the public database, *S. amazonensis*, *S. baltica* (five strains), *S. denitrificans*, *S. frigidimarina*, *S. halifaxensis*, *S. loihica*, *S. oneidensis*, *S. pealeana*, *S. piezotolerans*, *S. putrefaciens*, *S. sediminis*, *S. woodyi*, and four unidentified *Shewanella* strain (Dikow, 2011). The genome size of Shewanella ranges from 4.3 to 5.9 Mb, and 8–12 copies of the ribosomal RNA operon are present in each genome and harbor 1–3 plasmids in some strains. GC contents of each species are in agreement with previous data obtained using HPLC or Tm method, except for *S. piezotolerans* (Xiao et al. 2007; Wang et al. 2008). It is expected that the whole-genome sequence can provide useful information to elucidate metabolic pathways for some particular characteristics of *Shewanella*, e.g., TMAO reduction and metal reducing. The type II fatty acid biosynthesis pathway and the genes related to metal reduction in *S. piezotolerans* (Wang et al. 2008) and *S. oneidensis* MR-1, respectively, have been determined (Heidelberg et al. 2002; Kolker et al. 2005). Thus, whole-genome data can provide excellent raw material for the generation of hypotheses of historical homology that can be tested with phylogenetic analysis and compared with hypotheses of gene function (Kolker et al. 2005). Further analysis of whole-genome comparisons focusing on evolution in *Shewanella* has shown that no single orthologous copy of 16S rRNA exists across the species and that the relationships among multiple copies are consistent with 16S rRNA undergoing concerted evolution (Dikow, 2011).

As further studies after whole-genome sequencing, gene arrays can be routinely used to evaluate gene expression. Some studies have been performed based on *S. oneidensis* MR-1 (Beliaev et al. 2002a, b; Murray et al. 2001; Thompson et al. 2002; Gao et al. 2005). The work of Murray et al. (2001) has compared the expression of a number of *Shewanella*

species using an MR-1 array. The results suggested that although some genes are highly conserved, extensive modification has occurred, to the point that a single *Shewanella* array for environmental work is infeasible. Other reports, mainly the omics analysis, mark the start of a large effort to elucidate the global regulation mechanisms of this organism. This global approach that represents studies under various physiological conditions should provide some resolution about the mechanisms through which this organism interacts with its environment, including other species of bacteria (Nealson and Scott, 2006).

Plasmid and Page

Whole-genome analysis in *S. baltica* indicated the five strains sequenced harbored between one and three plasmids, sizing from 16kb to 120kb. As a matter of course, some cryptic plasmids were found in various *Shewanella* strains (Caro-Quintero et al. 2012). Detailed characteristics of these plasmids are little known; however, there is remarkable fact that *S. oneidensis* MR-1 harbors a 161kb of megaplasmid encoding genes for anaerobic respiration (Saffarini et al. 1994), indicating large-scale horizontal transfer of genes for anaerobic respiration and metal reduction via such a megaplasmid can occur. Such a possibility might be mechanisms for some of the major differences seen in the ecological group of the *Shewanella*. It is expected to elucidate the details of these plasmids and apply to industrial tools, such as shuttle vector and expression vector. While it was demonstrated that *S. oneidensis* MR-1 was an excellent vehicle for the expression of gene for heme protein (Ozawa et al. 2000, 2001). The advantages of using this strain is that the cell can be grown rapidly to high densities under aerobic conditions, then switched to anaerobic conditions, and large amounts of protein are produced, though utilization of this expression system are limited to produce cytochromes. For potentially valuable proteins needed in high amounts for analysis, such a system offers obvious advantage.

Lambda-like page was found in *S. oneidensis* MR-1 genome, which can be strongly related to biofilm formation (Heidelberg et al. 2002). While active filamentous M13-like phage was isolated from the deep-sea bacterium, *S. piezotolerans* WP3, which was induced at 4 °C compared to its expression at 25 °C (Wang et al. 2007; Jian et al. 2012). It is expected to provide a potential tool for genome engineering.

Phenotypic Analyses

The main features of the genus *Shewanella* are discussed here, since the family *Shewanellaceae* contains only one genus, *Shewanella*.

Shewanella (MacDonell and Colwell 1986) 355VP (Effective publication: MacDonell and Colwell 1985). Shew.a.nel'l.a. M. L. dim. Ending –ella; *Shewanella* named after James Shewan for his work in fisheries microbiology.

Type species: *Shewanella putrefaciens* (Lee et al. 1977) MacDonell and Colwell 1985.

General Description

Phenotypic characteristics are as follows: straight or curved rods. Endospores and microcysts are not formed. Motile by a single, unsheathed, polar flagellum. Gram negative. Cells do not accumulate poly-beta-hydroxybutyrate inclusion bodies and gas vesicles. Colonies are often pale tan to pink–orange, due to cytochrome accumulation. Oxidase and catalase positive. Chemoheterotrophic. Reduction of NO_3^- to NO_2^- and gelatin degradation positive. Facultatively anaerobic. Oxygen is used as the electron acceptor during aerobic growth. Anaerobic growth is predominantly respiratory; the oxidation of organic carbon compounds or H_2 is coupled to the reduction of various inorganic and organic electron acceptors, including NO_3^-, NO_2^-, Fe^{3+}, trimethylamine –N-oxide, fumarate, various sulfur compounds, and Mn^{4+}. May also be fermentative but rarely gas, producing acid from carbohydrates, such as D-glucose and *N*-acetylglucosamine. Most strains can degrade gelatin and form lipases, while certain species form chitinases, but in general do not attack starch, except for some strains. Able to form H_2S from thiosulfate. Some species do not require Na^+ ions for growth. Most species can grow at 4 °C. Some species are psychrophilic. Some species are psychrophilic and barophilic. In most species, the major isoprenoid quinones are Q7, Q8, and MK7, with small amounts of methyl-MK7. The major fatty acids are C13:0 iso, C14:0, C15:0 iso, C15:0, C16:1ω7c, C16:0, C17:1ω8c, and C18:1ω7c. Most species produce polyunsaturated fatty acid (PUFA). DNA base composition values for *Shewanella* vary from 38 to 54 mol%.

Differentiation of the Genus *Shewanella* from Other Genera

Characteristics that differentiate *Shewanella* from other members of the *Alteromonadales* and major marine bacteria are shown in ❯ *Table 31.1*. Due to the diversity of phenotypic-characteristics within the *Shewanella*, there are only few properties shared by all members of this genus, thus indicating that some of strains may be misidentified. Basically the bacteria showing the following phenotypic features may be member of *Shewanella*, especially food-related species; Gram-negative, rod, motile by a single unsheathed polar flagellum, oxidase and catalase positive, nitrate reduction and H_2S production positive, and negative for acid production from D-glucose in Hugh and Leifson test (some species are positive for oxidative acid production). The generally nonfermentative nature of *Shewanella* species in OF test distinguishes them from the *Vibrionaceae*. Fermentative species of *Shewanella*,

including *S. frigidimarina*, may be misidentified as *Vibrio*-related species; however, they have unsheathed flagellum and posses a mol% G+C values lower than those of most *Vibrio* species. Moreover, some species represented by *S. algae* can be confused with *Ferrimonas balearica*, but it is distinguishable based on following characteristics: hemolysis of sheep blood, gelatin degradation, tolerance to 10 % NaCl, and others (Vogel and Gram, 1994; Bowman, 2005). In ambiguous cases, 16S rRNA gene sequence and/or fatty acid profile provides direct identification (Wilkinson, 1988).

Morphology

Cells are generally rods, possessing either straight or slightly curved rod. Cell size is generally 1–3 μm long and 0.5–0.65 μm in diameter. In nutrient media, short filaments and, more rarely, helical forms may develop in older cultures. PHB inclusion or gas vesicles, endospore, and microcysts are not observed. Cytoplasmic inclusions, probably consisting of unknown function protein, have been found in thin sections, negatively stained, and freeze-fractured preparations of *Shewanella putrefaciens*. Cellular appendages, such as prosthecae or spinae, are not developed. Cells are motile by a single, unsheathed, polar flagellum.

Colony Morphology

Colonies on complex nutrient media, such as LB agar, marine agar, TSA, and others, have a pale tan to pink–orange or salmon-pink color due to strong accumulation of cytochrome proteins, though colony color is slightly different which depends on culture medium and culture age. Some strains, *S. hanedai* and *S. benthica* may not show typical color of colony due to less accumulation of cytochrome protein. In the case of melanin-like pigment producing strains, *S. colwelliana* and some strains of *S. hanedai*, colony may form dark brown pigment when growing on complex media or media containing L-tyrosine. Studies on *S. colwelliana* indicated that melanin-like pigmentation derives from L-tyrosine cataboliting to homogentisate, which in turn is polymerized to form the pigment pyomelanin (Facua and Weiner, 1993; Coon et al. 1994; Ruzafa et al. 1994; Kotob et al. 1995). Recent reports demonstrated that melanin compound secreted by S. algae strain was related to reduction of Fe (Turick et al. 2002, 2008). Shewanella violacea produces a violet pigment, 5,5'-didodecylamino-4,4'-dihydroxy-3,3'-diazodiphenoquinone-(2,2') (Kobayashi et al. 2007), but the mechanistic details are unknown. Most of the colonies of the *Shewanella* capable of growing show a black FeS precipitate on TSI agar medium due to production of H_2S (Vogel and Gram, 1994). Some strains are not able to form colony on standard plate count agar, even if supplemented with appropriate concentration of NaCl for them. *S. hanedai* and *S. woodyi* were originally reported as bioluminescence bacteria. Studies of the bioluminescence gene sequence, luxA, on *S. woodyi* have shown that the homology of luxA from *S. woodyi* and *S. hanedai* was low and *S. hanedai* luxA showed closer similarity to sequences appeared in *Vibrio* species (Makemson et al. 1997).

Fatty Acid Profiles

The major fatty acids (and their ranges of % composition) in *Shewanella* include C13:0 iso (1–16 %), C14:0 (1–12 %), C15:0 iso (4–24 %), C15:0 (1–8 %), C16:1 ω7c (16–55 %), C16:0 (5–31 %), C17:1 ω8c (1–15 %), and C18:1 ω7c (0–8 %). *Shewanella* species have a distinctive fatty acid pattern, as compared with other bacteria of the Gammaproteobacteria, in that they are rich in branched and odd-chain-length fatty acids (Wilkinson, 1989; Nichols et al. 1994; Rossello-Mora et al. 1995; Bowman et al. 1997; Russell and Nichols, 1999). Moreover, in most psychrotolerant and psychrophilic *Shewanella* species, eicosapentaenoic acid (EPA, C20:5 ω3), the levels of which range from 2 % to 22 %, were determined (Bowman et al. 1997; Bowman, 2005). The quantitative proportions of fatty acid components vary considerably among species, but it is difficult to judge with the data available whether these differences would be useful in species differentiation or whether they are merely due to variations in cultivation conditions. *Shewanella* possess both aerobic and anaerobic desaturatase pathways for fatty acid synthesis (Nicholas et al. 1992; Bowman 2005). *Shewanella* strains grown anaerobically thus form a different fatty acid profile than those grown aerobically (Nichols et al. 1997; Russell and Nichols, 1999; Venkateswaran et al. 1999). Additionally it is a problem that fatty acid profile easily varies depending on analytical method employed in each study, for instance, the results obtained using MIDI system are different from the data obtained from the traditional methods extracting cytoplasmic lipids and analyzing by GC and GC-MS. Some species belonging to the *Alteromonadales* are known to possess polyunsaturated fatty acid (PUFA), including EPA and docosahexaenoic (DHA, C22:6 ω3) (Delong and Yayanos, 1986; Yano et al. 1994; Bowman et al. 1998; Russell and Nichols, 1999). *Shewanella* can produce EPA, while *Colwellia* spp. and *Moritella* spp. produce DHA. Few other bacterial species known to possess EPA, including *Flexibacter polymorphus* (Johns and Perry 1997) and *Psychroflexus torquis* (Nichols et al. 1997a; Bowman et al. 1998c), are members of the flavobacteria division; however, DHA production is likely a specific ability in *Colwellia* and *Moritella*, belonging to *Alteromonadales*. Recent studies indicates that modulation of fatty acid composition is a common strategy for most organisms to ensure sufficient membrane fluidity by increasing the amount of low-melting-point fatty acids, such as monounsaturated fatty acid, PUFA, and branched-chain fatty acids (BCFA) (Delong and Yayanos, 1985, 1986; Russell and Nichols, 1999; Wang, et al. 2009; Nishida et al. 2010). Low temperature and high pressure exert similar effects on the biological membrane, with an irreversible change from a fluid, disordered state to a nonfluid, ordered state. The responses of bacteria to reduced temperature

or elevated pressure frequently entail the increased incorporation into membrane phospholipids of BCFA and PUFA. Due to compelling phenomenon, many studies focused on distribution of EPA in the genus and links production of EPA synthesis with cold and high-pressure adaptation. Several studies have detected EPA in unidentified marine bacteria with fatty acid profiles, which were very similar to *Shewanella* (Watanabe et al. 1996; Yazawa et al. 1988; Yazawa, 1996; Jostensen and Landfald, 1997; Yano et al. 1997; Mergaert et al. 2001; Gentile et al. 2003). Feature of EPA production in *Shewanella* is summarized in *Table 31.2*. Overall psychrophilic/psychrotolerant halophilic *Shewanella* are likely to produce EPA; however, EPA producers are phylogenetically spread in the genus, except for the species positioned in the cluster containing *S. algae* and species related to tropical organisms (*Fig. 31.1*). EPA levels decrease markedly when incubation temperatures are above its optimal growth temperature (Nichols et al. 1997b), and there seems to be a correlation between the optimal growth temperatures of *Shewanella* species and their inherent EPA levels in *S. gelidimarina* ACAM 456 and other Psychtophilic *Shewanella* (Bowman et al. 1997; Russell and Nichols, 1999). In *S. benthica* and *S. piezotolerans*, EPA levels increase until the hydrostatic pressure reaches the particular strain's growth pressure optimum (DeLong and Yayanos 1986; Wang, et al. 2009). Increased EPA levels are results from the homeoviscosity adaptation due to maintain cellular membranes fluidity (Morita, 1976; Nichols et al. 1994, 1996), an adaptation important for organisms living in perpetually cold and high-pressure environments. However, recent studies using high-pressure, time-resolved fluorescence anisotropy measurement have suggested that EPA prevents the membrane from becoming hyperfluid and maintains its stability against significant changes in pressure (Usui et al. 2012). Thus it is likely that EPA plays a role in stabilizing dynamic membrane structure in the deep-sea piezophile *Shewanella* (Sato et al. 2008; Kawamoto et al. 2009; Usui et al. 2012).

Because omega-3 fatty acid precursors cannot be synthesized de novo by most metazoans, bacteria and microalgae are thought to act as a dietary supply of these lipids (Temara et al. 1984; DeLong and Yayanos, 1986). The synthetic pathway of EPA has been partially characterized in *S. violacea* and appears to be derived from an aerobic desaturation pathway (Watanabe et al. 1997; Fang et al. 2004). EPA synthesize genes including a phosphopantetheinyl transferase gene and *pfaA, -B, -C*, and *-D* have already been cloned and sequenced and confirmed EPA expression occurs in transgenic marine organisms or *Escherichia coli* (Takeyama et al. 1997; Yu et al. 2000; Orikasa et al. 2004, 2009; Amiri-Jami and Griffiths, 2010). Recently, the type II fatty acid biosynthesis pathway in *Shewanella* was constructed, and intact EPA-synthesizing gene clusters have been shown to be conserved in all of the *Shewanella* genomes sequenced (Wang et al. 2008, 2009). Although mesophilic species do not appear to produce EPA and their fatty acid profiles are similar to those of *F. balearica*, another mesophilic, iron-reducing halophilic species isolated from sediment (Rossello-Mora et al. 1995), the EPA-synthesizing-gene cluster is coded in the genomes of some mesophilic *Shewanella: S. amazonensis, S denitrificans, S. loihica, S. oneidensis, and S. putrefaciens* (Wang et al. 2009). One peculiar and interesting study has reported the discovery of a long-chain polyunsaturated hydrocarbon, all-cis-3,6,9,12,16,19,22,25,28 hentriacontanonaene (C31:9) in mesophilic *Shewanella* strain and *S. amazonensis* strain SB2B^T, constituting 1–2% of the total fatty acid methyl ester and hydrocarbon fraction and was produced related to a decrease in growth temperature (Sugihara et al. 2010; Motoigi and Okuyama, 2011).

Lipids

The polar lipids of *Shewanella*, *S. putrefaciens*, *S. baltica*, and *S. algae* were consisted almost entirely of phosphatidylethanolamine and phosphatidylglycerol, with variable proportions of their lyso derivatives. This is a pattern typical of Gram-negative bacteria (Moule and Wilkinson 1987). A number of additional substituted lipids have been found in *S. putrefaciens* ATCC 8071, including two glycolipids, identified as β-D-glucopyranosyldiacylglycerol and β-D- glucopyranurono-syldiacylglycerol, and an ornithine amide lipid (Wilkinson 1968a, 1972; Wilkinson et al. 1973). However, detection of ornithine amide lipids and glycolipids likely depends on strain, growth condition, and experimental methods.

Quinones

The quinones found in *Shewanella* are ubiquinone-7 (11–51 %), ubiquinone-8 (31–58 %), and menaquinone-7 (5–52 %), with small quantities of methylmenaquinone-7 (therniokasniqyubibe-7, 0–6 %) also present, although only Q-7 and Q-8 were determined in some *Shewanella* species (Itoh et al. 1985; Moule and Wikinson 1987; Akagawa-Matsushita et al. 1992a). This pattern distinguishes *Shewanella* spp. from other related bacteria, such as *Alteromonas* and *Pseudoalteromonas*, which contain mostly Q-8 (Akagawa-Matsushita et al. 1992a). The role of quinines is not entirely elucidated, but menaquinones appear to play an important role in anaerobic respiratory activity in Fe(III) reduction (Myers and Myers, 1993; Saffarini et al. 2002). Interest in quinine physiology is increasing with advance of studies for metal reduction.

Lipopolysaccharides (LPS)

S. putrefaciens possesses R-type lipopolysaccharides, rather than the ladder-type LPS typically found in many other Gram-negative bacteria (Sleddjeski and Weiner 1991). The unique monosaccharide of LPS core region in *Shewanella* is 8-deoxy-8amino analogue of 3deoxy-D-manno-oct-ulosonic acid (Kdo), termed Kdo8N, which is connecting the core oligosaccharide with lipid A. The neutral and amino sugar content of the LPS core oligosaccharide of all strains tested contained of galactose and

heptose. In addition, *S. putrefaciens* ATGG 8071 contains glucose and 3-amino-3, 6-dideoxyglucose; both *S. putrefaciens* ATGG 8073 and *S. baltica* contain galactosamine; and *S. algae* contains quinovosamine. The chemical composition of the *O*-polysaccharide region varies among various species of *Shewanella*, *S. algae*, *S. fidelis*, *S. japonica*, *S. oneidensis*, and *S. pacifica*, and their detailed structures have been reported accompanying with development of analytical equipments (Shakshkov et al. 1997; Leone et al. 2007; Nazarenko et al. 2011). The lipid fraction of the LPS in *S. putrefaciens* (strains ATGG 8071 and 8073), *S. baltica* (strain ATCC 8072), and *S. algae* (NCIMB 11157) consists of (% range) C13:0 iso (4–9 %), C13:0 (6–13 %), C10:0 3OH (8–13 %), C12:0 3OH (11–16 %), C13:0 iso 3OH (8–14 %), C13:0 (13–21 %), and C14:0 3OH (7–9 %). In addition, *S. baltica* contains high levels of C12:0 iso 3OH (10 %) and C14:0 iso 3OH (8 %); these components are much less abundant in the other strains tested (0–2 %) (Moule and Wikinson 1989).

Polyamines

The major polyamines found in *Shewanella* species are putrescine and small amount of cadaverine, but cadaverine detection was likely to depend on strain or species. *S. benthica*, *S. hanedai*, and some strains of *S. putrefaciens* did not contain cadaverine. This pattern differs from those of *Pseudoalteromonas* and *Alteromonas*, which, in addition to putrescine and cadaverine possess spermidine. *Moritella marina*, by comparison, possesses only cadaverine (Hamana 1997), indicating similar profile of that of *Shewanella*. Polyamine profile of *Alteromonas macleodii* was different from that of other *Alteromonadales* with possession of two novel polyamines (2-hydroxyputrescine and 2-hydroxyspermidine). The knowledge of polyamine composition in *Shewanella* species is limited.

Energy Metabolism

Respiration

Shewanella species are chemoheterotrophic facultative anaerobes, with anaerobic growth typically of a respiratory nature, although some species can also grow fermentatively. During aerobic and anaerobic growth, *Shewanella* species can utilize a range of organic acids and some carbohydrates as sole sources of carbon and energy (Ringo et al. 1984). Glucose is catabolized via the Entner–Doudoroff pathway (Abu et al. 1994; Scott and Nealson,1994). In *Shewanella*, it is outstanding in its ability to use a variety of electron acceptors: oxygen, ferric iron (III), manganese (IV), TMAO, dimethyl sulfoxide, nitrate, nitrite, thiosulfate, fumarate, sulfite, elemental sulfur, and others, although denitrification is a strain-specific characteristic (Brettar et al. 2002). Most studies on the diverse modes of anaerobic respiration in *Shewanella* have concentrated on strains of *S. putrefaciens* and *S. algae*. As the result, recent experiments have suggested that it rather uses a fusion of the TCA cycle (a truncated TCA cycle) and the anabolic serine pathway (Scott and Nealson, 1994). Studies of electron acceptors for anaerobic respiration in *Shewanella* have demonstrated that anaerobic growth can also occur via the reduction of fumarate to succinate coupled with the oxidation of formate (Pinchuk et al. 2012). The fumarate reductase of *S. frigidimarina* NCIMB strain 400 is similar to a fumarate reductase flavoprotein found in *Wolinella succinogenes* (Simon et al. 1998). However, the mechanism of anaerobic growth in *Shewanella* is not fully elucidated, for example, a transposon mutant of *S. oneidensis* MR-1 lacks fumarate reduction ability, but other reductive pathways are not compromised (Myers and Myers 1997), indicating that the different modes of anaerobic respiration utilize different sets of enzymes. Genes for anaerobic respiration by *S. putrefaciens* strain MR-1 appear to be both chromosomal and borne on megaplasmids (Saffarini et al. 1994).

Fermentation

Most of *Shewanella* species are facultative anaerobes, with anaerobic growth typically of a respiration; however, some species can grow fermentatively using various carbohydrates (*Table 31.2*), for instance, *S. frigidimarina* and *S. benthica*, both of which can ferment D-glucose, and *S. gelidimarina*, which can ferment N-acetylglucosamine and chitin, but not D-glucose (MacDonell and Cowell 1985; Bowman et al. 1997). *S. gelidimarina* appears to be adapted almost specifically to chitin degradation, given its ability to utilize both aerobically and anaerobically only *N*-acetylglucosamine and chitin, out of a wide range of carbohydrates tested. Gas is not produced by these species during fermentation; however, *S. violacea*, a barotolerant species closely related to *S. benthica*, can ferment D-glucose and produce both acid and gas (Nogi et al. 1998b). The end products and relevant biochemistry of this process have not been studied.

TMAO Reduction

The ability of *Shewanella* to use trimethylamine-*N*-oxide (TMAO) as electron acceptor is a major reason for its importance in fish spoilage, as the reduced compound, TMA, has a characteristic fishy smell (Shewan 1971). Thus, *S. putrefaciens* can produce large amount of TMA under anaerobic rather than aerobic condition. In this research field, mechanism of TMAO reduction was studied using *S. putrefaciens* strains (Ringo et al. 1984; Debevere et al. 2001). Summary of TMAO reduction in the presence of lactate by *Shewanella* species is shown in *Fig. 31.2*. Respiration using TMAO or other compounds as electron acceptors is carried through a range of cytochromes of type *c* and to the final e^- carrier, the reductase. TMAO reductase has been localized to the periplasmic space (Dos Santos et al. 1988), whereas some experiments have shown the Fe(III) reductase to be located in the outer membrane (Shi et al. 2012).

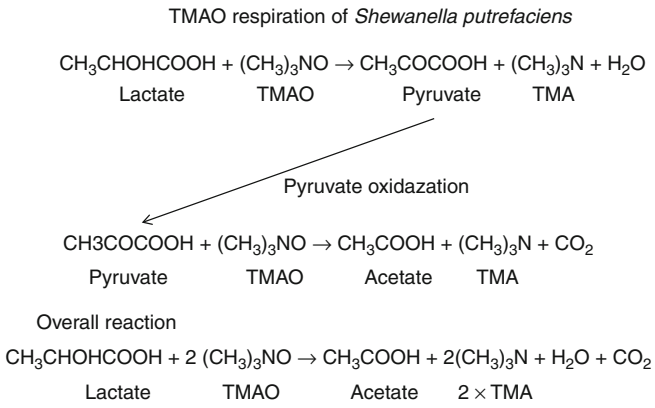

Fig. 31.2
Lactate oxidation with TMAO under anaerobic conditions in *Shewanella putrefaciens*

H_2S Production

Shewanella strains can grow anaerobically by reduction of various sulfur compounds to H_2S, including sulfur, thiosulfate, and sulfite (Semple and Westlake 1987; Perry et al. 1993; Moser and Nealson 1996). A partial lists of S compound reduction are shown: sulfur/polysulfide → H_2S, sulfite → H_2S, thiosulfate → H_2S, and dimethyl sulfoxide (DMSO) → dimethyl sulfide. In the case of food preservation, many studies in *S. putrefaciens*is were performed and indicated that a potent producer of volatile sulfides produces H_2S from cysteine in fish products. Other sulfides probably originate from methionine metabolism. During storage of fish, *S. putrefaciens* also degrades ATP-related compounds and is capable of producing hypoxanthine (a bitter-tasting component) from inosine monophosphate.

Metal Reduction

The special interest in metal-reducing bacteria is focused on its behavior in anaerobic environment contaminated by heavy metals such as iron, lead, and perhaps even uranium and reducing mechanisms of oxidized metals with insoluble solid form. Since *S. oneidensis* MR-1 belongs to a class of bacteria known as "Dissimilatory Metal-Reducing Bacteria (DMRB)" because of their ability to couple metal reduction with their metabolism, details of mechanisms for dissimilate reduction of metal are studied on this bacterium (please see the review article by Nealson and Scott 2006, in the Prokaryotes 3rd edition; Fredrickson et al. 2008; Shi et al. 2012). Due to this versatility of electron sinks, *Shewanella* may occur in many ecological niches, and it is believed to be important in nature for the turnover of Fe and Mn and, moreover, its contribution to geomagnetism and sedimentary diagenesis (Nealson and Saffarini, 1995; Lovley, 1996; Nealson and Scott, 2006; Fredrickson et al. 2008). The majority of *Shewanella* strains tested can grow anaerobically by coupling the oxidation of carbon compounds or H_2 (in the presence of a utilizable carbon source) to the reduction of Fe^{3+} to Fe^{2+} (Lovley and Phillips, 1989; Semple and Westlake, 1987; Nealson and Scott, 2006) or of Mn^{4+} to insoluble Mn^{3+} (Myers and Nealson, 1988; Nealson and Scott, 2006). Mn^{4+} and Fe^{3+} have redox potentials higher than that of sulfate and are capable of out-competing electron acceptors of lower potential, such as SO_4^{2} (used for sulfate reduction) and CO_2 (used for methanogenesis)(Nealson and Saffarini 1994; Nealson and Scott, 2006). Its ability to reduce Fe(III) and produce sulfides can be a cause of microbial corrosion of metals. During aerobic growth, the organism sequesters iron (for use in cytochromes) by use of low-molecular-weight iron chelators – so-called siderophores. The fact that *S. putrefaciens* produces a potent iron chelator, putrebactin, which is structurally similar to chelators formed by *Pseudoalteromonas haloplanktis* (bisucaberin) and *Bordetella* spp. (alcaligin) (Ledyard and Butler 1997). In the process of dissimilatory reduction of metal, as their cell envelopes are impermeable to metal (hydr)oxides that are poorly soluble in water, DMRB have to overcome this physical barrier. Thus, it is believed that *Shewanella oneidensis* MR-1 have developed electron transfer strategies that require multihaem c-type cytochromes (c-Cyts). Recent reports demonstrated the model of dissimilatory mechanism in *S. oneidensis* MR-1 as follows: multihaem c-Cyts CymA and MtrA transfer electrons from the inner membrane quinone/quinol pool through the periplasm to the outer membrane and then the type II secretion system imply in the reduction of metal (hydr) oxides, most likely by translocating decahaem c-Cyts MtrC and OmcA across outer membrane to the surface of bacterial cells where they form a protein complex (Myers and Myers, 1997, 2000, 2001, 2002, 2004; Nealson and Scott, 2006; Shi et al. 2006, 2007, 2012). In this case, the extracellular MtrC and OmcA can directly reduce solid metal (hydr)oxides. In the case of indirectly electron transport pathway between metal oxides and decahaem c-Cyts MtrC and OmcA, flavin compounds secreted from DMRB mediate electron transport (Shi et al. 2007). The mechanics of this bacterium's resistance and using of heavy metal ions is deeply related to its metabolism pathway web. Putative multidrug efflux transporters, detoxification proteins, extracytoplasmic sigma factors, and PAS-domain regulators are shown to have higher expression activity in presence of heavy metal (Shi et al. 2007).

Isolation, Enrichment, and Maintenance Procedures

General Isolation Methods

Most *Shewanella* species can be directly isolated from source material onto marine 2216E medium (MA, BD), trypticase soy agar (TSA, BD), nutrient agar (Oxoid), and other nutrient rich complex media without prior enrichment; however, the plate count agar, which is most common medium on the food hygiene

field, is sometimes not suitable for cultivation of *Shewanella* including food-related species, due to lack of Fe-containing minerals. Incubation temperature should be carefully selected for isolation due to the fact that most species is psychrophile, e.g., *S. gelidimarina* is not able to grow >25 °C. Other mesophilic and psychrotolerant species can be isolated at around 30 °C. Sodium chloride should be added to the media for *Shewanella* isolation, especially from marine environment samples, because some species require Na+ ion for growth or stimulate their growth by it. *Shewanella* colonies are often identifiable by their pale tan or pink-salmon color on MA.

Phenotypic Test

The ability to ferment carbohydrates is tested most effectively in the oxidation/fermentation medium of Leifson (1963), to which carbohydrates are added at a concentration of 0.5 % (w/v). This medium is more sensitive than the usual Hugh and Leifson medium because it contains phenol red instead of bromothymol blue. Other biochemical and nutritional traits can be tested using standard procedures, on which are carried out under suitable growth condition, e.g., appropriate incubation temperature and Na^+ –iron concentration. Commercial identification kits (API, Biology, and others) are also available for acceptance to routine biochemical tests. Baumann's minimal medium (Baumann et al. 1972) is used as a common basal medium for utilization tests, carbohydrate or amino acid.

Maintenance Procedures

In general, common and conventional methods can be used to preserve and maintain *Shewanella* in a carbohydrate-free medium, such as nutrient or marine agar in which they grow well, because these species are relatively robust. *Shewanella* species can be lyophilized using 20 % skim milk as a cryoprotectant. In addition, strains can sustain viability for over 6 months when frozen in liquid nitrogen or cryopreserved at −80°C in broth containing 20–30 % glycerol or DMSO. *S. algae* and *S. putrefaciens* strains can be maintained at 15–20oC in semi-solid agar medium containing 0.1 % Proteose peptone *S. putrefaciens* no.3 (Difco Laboratories), 0.1 % yeast extract, 0.05 % phytone, 0.02 % sodium thiosulfate, 0.005 % sodium sulfite, 0.004 % ferric citrate, and 0.3 % agar dissolved in 3:1 aged seawater (or artificial sea salts) and water: pH 7.6 (Simidu et al. 1990). Some species, *S. frigidimarina*, *S. gelidimarina*, and probably most of species, can be stored on marine agar slants or as heavy suspensions in sterile seawater at 2 °C for 12 months or longer.

Enrichment

A brief enrichment of samples in marine 2216 broth at 2 °C for 1–2 d is advantageous for the isolation of psychrophilic species such as *S. gelidimarina* (Bowman et al. 1997). *S. woodyi* and *S. hanedai* produce bioluminescence most effectively on marine agar, rather than on the standard glycerol-containing luminous medium normally used to detect bioluminescent *Vibrio* or *Photobacterium* spp. (Ruby et al. 1980; Hastings and Nealson 1977).

Food- and Clinical-Related Species

As it is regarded as a representative species of food and clinical bacteria, much research has focused on *Shewanella putrefaciens* and its relatives, e.g., Owen's DNA group I to IV. Several different media have been used for enumeration of *S. putrefaciens*, relying either on the ability of the organism to produce H_2S or its characteristic salmon-like pigmentation. *S. putrefaciens* and *S. algae* can be isolated semi-selectively from clinical and environmental samples on a medium containing 1 % NaCl, 0.1 % ox bile salts, and 1 % peptone (Nozue et al. 1992) and peptone iron agar, which is rich in peptones and contain ferrous sulfate. As *Shewanella* producing H_2S from peptone degradation will appear with a black precipitate of FeS just below and around the colony on peptone iron agar, the iron agar, Lyngby, also relies on the same basic principle; however, it has been modified in a number of ways: thiosulfate and L-cysteine are added to increase the pool of sulfur. *S. putrefaciens* degrades both the inorganic and organic sulfur sources (Gram et al. 2000). The pH of the medium is 7.4, which has a moderately stabilizing effect on the FeS. The medium is used as pour plate with a cover layer. This enhances FeS production. FeS is not stable and may be oxidized to Fe $(OH)_3$. Thus, the black precipitate will be oxidized if the agar plates are left for too long and/or at too high temperature. For routine purposes, incubation at 20–25 °C for 3–5 days is suitable. *S. putrefaciens* produced pink/reddish/brownish colonies when screened on modified Long and Hammer medium, probably due to production of colored cytochromes. Nonfermentative, H_2S-producing isolates (which are usually positive for ornithine decarboxylase) can be further selected on desoxycholate-hydrogen sulfide-lactose medium (Eiken Chemical Co., Ltd., Tokyo, Japan). Additional selection for *S. algae* includes incubation at 40 °C on nutrient agar containing 6 % NaCl. The identification of *S. algae* strains can be confirmed by their α-hemolytic activity on sheep blood agar; strains can also be selected and isolated by plating clinical samples onto *Salmonella–Shigella* agar (Nozue et al. 1992). *S. putrefaciens* and *S. algae* strains and several other *Shewanella* species can also be enriched and isolated with iron-, nitrate-, or sulfur-reducing media.

Balophilic or Barotolerant Species

Isolation of *S. benthica* involves a relatively straightforward method that can also be used to isolate barophilic species belonging to other genera (Sakiyama and Ohwada 1997); it is similar to methods employed in other studies (DeLong and Yayanos, 1985, 1986; Kato et al. 1995, 1996; DeLong et al. 1997; Yano et al. 1997). Small portions of sediment or seawater

filters are added to 0.5 × marine 2216 broth in small, sterile polyethylene bags (Whirl-Pak, Nasco, USA) and heat sealed. The bags are incubated at low temperatures (2–4 °C) in stainless steel pressure vessels under a hydrostatic pressure equivalent to that at the isolation site. (For further details on the use of high-hydrostatic-pressure culture equipment, see Yayanos et al. (1979, 1982)). Strains are isolated and purified using marine 2216 broth solidified with 2 % SeaPrep low-melting-point agarose (FTW Inc., Maryland, USA) or silica gel (Dietz and Yayanos 1978) in tubes sealed with silicon rubber double stoppers and pressurized to about 30–70 mPa (300–700 atm.). Individual colonies are then cut out of the medium with a scalpel and transferred to fresh marine 2216 broth.

Metal-Reducing Bacteria

Details for isolation of metal-reducing *Shewanella* are described in the Prokaryotes 3rd edition (Nealson and Scott 2006) and elsewhere (Nealson et al. 1991; Moser and Nealson 1996). Dissimilatory metal reduction, represented by iron and manganese reduction, is a common property of *Shewanella* species and can be used in their isolation. Briefly, samples are mixed with an equal volume of agar medium and then incubate anaerobically for enrichment of metal reducers; they are monitored daily and scored qualitatively for iron reduction. To keep anaerobic conditions in enrichment, purging with nitrogen gas or covering with upper layer of media with mineral oil is effective. The addition of H_2 to the headspace can substantially stimulate growth (Caccavo et al. 1992). After a secondary enrichment, samples that showed zones of strong metal reduction are streaked onto plates with similar media. Appropriate positive (*Shewanella* sp. MR- 1) and negative (*Escherichia coli* ATCC 25922) controls are performed. As the common medium for isolation of iron-reducing bacteria, LM medium are used: 0.02 % yeast extract, 0.01 % peptone, 0.6 % NaCl, 10 mM sodium bicarbonate, 10 mM HEPES, 5 mM lactate, 5 mM succinate, 5 mM glycerol, 1 mM acetate, 0.5 mM ferric chloride, 5 mM sodium molybdate, and ferrozine [3-(2-pyridyl)-5,6 bis (4-phenylsulfonic acid)-1,2,4 triazine], pH 7.2). Where ferric chloride is electron acceptor, which can substitute to other metal compounds, some of organic acids are supplemented as electron donors, lactate, succinate, acetate, etc. This medium supplemented with 0.02 % yeast extract can be used to test various metal-reducing properties and to enrich for metal-reducing strains, due to supporting boost growth (Lovley and Phillips 1988). In the case of studies for iron-reducing bacteria, a variety of iron electron acceptors can be used, including amorphic ferric oxide, ferric citrate, and ferric pyrophosphate (Lovley and Phillips 1986, 1988). These electron acceptors can be added separately or in combination. Reduction of Fe^{3+} to Fe^{2+} is indicated by the change of the rust-colored medium to a clear solution that contains white (smectite or vivianite) or black (FeS_2, pyrite) precipitates. A similar approach can be used with other electron acceptors, such as nitrates and MnO_2 (Lovley and Phillips 1988). After isolation of metal reducers, it can be necessary to measure metal reduction rate in these bacteria. The example for iron and manganese-reducing bacteria is as follows (Venkateswaran et al. 1998): LM growth medium containing 0.6 % NaCl and 20 mM lactate (Meyers and Nealson 1988) is used for metal reduction experiments. Amorphous manganese oxide and FeOOH are prepared as described (Lovely & Phillips, 1988). Cells grown aerobically in liquid medium are harvested by centrifugation and adjusted to an appropriate bacterial cell density and then inoculate to LM medium containing either ferric or manganese oxides. All media and solutions were deaerated by purging with nitrogen prior to the experiment. Samples were drawn at appropriate intervals, passed through a 0.2 pm filter (Millipore), and the resulting soluble (reduced) metal is measured by atomic absorption spectrometry (Burdige and Nealson 1985) or other measurement tools. To determine total Fe and Mn concentrations, nitric acid (1 % final concentration) was added to the unfiltered samples prior to measurement. Anaerobic sulfur reduction can be tested using a plate assay developed by Moser and Nealson (1996), which contains polysulfide. Colonies within clearing zones on the sulfur plates can then be transferred to nutrient or marine agar for further purification with aerobic incubation. Alternatively, direct isolation can be performed anaerobically on S0 reduction agar (Moser and Nealson 1996) with transfer of growth to nutrient or marine agar. Reduction of uranium (9–11, 14) and plutonium, whose redox chemistry is more complex than that of most other actinides, are also observed in *S. oneidensis* MR-1 (Fredrickson et al. 2000; Icopini et al. 2009), though this ability is limited on only some oxidation states of U and Pu. Therefore, it is expected to be utilized for the in situ immobilization of toxic metal elements.

Media for Metal-Reducing Bacteria

1. Iron-reducing medium consists (per liter distilled water) $NaHCO_3$, 2.5 g; $CaCl_2$ $2H_2O$, 0.1 g; KCl, 0.1 g; NH_4Cl, 1.5 g; NaH_2PO_4, 0.6 g; carbon source (acetate, lactate, citrate, etc.) 20 mM; trace element solution, 10 ml; and 200 mM amorphic ferric oxide. The medium is prepared with vigorous boiling and gassing with nitrogen to remove oxygen and dispensed into vials or tubes sealed with thick butyl rubber stoppers. Following autoclaving, the medium pH is about 6.7. The trace element solution (Bowman, 2005) contains (per liter distilled water) nitriloacetate, 1.5 g; $MgSO_4 \cdot 7H_2O$, 3 g; $MnSO_4 \cdot H_2O$, 0.5 g; NaCl, 1 g; $FeSO_4 \cdot 7H_2O$, 0.1 g; $CoCl_2 \cdot 6H_2O$, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $ZnSO_4 \cdot 7H_2O$, 0.1 g; $CuSO_4 \cdot 5H_2O$, 0.01 g; $Alk(SO_4)^2 \cdot 12H_2O$, 0.01 g; H_3BO_3, 0.01 g; $Na_2MoO_4 \cdot 2H_2O$, 0.01 g; $NiSO_4 \cdot 6H_2O$, 0.03 g; Na_2SeO_3, 0.02 g; and $Na_2WO_4 \cdot 2H_2O$, 0.02 g. The nitriloacetate is dissolved in 500 ml distilled water and the pH is adjusted to 6.5 by KOH. The remaining salts are added one at a time, and the volume is brought up to 1 l. Samples are added using Hungate techniques or in an anaerobic chamber. N_2/Co_2 (80:20) or $N_2/CO_2/H_2$ (80:10:10) can be used as the headspace atmosphere. For enrichment of

marine samples, the medium is supplemented with 27 g/1 NaCl and 3.7 g/1$MgCl_2$. Amorphic ferric oxide is prepared by neutralizing a 0.4 M $FeCl_3$ solution to pH 7 with NaOH. Ferric citrate can be used instead of amorphic ferric oxide and is added at 20 mM (with $CaCl_2{\cdot}2H_2O$ omitted to prevent precipitation).

2. Nitrate-reducing medium: similar to iron-reducing medium, but with an amorphic ferric oxide replaced by 20 mM KNO_3 ($CaCL_2{\cdot}2H_2O$ is omitted to prevent precipitation).
3. Manganese-reducing medium: similar to iron-reducing medium, but with amorphic ferric oxide replaced by 15 m M MnO_2. The latter is prepared by slowly adding 30 mM $MnCl_2$ to 20 mM $KMnO_4$ with constant stirring.
4. For sulfur-reduction plates, the basal medium used is similar to iron-reducing medium, but the amorphic ferric oxide is omitted and the medium is supplemented with 0.5 % casamino acids and 1.5 % agar. Immediately before dispensing the medium into plates, 40 mM polysulfide is added from a polysulfide stock (2.25 M total sulfur) prepared by adding 7.2 g sulfur flowers and 24 g $Na_2S{\cdot}9H_2O$ to 100 ml boiling water and stirring for 15 min. After adding the polysulfide, the medium is left in air overnight to allow for sufficient precipitation of sulfur globules and to shift the pH back to neutrality. The medium is then inoculated and incubated anaerobically.

Ecology

Food Spoilage *Shewanella*

Shewanella species as a food spoilage organism was first reported by Derby and Hammar (1931), and the number of such reports has continued to grow since then. As the primary species of interest to the food industry, *Shewanella putrefaciens* has been studied. Although the exact isolation sites of *S. putrefaciens sensu stricto* are unclear, *S. putrefaciens* and other food spoilers belonging to *Shewanella* are frequently isolated from a wide range of daily, poultry, beef, and seafood products (Shewan, 1971; Lee et al. 1977; Leroi et al. 1998; Stenstrom and Molin, 1990; Borch et al. 1996; Gram and Huss, 1996). Its importance stems from its role in spoilage of low-temperature stored protein-rich foods of high pH (e.g., marine fish, chicken). According to the recent papers, five *Shewanella* species (*S. baltica*, *S. algidipiscicola*, *S. glacialipiscicola*, *S. hafniensis*, and *S. morhuae*) isolated from marine fish were also reported to display strong fish spoilage activity. Of particular interest are the species that can grow well near 0 °C, such as *S. baltica*, *S. algidipiscicola*, *S. glacialipiscicola*, *S. hafniensis*, *S. morhuae*, *S. frigidimarina*, and *S. gelidimarina* and their potential role in food quality degradation, for instance, they can grow in cod juice at 0 ° C (Vogel et al. 2004). Thus, the *Shewanellae* become dominant organisms on stored marine fish materials and 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; Jorgensen and Huss, 1989; Gram et al. 1990, 1992, 1994, 1996, 1999; Gram, 1993; Stenstorm 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*. 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; Vogel et al. 2004, Satomi et al. 2006, 2007). The fact, apart from the ability to reduce TMAO and produce H_2S, fish spoilage *Shewanella* produces a range of degradative enzymes; the hydrolysis of DNA, ornithine decarboxylase, protease (casein and gelatin), RNase, and lipase. For a long time, *S. putrefaciens* have been considered the most important food spoiler in fishery industry; however, recent research by Vogel et al. (2005) has reported that *S. baltica* was the dominant spoilage species of Baltic Sea caught marine fish stored on ice. Since the main isolation source of *S. baltica* is coastal marine environments or deep-sea sediment, it is interesting whether it is epibiotic strains in the tissues of various marine animals or free living organisms in sea water. Genetic analysis of *S. baltica* (described in the study as *S. putrefaciens*) in Baltic Sea waters indicates the population is stable at the clonal level, and they appear to be correspondence with increased sulfur levels in the Baltic Sea and other locations (Nealson et al. 1991; Brettar and Hofle, 1993; Ziemke et al. 1997, Caro-Quintero et al. 2012). *S. algae* comprises the bulk of strains isolated from clinical specimens (Nozue et al. 1992; Vogel et al. 1997, 2000), and this species has also been isolated from various saline habitats, including salt marshes (Rossello-Mora et al. 1995), oil brine (Semple and Westlake 1987), surfaces of macroalgae (Simidu et al. 1990), seawater, and occasionally salted food products (Vogel et al. 1997). The true habitat is also unclear, though growth of them is stimulated by sodium ion.

Deep Sea, Psychrophilic, Epibiotic, and Bioluminescent *Shewanella*

Almost all marine-related *Shewanella* are halophilic and psychrophilic, reflecting their habitat. Some species are known as piezotrophic species, *S. benthica*, *S. piezotolerans*, *S. surugensis*, *S. abyssi*, *S. psychrophila*, *S. kaireiae*, and *S. violacea*. The first case of isolation of deep-sea *Shewanella* is that strains of *S. benthica* as well as *S. violacea* have been isolated from several deep-sea sites, including the Marianas Trench at a depth of nearly 11,000 m (Kato et al. 1995, 1996, 1998). This species has been found in water and sediment samples and organic detritus, but is most frequently associated with decaying deep-sea invertebrates, including amphipods and holothurians (DeLong et al. 1997). 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 (Bowman et al. 1997, Nichols et al. 1999; Wang et al. 2011; Usui et al. 2012) suggests a critical feature of the deep-sea cold- and pressure-tolerant microbes is their ability to produce (EPA), the levels of which range from 2 % to 20 %; it is likely to maintain membrane fluidity in cold environment. The strains adapted to pressure and cold temperature appear to be biochemically and phylogenetically related (Kato et al. 1997; Chikuma et al. 2007; Sato et al. 2008; Kawano et al. 2009; Aono et al. 2010; Nishida et al. 2010; Tamegai et al. 2011).

Several *Shewanella* species have been isolated from various marine animals (Leonaldo et al. 1999; Satomi et al. 2003; Hirota et al. 2005). As epibiotic species, *S. hanedai*, *S. woodyi*, *S. colwelliana*, *S. waksmanii*, *S. pneumatophori*, *S. marinintestina*, *S. schlegeliana*, and *S. sairae* have isolated from squid skin, fish surface, oyster, and fish intestine. While *S. pealeana* was isolated from the accessory nidamental gland of the squid, *Loligo pealei*, symbiotic relationships were speculated. Also, these traits appear to be associated with the ability of *Shewanellae* to survive as epibiotic strains in the tissues of various marine animals. The bioluminescent species *S. woodyi* (Makemson et al. 1997), as well as other novel *Shewanella* strains, has been shown to be associated with the ink and reproductive organs of certain squid (*Loligo* spp.) (Leonardo et al. 1999). *S. woodyi* has also been isolated from seawater and organic detritus collected at depths of 200–300 m in parts of the Mediterranean Sea. *S. colwelliana* has been isolated from cultured oysters and their vicinity in the Chesapeake Bay, United States (Weiner et al. 1988; Richards et al. 2008). Studies suggest that *S. cowelliana* can enter a symbiotic arrangement with a host oyster (Bonar et al. 1986). However, it does not fully understand the interaction between these marine organism and *Shewanella* species. Biochemical analysis of the epibiotic strains indicates that EPA can make up a significant portion of their unsaturated lipids (Satomi et al. 2003; Hirota et al. 2005). 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 (Nealson and Scott 2006). 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 pealeane* appeared not to produce detectable levels of EPA (Lenardo et al. 1999). 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. As other species isolated from marine creatures, *S. coralli, S. fodinae, S. irciniae, S. haliotis*, and *S. spongiae* have been reported; *S. coralli* and *S. fodinae* are from coral leaf. *S. spongiae* and *S. irciniae* are from sponges; *S. haliotis* is from an abalone. Although interaction between these bacteria and creatures are expected, there is no strong support for such a conjecture. Addition to it, the true habitations are ambiguous, because most species are consist of single isolate. Further ecophysiological studies are needed for these species.

The trait of bioluminescence is found in only two species of *Shewanellae*, *S. hanedai* and *S. woodyi*, both isolated from deep cold water or sometimes sea animals and both capable of growth at 4 °C. *S. hanedai* has also been isolated from Antarctic sea ice diatom communities, but was first isolated from sediments of the Arctic and Southern Oceans and from coastal areas of Canada (Jensen et al. 1980). 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 which is characteristic of most of the other *Shewanellae*.

Other Marine *Shewanella*

Most *Shewanella* species appear to be strictly marine in origin (*Table 31.2*). *S. amazonensis* has so far been isolated only from coastal marine muds off the Brazilian coast (Venkateswaran et al. 1998). Although *S. oneidensis* MR-1 has been isolated from Oneida Lake as freshwater lake, other strains of this species came from various isolation sources, including the Black Sea and clinical samples (Venkateswaran et al. 1999). Many *Shewanella* have been isolated from sea sediment or sea water, with various regions (Bowman et al. 1997; Ivanova et al. 2003a, b; Richards et al. 2008) and samples belonging to various temperature and redox profiles. Some species have been isolated from the Atlantic Ocean, near Halifax harbor in Canada as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) degrading bacteria (Zhao et al. 2005, 2006, 2007). These reports support that *Shewanella* is ubiquitously distributed in marine or marine-related environments. As the further studies, *S. frigidimarina* appears to be ubiquitous in the Antarctic marine environment and has been isolated from seawater, ice, sediments, and cyanobacterial mat communities of the southern ocean and continental, marine-derived lakes (Bowman et al. 1997, 2000). It has also been isolated from the deep sea (DeLong et al. 1997) and the North Sea (Reid and Gordon 1999), as indicated by 16S rDNA sequencing of isolates. *S. gelidimarina*, by comparison, has a much more restricted distribution and inhabits diatom-rich communities found in coastal Antarctic sea ice (Bowman et al. 1997).

Pathogenicity, Clinical Relevance

Shewanellae most commonly serve as secondary or opportunistic pathogens, and infections caused by them are rare. Nevertheless, there are reports of monomicrobial *Shewanella* infections; *S. algae* and *S. putrefaciens* have been implicated occasionally in polymicrobial bacteremia and septicemia and are associated with wide spectrum of clinical syndromes, such as cellulitis

in the context of other skin and soft tissue manifestations, arthritis, otitis media or otitis externa, respiratory distress, intra-abdominal infection, pneumonia, and empyema (Chen et al. 1997; Holt et al. 2005). With a few exceptions, *S. algae* and *S. putrefaciens* are susceptible to common antibiotics used to treat bacterial infections, although drug-resistant strains have been reported to emerge during the course of patient treatment (Chen et al. 1997: Holt et al. 2005; Kim et al. 2006). However, due to a lack of studies on human clinical specimens examined, the role of the *Shewanellae* in pathogenesis and clinical significance remain undefined. According to the case study analysis since 1963 when the first human clinical specimen was reported, *Shewanella* infection is correlated with an immunocompromised state, and liver disease appears to be a strong risk factor (Holt et al. 2005; Tsai et al. 2008). Virulence factors for *Shewanella* clinical isolates are largely unknown. However, Khashe and Janda (1998) have indicated that *S. algae* is more virulent than *S. putrefaciens*, and the authors speculated that the hemolytic activity of *S. algae* could be an important virulence factor. *Shewanella algae* is tolerant to bile salts and produces extracellular virulence factors such as siderophores and other exoenzymes . The production of tetrodotoxin, the pufferfish toxin, has also been reported (Simidu et al. 1987), but this finding has not been reproduced by other investigators. The most obvious source for human infection is exposure to seawater. Such contact has been reported in some case reports (Holt et al. 2005), and in a Danish study of ear infections, >80% of patients had been swimming in the sea shortly before symptoms developed (Vogel et al. 2000). The capability to form biofilms, which has been documented for *S. putrefaciens* and *S. oneidensis*, is likely associated with pathogenicity. In a Danish study of ear infections involving *S. algae*, 49 % of patients had ear tubes, and biofilm formation was a suspected pathogenicity factor in such cases (Vogel et al. 2000). Since taxonomic classification among "*S. putrefaciens*" is confusing for a long time, the easy and clear identification methods are needed to exact epidemiological analysis among the strains isolated from clinical specimens. As mentioned above, Owen et al. (1978) and recent studies demonstrated that *S. putrefaciens* known as the heterogeneous group was divided into four bacterial groups, characterized as Owen's groups I–IV and *S. alga* was corresponding to Owen's group IV (Ziemke et al. 1998; Venkateswaran et al. 1999; Vogel et al. 2000). The use of classical phenotypic characterization to distinguish between *S. algae* and other species (e.g., high G+C% (52–56 %), tolerance to 42 °C, and 10 % NaCl are characteristics of *S. alga* supported this affiliation with group IV. The semiautomated and automated identification systems used for identification often fail to identify *S. algae* as *S. putrefaciens* due to database not considering *S. algae* (Vogel and Gram, 1994; Domínguez et al. 1996). In earlier phenotypic studies of *S. putrefaciens*, at least two groups, or "Gilardi" biovars, were observed. Gilardi biovar 2 is equivalent to Owen's DNA hybridization group IV, *S. algae*, which can grow at 42 °C and tolerate 6 % NaCl, but cannot utilize glucose or maltose (Vogel et al. 2000). Gilardi biovars 1 and 3 include mostly food and environmental strains, which are psychrotrophic, saccharolytic, and unable to grow in the presence of 6 % NaCl (Gilardi 1972; Riley et al. 1972b; Richard et al. 1985; Khashe and Janda 1998).

A few studies have identified *S. algae* and *S. putrefaciens* as opportunistic pathogens in nonhuman species, though the literatures are limited in the aquaculture fields. In China and Taiwan, *S. algae* was isolated as causative agent of abalone mortality in hatchery ponds (Cai et al. 2006) and caused ulcer disease in the marine fish, *Sciaenops ocellata*, in China (Chen et al. 2003). *S. putrefaciens* was also identified as virulent bacteria to juvenile freshwater zebra mussels (Gu and Mitchell, 2002). Recently a highly pathogenicity of *S. marisflavi* to sea cucumber was reported (Li et al. 2010).

Application

Applications for *Shewanella* in current-generating devices include wastewater treatment, conversion of waste biomass, and bioremediation of chemical pollutant, radionuclides, toxic elements, harmful organics, etc.

Bioremediation of Metal Pollutants

Owing to the broad specificity of the *Shewanella* anaerobic reductase enzyme system, *S. oneidensis* MR-1 strains can reduce and mobilize toxic and radioactive metallic pollutants, including As, Co, Cr, Hg, Pu, Se, Tc, and U (Gorby and Lovley, 1992; Lovely, 1993; Caccavo et al. 1992, 1999; Lloyd and McCaskie, 1996; Ganesh et al. 1997; Fredrickson et al. 2002). Their ability has 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 introduction 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. 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 Halogenated and Nitramine Organics

In addition, utilization of halogenated organic compounds as the terminal electron acceptor during anaerobic respiration, also known as dehalorespiration, is a characteristic of some *Shewanellae*. *S. putrefaciens* 200 can reductively dehalogenate tetrachloromethane (CT), and *S. algae* BrY has the ability to transform CT via reduction of the redox-active vitamin B12, which acts as a catalyst in the reaction. *S. oneidensis* also reductively dehalogenates CT, polychlorinated biphenyls, gamma-hexachlorocyclohexane (lindane), 1,1,1-trichloroethane, and pentachloroethane (Petrovskis et al. 1994; Picardal et al. 1995; Cervini-Silva et al. 2003). Studies on dehalogenation of the above compounds have been performed and some degradation mechanisms have been proposed. Reduction of 1,1,1-trichloroethane and pentachloroethane has been described in conjunction with microbial reduction of iron-bearing clay minerals. Although they usually convert CT to chloroform that still remains harmfulness, advent researches may overcome these problems.

A unique feature of some species isolated from sediments, mainly in Halifax harbor, is the ability to attenuate and/or degrade cyclic nitramines such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)(Zhao et al. 2005), which is an explosive contaminant. Several *Shewanella* species have been isolated as RDX reducing bacteria, *S. atlantica*, *S. canadensis*, *S. halifaxensis*, and *S. sediminis*, but little is known about RDX degradation mechanisms. The full genome sequence has been determined in *S. halifaxensis* and *S. sediminis*; it is expected to elucidate complete mechanisms of RDX degradation.

Other unique characteristics are as follows. Some strains of *S. putrefaciens* degrade aromatic hydrocarbons, (e.g., benzene) and may have applications in environmental cleanups, although aromatic and heterocyclic compounds are not used in general. *Shewanella decolorationis* strains also degrade dye compounds, such as crystal violet, malachite green, methyl violet B, and naphthylaminesulfonic azo dye (Hong et al. 2007; Chen et al. 2010). *Shewanella algae* strains have been shown to form 4-epitetrodotoxin, which is a potent neurotoxin (Simidu et al. 1987, 1990; Nozue et al. 1992).

Biofuel Cell Application

A microbial fuel cell (MFC) or biological fuel cell is a bioelectrochemical system that drives a current by mimicking bacterial interactions found in nature. *Shewanella* can be used in MFC applications (Gorby et al. 2006; Hau and Gralnick, 2007; Qian and Morse, 2011); the main strain used in technique is *S. oneidensis* MR-1, using the ability to dissimilate various metals. A typical microbial fuel cell consists of anode and cathode compartments separated by a cation (positively charged ion) specific membrane. Energy can be harvested from biomass when bacteria oxidize organic compounds and utilize as electrode as a final electron acceptor. Development of MFC applications is expected, because it is one of the environmentally conscious and sustainable energy generating systems.

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32 The Family *Solimonadaceae*

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Abstract
The family *Solimonadaceae* was previously named *Sinobacteraceae* with the type genus *Sinobacter*. Later, the type species of *Sinobacter flavus* was reclassified as *Solimonas flava*, and the genus *Sinobacter* was united with *Solimonas*. Due to the defunct name *Sinobacter*, the family name *Sinobacteraceae* was replaced by *Solimonadaceae*. The family *Solimonadaceae* embraces three genera, i.e., *Solimonas*, *Panacagrimonas*, and *Fontimonas*, with the type genus *Solimonas*. Phylogenetically, the neighboring taxa of *Solimonadaceae* include *Nevskia*, *Hydrocarboniphaga*, *Alkanibacter*, *Steroidobacter*, *Renibacterium*, *Silanimonas*, *Salinisphaera*, and the members of the family *Xanthomonadaceae* (data not shown). Members of *Solimonadaceae* are defined by a wide range of morphological and chemotaxonomic properties, such as polar lipids, fatty acids, and respiratory quinones, which are used for the definition of genera and species. Members of the family are mainly found in soil and freshwater. Many species are described for their ability to decompose chemical pollutants such as atrazine, chlorinated hydrocarbons, and hexane. In this contribution, general aspects of the taxonomy (morphological, physiological, biochemical, phylogenetic, and chemotaxonomic properties), historical changes and growth requirements, habitat, and ecology characteristics for the members of the family *Solimonadaceae* are discussed and specified.

Taxonomy, Historical and Current

Short Description of the Family

Phylogenetically, the family is a member of the order *Xanthomonadales* (Saddler and Bradbury 2005), class *Gammaproteobacteria*. The family contains the genera *Solimonas* (Kim et al. 2007; Sheu et al. 2011), *Panacagrimonas* (Im et al. 2010), and *Fontimonas* (Losey et al. 2013), and the type genus is *Solimonas*. The genus *Solimonas* was originally described by Kim et al. (2007) and was emended by Sheu et al. (2011). In the study of Sheu et al. (2011), *Sinobacter flavus* (the type and sole species of *Sinobacter*) and *Singularimonas variicoloris* (the type and sole species of *Singularimonas*) were reclassified as *Solimonas flava* comb. nov. and *Solimonas variicoloris* comb. nov., based on a comparative experimental polyphasic taxonomic approach. The type genus *Sinobacter* of the family *Sinobacteraceae* was transferred to the genus *Solimonas* and reclassified as *Solimonas flava*. Subsequently, the family name *Solimonadaceae* replaced the name *Sinobacteraceae*, and the genus *Solimonas* was designated as the type genus. The family *Solimonadaceae* now embraces the three genera *Solimonas*, *Panacagrimonas*, and *Fontimonas*, embracing the six species of *Solimonas*, i.e., *S. flava*, *S. soli*, *S. aquatica*, *S. variicoloris*, *Panacagrimonas perspica*, and *Fontimonas thermophila* (Table 32.1).

Cells of the family are chemoorganotroph, Gram-staining negative and rods (long rods), nonmotile, or motile by means of one polar flagellum; aerobic to facultatively anaerobic, producing acid from only a few of carbohydrates. Endospores are not produced. Oxidase and catalase are positive. Ubiquinone-8 (Q-8) is the predominant respiratory quinone. Major fatty acids are $C_{16:0}$, $C_{18:1}\omega7c$, and/or summed feature 8 ($C_{18:1}\omega9c$, $C_{18:1}\omega7c$, or $C_{18:1}\omega6c$), but iso-$C_{16:0}$ sometimes also occurs as a major component. The set of 16S rRNA gene sequence signature nucleotides is comprised of pos. 143 (C), 220 (A), 289–311 (A-U), 317–336 (C-G), 369–392 (G-A), 514–537 (U-A), 560 (U), 508–761 (U-A), 778–804 (U-U), 1129–1143 (C-A), 1163–1173 (A-U), and 1268 (A). Genomic DNA G+C values range from 64.0 to 70.0 mol%. The polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, and some unidentified lipids. However, the polar lipids among the *Solimonadaceae* species are not consistent, and the polar lipid composition is not recommended as an essential

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Table 32.1
Type strain catalogue of the family *Solimonadaceae* and the GenBank accession numbers of the housekeeping sequences

Genus	Species	Deposited as	GenBank accession numbers of 16S rRNA (rpoB)
Solimonas	*S. soli*	DSM 21787^T, KCTC 12834^T, LMG 24014^T	EF067861 (HM357633)
	S. flava	DSM 18980^T, KCTC 12881^T, CCTCC AB 206145^T	EF154515 (HM357632)
	S. variicoloris	DSM 15731^T, LMG 22844^T	AJ313020 (HM357634)
	S. aquatica	BCRC 17835^T, LMG 24500^T	EU303271 (HM357631)
Fontimonas	*F. thermophila*	DSM 23609^T, CCUG 59713^T	JN415769
Panacagrimonas	*P. perspica*	KCTC 12982T, LMG 24239^T	AB257720

chemotaxonomic feature for describing a new genus in the family. Members are usually found in soil, chemical-polluted environment, and fresh spring water.

Genomic Basis of the Taxonomy

A neighbor-joining phylogenetic tree constructed by using the 16S rRNA gene sequences shows that members of the family *Solimonadaceae* belong to the class *Gammaproteobacteria* and clustered together with the established genera, *Alkanibacter*, *Nevskia*, and *Hydrocarboniphaga*. Three genera of *Solimonadaceae*, along with members of *Alkanibacter*, *Nevskia*, and *Hydrocarboniphaga*, formed a deep-rooting lineage within the class *Gammaproteobacteria*. The deep-rooting lineage was previously named by Zhou et al. (2008) as the "HNS (*Hydrocarboniphaga/Nevskia/Sinobacter*) clade," but now the "HNS" lineage should be renamed as *Hydrocarboniphaga/Nevskia/Solimonas*, because the type species of *Sinobacter flavus* was reclassified as *Solimonas flava* and the genera *Sinobacter* and *Singularimonas* were incorporated to the genus *Solimonas* (Fig. 32.1). On the basis of the phylogenetic analysis results, the separated "HNS" clade should not be included in the family *Xanthomonadaceae* within the order *Xanthomonadales* (Fig. 32.2). The 16S rRNA gene similarity values among the type strains of *Solimonas* are ranged from 99.7 % (DSM 15731^T and DSM 18980^T) to 96.2 % (LMG 24500^T and DSM 21787^T); the similarity values between type strain of *Fontimonas thermophila* and the type strains of *Solimonas* are from 93.1 % (DSM 23609^T and DSM 21787^T) to 94.1 % (DSM 23609^T and DSM 18980^T); the similarity values between type strain of *Panacagrimonas perspica* and the type strains of *Solimonas* are from 91.8 % (LMG 24239^T and DSM 21787^T) to 93.0 % (LMG 24239^T and LMG 24500^T). Furthermore, the 16S rRNA gene similarities between members of *Solimonadaceae* and the closest related genera *Alkanibacter*, *Nevskia*, and *Hydrocarboniphaga* within the "HNS clade" are around or lower than 90.0 %. Although the genera *Alkanibacter* (Friedrich and Lipski 2008), *Nevskia* (Famintzin 1892), *Hydrocarboniphaga* (Palleroni et al. 2004), and *Steroidobacter* (Fahrbach et al. 2008) were allocated to be the members of the family *Sinobacteraceae* by Gutierrez et al. (2012) in the phylogenetic analysis, there are no further evidences to support taxonomic results; therefore, the four genera were not included in the family *Solimonadaceae* of the present contribution. The GenBank and EzTaxon-e database (Kim et al. 2012) analyses based on the 16S rRNA gene sequences showed that the "HNS clade" belongs to a phylogenetic cluster mainly consisting of a special group of uncultured or unidentified bacterial strains from contaminated soil, freshwater, or other polluted or extreme environments. An interesting characteristic observed is that most members in the cluster showed degradation abilities to organic pollutants (data are available from GenBank database).

As described by Sheu et al. (2011), DNA-DNA hybridizations were performed fluorometrically by the method of Ezaki et al. (1989), and genomic DNA of *Solimonas soli* DSM 21787^T and *Sinobacter flavus* DSM 18980^T was labeled as probes for the hybridization reactions. The DNA-DNA relatedness values between type species *Solimonas soli* DSM 21787^T and *Sinobacter flavus* DSM 18980^T (46.5 %) and *Singularimonas variicoloris* DSM 15731^T (40.3 %) and *Solimonas aquatica* LMG 24500^T (39.1 %) were relative low. Comparatively speaking, *Sinobacter flavus* DSM 18980^T showed higher DNA-DNA relatedness value with *Singularimonas variicoloris* DSM 15731^T (51.5 %) but still showed lower values with *Solimonas soli* LMG DSM21787^T (39.7 %) and *Solimonas aquatica* LMG 24500^T (40.3 %), and all of these values were lower than the threshold value (70 %) for the recognition of genomic species (Stackebrandt and Goebel 1994).

Taxonomic Comments

The type and sole species of *Solimonas soli*, *Sinobacter flavus*, and *Singularimonas variicoloris* were described successively in a short period in 2007 and 2008 as three separated genera. The type species *Solimonas soli* was described by Kim et al. (2007) as Gram-negative, aerobic, oxidase-negative, catalase-positive, nonmotile rods; chemotaxonomically, the predominant respiratory quinone was Q-8, the major fatty acids were determined as $C_{16:0}$, $C_{18:1}$, and summed feature 3 (iso-$C_{16:1}$ I and/or $C_{14:0}$ 3-OH), and the DNA G+C content was reported as 40.5 mol%. The type species of *Sinobacter flavus* was described by Zhou et al. (2008) as Gram-negative, aerobic, oxidase-positive, catalase-negative, non-endospore-forming, nonmotile, long rods; chemotaxonomically, the predominant isoprenoid quinone

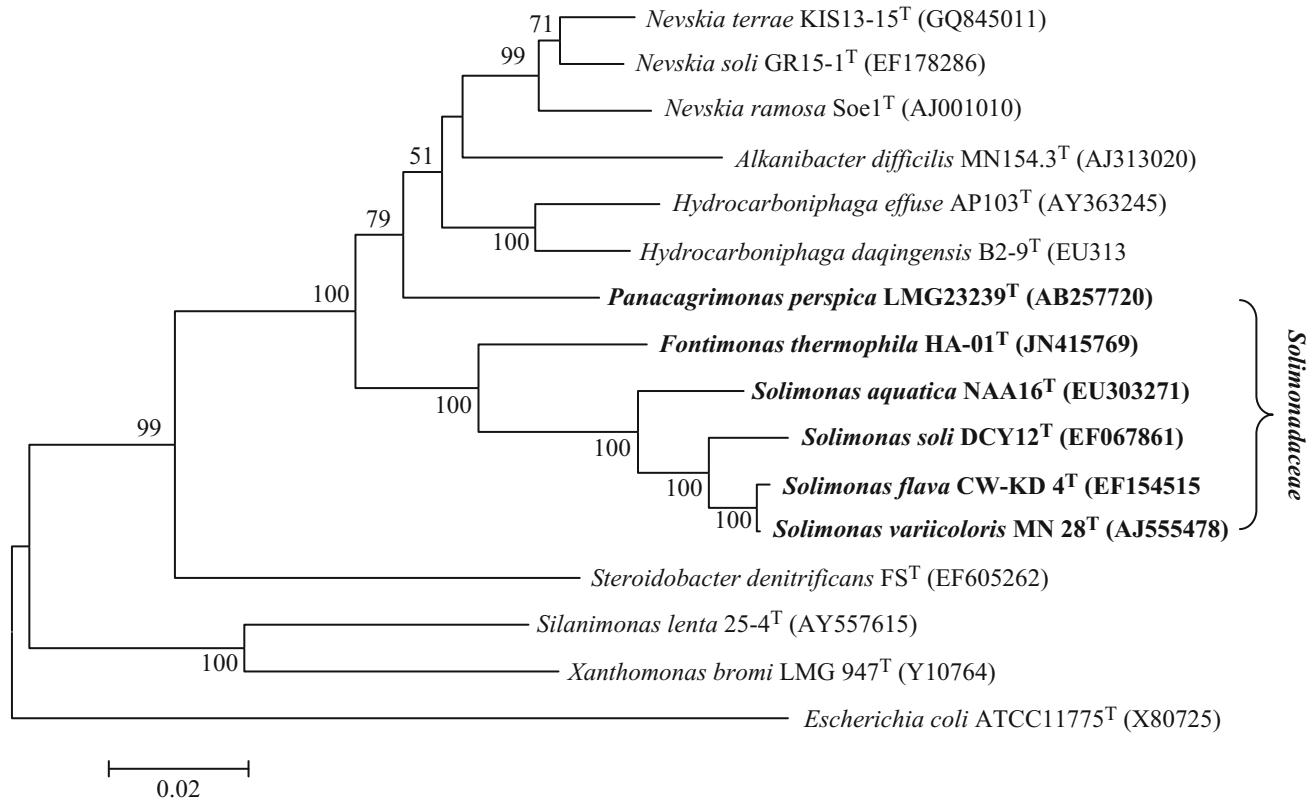

Fig. 32.1

Phylogenetic tree based on 16S rRNA gene sequence analysis, constructed using the neighbor-joining method showing the phylogenetic relations between *Solimonadaceae* and the closest taxa within the "HNS (*Hydrocarboniphaga/Nevskia/Solimonas*)" clade. Numbers on branch nodes are bootstrap values (1,000 resamplings, only values above 50 % are shown). Bar, 0.02 substitutions per nucleotide position

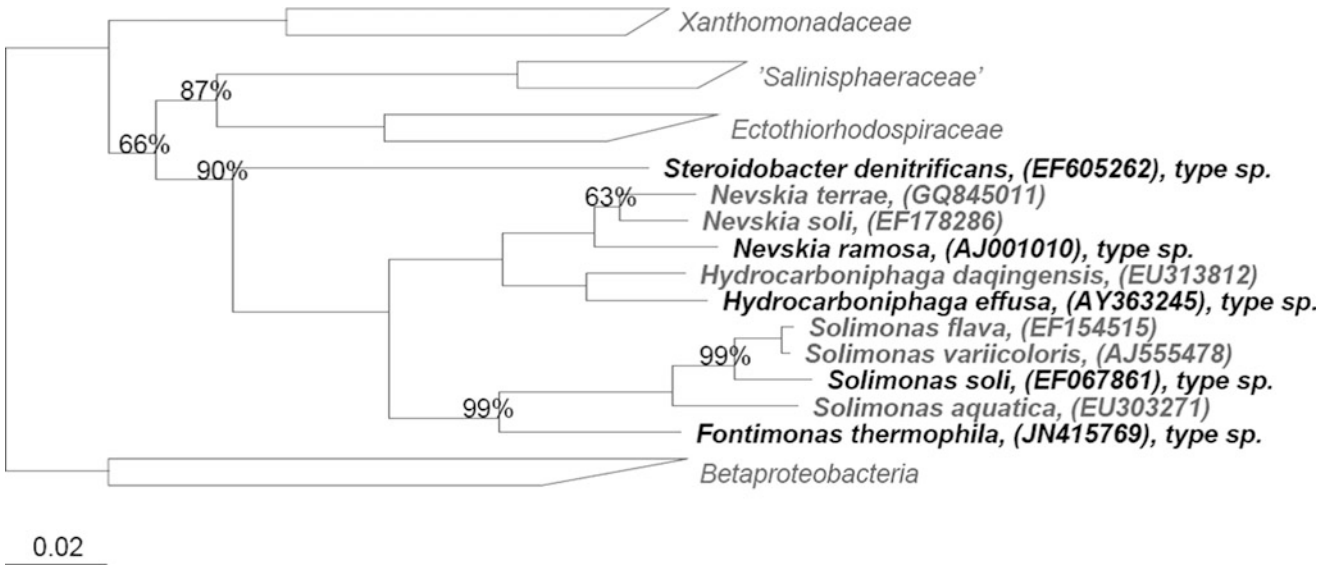

Fig. 32.2

Phylogenetic reconstruction of the family *Solimonadaceae* based on 16S rRNA and created using the maximum likelihood algorithm mRAxML (Stamatakis 2006). The sequence datasets and alignments were used according to the All-Species Living Tree Project (*LTP*) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). Numbers on branch nodes are bootstrap values (1,000 resamplings, only values above 50 % are shown). Bar, 0.02 substitutions per nucleotide position (Yarza et al. 2010)

was Q-8, the major fatty acids of $C_{16:0}$, $C_{18:1}\omega7c$, and summed feature 3 (iso-$C_{15:0}$ 2-OH and/or C16:1ω7c) were characterized, and the DNA G+C content was detected as 65.1 mol%. The type species *Singularimonas variicoloris* was described by Friedrich and Lipski (2008) as Gram-negative, aerobic, oxidase- and catalase-positive, non-endospore-forming rods; chemotaxonomically, the predominant respiratory quinone was Q-8, the major fatty acids of $C_{14:0}$ 2-OH, $C_{16:0}$, and $C_{18:1}$ cis-11 were determined, and the DNA G+C content was detected as 64.9 mol%. However, the 16S rRNA gene sequence similarity values among the type strains of the three species are more than 98 %, and the three species clustered as one separated phylogenetic branch. *Solimonas soli* DSM 21787^T shows 98.1 % similarity to *Sinobacter flavus* DSM 18980^T and 98.2 % to *Singularimonas variicoloris* DSM 15731^T. *Sinobacter flavus* DSM 18980^T even shows high similarity value of 99.7 % to *Singularimonas variicoloris* DSM 15731^T. Based on the closest phylogenetic relations among the three type species, *Sinobacter flavus* and *Singularimonas variicoloris* were transferred to the earliest described genus *Solimonas* as *Solimonas flava* and *Solimonas variicoloris* (Sheu et al. 2011). During the emended work of *Solimonas*, oxidase and catalase were detected positive for all type strains of *Solimonas*, the genomic DNA G+C content was reassessed as 68.4 mol% for *Solimonas soli*, the motility was observed for the *Solimonas soli*, and anaerobic growth was positive for *Solimonas flava* and *Solimonas variicoloris* (Sheu et al. 2011).

As the phylogenetic results discussed above, the evidences to support the genera *Alkanibacter*, *Nevskia*, *Steroidobacter*, and *Hydrocarboniphaga* as the members of the family *Solimonadaceae* are not robust enough, and the taxonomic position of the four genera is a debatable issue. In the NCBI taxonomic browser (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser), the genera *Alkanibacter*, *Nevskia*, *Steroidobacter*, and *Hydrocarboniphaga* are listed under previous family *Sinobacteraceae*, but the taxonomy of publications did not consider the four genera as the members of the family *Sinobacteraceae* or the renewed family *Solimonadaceae*, because of relatively low 16S rRNA gene similarities, phylogenetic distance, and insufficient arguments for taxonomic comparison (Zhou et al. 2008; Losey et al. 2013). These publications preferred to list the four taxa under genera incertae sedis, and at least the four closest related genera are not included in the family *Solimonadaceae* temporarily.

Phenotype of *Solimonadaceae* Species

Extensive information for the family *Solimonadaceae* is available from the type species *Solimonas soli*, *Panacagrimonas perspica*, and *Fontimonas thermophila*, as well as from *Solimonas flava*, *Solimonas variicoloris*, and *Solimonas aquatica*. Members of the *Solimonadaceae* are Gram-negative, rod- to long-rod (*Solimonas flava* and *Solimonas variicoloris*)-shaped, aerobic, or facultatively anaerobic (*Solimonas flava* and *Solimonas variicoloris*) cells. Members of genus *Solimonas* differ from those of the *Fontimonas* by low 16S rRNA gene sequence similarity values (the maximum similarity value is 94.1 % to *Solimonas soli*), major fatty acids, and thermophilic growth temperature; the genus *Solimonas* differs from the genus *Panacagrimonas* by low 16S rRNA gene sequence similarity values (the maximum similarity value was 93.0 % to *Solimonas aquatica*), major fatty acids, and NaCl tolerance.

Enzymatic Activities

Solimonas strains hydrolyze Tweens 20, 40, 60, and 80, corn oil, and starch, but not skimmed milk. According to the results of original studies, Zhou et al. (2008) described *Sinobacter flavus* (*Solimonas flava*) as oxidase positive and catalase negative, and Kim et al. (2007) described the *Solimonas soli* as oxidase negative and catalase positive. However, all the four species of *Solimonas* were determined as oxidase and catalase positive by Sheu et al. (2011). Members of *Solimonas* are positive for leucine arylamidase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase but negative for C_{14} lipase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Activities of arginine dihydrolase, urease, and β-galactosidase are negative for *Solimonas* strains; gelatin (gelatinase) and esculin are hydrolyzed by *Solimonas aquatica* and *Solimonas variicoloris*, but not hydrolyzed by *Solimonas flava* and *Solimonas soli*. Activities of oxidase and catalase are also positive for genera *Fontimonas* and *Panacagrimonas*; DNA and starch are not hydrolyzed by the species *Fontimonas thermophila* and *Panacagrimonas perspica*; alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, and naphthol-AS-BI-phosphohydrolase are positive, but lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase are negative for *Fontimonas thermophila*; gelatinase, and tryptophan deaminase are positive, but β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, and urease are negative for *Panacagrimonas perspica*.

Carbon Assimilation and Other Chemical Characteristics

Carbohydrates and organic acids of D-glucose, gluconate, adipate, and malate are assimilated as sole carbon and energy sources for *Solimonas*. Type strains of *Solimonas* are negative for nitrate reduction, indole production, glucose acidification, and assimilation of caprate and phenylacetate. Overall, *Solimonas* embraces a group of fastidious bacteria, and the nutritional spectrum of organic and inorganic substrates are narrow. For *Solimonas flava*, acid is produced from cellobiose, L-rhamnose, D-ribose, and sucrose; *N*-acetylglucosamine, cellobiose, D-glucose, glucosamine, D-ribose, L-rhamnose, starch, and

sucrose are utilized as sole carbon and energy in the API 50CHB test (bioMérieux), but the other 41 carbohydrates are not assimilated or fermented. *Solimonas soli* assimilates none of the growth substrates in the API 20NE (bioMérieux) and API ID 32GN (bioMérieux) test kits, which is quite unusual. When Luria-Bertani broth (LB, Oxoid) is added as growth factor, 3-hydroxybutyrate, acetate, adipate, gluconate, n-valerate, D-glucose, and L-histidine are assimilated in these micro-test systems (API biological test kits); if R2A medium (Difco) is added as growth factor, propionate, L-arabinose, L-fucose, D-sorbitol, L-proline, and L-serine are slowly and poorly utilized, but no substrate is assimilated in the presence of yeast extract as a growth factor. For *Solimonas variicoloris*, acid is produced from glycerol and sucrose; Tween 40, D-glucose, methyl pyruvate, monomethyl succinate, *cis*-aconitic acid, b-hydroxybutyric acid, a-ketobutyric acid, succinic acid, bromosuccinic acid, succinamic acid, alaninamide, L-alanyl glycine, L-glutamic acid, glycyl-L-glutamic acid, L-threonine, and urocanic acid are assimilated. For *Solimonas aquatica*, adipate, D-glucose, malate, maltose, gluconate, a-ketobutyric acid, pyruvic acid, adipic acid, malic acid, and D-saccharic acid are utilized as sole carbon and energy sources; L-aspartic acid, L-lysine, and L-methionine are utilized as sole carbon, nitrogen, and energy sources. Similar to *Solimonas*, only a few of organic substances (Tweens 40 and 80, α-keto valeric acid, alaninamide, L-alanine, alanyl glycine, glycyl-L-glutamic acid, and L-proline) are assimilated by *Fontimonas thermophila*. In contrast, *Panacagrimonas perspica* exhibits a wide nutritional spectrum, and the following carbohydrates and organic acids are able to utilize as growth substrates: D-arabinose, acetate, adipate, D-adonitol, aspartic acid, D-cellobiose, formic acid, D-fructose, D-fucose, fumaric acid, D-galactose, D-glucose, glutamic acid, glutamine, glutaric acid, glycerol, 3-hydroxybutyrate, isoleucine, malate, malonate, D-mannose, phenylacetate, proline, propionate, L-rhamnose, L-sorbose, suberate, succinic acid, D-sucrose, valerate, and valine.

Antibiotic Sensitivity

Based on the results of Sheu et al. (2011), the four type strains of *Solimonas* were susceptible to ampicillin (10 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), novobiocin (30 μg), rifampicin (5 μg), penicillin G (10 μg), streptomycin (10 μg), sulfamethoxazole (23.75 μg) plus trimethoprim (1.25 μg), ceftizoxime (30 μg), and tetracycline (30 μg). However, the results of Zhou et al. (2008) displayed that *Sinobacter flavus* (*Solimonas flava*) DSM 18980^T was resistant to gentamicin (10 μg), penicillin G (10 μg), and vancomycin (10 μg). Thus, the unambiguous susceptible antibiotics for *Solimonas* members are ampicillin, chloramphenicol, kanamycin, nalidixic acid, novobiocin, rifampicin, streptomycin, sulfamethoxazole plus trimethoprim, ceftizoxime, and tetracycline. Member of *Solimonas* showed highly sensitive properties to these frequently used antibiotic agents. Antibiotic sensitivity was not assessed for the genera *Fontimonas* and *Panacagrimonas* in the original studies (Im et al. 2010; Losey et al. 2013).

Chemotaxonomic Characteristics

The fatty acid pattern of *Solimonas* is defined by mainly straight-chain fatty acids, which is in accord with those of the closest phylogenetic neighbors (*Alkanibacter*, *Hydrocarboniphaga*, *Nevskia*, and *Steroidobacter*); the fatty acid patterns of *Solimonas* and closest phylogenetic neighbors are different from those of most genera in the family *Xanthomonadaceae* that are dominated by iso-/anteiso-branched fatty acids (Finkmann et al. 2000; Kim et al. 2006; Lee et al. 2005; An et al. 2005; Friedrich and Lipski 2008). At present, the only species of the family *Xanthomonadaceae* known to possess a straight-chain fatty acid pattern is *Xylella fastidiosa* which was isolated exclusively from the plant tissues (Wells et al. 1987; Mehta and Rosato 2001). According to currently available data, the pattern of *Solimonas* is quite complex, as usually more than 20 fatty acids are detected. The major fatty acids (>10 %) of *Solimonas aquatica* contained $C_{16:0}$, $C_{18:1}\omega7c$, and summed feature 3; the major fatty acids of *Solimonas soli* contained $C_{16:0}$ and $C_{18:1}\omega7c$; those of *Solimonas flava* contained $C_{16:0}$, iso-$C_{16:0}$, and $C_{18:1}\omega7c$, while those of *Solimonas variicoloris* contained $C_{14:0}$ 3-OH, $C_{16:0}$, and $C_{18:1}\omega7c$ (Table 32.2). Moderate contents (5.0 % < fatty acids <10 %) of *Solimonas aquatica* contained $C_{12:0}$, $C_{14:0}$ 3-OH, and iso-$C_{16:0}$; the moderate fatty acids of *Solimonas soli* and *Solimonas flava* contained $C_{14:0}$, $C_{14:0}$ 3-OH, and $C_{16:1}\omega5c$; the moderate fatty acids of *Solimonas variicoloris* contained $C_{12:0}$, $C_{14:0}$, and iso-$C_{16:0}$. Except for the branched fatty acid iso-$C_{16:0}$, all other iso-/anteiso-branched fatty acids in the *Solimonas* strains are lower than 3.0 %; this characteristic is significantly different from the typical members of *Xanthomonadaceae*. The fatty acid profiles of *Solimonas flava* (*Sinobacter flavus*) determined by Zhou et al. (2008), *Solimonas soli* determined by Kim et al. (2007), and *Solimonas variicoloris* (*Singularimonas variicoloris*) determined by Friedrich and Lipski (2008) were moderately different from the results of Sheu et al. (2011); perhaps different culture conditions (e.g., medium, temperature, and incubation time) and analysis methods may attribute to this discrepancy. Fatty acid profile of *Fontimonas thermophila* is similar to the *Solimonas* (Losey et al. 2013) which mainly contains $C_{16:0}$, iso-$C_{16:0}$, $C_{16:1}\omega5c$, and summed feature 8. The branched fatty acid iso-$C_{16:0}$ was detected as a major content for *Fontimonas thermophila*, and this is the biggest difference compared to most members of the *Solimonas*. Fatty acids of *Panacagrimonas perspica* mainly contained $C_{12:0}$, $C_{16:0}$, summed feature 3, and summed feature 8. Similar as *Solimonas soli*, the iso-/anteiso-branched fatty acids were not detected from the *Panacagrimonas perspica* (Im et al. 2010). According to the original study of Im et al. (2010), fatty acid $C_{12:0}$ was detected as the major content, and that characteristic is different from the genera of *Solimonas* and *Fontimonas*.

Polar lipid profiles for members of *Solimonadaceae* are quite complex; at least one of phosphatidylethanolamine,

Table 32.2

Genomic and phenotypic features that are useful in the differentiation of *Solimonadaceae* members

Characteristic	*Fontimonas*		*Solimonas*			*Panacagrimonas*
	F. thermophila	*S. aquatica*	*S. flava*	*S. soli*	*S. variicoloris*	*P. perspica*
Colony appearance	Transparent, yellow	Semiopaque	Yellow	Pale yellow	Yellow	White
Cell size (μm)	0.5–0.75 × 1.0–2.0	0.3–0.8 ×1.0	0.4–0.5 × 2.4–2.8	0.3–0.5 × 0.2–0.4	Reportedly long rods	1.2–1.8 × 2.0–4.0
Flagella	Single polar	Single polar	Nonmotile	Single polar	Nonmotile	Single polar
Anaerobic growth	−	−	+	−	+	−
Temperature range (optimum) (°C)	37–60 (45–50)	20–40 (25)	15–35 (25–30)	20–42 (30)	20–42 (30)	15–42 (30)
NaCl range (%), (optimum)	0–1.0 (0.0)	0–1.0 (0.5)	0–2.0 (0–1.0)	0–2.0 (0.5)	0–2.0 (0)	0–7.0 (ND)
Hydrolysis of:						
DNA	−	−	−	+	−	−
Esculin	ND	+	−	−	−	ND
Gelatin	ND	+	−	−	+	+
Polar lipids[a]	DPG, PG, PE, PL, APL (2)	DPG, PG, PE, PL (3), APL	DPG, PG, PE, PDE, PL (3), APL (2)	DPG, PG, PE, PDE, PL(3), APL (3)	DPG, PG, PE, PDE, PL (2), APL (4)	DPG, PG, PE, PL (2), APL (2), GL, L
DNA G+C content (mol%)	64.4	66.2	65.1	68.4	64.9	69.9
Major fatty acids	Iso-$C_{16:0}$, $C_{16:1\omega5c}$, Summed feature 8	$C_{18:1\omega7c}$, $C_{16:0}$, Summed feature 3	$C_{18:1\ \omega7c}$, $C_{16:0}$, iso-$C_{16:0}$	$C_{16:0}$, $C_{18:1\ \omega7c}$	$C_{18:1\ \omega7c}$, $C_{16:0}$, $C_{14:0}$ 3-OH	Summed feature 8, Summed feature 3, $C_{16:0}$ and $C_{12:0}$

[a]All species are rod shaped, Gram negative, oxidase and catalase positive, and negative for nitrate reduction and contain Q-8 as the predominant isoprenoid quinone. Data of *Solimonas aquatica* are obtained from Sheu et al. (2011), data of *Solimonas variicoloris* are obtained from Friedrich and Lipski (2008) and Sheu et al. (2011), data of *Solimonas flava* are obtained from Zhou et al. (2008), data of *Solimonas soli* are obtained from Kim et al. (2007) and Sheu et al. (2011), data of *Fontimonas thermophila* are obtained from Losey et al. (2013), and data of *Panacagrimonas perspica* are obtained from Im et al. (2010). Symbols: *DPG* diphosphatidylglycerol, *PG* phosphatidylglycerol, *PE* phosphatidylethanolamine, *PDE* phosphatidyldimethylethanolamine, *PL* unknown phospholipid (the number of unknown phospholipids), *APL* unknown aminophospholipids (the number of unknown aminophospholipids), *GL* unknown glycolipid, *L* unknown polar lipid. Summed feature 3 is composed of iso-$C_{15:0\ 2\text{-}OH}$ and/or $C_{16:1\ \omega6c}$ and/or $C_{16:1\ \omega7c}$. Summed feature 8 is composed of $C_{18:1\omega9c}$ and/or $C_{18:1\omega7c}$ and/or $C_{18:1\omega6c}$. + positive, − negative, *ND* not determined

phosphatidylglycerol, and diphosphatidylglycerol is usually detected as the major content; several contents of unidentified aminophospholipids and/or phospholipids and/or unknown lipids are always detected, and most members of the family contain one or more glycolipids (the data of glycolipids for *Solimonas* are not published). Based on the results of Sheu et al. (2011) and Friedrich and Lipski (2008), polar lipids of *Solimonas* are complex and different from species to species. The detailed polar lipid profiles of currently *Solimonas* species were outlined as below (Fig. 32.3): *Solimonas aquatica* exhibits polar lipids of phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, two uncharacterized aminophospholipids, and three uncharacterized phospholipids; *Solimonas soli* exhibits polar lipids of phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, four uncharacterized aminophospholipids, three uncharacterized phospholipids, and phosphatidyldimethylethanolamine; *Solimonas flava* (*Sinobacter flavus*) exhibits polar lipids of phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, three uncharacterized aminophospholipids, three uncharacterized phospholipids, and phosphatidyldimethylethanolamine; *Solimonas variicoloris* (*Singularimonas variicoloris*) exhibited polar lipids of phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, five uncharacterized aminophospholipids, two uncharacterized phospholipids, and phosphatidyldimethylethanolamine (Sheu et al. 2011). Phosphatidyldimethylethanolamine is absent from *Solimonas aquatica* but present in other species of *Solimonas*. The mutual contents for *Solimonas* are phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, two uncharacterized aminophospholipids (APL1, APL2), and two uncharacterized phospholipids (PL1, PL3). The major polar lipids of four *Solimonas* species (*Solimonas aquatica* contains PG, PE, and APL1; *Solimonas soli* contains DPG, PG, PE, APL1, and APL2;

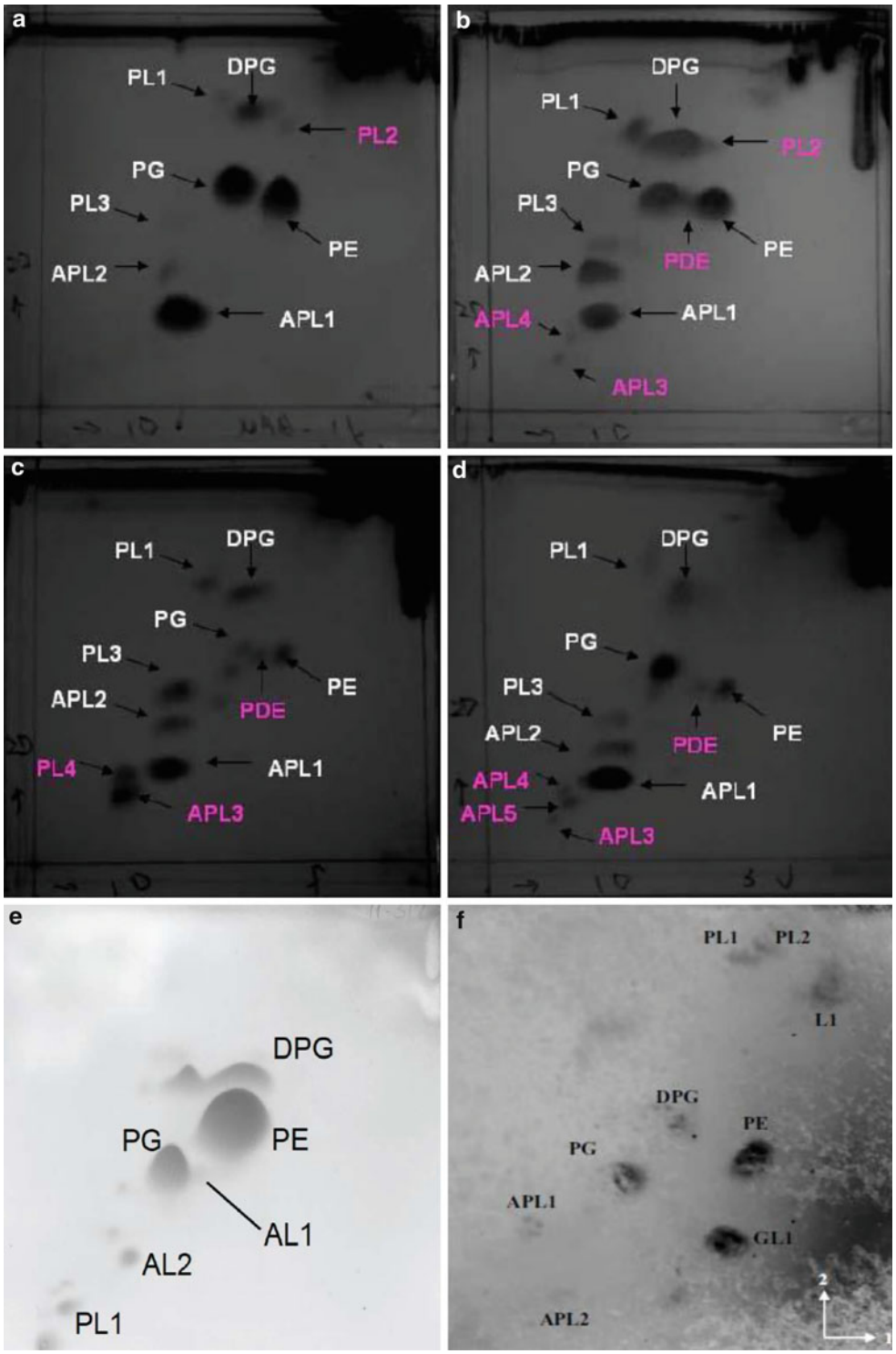

Fig. 32.3

Polar lipid patterns of *Solimonadaceae* species. Species: (a) *Solimonas aquatica*, (b) *Solimonas soli*, (c) *Solimonas flava*, (d) *Solimonas variicoloris*, (e) *Fontimonas thermophila*, (f) *Panacagrimonas perspica*. Data of (a–d) are obtained from Sheu et al. (2011), data of (e) are obtained from Losey et al. (2013), and data of (f) are obtained from Im et al. (2010). Abbreviations: *DPG* diphosphatidylglycerol, *PG* phosphatidylglycerol, *PE* phosphatidylethanolamine, *PDE* phosphatidyldimethylethanolamine, *PL* unknown phospholipids, *APL* (or *AL*) unknown aminophospholipid, *L* unknown lipids, *GL1* an unknown glycolipid

Solimonas flava contains APL1, APL3, and PL3; *Solimonas variicoloris* contains DPG, PE, PG, and APL1) are different from each other. These analyses suggest that the polar lipid profiles among the *Solimonas* species are not consistent enough; thus, the polar lipid composition should not be suggested as a diagnostic chemotaxonomic marker for the genus *Solimonas*. The polar lipids of diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylethanolamine are the predominant contents for *Fontimonas thermophila*, and an unknown phospholipid and two unidentified aminophospholipids are also determined as the minor composition (◉ *Fig. 32.3*). The polar lipid profile of *Panacagrimonas perspica* consisted of phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, an unknown glycolipid, two unknown aminophospholipids, two unknown phospholipids, and an unknown lipid (◉ *Fig. 32.3*).

Solimonas Kim et al. 2007, 2593[AL]

So'li.mo'nas. L. n. *solum* soil; L. fem. n. *monas* a unit, monad; N.L. fem. n. *Solimonas* a monad in soil

Members of the genus *Solimonas* are phenotypically characterized as Gram-negative, rod-shaped, aerobic (e.g., *Solimonas soli* and *Solimonas aquatica*), or facultatively anaerobic cells (e.g., *Solimonas flava* and *Solimonas variicoloris*). Endospores are not produced. Cells of some species (e.g., *Solimonas soli* and *Solimonas aquatica*) are motile by means of a single polar flagellum. The members of genus are chemoorganotrophs and oxidase and catalase positive. The major cellular fatty acids are $C_{16:0}$ and $C_{18:1}\omega7c$; summed feature fatty acids are usually detected as the major compositions. The fatty acid composition of the genus is moderate complex, and the iso-/anteiso-branched fatty acids in the *Solimonas* strains are lower than 3.0 % (except for the branched fatty acid iso-$C_{16:0}$). The polar lipid profiles and predominant polar lipids among the *Solimonas* species are not consistent. Consequently, the polar lipid compositions are not suggested as an important diagnostic feature for the genus of *Solimonas*. The predominant respiratory quinone is Q-8 which is considered as the common respiratory quinone for the order *Xanthomonadales*. The DNA G+C content is 64.9–68.4 mol%. The genus *Solimonas* is a member of the class *Gammaproteobacteria*, order *Xanthomonadales*, and family *Solimonadaceae*, with the type species of *Solimonas soli*. At the time of the writing, the validly described species are *Solimonas soli*, *Solimonas flava*, *Solimonas variicoloris*, and *Solimonas aquatica*.

Fontimonas Losey et al. 2013, 258[AL]

Fon'ti.mo'nas. L. adj. *fontus* from a spring, L. fem. N. *monas* a unit, monad; N. L. fem. n. *Fontimonas* a monad from a spring.

The *Fontimonas* was proposed by single strain DSM 23609^T and single species *Fontimonas thermophila*. The genus is phenotypically characterized as Gram-negative, rod-shaped, strictly aerobic cells. Endospores are not produced, and the type strain of *Fontimonas thermophila* is motile by means of a single polar flagellum. The member of genus *Fontimonas* is chemoorganotroph and oxidase and catalase positive. The major cellular fatty acids are $C_{16:0}$, iso-$C_{16:0}$, $C_{16:1}\omega5c$, and summed feature 8 ($C_{18:1}\omega9c$, $C_{18:1}\omega7c$, and $C_{18:1}\omega6c$). The branch fatty acid iso-$C_{16:0}$ is one of the major contents for *Fontimonas thermophila*, but other branch fatty acids are detected as the minor contents. The polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, an unknown phospholipid, and two unidentified aminophospholipid. The main respiratory quinone is Q-8, and the DNA G+C content is around 64.0 mol%. The genus *Fontimonas* is a member of the class *Gammaproteobacteria*, order *Xanthomonadales*, and family *Solimonadaceae*, with the type species *Fontimonas thermophila*.

Panacagrimonas Im et al. 2010, 265[AL]

Pa.na.ca.gri.mo'nas. N.L. fem. Panax -acis, scientific name of ginseng; ager agri, a field, monas a unit, monad; N.L. fem. gen. n. *Panacagrimonas*, monad of a ginseng field.

The genus is phenotypically characterized as Gram-negative, rod-shaped, strictly aerobic cells. Endospores are not produced, and the type strain of *Panacagrimonas perspica* LMG 24239^T is motile by means of a single polar flagellum. Bacteria of the genus are chemoorganotrophs and oxidase and catalase positive. NaCl is not needed for growth, but 7.0 % (w/v) NaCl is tolerated by the type species *Panacagrimonas perspica*. After 3-day incubation at 30 °C on R2A, colonies are 1–2 mm in diameter, smooth, flat, white, and irregularly shaped. The major cellular fatty acids are summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$), summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$), $C_{16:0}$, and $C_{12:0}$. The fatty acid $C_{12:0}$ was detected at the first time as major content for *Solimonadaceae*. The polar lipids are phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, an unknown glycolipid, two unknown aminophospholipids, two unknown phospholipids, and an unknown lipid. The main respiratory quinone is Q-8, and the DNA G+C content is around 70.0 mol%. The genus *Panacagrimonas* is a member of the class *Gammaproteobacteria*, order *Xanthomonadales*, and family *Solimonadaceae*, with the type species *Panacagrimonas perspica*.

Growth Condition Requirements

Solimonas strains grow on aerobic or facultatively anaerobic (e.g., *Solimonas flava* and *Solimonas variicoloris*). Slight distinctions are observed for growth temperatures among the *Solimonas* species; the temperature range was determined as 20–42 °C for *Solimonas soli*, 15–35 °C for *Solimonas flava*, 20–40 °C for *Solimonas aquatica*, and 20–42 °C for *Solimonas variicoloris*. Except for the type strain of *Solimonas aquatica*, the optimum temperature for *Solimonas* strains is 30 °C; the optimum temperature of *Solimonas aquatica* LMG 24500^T (25 °C) is slightly lower than the other *Solimonas* species. NaCl is not necessary for *Solimonas* strains, all type strains of *Solimonas* do not grow on the medium in which the NaCl concentration is higher than 2.0 %, and the optimum NaCl concentration is around 0–0.5 %. Strains of *Solimonas* always grow well without NaCl. The growth range of pH for *Solimonas flava* is 5.0–8.0

(optimum 6.0–7.0), *Solimonas soli* is 5.0–11.0 (optimum 7.0–9.0), and *Solimonas aquatica* is 7.0–8.0 (optimum 6.0–7.0), and pH 7.0 is commonly considered as the optimum pH for all strains of *Solimonas*. Type strains of *Solimonas* are a group of fastidious organisms, and the nutritional spectrum of organic or inorganic substrates is narrow, but special requirements are not observed. *Solimonas* strains can grow on complex media, such as Luria-Bertani, R2A and yeast-tryptone for *Solimonas flava*, R2A and nutrient agar for *Solimonas aquatica*, R2A and trypticase soy agar for *Solimonas variicoloris*, and R2A, Luria-Bertani agar, nutrient agar, and trypticase soy agar for *Solimonas soli*.

The species *Fontimonas thermophila* grows strictly aerobic conditions. Up to date, *Fontimonas thermophila* is the unique member of thermophilus bacterium of the family *Solimonadaceae*. Growth temperatures for the type strain *Fontimonas thermophila* are ranged from 37 °C to 60 °C, with optimum from 45 °C to 50 °C. NaCl is also not necessary for the *Fontimonas thermophila*, the type strain *Fontimonas thermophila* does not grow on the medium in which NaCl concentration is higher than 1.0 %, and the strain can grow better on complex media without NaCl. The pH range for *Fontimonas thermophila* is from 6.5 to 8.5 (optimum 6.5–7.0). Excepted for the temperature growth conditions, the pH and salt tolerance for *Fontimonas thermophila* are the same as *Solimonas* strains. *Fontimonas thermophila* can grow on complex media of Castenholz medium and R2A and trypticase soy agar, but not on Luria-Bertani agar, and special requirements are not observed (Losey et al. 2013). The nutritional spectrum of organic or inorganic substrates for *Fontimonas thermophila* is also narrow.

The species *Panacagrimonas perspica* grows in strictly aerobic conditions. Up to date, *Panacagrimonas perspica* is the unique member of NaCl-tolerant species of the family *Solimonadaceae*; the growth occurs at NaCl concentration not higher than 7.0 % (w/v). Growth temperatures for the type strain *Panacagrimonas perspica* LMG 24239^T are ranged from 15 °C to 42 °C, with optimum at 30 °C. NaCl is not necessary for the growth of *Panacagrimonas perspica*, and the strain can grow better on complex media without NaCl, but moderate NaCl concentration can be tolerated. The pH range for *Panacagrimonas perspica* is from 5.0 to 8.5 (optimum 6.5–7.0). Excepted for the NaCl tolerance trait, the pH and temperature characteristics for *Panacagrimonas perspica* are the same as *Solimonas* strains. *Panacagrimonas perspica* can grow on complex media of R2A, but not on Luria-Bertani agar, nutrient agar, trypticase soy agar, or MacConkey agar. Special requirements are not necessary (or discovered). Unlike the members of *Solimonas* and *Fontimonas*, the nutritional spectrum of organic or inorganic substrates for *Panacagrimonas perspica* is wide, and many carbon and nitrogen sources are utilized as described in the above paragraph.

Isolation and Maintenance

Solimonas soli was isolated from surface soil sample of an agricultural field in which ginseng was growing (Kim et al. 2007). One gram of (1 g) soil sample was immersed in 50 ml physiological saline solution, vortexed and serially diluted, and an aliquot (100 µl) was spread onto 10-fold-diluted R2A agar (Difco). Single colonies on agar plates were purified by transferring them onto new R2A plates and incubated for 5 days at 30 °C. The purified colonies were identified by 16S rRNA gene sequencing, and *Solimonas soli* DSM 21787^T was selected. Medium R2A contains per liter at pH 7.2: yeast extract 0.5 g, tryptone 0.5 g, casamino acid 0.5 g, glucose 0.5 g, soluble starch 0.5 g, K_2HPO_4 0.3 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, sodium pyruvate 0.3 g, and agar 15 g. The pure culture is maintained on R2A slant or preserved in a 20 % (v/v) glycerol solution in distilled water at −80 °C.

Solimonas flava was isolated from a farmland soil sample in Jiangsu Province, China, using a classic enrichment culture technique (Zhou et al. 2008; Rosenberg 1992). The farmland soil sample was collected near a chemical factory, and the soil had been contaminated by different kinds of herbicides and insecticides over a long period. Modified ISP 4 medium (contains per liter at pH 7.0: atrazine 0.2 g, K_2HPO_4 1.0 g, $MgSO_4 \cdot H_2O$ 1.0 g, NaCl 1.0 g, $(NH_4)_2SO_4$ 2.0 g, $CaCO_3$ 2.0 g, $FeSO_4$. H_2O 0.001 g, and $MnCl_2 \cdot 7H_2O$ 0.001 g), in which one of the carbohydrates (soluble starch) is substituted by atrazine as the sole source of carbon and energy, was selected as the isolation medium. Incubation was performed at 30 °C for 7 days, and the strain was preserved in a 20 % (v/v) glycerol solution in distilled water at −80 °C or as the lyophilized cells. Complex media of Luria-Bertani and yeast-tryptone are the good choice for the routinely cultivation.

Solimonas variicoloris was isolated from a hexane-treated biofilter of waste gas treatment (Friedrich and Lipski 2008). The type strain *Solimonas variicoloris* DSM 15731^T showed the hexane-degrading ability. The strain DSM 73151^T was enriched from 1 g filter material and cultivated in defined medium. Defined medium contains per liter at pH 7.0: K_2HPO_4 0.8 g, KH_2PO_4 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, $(NH_4)_2SO_4$ 1.0 g, trace element solution 1 ml, and vitamin solution 5 ml in double-distilled water. The trace element solution contains the following (per liter): Na_2-EDTA 3.0 g, $MnCl_2 \cdot 2H_2O$ 0.05 g, $CoCl_2 \cdot H_2O$ 0.19 g, $ZnCl_2$ 0.041 g, H_3BO_3 0.006 g, $NiCl_2 \cdot 6H_2O$ 0.024 g, $CuCl_2$ 0.002 g, and $NaMoO_4 \cdot 2H_2O$ 0.018 g, and adjusted the pH to 6.0. The vitamin solution contained the following (per liter): thiamine 0.01 g, nicotinic acid 0.02 g, pyridoxine hydrochloride 0.02 g, p-aminobenzoic acid 0.01 g, riboflavin 0.02 g, pantothenic acid 0.02 g, biotin 0.001 g, and cyanocobalamin 0.001 g, and adjusted the pH to 7.0. Liquid cultures 50 ml in volume were grown in screw-capped 100 ml flasks supplemented with 10 ml hexane. For solid medium, 15 g agar l^{-1} was included in the defined medium. Agar plates were incubated in 5 l desiccators; 1 ml hexane was placed in a separate beaker in the desiccator to supply hexane to the desiccator atmosphere. All incubations were performed at 30 °C. The type strain *Solimonas variicoloris* DSM 15731^T grows well on agar plates with hexane as the sole carbon source. In liquid minimal medium, *Solimonas variicoloris* DSM 15731^T shows only a weak growth or no growth at all.

Solimonas aquatica was isolated from a freshwater spring in Kaohsiung County, Taiwan (Sheu et al. 2011). Type strain LMG 24500^T of *Solimonas aquatica* was isolated by the R2A agar (Difco) at 25 °C for three days. Subcultivation was performed on R2A plates at 25 °C for 48–72 h. The pure culture is maintained on R2A slant or preserved in a 20 % (v/v) glycerol solution in distilled water at −80 °C or by lyophilization.

Fontimonas thermophila was isolated from a freshwater of artesian spring that erupts in the Hale Bathhouse in Hot Springs, AR, USA (Losey et al. 2013). Physical conditions of the Hale House Spring include a NaCl concentration of less than 10 mg · l^{-1}, a temperature of 63.7 °C, and a pH of 6.7 (Bell and Hays 2007). The type strain DSM 23609^T was isolated from the hot spring water that directly inoculated on the Castenholz agar plates (DSM medium no. 86), and the agar plates were incubated at 60 °C. Castenholz medium contains per liter at pH 8.2: nitrilotriacetic acid (Titriplex I) 0.1 g, $CaSO_4 \cdot 2\ H_2O$ 0.06 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, NaCl 8.0 mg, KNO_3 0.103 g, $NaNO_3$ 0.689 g, $Na_2HPO_4 \cdot 2H_2O$ 0.14 g, $FeCl_3 \cdot 6H_2O$ 0.47 mg, $MnSO_4 \cdot H_2O$ 2.2 mg, $ZnSO_4 \cdot 7H_2O$ 0.5 mg, H_3BO_3 0.50 mg, $CuSO_4 \cdot 5H_2O$ 25 μg, $Na_2MoO_4 \cdot 5H_2O$ 25 μg, $CoCl_2 \cdot 6H_2O$ 46 μg, tryptone 1 g, yeast extract 1 g, and distilled water 1,000 ml. The pure culture is routinely cultured on yeast extract-tryptone agar (contains 2 g · l^{-1} for each contents) at 60 °C and maintained on yeast extract-tryptone slant at 4 °C. Glycerol solutions (20 %, v/v) at −80 °C or lyophilization method are recommended for the long-term preservation.

Panacagrimonas perspica was originally isolated from soil in a ginseng field in Pocheon province in South Korea. The collected soil sample was well suspended with 50 mM phosphate buffer (pH 7.0) and spread on one-half-strength modified R2A agar. One-half-strength modified R2A agar contains per liter: tryptone 0.25 g, peptone 0.25 g, yeast extract 0.25 g, malt extract 0.125 g, beef extract 0.125 g, casamino acid 0.25 g, soytone 0.25 g, dextrose 0.5 g, soluble starch 0.3 g, xylan 0.2 g, sodium pyruvate 0.3 g, K_2HPO_4 0.3 g, $MgSO_4$ 0.05 g, $CaCl_2$ 0.05 g, and agar 15 g. After incubation, type strain of *Panacagrimonas perspica* was picked up and purified from the modified one-half-strength R2A. The pure culture is routinely cultured on R2A agar (Difco, USA) at 30 °C and maintained on R2A slant at 4 °C. Glycerol solutions (20 %, v/v) at −80 °C or lyophilization method are recommended for the long-term preservation.

Morphology

Colonies of *Solimonas soli* and *Solimonas flava* are circular, convex, and pale yellow-colored on LB agar or R2A agar and about 0.5–0.8 mm in diameter after 4–5-day cultivation at 30 °C. Colonies of the two species are ropy and not easily picked up with inoculation loops. Colonies for *Solimonas variicoloris* DSM 15731^T vary in color (yellow, orange, and brown) according to specific incubation time and cultural media; colonies on trypticase soy agar (TSA) are 1–3 mm in diameter and convex and are always surrounded by a flat, pale white rim, and the color is more intense in the center of the colony. Colonies of *Solimonas aquatica* LMG 24500^T are semiopaque, convex, circular, and smooth with entire edges, 1.2–1.5 mm in diameter on R2A agar after 4 days of incubation at 25 °C. Colonies of *Solimonas soli* and *Solimonas flava* are obviously smaller than that of *Solimonas variicoloris* and *Solimonas aquatica*, and the colony color of *Solimonas soli* and *Solimonas flava* is more consistent than other two *Solimonas* species. Soluble pigment is not produced by the members of genus *Solimonas*. After 2–4 days of cultivation on R2A at 45 °C, colonies of *Fontimonas thermophila* are approximately 1.0–1.5 mm in diameter, transparent, pale yellow, smooth, convex, and circular, and soluble pigment is not produced. *Panacagrimonas perspica* colonies grow on R2A agar plates for 3 days and are smooth, flat, white, and irregularly shaped with a diameter of 1–2 mm. Except for the species *Panacagrimonas perspica*, colonies are circular for all other present members of the family *Solimonadaceae*, and the motility of this group of bacteria is weak or nonmotile.

The cells of the *Solimonas flava* are characterized as nonmotile, and the type strain of *Solimonas soli* is motile by means of a single polar flagellum. Cells of the *Solimonas flava* are long rods (0.4–0.5 μm in width and 2.4–2.8 μm in length), but the cells of the *Solimonas soli* are rods (0.2–0.4 μm in width and 0.3–0.5 μm in length). Cells of *Solimonas variicoloris* are long rods, but the specific size range was not displayed in the original publication. Similar as type strain of *Solimonas soli*, cells of *Solimonas aquatica* also show motile ability by means of a single polar flagellum. Cells size of *Solimonas aquatica* is 0.3–0.8 mm in width and about 1.0 mm in length. Endospores are not produced, and pili or fimbriae are not observed for all members of the genus *Solimonas*. Cells of *Fontimonas thermophila* are non-spore forming, rod shaped, and motile. The cell size of *Fontimonas thermophila* is approximately 0.5–0.7 μm in width and 1.2–2.0 μm in length, and a single polar flagellum is detected, but pili or fimbriae are not discovered. Cells of *Panacagrimonas perspica* are also non-spore forming, rod shaped, and motile. The cell size of *Panacagrimonas perspica* is approximately 1.2–1.8 μm in width and 2.0–4.0 μm in length, and a single polar flagellum is detected, but pili or fimbriae are not discovered.

Physiology and Metabolism

Solimonas strains grow on aerobic or facultatively anaerobic conditions. The growth temperature range and the optimum temperature for *Solimonas* are mesothermal, and the pH ranges (optimum pH) are approximate to neutral. Except for the type strain of *Solimonas aquatica* (25 °C as the optimum temperature), 30 °C is usually considered as the optimum temperature. NaCl is not necessary for the growth, and *Solimonas* strains can grow well without NaCl, but the growth did not occur at the NaCl concentration higher than 2.0 %. A notable characteristic is that the *Solimonas* strains are a group of fastidious organisms, and only several organic substrates in the original researches were assimilated, but special requirements are not observed. *Fontimonas thermophila* is a strictly aerobic and thermophilus

bacterium. Unlike *Solimonas*, the growth temperature range (37–60 °C) and optimum temperature (45–50 °C) of the *Fontimonas thermophila* are relatively high. The type strain of *Fontimonas thermophila* is moderately sensitive to NaCl; growth is not observed when NaCl concentration is higher than 1.0 %. The pH range (6.5–8.5) and optimum pH (6.5–7.0) are approximate to neutral. Comparing with genera *Fontimonas* and *Solimonas*, *Panacagrimonas perspica* is strictly aerobic but moderately halotolerant bacterium. The growth temperature range (15–42 °C) and optimum temperature (30 °C) of the *Panacagrimonas perspica* are similar as *Solimonas*. However, the type strain of *Panacagrimonas perspica* can grow on medium at the NaCl concentration of 7.0 % (w/v), but growth is not observed at NaCl concentration of 8.0 %. The pH range (5.0–8.5) and optimum pH (6.5–7.0) are also approximate to neutral. Therefore, pH range and optimum pH for all members of family *Solimonadaceae* are approximate to neutral.

Some *Solimonas* species (or strains) show degradation ability to chemical pollutants (e.g., pesticides, chlorinated hydrocarbons, and alkane) and use these pollutants as the sole carbon and energy sources. *Solimonas flava* DSM 18980^T was isolated from multiple pesticide-polluted soil and showed atrazine degradation capability; the herbicide could be utilized as the sole carbon sources. Hexane could be utilized as the sole carbon source by type strain of *Solimonas variicoloris* which was isolated from an industrial biofilter. Although hexane is not the necessary growth factor for the type strain of *Solimonas variicoloris*, the type strain DSM 15731^T grows better when the culture medium is supplemented with hexane. Many *Solimonadaceae* phylogenetic neighbors also show the similar characteristics to the specific pollutants. For example, hydrocarbons are utilized by *Hydrocarboniphaga effuse* and *Hydrocarboniphaga daqingensis*; pentane, hexane, and decane are utilized by *Alkanibacter difficilis*; steroidal hormone is degraded by *Steroidobacter denitrificans*. However, the metabolism products of these pollutants have not been analyzed or purified.

Ecology

Members of the family *Solimonadaceae* are usually associated with soil (especially with polluted soil) and freshwater. Bacteria of *Solimonas* and *Fontimonas* are moderately sensitive to NaCl, and no strain of the two genera has been isolated from the saline soil or other saline environments. Quite a few of *Solimonadaceae* isolates and phylogenetic neighbors were obtained from polluted environments and associated with chemical pollutant degradation. Mesothermal and neutral conditions (excepted for *Fontimonas thermophila*) are the favorite habitat to the bacterial group of *Solimonas* and *Panacagrimonas*, whereas *Fontimonas thermophila* is thermophilic bacterium. Although *Panacagrimonas perspica* LGM 24239^T was not isolated from the saline environment, the type strain shows growth at the NaCl concentration up to 7.0 % (w/v), and *Panacagrimonas perspica* LGM 24239^T is the only strain that can grow under the NaCl concentration higher than 2.0 % (w/v).

The type strains of *Solimonas soli* and *Panacagrimonas perspica* are associated with agriculture field in which ginseng was growing, but little is known about the physical and chemical characteristics of the two soil samples. *Solimonas flava* is also isolated from farmland soil near a chemical factory. The farmland soil was multiple contaminated by herbicides and insecticides over a long period, and the insecticide atrazine can be degraded by type strain of the *Solimonas flava*. *Solimonas variicoloris* was isolated from a hexane-treated, full-scale biofilter for waste gas treatment, and hexane can be utilized by the type strain as the sole source of carbon and energy. Members of *Solimonadaceae* have also been isolated from freshwater. *Solimonas aquatica* was isolated from a freshwater spring, and *Fontimonas thermophila* was isolated from a freshwater of hot spring. Physical and chemical properties of the *Solimonas aquatica* habitat (freshwater spring) were not specified, and the physical conditions of the *Fontimonas thermophila* habitat (hot spring water) were determined at the temperature of 63.7 °C, pH of 6.7 with NaCl concentration of less than 10 mg $\cdot$ L^{-1}. The original thermophilic habitat of *Fontimonas thermophila* DSM 23609^T is the nomenclative basis for the type species of the genus *Fontimonas*.

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33 The Family *Succinivibrionaceae*

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Abstract
Succinivibrionaceae family encompasses two habitat-related groups of species. All species are Gram-negative, strictly anaerobic, non-spore-forming bacteria. They form a clearly separate line of descendent among *Gammaproteobacteria* members. The family is a phylogenetic neighbor of the family *Aeromonadaceae* being included in *Aeromonadales* order. The first group includes mainly ruminant inhabitant members from *Succinivibrio*, *Succinimonas*, and *Ruminobacter* genera. These species were usually found in ruminants. *Succinivibrio dextrinosolvens* and *Succinimonas amylolytica* are anaerobic and motile species found in animals with some grain in their diets. *Ruminobacter amylophilus* was initially classified as *Bacteroides amylophilus*, a starch-digesting bacterium from the bovine rumen. It can constitute up to 10 % of the bacterial populations of the rumen. On the other hand, the second group, the genera *Succinatimonas* and *Anaerobiospirillum*, is formed by species mostly isolated from mammalian feces. *Succinatimonas hippie* species was recently described and isolated from the human gastrointestinal tract. *Anaerobiospirillum* comprises two species: *A. succiniciproducens* and *A. thomasii*. In contrast to the rumen strains, these species were isolated from the throat and the colon of dogs (*A. succiniproducens*) and from diarrheal feces of humans and feces of cats and dogs (*A. thomasii*). *Anaerobiospirillum*-related bacteremia cases have clinical importance. Members of the family *Succinivibrionaceae* can ferment carbohydrates to succinate and acetate. Motile or not, they are chemo-organotrophic. Glucose and other carbohydrates are fermented with the production of succinate and acetate. Low amounts of formate and lactate may be produced. Nitrate is not reduced and catalase activity is negative. Fatty acid composition is well defined. The DNA G+C content varies between 39 mol% and 44 mol%. The type genus in *Succinivibrio*.

Taxonomy: Historical and Current

Suc.ci.ni.vib.ri.o.na' ce.ae M. L. masc. n. *Succinivibrio* type genus of the family; *-aceae* ending to denote a family; M. L. fem. pl. n. *Succinivibrionaceae* the *Succinivibrio* family.

The mol% G+C of the DNA ranges from 39 to 44.

Type genus: *Succinivibrio* (Bryant and Small 1956; Approved Lists 1980).

The family *Succinivibrionaceae* was created by Hippe et al (1999) based on 16S rRNA sequence analysis of Gram-negative, strictly anaerobic, non-spore-forming bacteria from the four different genera: *Anaerobiospirillum, Ruminobacter, Succinimonas*, and *Succinivibrio*. All four type species clustered with members of the *Gammaproteobacteria* class. Species of *Succinomonas* (*S. amylolytica*) and *Succinovibrio* (*S. dextrinosolvens*) formed a separate line of descendent with *Ruminobacter amylophilus* and *Anaerobiospirillum* species (*Fig. 33.1*). The 16S rRNA similarity values for the type species of the four genera range between 87.1 % and 93.6 %, the latter value obtained for the pair *A. succiniproducens* and *A. thomasii* (Hippe et al. 1999). Stackebrandt and Hespell (2006) reported that the phylogenetic distance between the type species of the family was 97.7–92.7 %. Hippe and coworkers suggested that *Anaerobiospirillum, Ruminobacter, Succinimonas*, and *Succinivibrio* genus were affiliated in the new family *Succinivibrionaceae* (Hippe et al. 1999). As members of the four genera are phylogenetically related to *Aeromonas* spp., the second edition of *Bergey's Manual of Systematic Bacteriology* affiliated this family in *Aeromonadales* order (Garrity et al. 2005; *Fig. 33.1*).

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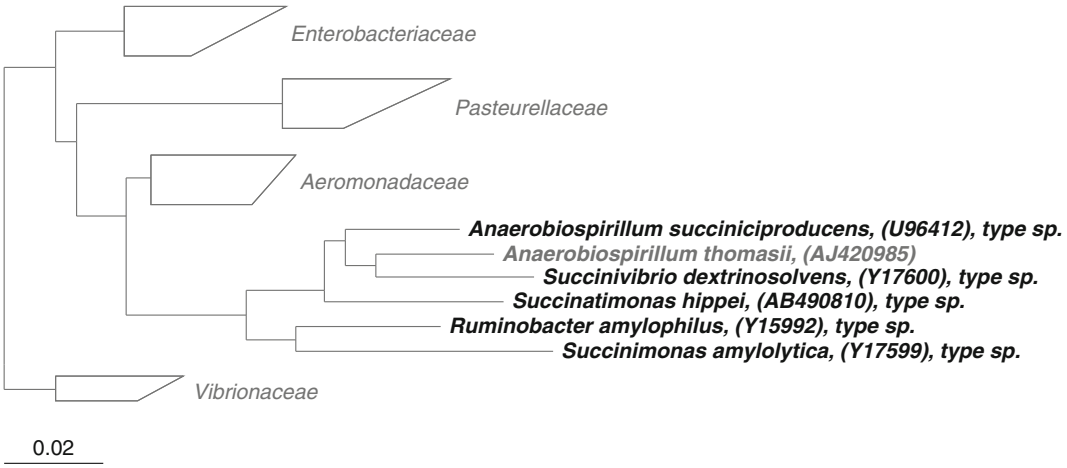

Fig. 33.1

Phylogenetic reconstruction of the family *Succinivibrionaceae* based on the 16S rRNA sequences. Tree was created using the Neighbor-Joining algorithm (Saitou and Nei 1987) with Jukes-Cantor correction (Jukes and Cantor 1969). Sequences were aligned according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high quality type strain sequences proportionally distributed among the different bacterial and archaeal phyla. Additionally, a 40 % maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. *Bold characters* highlight type species. Scale bar indicates estimated sequence divergence

The family is a phylogenetic neighbor of the family *Aeromonadaceae* and of a cluster consisting of the genera *Oceanisphaera, Oceanimonas* and *Ferrimonas* (Stackebrandt and Hespell 2006). All species described so far can be identified by their 16S rRNA gene sequence (Tee et al. 1998; Tajima et al. 2001; Misawa et al. 2002; De Cock et al. 2004; Li et al. 2012). Similarity of sequences between *Succinivibrionaceae* members and neighbor genera range between 84.6 % and 88.5 %. Species of this family can be described unambiguously by their 16S rRNA sequence (Tee et al. 1998; Misawa et al. 2002). Additional phylogeny analysis of the ATP/ADP-dependent phosphoenolpyruvate carboxykinase amino acid sequence indicated that *Anaerospirillum succiniproducens* branched with those of other members of *Gammaproteobacteria*, confirming the 16S rRNA-based phylogeny (Laivenieks et al. 1997). *Succinivibrio dextrinosolvens* (Bryant and Small 1956) and *Succinimonas amylolytica* (Bryant et al. 1958) are anaerobic and motile species that inhabit the rumen of sheep and cattle. Both species were originally affiliated as *Bacteroidaceae* family too, as the type species of the *Anaerobiospirillum*, *A. succiniproducens* (Davis et al. 1976). The genus *Anaerobiospirillum* comprises two species: *A. succiniciproducens* and *A. thomasii* (Malnick 1997). In contrast to the rumen strains, the *Anaerobiospirillum* species were isolated from the throat and the colon of dogs (*A. succiniproducens*) and from diarrheal feces of humans and feces of cats and dogs (*A. thomasii*) (Malnick 1997). Other studies have shown that both species are found as part of the fecal microbiota in cats and dogs (Davis et al. 1976; Malnick et al. 1990; Rossi et al. 2008).

Ruminobacter amylophilus was initially classified as *Bacteroides amylophilus*, a starch-digesting bacterium from the bovine rumen (Hamlin and Hungate 1956), which was included in *Bacteroidaceae* family. *Bacteroides amylophilus* was different from *Bacteroides* spp. by fatty acid composition and absence of sphingophospholipids (Miyagawa et al. 1979; Shah and Collins 1983). On the basis of phenotypic and metabolic conditions, *Ruminobacter* can be distinguished from members of *Bacteroides* and many other Gram-negative genera (Holdeman et al. 1986). Comparative analysis of 16S rRNA sequence revealed that *B. amylophilus* was from *Gammaproteobacteria* class while authentic *Bacteroides* formed a coherent cluster. In 1986, Stackebrandt and Hippe suggested the transference of *B. amylophilus* to a new genus *Ruminobacter*, as *Ruminobacter amylophilus* comb. nov, what was validated in 1987. Phylogenetic analyses linked R. amylophilus to Succinivibrionaceae group (Stackebrandt and Hippe 1986; Stackebrandt 2005).

Recently, during the course of several intensive cultivation trials aimed at isolating so-called unculturable or as-yet-uncultured bacteria from the human gastrointestinal tract (Sakon et al. 2008; Morotomi et al. 2008, 2009; Nagai et al. 2009), a novel member of the family *Succinivibrionaceae* was isolated from the feces of a healthy Japanese adult. 16S rRNA sequence analysis showed that strain YIT 12066T was most closely related to members of the family *Succinivibrionaceae* (*Fig. 33.1*), with sequence similarity of 92–87 %. The DNA G+C content was 40.3 mol%. However, some phenotypic characteristics such as cellular morphology and the major fatty acid profile of strain YIT 12066T were markedly different from those of other members of the

family. It was suggested that strain YIT 12066T represents a novel species in a new genus, *Succinatimonas*, for which the name *Succinatimonas hippie* gen. nov., sp. nov. was proposed (Morotomi et al. 2010).

Currently, this family includes five genera: *Succinivibrio*, *Succinimonas, Ruminobacter*, and *Succinatimonas* are monospecific and the type species are *Succinivibrio dextrinosolvens, Succinimonas amylolytica, Ruminobacter amylophilus, a*nd *Succinatimonas hippie*, respectively (*Fig. 33.1*). The genus *Anaerobiospirillum* currently contains two well-described species: *A. succiniciproducens* and *A. thomasii*. During the last decade, new *Anaerobiospirillum sp.* strains were isolated from cats and dogs (Misawa et al. 2002; de Cock et al. 2004). Culture-independent studies reported several 16S rDNA sequences closely related to *Ruminobacter amylophilus* and *Succinivibrio dextrinosolvens* species in porcine colon microbiota (Li et al. 2012) and ruminal samples of cattle (Wise et al. 1999; Tajima et al. 2001).

Habitat and Ecology

The complex rumen microbiome plays essential roles in digesting feeds and supplying nutrients to host animals. To positively affect rumen functions, dietary interventions have been attempted to modulate this microbiome (Calsamiglia et al. 2007). End-products of the overall rumen fermentation are the result of the metabolic activity of the rumen inhabitants and are important to the health and productivity of the animal. The importance of the activity of *Succinivibrionaceae* bacteria in the rumen can be inferred from its large numbers under certain circumstances and from knowledge of the roles of its end-products (acetate, succinate, formate, and lactate). Acetate is readily absorbed from the rumen and subsequently used in fatty acid metabolism. Succinate and formate, on the other hand, are considered intermediates of rumen fermentation which are further metabolized by other organisms. Succinate is thought to be an important intermediate for propionate formation (Russell and Hespell 1981; Scheifinger and Wolin 1973), with the latter being readily absorbed from the rumen for gluconeogenesis. Formate has been shown to be metabolized to methane by rumen methanogens (Hungate et al. 1970; Lovley et al. 1984). Lactate is also an intermediate of the overall rumen fermentation and, when present in excess, has been correlated with acidosis (Russell et al. 1979).

Species of *Succinivibrio*, *Succinimonas*, and *Ruminobacter* genera are inhabitants of ruminant animals. They colonize their hosts soon after host organisms are born. Their population numbers are sensitive to changes in diet, age, and environment. *Succinivibrio dextrinosolvens* and *Succinomonas amylolytica* are often isolated from rumen of sheep and cattle in which they play an ecologically important role as starch digests, producing acetic and succinic acids as the main fermentation end-products from carbohydrates (Hespell 1992). *Succinimonas amylolytica* was a large portion of total ruminal bacterial when starch in the form of a grain mixture was present in the diet (Bryant et al. 1958). *Succinivibrio dextrinosolvens* is often the predominant isolate from the rumen when the diet of the animal is high in starch and with other diets containing large amounts of fast-fermentable carbohydrates (Bryant and Small 1956; Bryant et al. 1958; Bryant 1970; Wozny et al. 1977). Moreover, *Succinivibrio dextrinosolvens*-related sequences were detected in a study about Proteobacteria population in rumen fluid from ruminally fistulated Holstein cow, with the use of 16S rDNA sequence–specific primers (Wise et al. 1999).

The occurrence of *Succinovibrio* and *Succinimonas* species in habitat other than the rumen or gastrointestinal tract from animal was not well documented. Still, *Succinivibrio* strains could be isolated from human blood suffering bacteremia (Southern 1975; Porschen and Chan 1977). In all cases, patients had gastrointestinal disorders and thus loss of its integrity might have permitted these bacteria to enter into blood, which suggests a low abundance presence of *Succinivibrio* in the colon of humans (Stackebrandt and Hespell 2006).

Li et al (2012) described the occurrence of *Succinivibrio* in porcine colon using 16S rRNA gene (*rrs*)-based and whole-genome shotgun (WGS) sequencing. They studied the community shift in porcine colon microbiota induced by a gastrointestinal nematode infection. The relative abundance of *Succinivibrio*-16S rDNA sequences decreased from 3.6 % in control pigs to 0.4 % in infected pigs. Changes in relative abundances of *Succinivibrio* and *Mucispirillum*, for example, may relate to alterations in carbohydrate metabolism and niche disruptions in mucosal interfaces induced by parasitic infection, respectively (Li et al. 2012). A study about the impact of feed efficiency and diet on adaptive variations in the bacterial community in the rumen fluid of cattle identified three phylotypes belonging to the genera *Robinsoniella*, *Eubacterium*, and *Succinivibrio* sp. which were associated to residual feed intake (RFI) (Hernandez-Sanabria et al. 2011).

Ruminobacter amylophilus occurs occasionally in rumen contents of cattle and could be found in ovine rumen too. When present in bovine rumen, they may be the predominant starch digester and correspond to 10 % of the total bacterial population. Since its first cultivation from the bovine rumen, as *Bacterioidetes amylophilus*, several similar lineages have been isolated frequently from rumen (Hamlin and Hungate 1956; Blackburn and Hobson 1962; Bryant and Robinson 1961; Caldwell et al. 1969; Holdeman et al. 1977). Population dynamism of *Ruminobacter amylophilus* and *Succinivibrio dextrinosolvens* in rumen fluid was analyzed by the use of real-time PCR. Both species were detected by 16S rDNA sequence–specific primer. Both species populations were high before and 3 days after the changes in the diet of cows from hay to grain. Cell numbers decreased significantly only after 28 days (Tajima et al. 2001). Stiverson and coworkers (2011) assessed the importance of select cultured and uncultured bacteria in the rumen and the effect of diets and ruminal fractions. *Ruminobacter amylophilus* population was quantified in fractionated ruminal samples from sheep fed with hay alone or hay plus corn, using specific quantitative PCR (qPCR). *R. amylophilus* was found in relatively high abundance in the adherent fraction of

the sheep fed with the hay/corn diet, corresponding to its ability to utilize starch (Stiverson et al. 2011).

Members of *Anaerobiospirillum* genus have been isolated from the throat and feces of health dogs and cats (Davis et al. 1976; Malnick et al. 1990; Malnick 1997), and diarrheal feces (Malnick et al. 1983; Misawa et al. 2002) and blood (Malnick et al. 1989; Rifkin and Opdyke 1981; Yuen et al. 1989; Tee et al. 1998) of humans. Both species are found as part of the fecal microbiota in cats and dogs (Davis et al. 1976; Malnick et al. 1990; Rossi et al. 2008). Although *A. thomasii* comprises human and animal strains isolated during routine diagnostic examination of blood and fecal samples, only *A. succiniciproducens* strains had been associated with septicemia in humans (Malnick 1997).

Clinical Relevance

Many strains of the *Anaerobiospirillum* genus were reported as associated to gastroenteritis cases in cats and dogs (Malnick et al. 1990; Malnick 1997; Misawa et al. 2002) and diarrheal feces of humans, and related to septicemia in humans (Malnick et al. 1983, 1989; Rifkin and Opdyke 1981; Yuen et al. 1989; Malnick 1997; Tee et al. 1998). Bacteremia in humans appears to be found almost exclusively in subjects with underlying disease, and immunosuppression is a risk factor (McNeil et al. 1987). *Anaerobiospirillum succiniciproducens* has been reported as a rare cause of bacteremia, particularly in immunocompromised hosts (Tee et al. 1998; Pienaar et al. 2003; Fadzilah et al. 2009), and diarrheal illness (Malnick et al. 1990). As gastrointestinal symptoms are the most common accompanying symptoms of bacteremia, the gastrointestinal tract is believed to be the portal of entry. Most patients with *A. succiniciproducens* bloodstream infection have underlying disorders such as alcoholism, malignancy, atherosclerosis, surgery, diabetes mellitus, and poor dentition (McNeil et al. 1987). Strains of this species were isolated from blood of AIDS patient in Australia and Europe. It was also rarely associated with bacteremia in USA, Europe, Africa, and Asia, suggesting a global distribution (McNeil et al. 1987; Goddard et al. 1998; Pienaar et al. 2003).

An uncommon infection of *Anaerobiospirillum* was described in a 68-year-old man with follicular lymphoma presenting with fever and chills (Sarvepalli et al. 2012). Identification of *Anaerobiospirillum succiniciproducens* was based on 16S rRNA sequencing. It was resistant to clindamycin and metronidazole, which are among the most commonly prescribed agents for anaerobic infections, and optimal therapy has not been established. *A. succiniciproducens* was reported to be susceptible to chloramphenicol, cephalosporins, fluoroquinolones, carbapenems, and penicillin/β-lactamase combinations (Kelesidis et al. 2010; Kelesidis 2011). Kelesidis and coworkers (2010) described the first case of bloodstream infection (BSI) due to *A. succiniciproducens* in an asymptomatic elderly male with poor dentition that was treated with levofloxacin.

Other studies have found that *A. succiniciproducens* is often resistant to metronidazole and erythromycin (McNeil et al. 1987; Yuen et al. 1989; Tee et al. 1998; Pienaar et al. 2003), antibiotics that are empirically used for suspected infections with anaerobes and *Campylobacter* species, respectively. Thus, it is important for diagnostic laboratories to identify *A. succiniciproducens*, and especially to distinguish it from *Campylobacter* species, which have a similar Gram stain and colony appearance, but are oxidase- and catalase-positive. Isolation on Skirrow media is not specific for *Campylobacter* species, as some strains of *A. succiniciproducens* may grow (Malnick et al. 1990). Commercial identification systems using biochemical tests may help, and 16S rRNA sequencing is definitive.

Misawa and coworkers (2002) reported a case of bloody diarrhea in a puppy from which an *Anaerobiospirillum* species was isolated in combination with other species of spiral bacteria. The presence of *Anaerobiospirillum sp.* in ileum of cats was demonstrated on the basis of ultrastructural morphology of spiral bacteria associated with intestinal lesions and PCR amplification of a genus-specific 16S rRNA gene from affected tissues from each cat (De Cock et al. 2004). Comparative sequence analysis of partial 16S rRNA gene sequences obtained from these samples revealed that identified *Anaerobiospirillum spp.* were different from the previously cultured canine reference.

Reports of *Succinivibrio* species and *Succinomonas* spp. causing bacteremia was rare (Southern 1975; Porschen and Chan 1977; Johnson and Finegold 1987). The clinical significance of the organism remains unclear.

Phenotypic and Metabolic Analyses

The Properties of the Genera and Species of *Succinivibrionaceae*.

Members of family *Succinivibrionaceae* are Gram-negative short, or oval to long, or curved to helical, rods, non-spore-forming bacteria that ferment carbohydrates to succinate and acetate (Garrity et al. 2005). Motile (*Succinivibrio, Anaerobiospirillum* and *Succinimonas*) or nonmotile (*Ruminobacter* and *Succinatimonas*), they are chemo-organotrophic and strictly anaerobic. Glucose and other carbohydrates are fermented with the production of succinate and acetate; low amount of formate and lactate may be produced. CO_2 uptake is positive, but gas is not produced. Catalase-negative. Nitrate is not reduced. Major fatty acids are saturated (35–66 %) and unsaturated (19–59 %) straight-chained, even-numbered fatty acids, and 16:0 and 18:0 3-OH fatty acids (4–11 %); iso- and anteiso-branched fatty acids, cyclopropane fatty acids and odd-numbered fatty acids are absent (Moore et al 1994; Hippe et al. 1999; Morotomi et al. 2010). ❯ *Table 33.1* shows the main discriminating properties from members of the family *Succinivibrionaceae*.

Succinivibrio

It includes anaerobic, non-spore-forming, gram-negative, curved rods (usually 0.3–0.5 × 2–4 μm) with monotrichous

Table 33.1
Major discerning characteristics of members of the family *Succinivibrionaceae*

Characteristic	*Succinivibrio dextrinosolvens*	*Succinimonas amylolytica*	*A. succiniproducens*	*A. thomasii*	*R. amylophilus*	*Succinatimonas hippei*
Isolation source	Rumen of cattle and sheep, a few cases of human bacteremia	Rumen of cattle and sheep	Human clinical samples, feces of cats and dogs	Feces of cats and dogs, diarrheal feces of humans	Rumen of cattle	Human feces
Morphology	Curved rods helically twisted, single polar flagellum	Short, straight rods to coccobacilli, single polar flagellum	Helical rods with bipolar tufts of flagella	Helical rods with bipolar tufts of flagella	Oval to long rods, without flagella	Straight rods without flagella
Motility	+	+	+	+	–	–
Oxygen sensitivity	Strictly anaerobic	Strictly anaerobic	Microaerobic	Microaerobic	Strictly anaerobic	Strictly anaerobic
DNA G+C content (mol%)	NA	NA	44[a]	39–42	40–42	40.3
Major fermentation end-products	Succinate, acetate, and formate[b]	Succinate, acetate	Succinate, acetate	Succinate, acetate	Succinate, acetate, and formate[b]	Succinate, acetate
Fermentation of carbohydrates	ND	ND	Fructose, glucose, lactose, maltose, sucrose, raffinose, inulin, β-D-galactoside, and α-D-glucoside	Adonitol, galactose, glucose, and maltose	Dextrin, glycogen, maltose, and starch	Glucose, D-xylose
Negative for	Catalase; nitrate reduction; production of indole, acetoin, or hydrogen sulfide	ND	Catalase; oxidase and nitrate reduction; gelatin processing; production of indole; meat digestion; hydrolyze esculin, hippurate, or urea; lipase activity	Catalase; oxidase and nitrate reduction; hydrolysis of esculin, hippurate, or urea	Cellulose milk and meat digestion	Catalase, oxidase, urease, indole production, nitrate reduction, and hydrolysis of aesculin and gelatin

Data retrieved from Bryant and Small (1956), Bryant et al. (1958), Davis et al. (1976), Malnick (1997), Stackebrandt and Hippe (1986), Stackebrandt and Hespell (2006), Morotomi et al. (2010)
[a]Data for the type strain
[b]Lactate is sometimes formed

polar flagellation. *Succinovibrio dextrinosolvens* (type strain is ATCC 19716 = DSM 3072) cells are motile and appear singly or in pairs. Motility is translational with a vibrating movement. Newly isolated strains commonly form helical or twisted filaments of 2–4 coils composed of cells (Stackebrandt and Hespell 2006). *Succinivibrio* strains ferment glucose with the production of large amounts of acetic and succinic acid (Bryant and Small 1956; Scardovi 1963).

Growth occurs at 30–40 °C but not at 22 °C or 45 °C. The addition of CO_2 or bicarbonate to the growth medium allows better growth. *Succinivibrio dextrinosolvens* produce succinic, acetic, and formic acids in a rumen fluid-glucose medium. It ferments glucose, D-xylose, L-arabinose, maltose, galactose, fructose, sucrose, mannitol, dextrin, and pectin. It is negative to photolytic activities, nitrate reduction, catalase, and production of indole, acetoin, and hydrogen sulfide (Bryant and Small 1956). Enzymatic activities of the Embden-Meyerhof pathway are present in cell-free extracts but enzymes of the hexose monophosphate pathway are absent (Scardovi 1963; O'Herrin and Kenealy 1993). Pathways for ammonia assimilation and regulation were described in *S. dextrinosolvens* by Patterson and Hespell 1985.

Succinimonas

It includes Gram-negative, anaerobic strains from a single species that break down starch. Cells are motile with a smooth rod shape or coccobacillus (usually 1–1.5 × 1.5–3 μm). They appear singly or in pairs but can form clumps in older cultures. In wet-mount preparations, most cells show some translational motility degree, which stops when oxygen is present.

The flagellar organization is polar or monotrichous. *Succinomonas amylolytica* (the type strain is ATCC 19206 = DSM 2873; Bryant et al. 1958) can ferment carbohydrates and produce high levels of succinic acid. In addition to succinic acid, a small production of acetic acid was found with large uptake of carbon dioxide. Small amounts of acetoin and/or propionate can be formed, but not hydrogen gas, formate, lactate, or ethanol. It has a limited range of fermentable substrates, only glucose, maltose, dextrin, and starch. Temperature of growth ranges between 30 °C and 39 °C but no growth occurs at 22 °C or 45 °C (Bryant 2005).

Ruminobacter

Ruminobacter amylophilus (ATCC 29744 = DSM 1361 as the type strain) are Gram-negative, nonmotile cells. They are larger (0.9–1.2 × 1–8 μm) than other *Succinivibrionaceae* species. The shape of the cells is oval to long rods with tapered or round ends and tend to form swollen and irregular curved form (Hamlin and Hungate 1956; Cato et al. 1978). *R. amylophilus* produces acetate, formate, and succinate as major fermentation products, and trace amounts of lactate and ethanol can be formed. It is not able to grow on glucose (Hamlin and Hungate 1956), but ferments a variety of carbohydrates. This species differs phenotypically slightly from the other two starch-fermenting species (*S. dextrinosolvens* and *S. amylolytica*) in morphology and some physiological properties (Hespell 1992; Malnick 1997; *Table 33.1*). Rumen fluid is not required for growth, but ammonia is essential as nitrogen. Growth occurs near 37 °C. *Ruminobacter amylophilus* can fix carbon dioxide and assimilate ammonia (Bryant and Robinson 1961).

Anaerobiospirillum

The genus *Anaerobiospirillum* currently contains two species: *Anaerobiospirillum succiniciproducens* (type strain is ATCC 29305 [= CCUG 21558 = CCUG 24194 = DSM 6400 = LMG 7826 = NCTC 11536]) and *Anaerobiospirillum thomasii* (strain A273/88 [= ATCC 700432 = CCUG 46380 = DSM 11806 = NCTC 12467] as the type strain). Cells of these species are non-endospore-forming helical rods with rounded ends (usually 0.6–0.8 μm × 3–15 μm) that are motile by bipolar tufts of flagella (Malnick 1997). Cells generally occur singly and can reach up to 32-μm in length (*A. thomasii*). Both species are inhabitants of the fecal microbiota in cats and dogs (Davis et al. 1976; Malnick et al. 1990; Rossi et al. 2008) and can be isolated from diarrheal feces (Malnick 1997) or rarely from blood of humans (McNeil et al. 1987; Goddard et al. 1998; Pienaar et al. 2003; Kelesidis et al. 2010; Kelesidis 2011). They are defined as strictly anaerobic. In contrast, the phylogenetically most closely related *Anaerobiospirillum* species have been isolated under microaerobic conditions (Malnick et al. 1990; Rossi et al. 2008). Members of this genus are catalase- and oxidase-negative and do neither hydrolyze esculin, hippurate, or urea, nor reduce nitrate. They ferment carbohydrate and form succinic and acetic acids as major products of glucose fermentation. Traces of lactic and formic acids may also be formed. Temperature of growth ranges from 33 °C to 43 °C, with an optimum of 37 °C. Hemolytic activity is not present.

Succinatimonas

The type species of this monospecific genus is *Succinatimonas hippei*. The type strain is YIT 12066T (=DSM 22608T = JCM 16073T), isolated from human feces. *Succinatimonas hippei* isolate YIT 12066T are Gram-negative, strictly anaerobic, non-spore-forming, nonmotile rods (0.5–1.4 × 1.4–4.8 μm). Growth was observed under strictly anaerobic conditions, but not under aerobic or microaerobic conditions. Under anaerobic conditions, growth of the isolate was most rapid at 35–40 °C, weak at 33 °C, scanty at 43 °C, and did not occur below 30 °C or above 45 °C. Similar growth temperature ranges have been observed for other species of the *Succinivibrionaceae*. No helical structures or flagella were observed by electron microscopy (Morotomi et al. 2010).

Acids were produced from glucose, maltose, and D-xylose. The major end-products of glucose fermentation in PYG broth were succinate (16.4 mM) and acetate (11.6 mM). Small amounts of formate and lactate were also detected. The isolate was negative for catalase, oxidase, urease, indole production; nitrate reduction; and hydrolysis of aesculin and gelatin. *Succinatimonas hippei* isolate was not resistant to 20 % bile and non-hemolytic. The major fatty acid components (>10 % of total fatty acids) were $C_{14:0}$ (31.2 %), $C_{18:1}\omega7c$ (18.2 %), $C_{18:1}$ ω 9c (14.1 %), $C_{16:1}\omega7c$ (13.7 %), and $C_{16:0}$ (13.0 %). Small amounts of $C_{14:0}$ 3-OH (4.5 %) and $C_{15:0}$ (1.0 %) were also detected (Morotomi et al. 2010). The fatty acid profile was distinct from those members of other genera of the family *Succinivibrionaceae*. In particular, two of the major cellular fatty acids in *S. hippie* strain [$C_{14:0}$(31.2 %) and $C_{18:1}\omega9c$ (14.1 %)] were present only in small amounts in *Anaerobiospirillum succiniciproducens* (3 % and 2 %, respectively; Moore et al. 1994). Metabolic properties were determined by using the commercially available API test systems (API Rapid ID 32A and API ZYM) and compared to type species from other genera of the *Succinivibrionacea* family (*Table 33.1*; Morotomi et al. 2010).

Metagenome Data

16S rRNA gene-based analysis and whole-genome shotgun (WGS) sequencing of porcine colon microbiota in response to *Trichuris suis* (whipworm) infection described the occurrence of *Succinivibrio* and *Ruminobacter* sequences. The 10 most abundant genera accounted for about 90 % of all 16S sequences that were positively assigned to any genus in control pigs. *Prevotella* was the most abundant with 62.3 % and 68.2 % of 16S sequences

assigned in control and infected pigs, respectively. The second most abundant genus was *Oscillibacter* (7.8 % in control pigs), followed by *Treponema* (7.5 %), *Succinivibrio* (3.6 %), *Anaerovibrio* (2.2 %), and *Roseburia* (1.9 %) (Li et al 2012).

Metagenome analysis of foregut microbiome of the Tammar wallaby revealed several taxonomic units (OTUs) within the family *Succinivibrionaceae*. They have isolated a dominant bacterial species from the wallaby microbiota from *Succinivibrionaceae* family that was implicated in lower methane emissions from starch-containing diets (Pope et al. 2011).

Taxonomic evaluation showed the microbiome is mainly composed of bacteria from phyla Firmicutes, Bacteroidetes, and Proteobacteria. Approximately 77 % of the Proteobacteria sequences (9 % of all sequences recovered in the 16S rRNA clone library) were assigned to just two deeply branched OTUs within the family *Succinivibrionacceae* (referred to Whallaby Group 1—WG1). The closest cultured relatives of WG1 include *Succinivibrio*, *Ruminobacter*, and *Anaerobiospirillum spp.*, although WG-1 does not share more than 93 % sequence identity to the 16S rRNA genes of the described species. The isolation of this lineage (WG-1) was achieved by using a partial metabolism reconstruction from binned metagenomic data (nitrogen and carbohydrate utilization pathways and antibiotic resistance). Cultivation-based strategies were stabilized to cultivate axenic WG-1 cultures. Pure-culture studies confirm that the bacterium is capnophilic and produces succinate, further explaining a microbiological basis for lower methane emissions from macropodids (Pope et al. 2011).

Genome Sequences

The project of Tammar wallaby foregut microbiome described the genome composition of the dominant bacterial species WG-1 (Pope et al. 2011). The partial genome of WG-1 (WG-1 M), which includes 2 Mb, was analyzed using IMG/M (Markowitz et al. 2008), KEGG (Kanehisa et al. 2008), and RAST (Aziz et al. 2008) databases for partial metabolic reconstruction and prediction of some of its functional capabilities (*Fig. 33.2*). WG-1 M was predicted to use starch as carbon source and urease gene cluster (transport and catabolism) was found. Three genes encoding putative GH13 alpha-amylase genes, as well as genes encoding putative glucose and maltose transporters, were identified. Additionally genes encoding a phosphoenolpyruvate (PEP) carboxykinase, pyruvate formate lyase (PFL), and acetate kinase (AK) were found (*Fig. 33.2*). The affiliation with *Succinivibrionaceae* family was confirmed by hallmarks on tricarboxylic acid cycle (TCA) genes. Based on founds, Pope and employees suggested that WG-1 would be similar to *Anaerobiospirillum succiniproducens* in that bacteria employs an anaplerotic reaction to produce oxaloacetate from PEP, with the subsequent reduction of oxaloacetate to succinate as a principal fermentation end-product (Samuelov et al. 1999; Pope et al. 2011). Partial metabolic reconstruction was used to develop a strategy for WG-1 lineage isolation from Tammar wallaby digestive samples and cultivation. The genome of WG-1 was sequenced, resulting in 43 scaffolds comprising 2.9 Mb with 2,403 putative ORFs. They concluded that although the abundance of WG-1 was variable in samples collected in winter and spring, their results showed that these bacteria would be numerically dominant when the plane of nutrition was rich in starches and soluble sugars. An environment favoring large number of WG-1 would not only contribute to substrate oxidations and reductions remaining closely coupled, with little methane being formed, it would also ensure that more digestible energy is available to the host animal for nutrition (Pope et al. 2011).

The *Succinatimonas hippei* type strain YIT 12066 genome was completely sequenced as a reference genome for the Human Microbiome Project (http://commonfund.nih.gov/hmp/overview.aspx) established by the National Institute of Health (NIH; http://www.nih.gov/). It was submitted in 2011 by the Genome Sequencing Center (GSC) of Washington University School of medicine (http://genome.wustl.edu/). The project accession number is AEVO00000000 and the first assembly version (ASM18819v1) has the accession number AEVO01000000, and consists of sequences AEVO01000001-AEVO01000169. *Succinatimonas hippie* genome includes 2.3 Mb allocated in 169 scaffolds (141 disposable yet): 2,224 genes and 2,169 proteins were annotated. Actually, 3 rRNAs and 48 tRNAs were detected in the genome (Weinstock et al. 2011).

The whole-genome shotgun projects of *Succinimonas amylolytica* (DSM 2873 type strain) and *Anaerobiospirillum succiniciproducens* (DSM 6400 type strain) were submitted in 2013 by the DOE Joint Genome Institute consortium. These projects are part of the GEBA (Genomic Encyclopedia of Bacteria and Archaea) initiative to sequence genomes that fill phylogenetic gaps, generating reference genomes for every major and minor group of bacteria and archaea. Currently sequenced genomes have provided highly uneven coverage of the type strain material present in public culture collections. The Microbial Earth Project is an initiative of the Microbial Genomics Program of the DOE Joint Genome Institute that focuses on 1,000 Type strains from the DSMZ and ATCC repositories (http://www.jgi.doe.gov/). Actually, the accession number of *A. succiniciproducens* genome project is PRJNA188817 but no data is available.

The accession number of *S. amylolytica* genome is ARKF00000000 (GI:481756082; PRJNA182396) and the first assembly version (ASM37840v1) of the project has the accession number ARKF01000000.1. It consists of sequences ARKF01000001-ARKF01000152 and includes 23 disposable scaffolds. This genome currently comprises 3.6 Mb with a GC content of 48 %. No protein annotation was found (Eisen et al. 2013).

Applications

Succinic acid is a dicarboxylic acid produced as an intermediate of the tricarboxylic acid cycle and also as one of the fermentation products of anaerobic metabolism (Zeikus 1980). Succinic acid

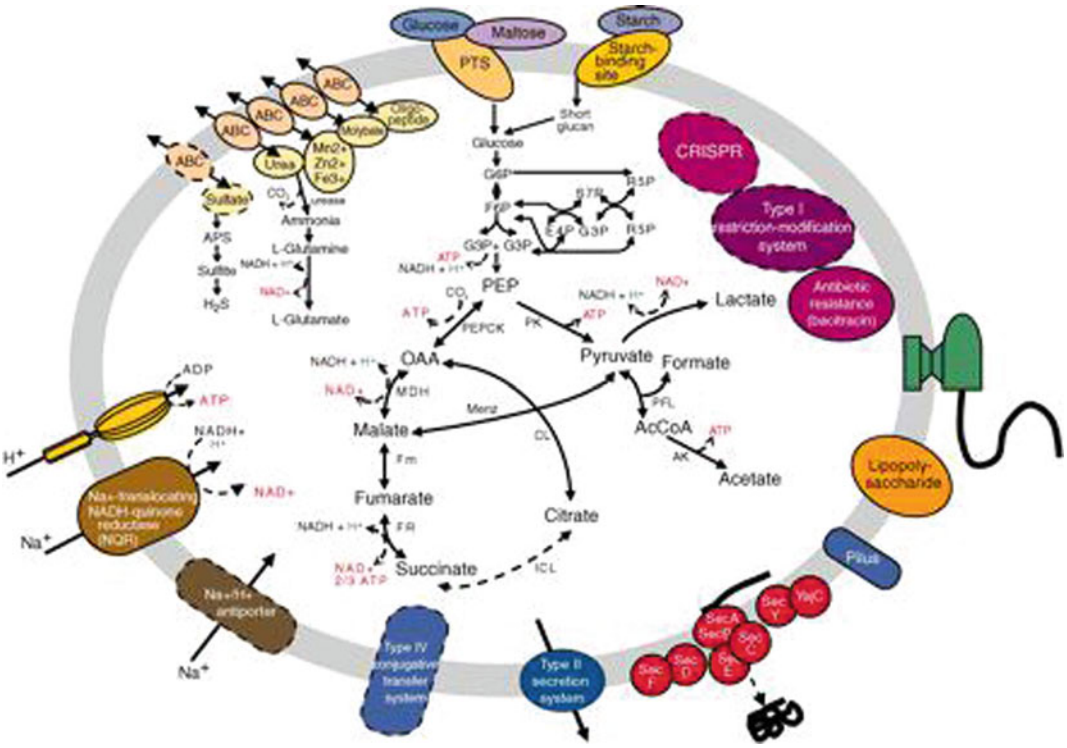

Fig. 33.2

Pope et al. 2011. Selected metabolic features of WG-1 phylogroup as inferred from genome comparisons. The assumption that phosphoenolpyruvate (*PEP*) serves as the branch point in WG-1 to the formate-, acetate-, and lactate-producing C_3 pathway and the succinate-producing C_4 pathway is based on data from McKinlay et al (2007). Broken border lines indicate annotations identified only in the WG-1 isolate genome sequence. Abbreviations: *AcCoA* acetyl-coenzyme A, *AK* acetate kinase, *APS* adenylylsulfate, *CL* citrate lyase, *E4P* erythrose-4-phosphate, *F6P* fructose-6-phosphate; *Fm* fumarase, *FM* fumarase reductase, *G3P* glyceraldehyde-3-phosphate, *G6P* glucose-6-phosphate, *ICL* isocitrate lyase, *MDH* malate dehydrogenase, *Menz* malic enzymes, *OAA* oxaloacetate, *PEPCK* PEP carboxykinase, *PFL* pyruvate formate lyase, *PK* pyruvate kinase, *PTS* phosphotransferase system, *S7P* sedoheptulose-7-phosphate

has recently emerged as an important chemical because it can be used for the manufacturing of synthetic resins and biodegradable polymers and as an intermediate for the synthesis of various chemicals (McKinlay et al. 2007). Among the succinic acid–producing microorganisms, the strict anaerobic bacterium *A. succiniciproducens* has been considered one of the most attractive succinic acid producers because it is able to utilize several renewable resources such as whey (lactose) (Lee et al. 2000), glycerol (Lee et al. 2001), wood hydrolysates (Lee et al. 2003), and galactose (Lee et al. 2008). Between 60 % and 90 % succinate yield was achieved depending on the production process. During continuous fermentation of glucose by *A. succiniciproducens*, succinic and lactic acid formation was found to strongly depend on the level of CO_2 (Samuelov et al. 1991). Under conditions of excess CO_2, more than 90 % of the whey lactose was consumed and the product ratio of succinate to acetate was 4:1. Lactate was the main end-product when no excess CO_2 was provided (Samuelov et al. 1999). Higher amounts of succinate were obtained (93–95 %) when yeast extract, polypeptones, and glucose were added to whey and the ratio of succinic acid to acetic acid was increased to 5.1:1 (Lee et al. 2000). When glycerol was the carbon source, the yield of succinic acid reached 133 % (succinic acid to acetic acid ratio of 25.8:1). In the presence of yeast extract, the yield reached 160 % (succinic acid to acetic acid ratio of 31.7:1) (Lee et al. 2001). When grown on minimal salts medium, wood hydrolysates, and corn step liquor, the succinate yield was 88 % glucose (Lee et al. 2003). Lee and coworkers studied the cell growth kinetics and succinic and acetic acid formation by *A. succiniciproducens* during continuous culture to determine the optimal operation conditions for the continuous production of succinic acid with minimal formation of acetic acid as a by-product. The formation of acetic acid as a by-product needs to be examined as well, because it affects the process of separating succinic acid from culture media (Lee et al. 1999). They showed that the production of succinic and acetic acids is growth-associated and is enhanced by increasing the glucose concentration in the feed medium without additional by-product formation. The higher maintenance requirement and lower biomass yield relative to other anaerobic bacteria are unique to *A. succiniciproducens*. The maximum productivities of succinic acid obtained were 5.5 g/l/h and 6.1 g/l/h at 19 g/l and 38 g/l of glucose, respectively. Although these results will be important in the design of novel bioprocesses for succinic acid production by *A. succiniciproducens*, further studies will be required to increase the acid-tolerance of this system or to reduce acetic acid formation (Lee et al. 2009).

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34 The Family *Thermithiobacillaceae*

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Abstract
The *Thermithiobacillaceae* comprise the second family of the *Acidithiobacillales* and are currently represented by one genus and its type species, *Thermithiobacillus tepidarius*. Unlike the *Acidithiobacillaceae, T. tepidarius* is neutrophilic and is also distinguished from the *Acidithiobacillus* species by its 16S rRNA gene sequence. It is an obligately chemolithotrophic moderate thermophile, oxidizing inorganic sulfur compounds for energy, and growing at 20–52 °C, with an optimum of 43–45 °C, at pH 6.0–7.5. Two other putative *Thermithiobacillus* strains are known, *T. tepidarius* strain Parker M (Boden et al. Arch Microbiol 194:187–195, 2012) and *Thiobacillus* sp. strain C (Chang et al. J Microbiol (Korea) 35:165–171, 1997), whose properties are described. The genome of the type strain has not yet been sequenced.

Taxonomy, Historical and Current

The *Thermithiobacillaceae* currently comprise Family II of Order II (the *Acidithiobacillales*) of the *Gammaproteobacteria* (Garrity et al. 2005a, b; Kelly and Wood 2005a). The family was defined using the 16S rRNA gene sequence of the type genus, *Thermithiobacillus tepidarius*, as the phylogenetic basis for classification (Fig. 34.1). The *Thermithiobacillaceae* are distinguished from the only other family in the Order, the *Acidithiobacillaceae* (Garrity et al. 2005c), on the basis of 16S rRNA gene sequences (Fig. 34.1), and physiological differences, such as widely differing pH optima for growth, as all the *Acidithiobacillus* species are acidophilic (Kelly and Wood 2005b; and this volume). The single currently established genus, *Thermithiobacillus*, was created by Kelly and Wood (2000) in the reorganization of the genus *Thiobacillus*, which previously contained examples of each of the *Alpha-*, *Beta-*, and *Gammaproteobacteria*. While currently assigned to the *Gammaproteobacteria*, the taxonomic position of *Thermithiobacillus* has been a subject of some uncertainty since the evolutionary relationships of the sulfur-oxidizing bacteria were examined in detail using 16S rRNA phylogeny (Lane et al. 1992). Initially, *T. tepidarius* was assigned to the *Betaproteobacteria*, close to the beta-gamma root (Lane et al. 1992), but was subsequently transferred to the *Gammaproteobacteria* (Kelly and Wood 2000; Kelly et al. 2005). Currently, this assignment is again under review, as the *Acidithiobacillales*, represented by *At. ferrooxidans*, seem to be outside both the *Beta-* and *Gammaproteobacteria* and probably represent a novel Class within the *Proteobacteria* (Williams and Kelly 2013; Williams et al. 2010). This probability is discussed more fully in our chapter on the *Acidithiobacillaceae* in this volume.

Molecular Analyses

As yet the genome of *T. tepidarius* has not been sequenced, but is scheduled to be analyzed as part of Genomic Encyclopedia of Bacteria and Archaea "Tree of Life" program (N. Kyrpides; DOE, Joint Genome Institute). The GC content of the genomic DNA is 66.6 %, and the accession number for its 16S rRNA gene sequence is AJ459801. Apart from the other putative *Thermithiobacillus* strains discussed below, its only phylogenetic relatives are other *Acidithiobacillales*, in the genus *Acidithiobacillus* (Fig. 34.1). The comparative 16S rRNA gene sequence identities are, however, quite low: the moderate thermophile *At. caldus, At. thiooxidans*, and *At. albertensis* at 91.1–91.3 % with *At. ferrivorans* and *At. ferrooxidans* at 90.4–90.5 %. Identities to the other chemolithotrophic thiosulfate oxidizers, *Thiobacillus thioparus* (*Betaproteobacteria*) and *Paracoccus versutus* (*Alphaproteobacteria*), were only 84.5 % and 78.8 %, respectively (authors' BLAST2 data). This confirms the phylogenetic basis for placing both genera in the *Acidithobacillales*, with separation of the two families based on 16S rRNA and their marked phenotypic differences.

Phenotypic Analyses

The type and only validated species (DSM 3134^T, ATCC 43215^T) of *T. tepidarius* is a Gram-negative, moderately thermophilic, and obligately chemolithoautotrophic aerobe that uses the

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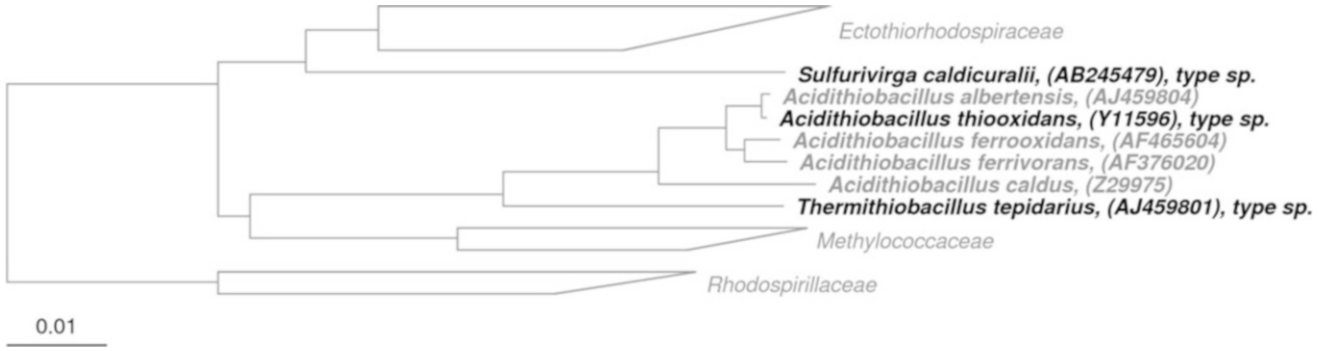

Fig. 34.1

Phylogenetic reconstruction of the family *Thermithiobacillaceae* based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes-Cantor correction. The sequence datasets and alignments were used according to the All-Species Living Tree Project (*LTP*) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high quality type strain sequences proportionally distributed among the different bacterial and archaeal phyla. In addition, a 40 % maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. *Scale bar* indicates estimated sequence divergence

oxidation of inorganic sulfur compounds (sulfide, thiosulfate, trithionate, or tetrathionate) as its sole source of energy for growth and fixes carbon dioxide for biosynthesis using the Calvin-Bassham-Benson cycle. It also oxidizes sulfur, sulfite, hexathionate, and heptathionate to sulfate. Its cells are nonspore-forming small rods, 0.2–0.4 μm × 0.6–1.0 μm in size, that are motile by means of a single polar flagellum; colonies on thiosulfate or tetrathionate agar at 43 °C are 1–2 mm in diameter, smooth and circular, becoming white or yellow with precipitated sulfur. Its optimum temperature is 43–45 °C, with a range for growth of 20–52 °C, with no growth at 15 °C or 55 °C; the optimum pH range for growth is pH 6.0–7.5, with a range of pH 5.2–8.0 (Wood and Kelly 1985, 1986). Its respiratory quinone is ubiquinone Q-8, as is also seen in the *Acidithiobacillus* species.

In batch culture in liquid medium with 20 mM thiosulfate, quantitative production of tetrathionate occurs (20 mM $Na_2S_2O_3$ → 10 mM $Na_2S_4O_6$), with a significant rise in pH, before growth continues, supported by the oxidation of the tetrathionate to sulfate, with a fall to pH 4.8–5.0, possibly with concomitant sulfur deposition.

$$4\ Na_2S_2O_3 + O_2 + 2\ H_2O \rightarrow 2\ Na_2S_4O_6 + 4\ NaOH$$

$$2\ Na_2S_4O_6 + 7\ O_2 + 6\ H_2O \rightarrow 2\ Na_2SO_4 + 6\ H_2SO_4$$

In pH-controlled batch culture held at pH 8.5, stoichiometric production of tetrathionate occurred but no further growth or oxidation to sulfate was possible (Wood and Kelly 1986). Substrate-limited continuous flow chemostat cultures (pH 7.0, 44 °C) on thiosulfate do not accumulate tetrathionate; do not precipitate sulfur during growth on sulfate, thiosulfate, trithionate, or tetrathionate; and do not produce detectable intermediates during tetrathionate oxidation (Wood and Kelly 1986).

Two other putative strains of *Thermithiobacillus* are known: the first of these is strain ParkerM (Boden et al. 2012), which is strain M79 of Parker and Prisk (1953). This was originally deposited as *Thiobacillus thioparus* (NCIMB 8349), but was reclassified as *Thermithiobacillus* by Boden et al. (2012). The 16S rRNA gene sequence of strain ParkerM (HM173631) showed 99.4 % identity to that of the type strain, suggesting it to be *T. tepidarius*. Similarly to the type strain, it showed only 92.4 % identity to *At. caldus*, 91.5 % to *At. thiooxidans*, 85.0 % to *T. thioparus*, and 79.6 % to *P. versutus*. It converted about 92 % of 40 mM thiosulfate to tetrathionate, with pH rising to pH 7.8, but little sulfate was subsequently produced in the study by Parker and Prisk (1953), possibly because pH 7.8 was inhibitory to further oxidation of 20 mM tetrathionate, as observed for 10 mM tetrathionate at pH 8.5 with the type strain (Wood and Kelly 1986). Boden et al. (2012), using more favorable growth conditions and 20 mM thiosulfate, showed conversion of the intermediate tetrathionate to sulfate, with a fall to about pH 5.2, as seen with the type strain.

The second candidate strain identified as a probable *Thermithiobacillus* species is strain C isolated from mine wastewater in Korea by Chang et al. (1997), using 30 mM thiosulfate as the growth substrate for isolation. This strain was a nonmotile, small rod, similar to the type strain in size (0.2 μm × 1.2–1.5 μm), with optimal growth on thiosulfate at 37 °C (range 25–42 °C) and pH 7.0 (range pH 4.5–8.5), and could also grow on sulfide. Its growth rates were similar at 30 °C, 37 °C, and 42 °C, consistent with moderate thermophily, although the biomass production at 30 °C or 42 °C was depressed by 11 % and 29 % of that at 37 °C respectively, possibly due to the longer lag phases observed at 30 °C and 42 °C (Chang et al. 1997). Like the type strain, it contained ubiquinone Q-8. The 16S rRNA gene sequence of strain C (AF023264) showed 98.3 % identity to the type strain sequence and 99.0 % identity to that of strain ParkerM (authors' BLAST2 analyses). As with the other two strains, its more distant phylogenetic relatives were

other *Acidithiobacillales*, namely, the moderately thermophilic *At. caldus* (92.0 %) and the extremely acidophilic *At thiooxidans* (91.5 %). This strain might represent a second species of *Thermithiobacillus*, but no further information about it has been published. The organism was not deposited with a culture collection and is now unavailable (S. W. Kim, personal communication, 2012).

Isolation, Culture Conditions, Media, and Maintenance Procedures

The type strain was isolated by incubating water samples (pH 7.3, 43 °C) from the inflow of the Great Bath in the Roman baths in the City of Bath (UK) in a medium containing 20 mM thiosulfate and isolating a pure culture by single colony isolation from agar plate cultures. The medium for isolation and for growth in batch and chemostat culture was described by Wood and Kelly (1985) and contained (per liter) $Na_2S_2O_3$ (20 mM) or $K_2S_4O_6$ (10 mM), KH_2PO_4 (4.0 g), K_2HPO_4 (4.0 g), NH_4Cl (0.4 g), $MgSO_4{\cdot}7H_2O$ (0.8 g), and trace metals (Wood and Kelly 1985, 1986; Kelly and Wood 1998). The medium should be prepared from stock solutions A, B, and T, prepared as follows (g L^{-1}):

A	
KH_2PO_4	16
K_2HPO_4	16
B	
NH_4Cl	10
$MgSO_4{\cdot}7H_2O$	20
T	
The description of this is given in detail by Kelly and Wood (1998) and contains (g) $ZnSO_4{\cdot}7H_2O$ (5); $CaCl_2$ (5; or $CaCl_2{\cdot}2H_2O$, 7.34); $FeSO_4{\cdot}7H_2O$ (5); $MnCl_2{\cdot}6H_2O$ (2.5); $CoCl_2{\cdot}6H_2O$ (0.5); ammonium molybdate (0.5); and $CuSO_4{\cdot}5H_2O$ (0.2), dissolved individually in water (30–40 mL) and mixed into a solution of 50 g disodium ethylenediaminetetraacetic acid (EDTA) containing 9 g NaOH. Adjust mixture to pH 6.0 with 1 M NaOH and make up to one liter with distilled water. This should be stored in a dark bottle at room temperature and any color change is ignored	

Liquid medium is prepared in two parts as follows:

Part 1	
$Na_2S_2O_3{\cdot}5H_2O$	5 g [or $K_2S_4O_6$ 3 g]
Solution B	40 mL
Solution T	10 mL
Distilled water	700 mL
Part 2	
Solution A	250 mL

Autoclave each at 110 °C for 10 min and combine aseptically when cool. Medium is initially at about pH 7.0.

For agar medium (per liter), the following should be mixed and autoclaved at 110 °C for 10 min:

$Na_2S_2O_3{\cdot}5H_2O$	5 or 10 g [or $K_2S_4O_6$ 3 g]
Solution A	250 mL
Solution B	40 mL
Solution T	10 mL
Saturated bromocresol purple	2 mL
Agar	10 or 15 g
Distilled water	700 mL

During growth with thiosulfate on agar plates at 43 °C, there will initially be an increase in pH, shown by the indicator color changing from violet to purple, as tetrathionate is formed; subsequently the oxidation of the tetrathionate leads to acidification, turning the indicator yellow. The color change with tetrathionate medium will simply be acidification as tetrathionate is oxidized.

Growth of colonies on agar, or growth in liquid, is visible within 2–4 days. Agar plate stock cultures can be maintained at 4 °C for several weeks before subculture, and long term storage can be achieved by freeze-drying or deep-freezing.

Ecology

The type strain was isolated from a thermal spring (43 °C) using 20 mM thiosulfate as an enrichment substrate (Kelly and Wood 2005a; Wood and Kelly 1985, 1986). Its distribution is unknown, but it is likely to occur widely in thermal spring waters consistently at 40–50 °C, where reduced sulfur compounds are available, and seems also to occur in corroding concrete and mine drainage (see above for other putative strains).

Pathogenicity, Clinical Relevance, Application, and Biodeterioration

No pathogenic effects are known or are likely to be found for this obligate chemolithoautotroph, and apart from its ability to oxidize inorganic sulfur compounds, it offers no obvious practical applications. As the ParkerM strain was isolated from corroding concrete (Boden et al. 2012; Parker and Prisk 1953), it seems likely that *T. tepidarius* could be part of the moderately acid-tolerant sulfur-oxidizing community that initiates sulfide and sulfur compound oxidation in concrete pipes, leading ultimately to corrosion and collapse.

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35 The Family *Thioalkalispiraceae*

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Abstract
The family *Thioalkalispiraceae* is accommodated in the order *Chromatiales* of the class *Gammaproteobacteria* of the phylum Proteobacteria for the chemolithoautotrophic bacteria oxidizing sulfur compounds. Four species of three genera, *Thioalkalispira*, *Thiohalophilus*, and *Thioprofundum*, are classified in this family to date. The organisms have been isolated from a soda lake, hypersaline lake, or marine samples and require a certain concentration of NaCl for growth. The organisms of this family are obligate chemolithoautotroph. Thiosulfate is oxidized by all species, but capability of oxidation of elemental sulfur and sulfide is different in species. The species use oxygen as an electron accepter. The members are mesophilic or moderate thermophilic and neutrophilic except *Thioalkalispira microaerophila*, which is alkaliphilic. Photosynthetic growth is not reported.

Taxonomy, Historical and Current

Thioalkalispira was proposed as a new genus for a moderate alkaliphilic chemolithoautotrophic bacterium isolated from a soda lake in Egypt by Sorokin et al. (2002) as a member of the class *Gammaproteobacteria*. Sorokin et al. (2007) followed their study by proposing a new genus *Thiohalophilus* for a halophilic nitrite-reducing chemolithotrophic isolate from a hypersaline lake in Russia. However, the higher taxa for the two genera were not discussed. *Thioalkalispira microaerophila* was tentatively classified as a member of the family *Ectothiorhodospiraceae* in the order *Chromatiales* based on the taxonomic outline of *Bergey's Manual of Systematic Bacteriology* (http://www.bergeys.org/outline.html). The genus *Thioprofundum* was proposed by Takai et al. (2009) in the course of the study on the chemolithoautotrophic piezophilic bacteria from a deep-sea hydrothermal vent chimney. Mori et al. (2011) studied the taxonomy of an isolate from a hydrothermal vent and classified the isolate in the genus *Thioprofundum*. The accumulation of these culturable strains accompanying with the data of clones from marine samples led the conclusion of establishment of an independent family *Thioalkalispiraceae* for these three genera in the order *Chromatiales* (Mori et al. 2011). All the known species of this family are composed by the new isolates, and no species were transferred from other genera as new combinations.

Short Description of the Family

Thioalkalispiraceae Mori, Suzuki, Urabe, Sugihara, Tanaka, Hamada, and Hanada 2012, 2416[VP]

Thi.o.al.ka.li.spi.ra'ce.a.e. N.L. fem. n. *Thioalkalispira*, the type genus of family; suff. -*aceae*, ending to denote a family; N.L. fem. pl. n. *Thioalkalispiraceae* the family of the genus *Thioalkalispira*.

The members of the family *Thioalkalispiraceae* form an independent cluster in the order *Chromatiales* of the class *Gammaproteobacteria* of the phylum Proteobacteria by the phylogenetic analysis based on the 16S rRNA gene sequences.

The species of this family have the Gram-negative type cell envelope. They are strictly chemolithoautotrophic,

E. Rosenberg et al. (eds.), *The Prokaryotes – Gammaproteobacteria*, DOI 10.1007/978-3-642-38922-1_399,

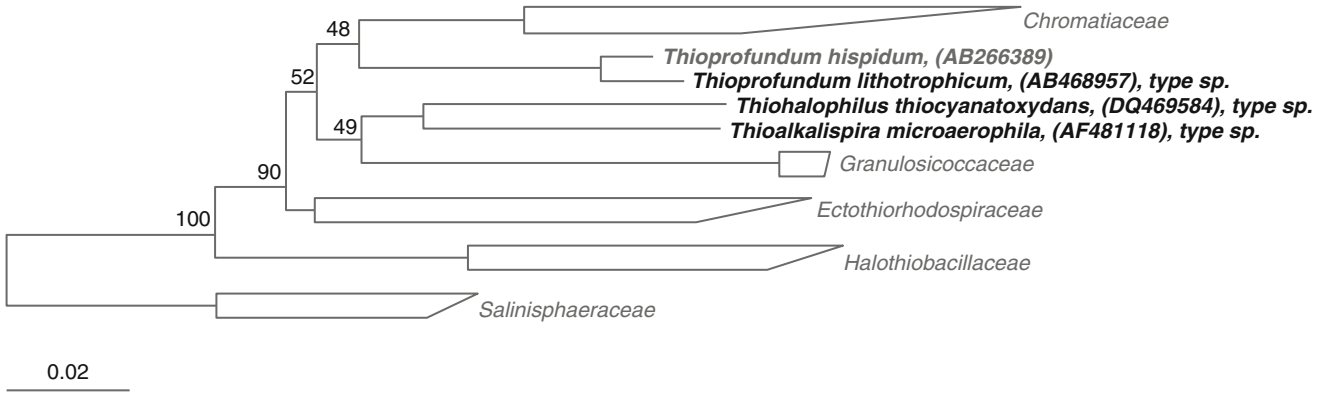

Fig. 35.1

Phylogenetic tree of the family *Thioalkalispiraceae* and the related organisms in the order *Chromatiales* based on their 16S rRNA gene sequences using the neighbor-joining algorithm with the Jukes-Cantor correction. The vales at the nodes represent the bootstrap values. The scale bar indicates estimated sequence divergence

non-photosynthetic, and mesophilic or moderately thermophilic growing optimally not higher than 50 °C. Growth occurs by sulfur oxidation and carbon dioxide fixation. Members of the family are moderately halophilic and isolated from marine and saline environments.

The genomic DNA G+C content is 59–66 mol%. The type genus is *Thioalkalispira*.

Phylogenetic Structure of the Family and Its Genera

The phylogenetic relationship based on the 16S rRNA genes is shown in *Fig. 35.1*.

In addition to the species of the family, clones obtained from marine samples are included (Mori et al. 2011). The independent cluster is formed by the members of the family *Thioalkalispiraceae* in the order *Chromatiales* which contains the families *Chromatiaceae*, *Ectothiorhodospiraceae*, and *Halothiobacillaceae*.

Molecular Analysis

The family also contains uncharacterized marine denitrifying sulfur-oxidizing isolates, strains OAII2 and NDII.1 (Meyer et al. 2007). The similarities between valid species of the family *Thioalkalispiraceae* are less than 92.8 %. The family is closely related to the cluster of environmental clones including marine invertebrate symbionts but completely separated from the cluster on the 16S rRNA gene analysis (Mori et al. 2011).

DNA-DNA Hybridization Studies

The genera of the family *Thioalkalispiraceae* consisted of single species except the genus *Thioprofundum*. Intrageneric DNA-DNA hybridization (DDH) was carried out by the microtiter plate method of Ezaki et al. (1989) between the two species of the genus *Thioprofundum* and showed low values (11–21 %) (Mori et al. 2011). DDH of *Thioalkalispira microaerophila* ALEN 1^T with the type strains of the genera *Thioalkalimicrobium* and *Thioalkalivibrio* were carried out by the method of De Ley et al. (1970) and showed low values equivalent to the background (Sorokin et al. 2002).

Genome Analysis

The whole genome sequences of the members of the family *Thioalkalispiraceae* have not been studied to date.

Bacteriophages

Bacteriophages of the members of this family have not yet been known.

Phenotypic Analysis

Thioalkalispira Sorokin, Tourova, Kolganova, Sjollema, and Kuenen 2002 2181^{VP}

Thi.o.al.kal.i.spi'ra. Gr. n. *theion* (Latin transliteration thium), sulfur; N.L. n. *alkali* (from Arabic *al-qalyi*, the ashes of saltwort), soda ash; L. fem. n. *spira* spiral; N.L. fem. n. *Thioalkalispira* sulfur alkaline spiral.

Thioalkalispira strains are obligately chemolithoautotrophic. Cells have *Spirillum*-like morphology with dimensions 0.3–0.45 × 1–4 μm (occasionally up to 15 μm in length). Motile by means of a single polar flagellum. Cell wall is Gram-negative type. Intracellular sulfur globules and polar polyphosphate

granules are deposited. Sulfide, polysulfide, elemental sulfur, and thiosulfate are oxidized to sulfate. *Thiomicrospira* strains are obligate aerobe or microaerophile and able to grow only under reduced oxygen concentrations (<2 %). Nitrate and sulfur are reduced to nitrite and sulfide, respectively, without growth. Alkaliphilic, with the pH range for growth between 8 and 10.4 (optimum around pH10). Moderately halophilic, with total Na^+ content suitable for growth between 0.2 and 1.4 M (optimum 0.5 M).

The type species is *Thioalkalispira microaerophila* (Sorokin et al. 2002). The type strain of the type species is ALEN 1 (=DSM 14786 = UNICEM 212). The type strain was isolated from hypersaline alkaline lake Fazda (Wadi Natrun, Egypt). DNA G+C content of the type strain is 58.9 mol% (*T*m).

Thiohalophilus Sorokin, Tourova, Bezsoudnova, Pol, and Muyzer 2007, 449[VP]

Thi.o.ha.lo.phi.lus. Gr. n. *theion* (Latin transliteration thium), sulfur; Gr. n. *hals* halos, salt; N.L. adj. *philus* -a -um (from Gr. adj. *philos* -e -on), friend, loving; N.L. masc. n. *Thiohalophilus* sulfur and salt loving. The names were effectively published by Sorokin et al. (2007) and validly published in the Validation List No. 117.

The cells are nonmotile long rods with a Gram-negative type cell envelope. Obligately chemolithoautotrophic and facultatively anaerobic. Utilize reduced sulfur compounds, including thiocyanate, as the electron donor with oxygen or nitrite as the electron acceptors. Moderately halophilic.

Type species: *Thiohalophilus thiocyanatoxydans* corrig (Sorokin et al. 2007). Species epithet "*thiocyanoxydans*" in the original paper (Sorokin et al. 2007) was corrected to "*thiocyanatoxydans*" by Lists editor of IJSEM (Validation list No 117 2007). The type strain of the type species is HRhD 2 (=DSM 16326 = UNIQEM U231). The type strain was isolated from hypersaline lake in Kulunda Steppe, Russia. The G+C content of the type strain is 58.2 mol%.

Further characteristics of this genus shown as those of the single species *T. thiocyanatoxydans* are as follows: sulfide, thiosulfate, and thiocyanate are used as the electron donor producing sulfate as the final oxidation product. Thiosulfate is degraded through the "carbonyl sulfide (COS) pathway" with the thiocyanate hydrolase-type enzyme (Katayama et al. 1992, 1998).

Thioprofundum Takai, Miyazaki, Hirayama, Nakagawa, Querellou, and Godfroy 2010 1991[VP]

Thi.o.pro'fu.n.dum. Gr. n. *thios* sulfur; L. n. *profundus* deep; N.L. neut. n. *Thioprofundum* sulfuroxidizer from deep sea.

Effective publication of this genus is Takai et al. (2009) and validly published in the Validation list No.134 (2010).

Cells are bent to spiral rod. Facultatively anaerobic and piezophilic. Thermophilic and neutrophilic. Chemolithoautotrophic. Able to utilize reduced sulfur compounds as electron donors and nitrate and molecular oxygen as electron acceptors. NaCl is absolutely required for growth.

G+C content of genomic DNA is about 66 %. Major cellular fatty acids are $C_{16:0}$, $C_{16:0}$-methyl[1], $C_{18:0}$, and $C_{18:1}$. Based on 16S rRNA gene analysis, the genus *Thioprofundum* is related to the endosymbionts of the deep-sea animals within the *Gammaproteobacteria*.

The type species is *T. lithotrophica*. The type strain of the type species is strain 106 (=JCM 14586[2], = DSM 19353). The type strain of the type species was isolated from a black smoker chimney of the TAG field in the Mid-Atlantic Ridge. The G+C content of the type strain is 65.7 mol% (*Tables 35.1* and *35.2*).

Isolation, Enrichment, and Maintenance Procedures

Isolation and Enrichment

Isolation of the organisms of the family *Thioalkalispiraceae* has been achieved essentially by enrichment culture using chemolithoautotrophy by oxidizing sulfur with some specific modification in consideration of the characteristics of the samples used for isolation. Although the members of this family are facultative anaerobe, their optimal concentration of O_2 for growth is low (2 % for the genus *Thiohalophilus*).

Thioalkalispira microaerophila ALEN 1^T was isolated by the procedure for anaerobic phototrophs described by Imhoff et al. (1979) using the salt concentration and pH in consideration of those of the isolation samples, namely, 220–360 g/L and pH 9.2–10.3, respectively, for haloalkaliphilic condition from samples of soda lakes. Thiosulfate (40–80 mM) is added for the electron donor and nitrate (3 mM as KNO_3) for the nitrogen source. Solid alkaline medium was prepared by mixing equal volume of 4 % agar and double-strength mineral base at 50 °C. Enrichments for denitrifying sulfur-oxidizing bacteria are performed in 100 ml serum bottles with butyl rubber stoppers and filled with 50 ml of alkaline base with 20 mM thiosulfate and 30 mM nitrate. Anoxic conditions were achieved by five cycles of evacuation-argon flushing with active degassing of the liquid (Sorokin et al. 2001). Micro-oxic incubation was performed in closed 3.5 l jars filled with argon containing 1 % O_2.

For isolation of *Thiohalophilus thiocyanatoxydans*, a certain concentration (0.5–4.0 M) of NaCl was added to the basal medium for chemolithoautotroph containing thiosulfate or thiocyanate as the substrate.

Piezotrophic cultivation technique (Takai et al. 2008) was employed for isolation of strain 106^T, the type strain of

[1] Takai et al. (2009) identified as $C_{17:0}$anteiso. However, Mori et al. (2011) reported that it is methyl-branched $C_{16:0}$ but that it is different from $C_{17:0}$anteiso.
[2] The accession number of the type strain in JCM was incorrectly cited (Takai et al. 2009) but corrected by the Validation List No. 134 (2010).

Table 35.1
Cultural and physiological characteristics of the type strains of the species of the family *Thioalkalispiraceae*

Characteristics	*Thioalkalispira microaerophila* ALEN 1^T	*Thiohalophilus thiocyanatoxydans* HRhD2^T	*Thioprofundum lithotrophicum* 106^T	*Thioprofundum hispidum* gps61^T
Morphology	Spiral rod	Rod	Short bent to long spiral rod	Rod
Cell size	0.3–0.45 × 1–4 μm	0.35–0.4 × 2–6 μm	0.4–0.6 × 1–20 μm	0.3 × 1.5–2.0 μm
Motility	Motile with single polar flagellum	Nonmotile	Motile with a polar flagellum	Nonmotile / Single thick polar flagellum
Intracellular deposit	Sulfur globules and polar polyphosphate granules	ND	ND	–
Requirement for oxygen	Microaerobic (<2 %)	Facultatively anaerobic	Anaerobic to microaerobic	Facultatively anaerobic
Photosynthesis	–	ND	ND	–
Electron acceptors	O_2	O_2, NO_2^-	O_2, NO_3^-	O_2, NO_3^-
Electron donors	S^{2-}, S_8^{2-}, S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$	S^{2-}, $S_2O_3^{2-}$, thiocyanate	S^{2-}, S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$,	S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$
Catalase	+	ND	ND	–
Oxidase	+	ND	ND	+
Optimum temperature for growth (°C) (growth range)	30	30	50 (30–55)	39 (29–43)
Optimum pH for growth (growth range)	10 (8–10.4)	7.5 (6.5–8.2)	7.0 (5.8–7.6)	7 (6–8)
Optimum NaCl concentration for growth (growth range)	0.5 M (0.2–1.4 M)	1.5 M (1.0–4.0 M)	0.5 M (0.2–0.72 M)	0.34 M (0.17–0.68 M)
Source of isolation of the type strain	Lake Fazda of Wadi Natrun area, Egypt	Hypersaline lake in Kulunda Steppe, Russia	TAG field in the Mid-Atlantic Ridge	Suiyo Seamount on the Izu-Bonin Arc in western Pacific Ocean

Thioprofundum lithotrophica that grows under normal pressure. In contrast, different from strain 106^T, co-isolated strain 108 showed obligate piezotrophy (Takai et al. 2009).

Thioprofundum hispidum gps61^T was isolated using the AP8SO1 medium which consisted of the basal medium (*Table 35.3*) supplemented with 5 mM $Na_2S_2O_3$. The cultivation condition was under an atmosphere of $N_2/CO_2/O_2$ (75 : 20 : 5, v/v/v;150 kPa) in a gas-tight vial at 50 °C. Repeated transfer to the same medium by observing the presence of bacteria and appearing elemental sulfur. The enrichment was followed by increasing the concentration of thiosulfate into 20 mM and cultivation at 37 °C with $N_2/CO_2/O_2$ (60 : 20 : 20, v/v/v;150 kPa). Finally, the organism was purified by repeated dilution and confirmation of no growth on heterotrophic media.

Ecology

Various chemolithoautotrophic prokaryotes inhabit marine environments and others where the substrates for such chemolithoautotrophic growth are supplied. Hydrothermal vents in a deep sea or hypersaline environments are to be common sources for the organisms of this family so far. Strains of the genera *Thioalkalispira* and *Thiohalophilus* were from terrestrial while those of the genus *Thioprofundum* were of marine origin. The genus *Thioalkalispira* isolated from an alkaline soda lake was alkaliphilic, while the other members of this family that were neutrophilic. The type strain of *Thioalkalispira microaerophila* was isolated only from Lake Fazda of Wadi Natrun area, Egypt, and not from the other samples so far studied.

Thioprofundum lithotrophica was isolated from a black smoker chimney of the TAG field in the Mid-Atlantic Ridge. *Thioprofundum hispidum* was isolated from the surface of a rock sample collected from a hydrothermal field of Suiyo Seamount on the Izu-Bonin Arc in the western Pacific Ocean. *Thioprofundum lithotrophica* is moderately thermophilic and optimally grows at 50 °C, whereas *Thioprofundum hispidum is mesophilic* and growth is not observed at 50 °C. Sensitivity to oxygen is different in two species: *Thioprofundum lithotrophica* is microaerobic but *Thioprofundum hispidum* can grow under 20 % oxygen condition.

Table 35.2
Taxonomic characteristics of the type strains of the species of the family *Thioalkalispiraceae*

Characteristics	*Thioalkalispira microaerophila*	*Thiohalophilus thiocyanatoxydans*	*Thioprofundum lithotrophicum*	*Thioprofundum hispidum*
Type strain	ALEN 1^T	HRhD2^T	106^T	gps61^T
	=DSM 14786 = UNICEM 212	=DSM 16326 = UNIQEM U231	=DSM 19353 = JCM 14586	=DSM 18546 = NBRC 101261
Major cellular fatty acids (minor components)[a]	ND[b]	$C_{16:0}$, $C_{16:1\omega7c}$, $C_{17:1\omega5}$iso	$C_{16:0}$, $C_{16:1\omega7c}$, $C_{17:0}$anteiso, $C_{18:1\omega7c}$ ($C_{18:0}$)[c]	$C_{16:0}$. $C_{17:0}$br[d] ($C_{16:1\omega7c}$, $C_{18:1\omega7c}$, $C_{15:0}$, $C_{14:0}$)
16S rRNA gene sequence	AF481118	DQ469584	AB468957	AB266389
DNA G+C content (mol%)	58.9	58.2 (Tm)	65.7	62.9

[a]Analytical method is based on MIDI System (Sasser 1990; MIDI 2002)
[b]Not determined
[c]Cells grown at 16 MPa
[d]Methyl-branch hexadecanoic acid

Table 35.3
Composition of AP8SO1 medium

(i) Basal medium			
KH_2PO_4	0.6 g		
K_2HPO_4	0.11 g		
$MgCl_2$ $6H_2O$	3.05 g		
$CaCl_2$ $2H_2O$	0.15 g		
$(NH_4)_2SO_4$	0.66 g		
NaCl	30 g		
$Na_2S_2O_3$ $5H_2O$	5 g		
Trace elements solution[a]	2 ml		
Vitamin solution[b]	2 ml		
$NaHCO_3$	2.52 g		
Distilled water	1 L		
(ii) Trace elements solution and vitamin solution			
[a]Trace elements solution		**[b]Vitamin solution**	
Nitrilotriacetic acid (NTA)	12.8 g	Biotin	2 mg
$FeCl_3$ $6H_2O$	1.35 g	Folic acid	2 mg
$MnCl_2$ $4H_2O$	0.1 g	Pyridoxine HCl	10 mg
$CoCl_2$ $6H_2O$	0.024 g	Thiamine HCl	5 mg
$CaCl_2$ $2H_2O$	0.1 g	Riboflavin	5 mg
$ZnCl_2$	0.1 g	Nicotinic acid	5 mg
$CuCl_2$ $2H_2O$	0.025 g	Ca-pantothenate	5 mg
H_3BO_3	0.01 g	*p*-Aminobenzoic acid	1 mg
Na_2MoO_4 $2H_2O$	0.024 g	Vitamin B_{12}	0.01 mg
NaCl	1 g	Distilled water	1 L
$NiCl_2$ $6H_2O$	0.12 g		
Na_2SeO_4	0.004 g		
Na_2WO_4	0.004 g		
Distilled water	1 L		

Maintenance Procedure

The strains are maintained by freezing at −80 °C in a freezer or desirably at −196 °C in a vapor phase of liquid nitrogen for long-time preservation. Cells are suspended in 10 % dimethyl sulfoxide with an appropriate concentration of NaCl for the strains. Some strains may be preserved by freeze- or liquid-drying methods.

Pathogenicity and Clinical Relevance

The members of this family were isolated from natural environments such as hypersaline and hydrothermal vent of deep sea. No pathogenicity for plants and animals has been known to date.

Application

The members of this family were isolated from natural environments and used for the physiological studies. There are no reports of application using the organisms of this family.

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36 The Famlily *Vibrionaceae*

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Abstract

Vibrionaceae embraces the genera *Vibrio* (1854), *Photobacterium* (1889), *Salinivibrio* (1996), *Enterovibrio* (2002), *Grimontia* (2003), and *Aliivibrio* (2007). Totally 131 species are described currently. These described species are mainly marine origin, but important human pathogens, *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, are included. Many obligate fish/shellfish pathogens (e.g., *V. anguillarum*, *P. damselae* subsp. *piscicida*) are also included. Strains showing zoonotic features (between human and fish/shellfish) are known. On the contrary of pathogenic feature of some vibrio species/strains, *A. fischeri* are known to be mutual symbiont, and many vibrios show beneficial or commensal association against marine animal hosts. *Vibrio* species is defined as a group of strains forming rods with polar flagella enclosed in a sheath, facultative anaerobic metabolism, capable of fermenting D-glucose and growth at 20 °C. Primarily aquatic, most species are oxidase positive, reduce nitrate to nitrite, require Na^+ for growth, and ferment D-fructose, maltose, and glycerol. Each vibrio species is further identified by an array of over 100 phenotypic tests; however, there is not an operational definition for genera within the vibrios. *Vibrionaceae* species are metabolically versatile; species showing gas production, nitrogen fixation, phototrophy, and nonmotile are increasing. *Vibrionaceae* species may be better defined on the basis of amplified fragment length polymorphism (AFLP) and multilocus sequence analysis (MLSA), more recently by genome comparison. Strains of the same species (including the type strain) share more than 60 % mutual AFLP band pattern similarity and more than 95 % similarity in MLSA (using the loci *rpoA*, *recA*, *pyrH*, *ftsA*, *topA*, *mreB*, *gyrB*, and *gapA*). More importantly, strains of the same species and species of the same genus will form monophyletic groups on the basis of MLSA or genome comparison.

Taxonomy, Historical and Current

Short Description of the Family

Vib.ri.o.na'ce.ae. M.L. masc. n. *Vibrio* type genus of the family; -aceae ending to denote family; M. L. fem. Pl. n. *Vibrionaceae* the family of bacteria.

One hundred thirty-one species are described in this family (Table 36.1), which is consisted of six genera *Vibrio* (Pacini, 1854), *Photobacterium* (Beijerinch, 1889), *Salinivibrio* (Mellado et al. 1996), *Enterovibrio* (Thompson et al. 2002), *Grimontia* (Thompson et al. 2003), and *Aliivibrio* (Urbanczyk et al. 2007). *Vibrio aestuarianus*, *Photobacterium damselae*, and *Salinivibrio costicola* involve subspecies. Vibrio species is defined as a group of strains forming small (0.5–0.8 1.4–2.6 μm) rods with polar flagella enclosed in a sheath, facultative anaerobic metabolism, capable of fermenting D-glucose and growth at 20 °C. Primarily aquatic, most species are oxidase positive, reduce nitrate to nitrite, require Na^+ for growth, and ferment D-fructose, maltose, and glycerol. Each vibrio species is further identified by an array of over 100 phenotypic tests. There is not an operational definition for genera within the vibrios. *Vibrionaceae* species are metabolically versatile; species descriptions showing gas production, nitrogen fixation, phototrophy, and nonmotile, which have been believed to be atypical phenotype for *Vibrionaceae*, are accumulated. In our hands, vibrio species may be better defined on the basis of AFLP and MLSA. Strains of the same species (including the type strain) share more than 60 % mutual AFLP band pattern similarity and more than 95 % similarity in MLSA (using the loci *rpoA*, *recA*, *pyrH*, *ftsA*, *topA*,

Table 36.1
List of species of *Vibrionaceae*

Species name	Year proposed	Type strain	16S rRNA accession number	Representative genome sequenced strain (accession number/BioProject and status)	mol% G+C	Place and date of isolation	Source
Vibrio cholerae[a]	1854	LMG 21698	X76337	N16961 (NC_002505, NC002506, Complete)	47–49	Asia	Clinical
V. aerogenes	2000	LMG 19650 CAIM 906 CECT 7868	AF124055	CECT 7868 (PRJNA171492)	45.9	Nanwan bay, Taiwan	Sediment of sea-grass bed
V. aestuarianus subsp. *aestuarianus*	1983	LMG 7909 CAIM 592	X74689	NBRC 15629 (PRJDB354)	43–44	Oregon, USA	Oyster
V. aestuarianus subsp. *francensis*	2008	LMG 24517	AJ845017		NA	Argenton, France, 2002	Diseased oyster hemolymph
V. agarivorans	2001	LMG 21449 CAIM615	AJ310647		44.8	Valencia, Spain	Seawater
V. alfacsensis[c]	2012	CAIM 1831 DSM 24595	JF316656		43.5–45.1	IRTA, Ebro delta, Catalonia, Spain, 2006	Gill from a healthy sole (*Solea senegalensis*)
V. alginolyticus	1961, 1968[b]	LMG 4409 CAIM 516	X56576	40B (ACZB00000000, Draft) 12G01 (AAPS000000, Draft)	45–47	Japan	Spoiled horse mackerel (*Trachurus trachurus*)
V. anguillarum	1909	LMG 4437 CAIM 696	X16895 AM235737	775 (NC_015633, NC_016537, Complete)	43–46	Norway	Diseased cod (*Gadus morhua*)
V. areninigrae	2008	CAIM 1411 JCM 14949	EU143360		NA	Soesaggak, Jeju island, Korea	Black sand
V. artabrorum	2011	CAIM 1845 CECT 7226 LMG 23865	CECT 7226	EF599164	44.4	Galicia, Spain, 2005	Clam (*Ruditapes philippinarum*)
V. atlanticus	2011	CAIM 1847 CECT 7223 LMG 24300	EF599163		44.2	Galicia, Spain, 2005	Clam (*Ruditapes philippinarum*)
V. atypicus	2010	LMG 24781	FJ009624		44.4	China	The gut of the Chinese prawn (*Penaeus chinensis*)
V. azureus	2009	NBRC 104587 CAIM 1457	AB428897	NBRC 104587 (PRJDB356)	45.2–45.5	Japan	Seawater
V. brasiliensis	2003	LMG 20546 CAIM 495	AJ316172	LMG 20546 (AEVS00000000, Draft)	45.9	LCMM, Florianópolis, Brazil, 1999	Bivalve larvae (*Nodipecten nodosus*)
V. breoganii	2009	CAIM 1829 CECT 7222 LMG 23858	EF599161	1C10 (PRJNA169653)	44.4	Galicia, Spain	Grooved carpet shell (*Ruditapes decussatus*)

Table 36.1 (continued)

Species name	Year proposed	Type strain	16S rRNA accession number	Representative genome sequenced strain (accession number/BioProject and status)	mol% G+C	Place and date of isolation	Source
V. campbellii	1971, 1981[b]	LMG 11216 CAIM 519	X56575	ACTT BAA 1116 (NC_009783, NC_009784, NC_009777, Complete)	45.9– 47.2	Hawaii, USA	Seawater
V. caribbeanicus	2012	DSM 23640	GU223601	ATCC BAA 2122 (AEIU00000000, Draft)	41.6	West coast of Curacao, 2005	Marine sponge (*Scleritoderma cyanea*)
V. casei	2010	CAIM 1451 DSM 22364 LMG 25241	FJ968722		41.8	Fromagerie de l'Ermitage, Bulgnéville, Lorraine,	Smear-ripened cheese surface
V. chagasii	2003	LMG 21353 CAIM 431	AJ316199		44.6	AARS, Austevoll, Norway, 1997	Gut of turbot larvae (*Scophthalmus maximus*)
V. celticus	2011	CAIM 1849 CECT 7224 LMG 23850	EF599162		NA	Galicia, Spain, 2005	Clam (*Ruditapes philippinarum*)
V. cincinnatiensis	1986	LMG 7891 CAIM 607	X74698		45	Ohio, USA	Human blood and cerebrospinal fluid
V. comitans	2007	CAIM 813 NBRC 102076	DQ922915	NBRC 102076 (PRJDB357)	45–48	Japan, 2005	Guts of wild-caught abalone (*H. discus discus, H. gigantea* and *H. madaka*)
V. communis	2011	CAIM 1816 LMG 25430	GU078672		45.2–46.0	Praia Grande, Sao Paolo, Brazil, 2005	Mucus of an apparently healthy coral (*Mussismilia hispida*)
V. coralliilyticus	2003	LMG 20984 CAIM 616	AJ440005	ATCC BAA 450 (ACZN00000000, Draft)	45.6	Indian Ocean, near Zanzí bar, 1999	Diseased Pocillopora damicornis
V. crassostreae	2004	LMG 22240 CAIM 1405	AJ582808 EF094887	9ZC13 (AJYZ00000000, Draft)	NA	IFREMER, La Tremblade, France	Hemolymph of diseased reared oysters (*Crassostera gigas*)
V. cyclitrophicus	2001	LMG 21359 CAIM 596	DQ481610 AM162656	ZF205 (AIDO00000000, Draft)	38.8	Washington, USA	Creosote-contaminated sediment
V. diabolicus	1997	LMG 19805	X99762		45.6	East pacific rise, 1991	Dorsal integument of polychaete (*Alvinella pompejana*)
V. diazotrophicus	1982	LMG 7893 CAIM 613	X74701		46–47	Nova Scotia, Canada	Sea urchin (*Strongylocentrotus* sp.)
V. ezurae	2009	LMG 19970 CAIM 905	AY426980	NBRC 102218 (PRJDB359)	43.6–44.8	Kanagawa, Japan, 1999	Gut of abalone (*Haliotis diversicolor supertexta*)
V. fluvialis	1981	LMG 7894 CAIM593	X74703 X76335	NBRC 103150 (PRJDB353)	49.3–50.6	Bangladesh	Human faeces
V. fortis	2003	LMG 21557 CAIM629	AJ514916		45.6	Ecuador, 1996	Shrimp (*Litopenaeus vannamei*) larvae

V. furnissii	1984	LMG 7910 CAIM 518 CIP 102972	X76336	NCTC 11218 (NC_016602, NC_016628, Complete) CIP 102972 (NZ ACZP00000000, Draft)	49.9–50.9	Japan	Human faeces
V. gallaecicus	2009	CAIM 1814 CECT 7244	EU541605		NA	Ria de Vigo, Galicia, Spain	Manila clams (*Ruditapes philippinarum*)
V. gallicus	2004	LMG 21878 CAIM 903 CIP 107963	AY257972	CIP 107963 (unpublsihed, Draft)	43.6–44.3	Brest, France, 2001	French abalone Haliotis tuberculata
V. gazogenes	1980, 1981[b]	LMG 19540	X74705	NBRC 103151 (PRJDB352)	47.1	Massachusetts, USA	Mud from a salt marsh
V. gigantis	2005	LMG 22741	AJ582810 EF094888		NA	FREMER, La Tremblade, France	Hemolymph of diseased reared oyster (*Crassostrea gigas*)
V. hangzhouensis	2009	CAIM 1448	EU082035		44.9	East China sea	Sediment
V. halioticoli	1998	LMG 18542 CAIM 283 JCM 21271	AB000390	JCM 21271 (Unpublished, Draft) NBRC 102217 (PRJDB351)	41.6–43.1	Kumaishi, Japan, 1991	Gut of abalone (*Haliotis discus hanai*)
V. harveyi	1936, 1981[b]	LMG 4044 CAIM 513	X74706 AY750575	1DA3 (NZ_ACZC_00000000, Draft) HY01 (NZ AAWP00000000, Draft) S20 (Unpublished,	46–48	Massachusettes, USA, 1935	Dead amphipod (*Talorchestia* sp.)
V. hepatarius	2003	LMG 20362 CAIM 693 CAIM 1843 LMG	AJ345063		45.5	CENAIM, Ecuador, 2000	Digestive gland of white shrimp *(Litopenaeus vannamei)*
V. hippocampi	2010	CAIM 1843 LMG 25354	FN421434		49.3	Toralla, Galicia, Spain	Faeces of wild seahorse (*Hippocampus guttulatus)*
V. hispanicus	2004	LMG 13240 CAIM 525	AJ316178 AY254040		42.8	Barcelona, Spain, 1990	Seawater from a aquaculture system
V. ichthyoenteri	1996	LMG 19664 CAIM 597	AJ437192 AJ421445	ATCC 700023 (AFWF00000000, Draft)	43–44	Hiroshima, Japan	Gut of diseased japanase flounder *(Paralichtys olivaceus)*
V. inhibens[c]	2012	DSM 23440 CECT 7692	FN687911		45.6	Northwest Spain	Faeces of wild long-snouther seahorse (*Hippocampus guttulatus*)
V. inusitatus	2007	CAIM 1811 LMG 23434 NBRC 102082	DQ922920	NBRC 102082 (PRJDB349)	43.1–43.7	California, USA, 2005	Gut of the Californian red abalone *(Haliotis rufescens)*
V. jasicida	2012	LMG 25398	AB562589		45.0–47.1	New Zealand, 1999.	Rock lobster (*Jasus verreauxi*)
V. kanaloae	2003	LMG 20539 CAIM 485	AJ316193 AM162657	5S–149 (AJYX00000000, Draft)	44.5	IFREMER, France, 1998	Diseased oyster larvae (*Ostrea edulis*)
V. lentus	2001	LMG 21034 CAIM 907	AJ278881		44	Valencia, Spain	Oysters in the Mediterranean coast
V. litoralis	2007	CAIM 315 DSM 17657 KCTC 12520	DQ097523		NA	Dae-Chun, Korea	Yellow sea tidal flat

Table 36.1 (continued)

Species name	Year proposed	Type strain	16S rRNA accession number	Representative genome sequenced strain (accession number/BioProject and status)	mol% G+C	Place and date of isolation	Source
V. mangrovi	2011	CAIM 1391 LMG 24290	EU144014		45.4	Pichavaran mangroves, Tamil Nadu, India, 2006	Roots of mangrove-associated wild rice (*Porteresia coarctata Takeoka*)
V. marisflavi	2011	LMG 25284 FJ847833		CECT 7928 (PRJNA171491)	42.5	Yellow Sea Cold Water Mass, China	Seawater
V. maritimus	2012	CAIM 1455 LMG 25439	GU929925		46.3	Portinho beach, Sao Paulo, Brazil, 2005	Mucus of apparently healthy coral Palythoa caribaeorum
V. mediterranei	1986	LMG 11258 CAIM 316	X74710	AK1 (ABCH00000000, Draft)	42–43	Valencia, Spain	Coastal seawater
V. metecus[c]	2010	RC341	ACZT101000016	RC341 (ACZT00000000, Draft)	46	Chesapeake Bay, USA	Seawater
V. metschnikovii	1888	LMG 11664 CAIM 317	X74711	CIP 69.14 (ACZO00000000, Draft)	44–46	Asia	Diseased fowl
V. mimicus	1982	LMG 7896 CAIM 602	X74713	MB 451 (ADAF00000000, Draft) VM223 (ADAJ00000000, Draft)	NA	North Carolina, USA	Infected human ear
V. mytili	1993	LMG 19157 CAIM 528	X99761		45.6	Valencia, Spain	Bivalve (*Mytilus edulis*)
V. natriegens	1961, 1981[b]	LMG 10935 CAIM 12	X74714	NBRC 15636 (PRJDB346)	46–47	Sapelo Island, USA	Salt marsh mud
V. navarrensis	1991	LMG 15976 CAIM 609	X74715	08–2462 (PRJNA43075)	45–47	Villa Franca, Navarra, Spain, 1982	Sewage
V. neonatus	2005	LMG 19972 CAIM 1747 JCM 21521	AY426979	JCM 21521 (Unpublished, Draft)	42.1–43.9	Kanagawa, Japan, 1999	Gut of abalone (*Haliotis discus discus*)
V. neptunius	2003	LMG 20536 CAIM 532	AJ316171		46	LCMM, Florianópolis, Brazil, 1998	Bivalve larvae (*Nodipecten nodosus*)
V. nereis	1980, 1981[b]	LMG 3895 CAIM 322	X74716	NBRC 15637 (PRJDB347)	46.4–47.4	Hawaii, USA	Seawater
V. nigripulchritudo	1971, 1981[b]	LMG 3896 CAIM 323 ATCC 27043	X74717	ATCC 27043 (AFWJ00000000, Draft)	46–47	Hawaii, USA	Seawater
V. ordalii	1982	LMG 13544 CAIM 608 ATCC 33509	X74718	ATCC 33509 (AEZC00000000, Draft)	43–44	Washington, USA, 1973	Diseased coho salmon (*Oncorhynchus rhoddurus*)
V. orientalis	1983	LMG 7897 CAIM 332 CIP 102891	X74719	CIP 102891 (ACZV00000000, Draft)	45.6	Yellow Sea, China	Seawater

V. owensii	2010	CAIM 1854 JCM 16517	GU018180	CAIM 1854 (PRJNA174229)	45.3–45.9	Queensland, Australia	Diseased cultured lobster (*Panulirus ornatus)*
V. pacinii	2003	LMG 19999 CAIM 530	AJ316194		44.9	Dahua, China, 1996	Healthy shrimp larvae (*Penaeus chinensis)*
V. parahaemolyticus	1951, 1963, 1980[b]	LMG 2850 CAIM 320	X56580 AF388386	RIMD 2210633 (NC_004603, NC_004605, Complete)	46–47	Japan, 1950	Diseased human
V. parilis[c]	2010	RC586	ABD01000014	RC586 (ADBD00000000, Draft)	46	Chesapeake Bay, USA	Seawater
V. pectenicida	1998	LMG 19642 CAIM 594	Y13830		39–41	Brittany, France, 1991	Diseased bivalve larvae (*Pecten maximus)*
V. pelagius	1971, 1981[b]	LMG 3897 CAIM 324	X74722 AJ293802		45–47	Hawaii, USA	Seawater
V. penaeicida	1995	LMG 19663 CAIM 285	AJ437191 AJ421444		46.2–47.0	Kagoshima, Japan	Diseased kuruma prawn (*Penaeus japonicus*)
V. plantisponsor	2012	CAIM 1392 LMG 24470	GQ352641		41.8	Pichavaran mangroves, Tamil Nadu, India	Roots of mangrove-associated wild rice (Porteresia coarctata Takeoka) Healthy bivalve larvae (Nodipecten nodosus)
V. pomeroyi	2003	LMG 20537 CAIM 578	AJ491290		44.1	LCMM, Florianópolis, Brazil, 1998	Healthy bivalve larvae (*Nodipecten nodosus*)
V. ponticus	2005	CAIM 1731 CECT 5869	AJ630103		NA	Mediterranean coast, Spain, 1986	Gilthead Sea Bream (*Sparus aurata*), Mussels and Seawater
V. porteresiae	2008	CAIM 662 DSM 19223 LMG 24061 MSSRF 30	EF488079	MSSRF 30 (Unpublished, Draft)	44.4	India	Wild rice (*Porteresia coarctata* Tateoka)
V. proteolyticus	1964, 1982[b]	LMG 3772 CAIM 511	X74723	NBRC 13287 (PRJDB344)	50.5	USA	Intestine of isopod (*Limnoria tipunctala*)
V. rarus	2007	CAIM 1812 NBRC 102084	DQ914239	NBRC 102084 (PRJDB342)	43.8	California, USA, 2005	Gut of the Californian red abalone (*Haliotis rufescens*)
V. rhizosphaerae	2007	CAIM 663 LMG 23790	DQ847123		51.0–51.3	Pichavaram, India	Rhizosphere region of mangrove associated Tateoka wild rice (*Porteresia*)
V. rotiferianus	2003	LMG 21460 CAIM 577	AJ316187	DAT722 (AFAJ00000000, Draft)	44.5	ARC, Ghent, Belgium, 1999	Rotifer in recirculation system (*Brachionus plicatilis*)
V. ruber	2003	LMG 21676 CAIM 902	AF462458	LMG 21676 (Unpublished, Draft)	45.8	Keelung, Taiwan	Seawater
V. rumoiensis	1999	LMG 20038 CAIM 598	AB013297	1S-45 (AJYK00000000, Draft)	43.2	Japan	Drain pool of a fish processing plant
V. sagamiensis	2011	NBRC 104589	AB428909	NBRC 104589 (PRJDB343)	43.7–43.8	Sagami Bay, Japan	Seawater

Table 36.1 (continued)

Species name	Year proposed	Type strain	16S rRNA accession number	Representative genome sequenced strain (accession number/BioProject and status)	mol% G+C	Place and date of isolation	Source
V. scophthalmi	1997	LMG 19158 CAIM 75	U46579	LMG 19158 (AFWE00000000, Draft)	45.3	Spain	Turbot juvenile (*Scophthalmus maximus*)
V. sinaloensis	2008	CAIM 797 CECT 7298	DQ451211	DSM 21326 (AEVT00000000, Draft)	NA	Mexico, 2003	Spotted rose snapper (*Lutjanus guttatus*)
V. splendidus	1900, 1981[b]	LMG 19031 CAIM 319	X74724 AJ515230	LGP32 (NC_011744, NC_011753, Complete) 12B01 (AAMR00000000, Draft)	45–46	North Sea	Marine fish
V. stylophorae	2011	LMG 25357	GQ281380		47.8	Kenting, Taiwan	Reef building coral *Stylophora pistillata*
V. superstes	2003	LMG 21323 CAIM 904	AY155585	NBRC 103154 (PRJDB341)	48.0–48.9	Australian Coast, 2000	Gut of abalone (*Haliotis laevigata* and *H. rubra*)
V. tapetis	1996	LMG 19706 CAIM 603	Y08430		43.2	Landeda, France	Clam (*Tapes philippinarum*)
V. tasmanienis	2003	LMG 20012 CAIM 634	AJ316192	1F-267 (AJZO00000000, Draft)	44.7	MPL, Tasmania, Australia	Atlantic salmon (*Salmo salar*)
V. tubiashii	1984	LMG 10936 CAIM 313 ATCC 19109	X74725	ATCC 19109 (AFWI00000000, Draft)	43–45	Milford Connecticut USA	Hard clam (*Mercenaria mercenaria*)
V. variabilis	2011	CAIM 1454 LMG 25438	GU929924		46.8	Preta beach, Sao Paulo, Brazil, 2005	Mucus of apparently healthy coral *Palythoa caribaeorum*
V. vulnificus	1979, 1980[b]	LMG 13545 CAIM 610	X76333	CMCP6 (NC_004459, NC_004460, Complete)	46–48	USA	Human wound infection
V. xiamenensis	2012	DSM 22851	GQ397859		46	Xiamen, Fujian, China	Mangrove soil
V. xuii	2003	LMG 21346 CAIM 467	AJ316181		46.6	Dahua, China, 1995	Shrimp culture water
V. zhanjiangensis[c]	2012	DSM24901	JF931139		38.7	Zhanjiang, China	Shrimp faming pond water
Photobacterium phosphoreum[a]	1878, 1889[b]	LMG 4233 CAIM 328	D25310		41–42	Hawaii, USA	Seawater
P. angustum	1979	LMG 8455 CAIM 908	D25307	S14 (AAOJ00000000, Draft)	40.7–41.3	Hawaii, USA	Seawater
P. aphoticum	2011	CECT 7614	FN796493		NA	Malvarrosa beach, Valencia, Spain, 2008	Mediterranean coastal seawater
P. aplysiae	2005	KCTC 12383	AY781193		45	Mogiyeo, South Sea, Korea	Eggs of Sea Hare (*Aplysia kurodai*)

P. aquimaris	2009	CAIM 1844 KCTC 22356	AB428873		42.2–42.9	Sagami bay, Japan	Seawater
P. atrarenae[c]	2011	KCTC 23265	HM452945		53.6	Jeju Island, Korea	Black sea sand
P. damselae subsp. *damselae*	1982, 1991[b]	LMG 7892 CAIM 331	AB032015	CIP 102761 (ADBS00000000, Draft)	43	USA	Diseased damsel fish (*Chromis punctipinnis*)
P. damselae subsp. *piscicida*	1995[b]	ATCC 51736, NCIMB 2058	X78105	DI21 (PRJNA168653)	41.1–41.3	Japan, 1970	Yellowtail (*Seriola queradiata*) with pseudotuberculosis
P. frigidiphilum	2005	KCTC 12384	AY538749		43.8	Edison seamount, Western Pacific	Deep-sea sediments (1,450 m)
P. gaetbulicola	2010	KCTC 22804	GQ260188		50.6	West coast, Korea	Tidal flat sediment
P. ganghwense	2006	CAIM 512 KCTC 12328	AY960847		44	Ganghwa island, Korea	Seawater
P. halotolerans	2006	CAIM 1804 LMG 22194 CECT 5860	AY551089		49.8	Lake Martel, Mallorca, Spain	Subterranean saline lake water
P. iliopiscarium	1995, 1999[b]	LMG 19543	AB000278 AY643710		39	Norway	Gut of fish
P. indicum	1969, 2004[b]	CAIM 1856 ATCC 19614	AB159513 AB016982		40	Indian Ocean	Marine mud (−400 m)
P. jeanii	2010	CAIM 1817 LMG 25436	GU065210		45.5	Sao Sebastiao channel, Brazil	Mucus of coral (*Palythoa caribaeorum*)
P. kishitanii	2007	CAIM 1852 LMG 23890	AY341439		40.2	Manazuru, Japan, 1982	Light organ of deep-water fish (*Physiculus japonicus*)
P. leiognathi	1967	LMG 4228 CAIM 327	X74686	Svers.1.1 (BACE00000000, Draft)	42–44	Thailand	Leiognathidae fish (Family *Leiognathidae*)
P. lipolyticum	2005	CAIM 15 KCTC 10560BP	AY554009		47	Kaehwa-do, Korea	Intertidal sediment at the Yellow Sea
P. lutimaris	2007	CAIM 1851 KCTC 12723	DQ534014		48.3	Saemankum, Korea	Tidal flat sediment
P. profundum	1998	LMG 19446	D21226	SS9 (NC_006370, NC_006371, NC_005871, Complete)	42	Ryukyu Trench, Japan	Sediment
P. rosenbergii	2005	CAIM 911 LMG 22223	AJ842344		47.6	Magnetic Island, Australia, 2003	Tissue extract of bleached coral (*Pachyseris speciosa*)
P. swingsii	2010	CAIM 1393 CECT 7576	GQ386822		46.7–48.7	Taliarte bay, Gran Canaria, Canary Islands, Spain,	Haemolymph of a healthy spider crab (*Maja brachydactyla*)
Salinivibrio cosicola subsp. *costicola*[a]	1938, 1996[b]	LMG 11651 CAIM 910	X95527 X74699		50	Australia	Bacon curing brine
S. costicola subsp. *alcaliphilus*	2005	ATCC BAA-952 CAIM 741 DSM 16359	AJ640132		49.3	Campania, Italy	Saltish spring with algal mat

Table 36.1 (continued)

Species name	Year proposed	Type strain	16S rRNA accession number	Representative genome sequenced strain (accession number/BioProject and status)	mol% G+C	Place and date of isolation	Source
S. costicola subsp. *vallismortis*	2000	CAIM 735 DSM 8285	AF057016		50	Death Valley, California, USA	Hypersaline pond
S. proteolyticus	2008	CAIM 1855 DSM 19052	DQ092443		49.5	Iran	Bakhtegan hypersaline lake
S. sharmensis	2011	DSMZ 18182	AM279734		51	Ras Mohammed park, Egypt	Saline lake
S. siamensis	2009	JCM 14472 PCU 301	AB285018		49	Thailand	Fermented fish (pla-ra)
Enterovibrio norvegicus[a]	2002	LMG 19839 CAIM 430	AJ316208	FF-33 (AJYD00000000, Draft)	47.1–47.9	AARS, Austevoll, Norway, 1997	Gut of turbot (*Scophthalmus maximus*) larvae
E. calviensis	2002, 2009[b]	LMG 21294 CAIM 595	AF118021	1F-211 (AJYG00000000, Draft)	49.5	Bay of Calvi, France	Seawater of the Mediterranean sea
E. coralii	2005	CAIM 912 LMG 22228	AJ842343		48.2	Magnetic Island, Australia, 2002	Water extract of bleached coral (*Merulina ampliata*)
E. nigricans	2009	CAIM 661 CECT 7320	AM942722		47.9	Catalonia, Spain	Cultured Gilthead seabream (*Sparus aurata*),
Grimontia hollisae[a]	1982, 2003[b]	LMG 17719 CAIM 625	AJ514909		48.5–51.0	Maryland, USA	Human faeces
Aliivibrio fischeri [a]	1889, 2007[b]	LMG 4414 CAIM329	X74702	ES114 (NC_006840, NC_006841, NC_006842, Complete)	39.3 –41.3	Massachusetts, USA, 1933	Dead squid
A. finisterrensis	2010	CECT 7288 LMG 23869	EU541604		38–42	Galicia, Spain, 2004–2005	Manila clam (*Ruditapes philippinarum*)
A. logei	1980, 2007[b]	LMG 19806 CAIM 318	X74708 AJ437616	5S-186 (AJYJ00000000, Draft)	41.2	USA	Gut of arctic scallop
A. salmonicida	1986, 2007[b]	LMG 14010 CAIM 321	X70643	LFI1238 (FM178379-FM178384, Complete)	42	Norway	Diseased atlantic salmon (*Salmo salar*)
A. sifiae	2011	NBRC 10501	AB464964		40.2	Harumi pier, Tokyo, Japan, 2007	Surface seawater
A. wodanis	2000, 2007[b]	LMG 21011	AJ132227		40	Norway, 1988	Salmon (*Salmo salar*) suffering of winter ulcer

[a]Type species of each genus

[b]Year of the latest transfer to new taxa

[c]Species has not yet listed in the List of Prokaryotic names with Standing in Nomenclature (13 December 2012)

mreB, *gyrB*, and *gapA*). More importantly, strains of the same species and species of the same genus will form monophyletic groups on the basis of MLSA or genome comparison.

Historical and Current Status of the Family

Until the middle of the 1900s, bacterial taxonomy was dominated by morphological studies that tried to group strains on the basis of very few phenotypic features, e.g., flagellation, morphology, and curvature of the cells, and cultural aspects. Obviously, classification and identification based on these features ran into several problems. For instance, it realized that the genus *Vibrio* was quite artificial on the basis of morphological and biochemical features and it was concluded that at least three genera existed among the species examined (Davis and Park 1962). In the 7th edition of *Bergey's Manual of Determinative Bacteriology* (Breed et al. 1957), the genus *Vibrio* belonged to the family *Spirillaceae* and consisted of 34 species which, with the exception of *V. cholerae* (= *V. comma*) and *V. metschnikovii*, were later reclassified into other genera, e.g., *Campylobacter* (*C. fetus*, *C. jejuni*, *C. sputorum*), *Comamonas* (*C. terrigena*), and *Pseudomonas* (*P. fluorescens*), or no longer accepted as validly described species according to the Approved List of Bacterial Names (Skerman et al. 1980). The genus *Photobacterium* harbored one species, i.e., *P. phosphoreum*, and was allocated into the genus *Bacterium* of the family *Bacteriaceae* (Breed et al. 1957).

The foundation of the modern vibrio taxonomy was laid by a number of numerical (phenetic) and/or polyphasic taxonomic studies in the 1970s and 1980s (Tubiash et al. 1970; Citarella and Colwell 1970; Colwell 1970; Reichelt and Baumann 1973; Baumann and Baumann 1973, 1977; Fujino et al. 1974; Reichelt et al. 1976; Lee et al. 1981; Baumann et al. 1971, 1980, 1983; Baumann and Schubert 1983; West et al. 1983). The rationale of these studies was to group strains on the basis of their ability to utilize different compounds (ca. 50–150) as sources of carbon and/or energy, enzyme activity (e.g., gelatinase, chitinase, DNase), salt tolerance, luminescence, growth at different temperatures, antibiograms, DNA base composition, morphological features, and other biochemical tests (e.g., oxidase, catalase, Voges–Proskauer, indole, nitrate reduction, arginine dihydrolase, and lysine and ornithine decarboxylases). Phenotypic clusters having about 80 % similarity were found to correspond to DNA–DNA homology clusters having more than 80 % similarity (Baumann and Baumann 1977). This suggests that for the *Vibrionaceae* taxonomy, one should use 80 % DNA–DNA similarity as the limit for species definition instead of the canonical 70 % proposed by Wayne (Wayne et al. 1987).

In the 8th edition of the *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbons 1974), the family *Vibrionaceae*, which was proposed by Véron (1965), comprised *Vibrio* and *Photobacterium* along with *Beneckea*, *Aeromonas*, *Plesiomonas*, and *Lucibacterium*. Baumann (Allen and Baumann 1971) proposed the genus *Beneckea* to encompass vibrios (i.e., *B. campbellii*, *B. neptuna*, *B. nereida*, and *B. pelagia*) isolated from the marine environment which required Na^+ for growth. In subsequent studies, Baumann proposed that *Beneckea* species and *Lucibacterium harveyi* should be reallocated to the genus *Vibrio* (Baumann and Baumann 1977; Baumann et al. 1980, 1983). *Aeromonas* and *Plesiomonas* should be placed into other families and *V. costicola* in another genus (Baumann and Baumann 1977; Baumann et al. 1980, 1983). DNA–DNA relatedness studies among *Vibrio* and *Photobacterium* species underpinned the taxonomy of these groups (Reichelt et al. 1976; Baumann and Schubert 1983; Baumann et al. 1984). These studies revealed a core group of related vibrios, i.e., the *Vibrio harveyi* group consisting of *V. harveyi*, *V. campbellii*, *V. natriegens*, *V. alginolyticus*, and *V. parahaemolyticus*. *V. harveyi* and *V. campbellii* were found to have 61–74 % DNA–DNA similarity, while *V. parahaemolyticus* and *V. alginolyticus* had 61–67 %. Biotypes I and II for each *V. splendidus* and *V. pelagius*, respectively, were proposed (Reichelt et al. 1976), but it was suspected that these biotypes could be in fact different species. The biotypes I and II of *V. splendidus* and *V. pelagius* showed a maximum of 61 % and 58 % DNA–DNA similarity, respectively. These biotypes were clearly distinguishable by phenotypic features. Nevertheless, the biotype designation has been misused still today. Obviously, the two biotypes of *V. vulnificus* should be abolished (Arias et al. 1997b). They should be considered as different species according to the current species definition (Stackebrandt 2002).

On the basis of immunological reaction of antigens and antisera of glutamine synthetase (GS), superoxide dismutase (SOD), and alkaline phosphatase (AP), Baumann et al. (1980, 1983) concluded that Beneckea species, *Photobacterium fischeri* and *P. logei*, should be transferred to the genus *Vibrio* (Baumann et al. 1980); see also the 10th edition of *Bergey's Manual of Systematic Bacteriology* (Krieg and Holt 1984). They also mentioned that they applied a certain "subjective judgment" about the limits of the genus *Vibrio* because they found this genus was highly diverse. Several species, e.g., *V. cholerae*, *V. fischeri*, *V. logei*, and *V. costicola* (now *Salinivibrio costicola*), were distantly related to each other and to the *Beneckea* species. *V. fischeri* and *V. logei* are still considered members of the genus *Vibrio*.

According to the *Bergey's Manual of Systematic Bacteriology* (2002), the family *Vibrionaceae* comprises six genera, i.e., *Allomonas* (1 sp.), *Catenococcus* (1 sp.), *Enterovibrio* (1 sp.) *Listonella* (2 spp.), *Photobacterium* (6 spp.), *Salinivibrio* (1 sp.), and *Vibrio* (59 spp.). The genera *Allomonas* (Kalina et al. 1984) and *Enhydrobacter* (Staley et al. 1987) were tentatively allocated to the family *Vibrionaceae* based on phenotypic features. *Allomonas enterica* and *V. fluvialis* are very similar in the 16S rRNA sequence, DNA–DNA similarity, and phenotypically and therefore have been suggested to be a junior synonym of the latter species (Farmer 1986). It was suggested that *Enhydrobacter aerosaccus* belongs to the family *Moraxellaceae* due to the high phenotypic and 16S rRNA sequence similarity with *M. osleoensis* (Thompson 2003).

Thompson and coworkers (Thompson 2003; Thompson et al. 2004a, b) suggested that the current family *Vibrionaceae* should be split into four families, *Enterovibrionaceae*, *Photobacteriaceae*, *Salinivibrionaceae*, and *Vibrionaceae* (Supplementary Table 36.1). The new family *Vibrionaceae* would then

Supplementary Table 36.1

Characteristics of *Vibrionaceae* related families (Thompson et al. 2004c) based on phylogenetic analyses of concatenated 16S rRNA, *recA* and *rpoA* gene sequences

	Enterovibrionaceae	*Photobacteriaceae*	*Salinivibrionaceae*	*Vibrionaceae*
Genera and number of species	*Enterovibrio* (2)	*Photobacterium* (10)	*Salinivibrio* (1)	*Vibrio* (64)
	Grimontia (1)			*Listonella* (2)[a]
D-mannitol fermentation	–	–	V	V
Voges-Proskauer	–	V	+	V
Indole	V	–	–	V
Arginine dihydrolase	V	+	–	V
Ornithine decarboxylase	–	–	–	V
PHB accumulation	–	+	–	V
Growth at				
20 % NaCl	–	–	+	–
45 °C	–	–	+	–
Presence of				
16:1 ω9c	+	–	+	V
18:1 ω9c	+	–	–	V

[a]It is commonly accepted that *Listonella* is no longer a valid genus

comprise only species of the genus *Vibrio*. The splitting of vibrios into four families was based on 16S rRNA, *rpoA*, *recA*, and *pyrH* gene sequences and phenotypic features.

"*Vibrionales*" is used in the most recent outline of the *Bergey's Manual of Systematic Bacteriology*, 2nd edition (2005), and consisted of a single family *Vibrionaceae* comprising three genera, *Vibrio* (44 spp.), *Photobacterium* (6 sp.), and *Salinivibrio* (1 sp.), based on 16S rRNA phylogeny (Farmer and Janda 2005). At current, the numbers of *Vibrionaceae* species increase to nearly 2.5-folds (*Table 36.1*) than those described in the 2005 year Bergey's edition; the phylogenetic picture must be considered based on not only single 16S rRNA phylogeny but also multilocus gene phylogeny and/or phylogenomic approach (supertree). One challenge to elucidate evolutionary history in *Vibrionaceae* is conducted based on MLSA, and at least 14 distinctive clades are proposed (Sawabe et al. 2007a) (see also Phylogenetic Structure of the Family and Its Genera section).

For up-to-date status on the number and changes of the approved species of vibrios, please consult the List of Bacterial Names with Standing in Nomenclature (http://www.bacterio.cict.fr/index.html) and the Bacterial Nomenclature up-to-date at the German Collection of Microorganisms and Cell Cultures (http://www.dsmz.de/bactnom/bactname.htm).

Phylogenetic Structure of the Family and Its Genera

The phylogenetic structure of vibrios has been laid in the early 1990s (Dorsch et al. 1992; Kita-Tsukamoto et al. 1993; Ruimy et al. 1994). The almost complete 16S rRNA sequences of 10 *Vibrio* species revealed a *Vibrio* core group (*V. harveyi*-related species) and also showed that *V. hollisae* should be allocated into a new genus (Dorsch et al. 1992). A comprehensive phylogenetic study of the *Vibrionaceae* was accomplished by Kita-Tsukamoto (Kita-Tsukamoto et al. 1993) and coworkers. They sequenced a fragment of the 16S rRNA sequences (around 450 nt) of 50 species, including most known vibrios, and species of *Aeromonas*, *Deleya*, *Escherichia*, *Marinomonas*, *Pseudomonas*, and *Shewanella*. The main outcomes of this study were (i) the circumscription of species (at least 99.3 % 16S rRNA similarity), genera (95–96 %), and family (90–91 %) borders within the *Vibrionaceae* and (ii) the delineation of seven main groups of *Vibrionaceae* species that would correspond to different genera or families. Subsequently, *V. costicola* was transferred into *Salinivibrio costicola* (Mellado et al. 1996), *V. marinus* into *Moritella marina* (Urakawa et al. 1999), and *V. iliopiscarius* into *Photobacterium iliopiscarius* (Urakawa et al. 1998). *V. hollisae* was transferred into *Grimontia hollisae* (Thompson et al. 2003). Genus "*Listonella*" is proposed as a later heterotrophic synonym based on the 16S rRNA gene phylogeny and genome features (Thompson et al. 2011), and now *V. anguillarum* and *V. plagius* should be used in place of *L. anguillarum* and *L. pelagia*.

A consensus view emerged from these studies: vibrios were highly heterogeneous. According to Kita-Tsukamoto, *V. cholerae* and *V. mimicus* would correspond to a genus on their own (Kita-Tsukamoto et al. 1993). *V. fischeri*, *V. logei*, *V. salmonicida*, and relatives should be elevated to the genus rank. In both cases, the status of these *Vibrio* species has not yet been fully determined. If *V. cholerae* and *V. mimicus* and the *V. fischeri*-related group are to be elevated to the genus level, then one might argue the revival of *Beneckea* to encompass all other remaining

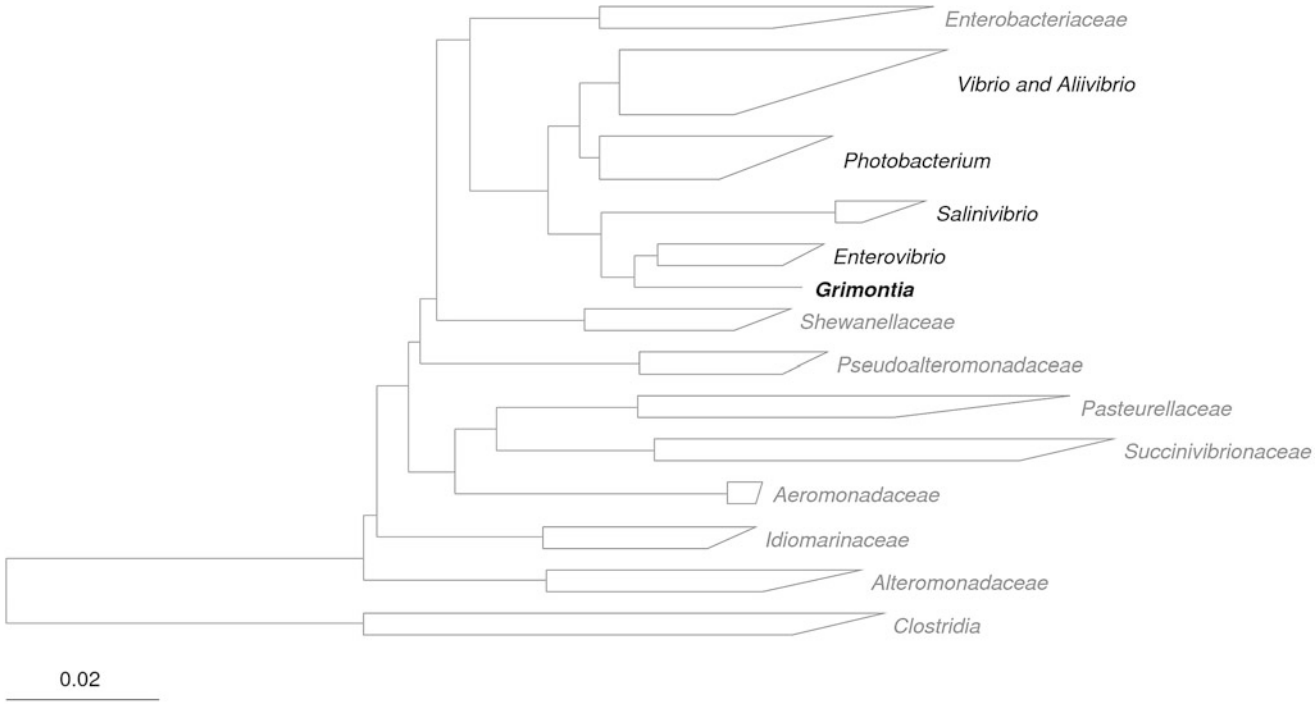

Fig. 36.1

Phylogenetic reconstruction of the family *Vibrionaceae* based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes-Cantor correction. The sequence datasets and alignments were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high quality type strain sequences proportionally distributed among the different bacterial and archaeal phyla. Scale bar indicates estimated sequence divergence

vibrios, an idea which was originally laid down by Bauman (Allen and Baumann 1971). As recently *V. fischeri*, *V. logei*, *V. salmonicida*, and *V. wodanis* are transferred into *Aliivibrio* gen. nov. by 16S rRNA gene phylogeny, MLSA, and phenotypic characterization (Urbanczyk et al. 2007); more accurate phylogenetic pictures for *Vibrionaceae* must be reconstructed.

The most recent phylogenetic tree for all currently known 131 *Vibrionaceae* species and one describing species (*Vibrio tritonius* sp. nov.) based on 16S rRNA gene according to the ALL-Species Living Tree Project (LTP) database (Yarza et al. 2010) is shown in ❯ *Figs. 36.1–36.3*. All species are belonged into a single cluster forming *Vibrionaceae* on the basis of the well-cured LTP database and neighbor-joining algorithm (❯ *Figs. 36.1–36.3*). The family *Vibrionaceae* is moderately related to the family *Enterobacteriaceae* (❯ *Fig. 36.1*). *Shewanellaceae*, *Pseudoalteromonadaceae*, *Aeromonadaceae*, *Pasteurellaceae*, and *Succinivibrionaceae* appear as sister clades of these two families, while clades of *Alteromonadacea* and *Idiomarinaceae* are branching slightly deeper. In more detail pictures of these genera, each genus of *Photobacterium*, *Enterovibrio*, *Grimontia*, and *Salinivibrio* seems to form each cluster (❯ *Fig. 36.3*). However, the genus *Aliivibrio* is nested within the cluster of the genus *Vibrio* (❯ *Fig. 36.2*); nevertheless, *V. fischeri*, *V. logei*, *V. salmonicida*, and *V. wodanis* are reclassified as *Aliivibrio* gen. nov. recently (Urbanczyk et al. 2007). In our further analysis on the phylogeny of the genus *Aliivibrio* using the latest 16S rRNA gene sequence data set, the genus formed a robust clade (>99 % bootstrap support by NJ; MP and ML), but it is located at the terminal branch of *Vibrio halioticoli* and/or the related species clusters. So, on the basis of the 16S rRNA gene tool, it is hard to say each genus in *Vibrionaceae* is supported as a robust clade; it seems to be polyphyletic.

New phylogenetic insights have been obtained by other chronometers (Zeigler 2003; Lerat et al. 2003). A comprehensive list containing the so-called bacterial core genes, which may be useful for phylogenetic analyses of vibrios, has been compiled (Gevers et al. 2000). As expected from these in silico studies, phylogenies based on 23S rRNA (Macián et al. 2000a, b), *gyrB* (Faury et al. 2004), *hsp60* (Kwon et al. 1995), *recA* (Thompson et al. 2004), and *rpoA* (Thompson et al. 2005b) correlate well with the 16S rRNA tree of vibrios. Concatenated sequence analysis of the *recA*, *rpoA*, 16S rRNA, and *pyrH* provide more clear separation of clusters belonging into genera *Vibrio*, *Photobacterium*, *Enterovibrio*, *Grimontia*, *Salinivibrio*, and *Aliivibrio* with higher robustness. For 16S rRNA values above 98 %, there was a wide range of *recA* similarities (Thompson et al. 2004b), varying from 83 % to 99 %. *Grimontia hollisae* and *Photobacterium* were distantly related to *Vibrio* (66.3 % and 70.5 % *recA* sequence similarity, respectively). *rpoA* and *recA* gene sequences suggest

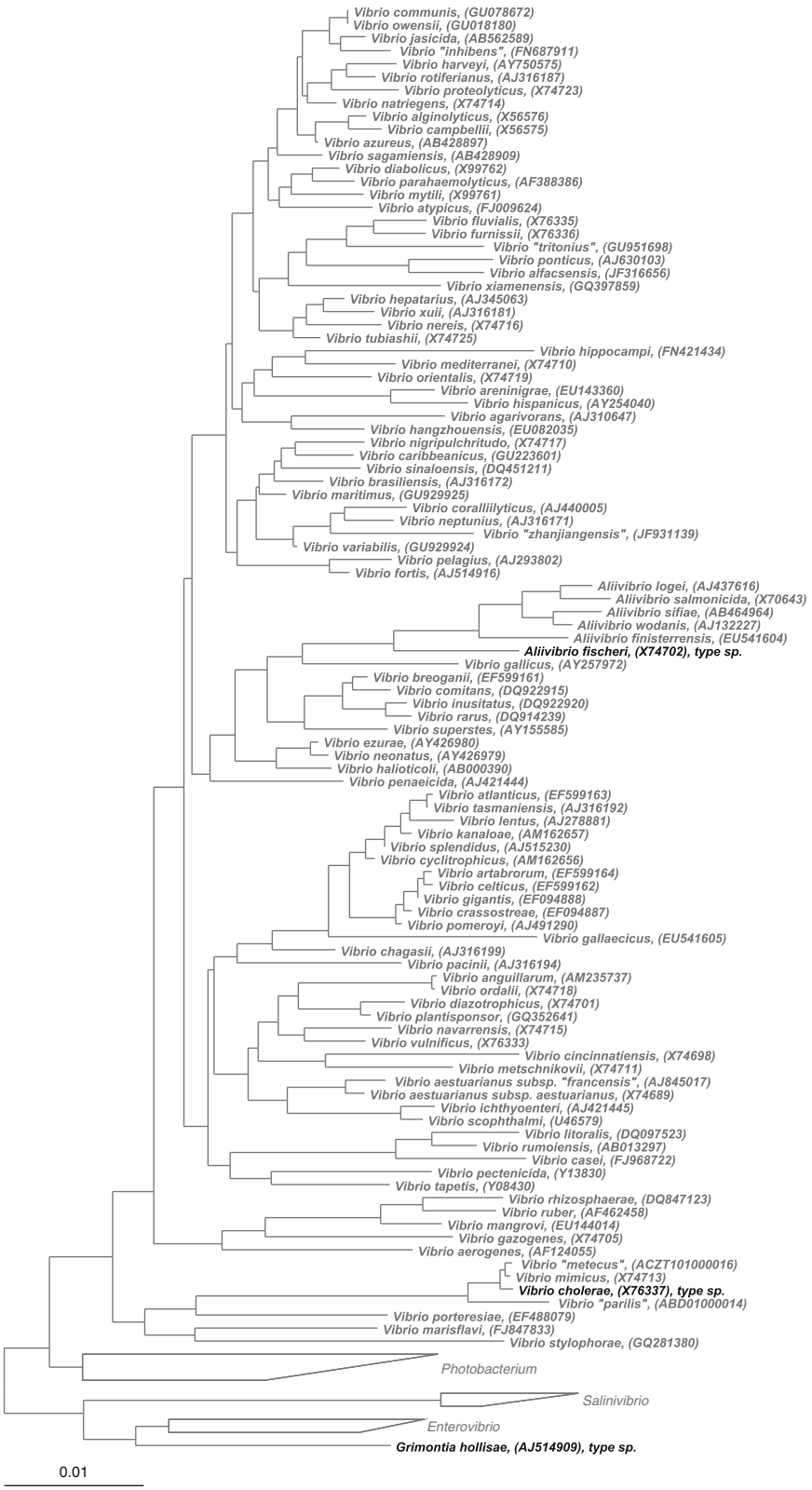

Fig. 36.2

Phylogenetic reconstruction of the genera *Vibrio* and *Aliivibrio* based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes-Cantor correction. The sequence datasets and alignments were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high quality type strain sequences proportionally distributed among the different bacterial and archaeal phyla. Scale bar indicates estimated sequence divergence

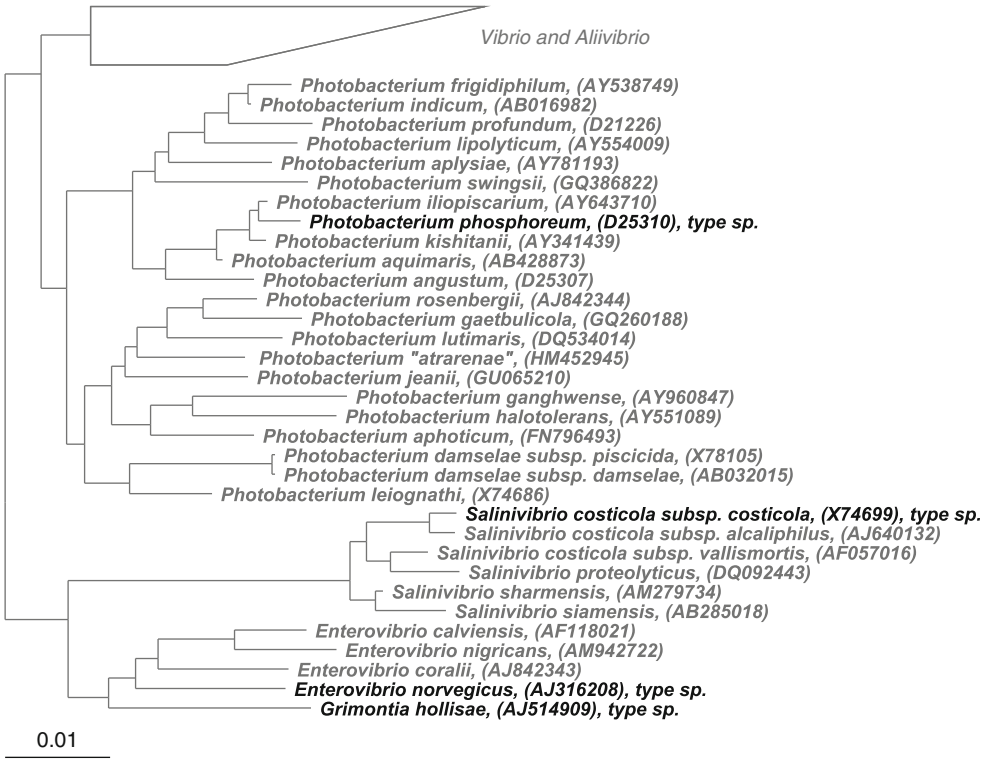

Fig. 36.3

Phylogenetic reconstruction of the genera *Photobacterium*, *Salinivibrio*, *Enterovibrio* and *Grimontia* based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes-Cantor correction. The sequence datasets and alignments were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree). The tree topology was stabilized with the use of a representative set of nearly 750 high quality type strain sequences proportionally distributed among the different bacterial and archaeal phyla. Scale bar indicates estimated sequence divergence

that *V. fischeri*-related species are more closely related to *Photobacterium* than to *Vibrio*. This analysis was a fundamental data for the proposal of four kinds of family nov. (*Vibrionaceae*, *Photobacteriaceae*, *Salinivibrionaceae*, and *Enterovibrionaceae*) for vibrio taxonomy (Thompson et al. 2004a, Supplementary *Table 36.1*).

Sawabe et al. (2007a) propose the use of a split decomposition analysis using multilocus gene sequence data set to define more robust clades in *Vibrionaceae*. Based on nine genes (i.e., *ftsA*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, *rpoA*, *topA*, and 16S rRNA) multilocus sequence analysis of 78 type strains belonged to *Vibrionaceae*; at least 14 monophyletic clades are found with a significant bootstrap support. The species within each clade shared >20 % DDH, <5 % GC variation (mol %), >85 % MLSA sequence similarity, and >89 % average amino acid identity (AAI) (Sawabe et al. 2007a). The analysis is capable of an elucidating minimum evolutionary unit in *Vibrionaceae* as the "clade," but more robust phylogenetic relationships among the clades are remained to be veiled.

The most recent MLSA phylogenetic tree based on eight housekeeping genes (*ftsA*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, *rpoA*, *topA*), which are available currently from 85 strains of *Vibrionaceae* (including 79 known and describing species), is shown in *Fig. 36.4*. At least two distinct clades were found; one involved the genera *Photobacterium* and *Aliivibrio* nesting within the large *Vibrio* species, and the other was robust clade formed by the genera *Enterovibrio* and *Grimontia*. While we still face lack of gene data in *Photobacterium* spp., *Salinivibrio* spp., and *V. gazogenes* and the related species, the MLSA results shown in *Fig. 36.4* could also make a good elucidation of the presence of at least 18 robust clades supported by three distinctive phylogenetic analyses (NJ with >90 % bootstrap support, ML, and MP), but still lack the phylogenetic interconnections between those robust clades. The MLSA approach also provided possible taxonomic positions among recently genome sequenced *Vibrionaceae* strains, *Vibrio* genomosp. F6 and F10 (Preheim et al. 2011) (details are described in "Ecology" section), EJY3 (Roh et al. 2012), Ex25, N418, LGP32, and *V.* '*tritonius*' AM2 (Sawabe et al. 2013). There are still many orphan species (*V. proteolyticus*, *V. nigripluchritude*, *V. meditenanei*, *V. tapetis* and so on). Further descriptions of the closest neighbor species to these orphans could reveal better picture of the evolutionary tree and/or network in *Vibrionaceae*.

In conclusion, the introduction of MLSA or genome base taxonomy on *Vibrionaceae* could make new promising future insights into the better and fine-scale solutions in the vibrio taxonomy and phylogeny.

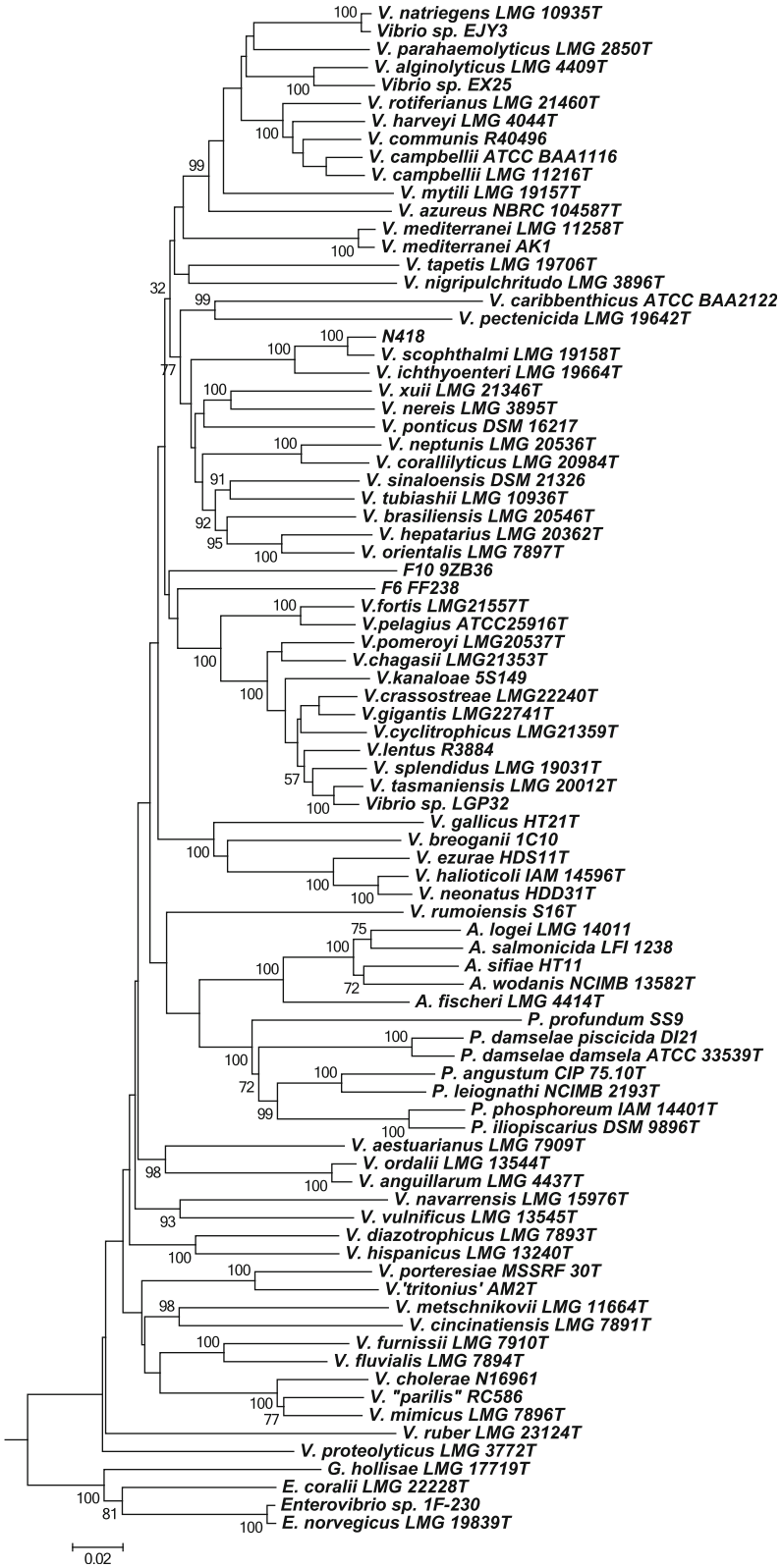

Fig. 36.4

The molecular phylogenetic tree for *Vibrionaceae* using the most recent nucleotide sequences based on 8 gene multilocus gene sequences. The evolutionary history was inferred using the Neighbor-Joining (NJ) method, the Maximum Likelihood (ML) method, and the Maximum Parsimony (MP) method. Based on the optimal tree with the sum of branch length = 5.00019540 by NJ, nodes supported by ML (the highest log likelihood (−136166.1863)) and MP are combined. These nodes are only shown with the bootstrap (1,000 replicates)

New Era of Genomic Taxonomy of Vibrios: 1970s Numerical Taxonomy → 1980s Polyphasic Taxonomy → 2000s Genomic Taxonomy

Whole microbial genome sequence launched microbial taxonomy into a new era, with the possibility of establishing systematics on the basis of complete genomes, called genomic taxonomy (Coenye and Vandamme 2003, 2004; Coenye et al. 2005). The genomic taxonomy is defined on the basis of comparative genomics, including multilocus sequence analysis (MLSA), supertree, average amino acid identity (AAI), average nucleotide identity (ANI), genomic signatures, codon usage bias, metabolic pathway content, and core and pan-genome analysis. In silico methods for the comparison of genome sequences can be used to replace the DNA–DNA hybridization (DDH), the gold standard technique for species definition in prokaryotes (Auch et al. 2010). The main goal of the genomic taxonomy is to extract taxonomic and phylogenetic information from WGS that can be used to establish a solid approach for the identification and classification of prokaryote species.

In Vibrios, the birth of the genomic taxonomy occurred with a series of papers that attempted to use multilocus sequence analysis (MLSA) (Sawabe et al. 2007a; Thompson et al. 2005b, 2006, 2007a, b, 2008). These studies allowed the establishment of rapid and powerful identification systems through the Internet. Currently there are MLSA schemes for most of the human pathogens. Establishing a universal MLSA will not be possible though. Studies accomplished so far have shown that the resolution of different markers varies according to the taxonomic groups. For instance, the *recA* gene is very useful to differentiate closely related species of *Burkholderia* (Payne et al. 2005), but it is not appropriate for some closely related vibrio species (Thompson et al. 2007b). Clearly, genes have different molecular clocks in different microbes, indicating the need of a multigene approach. With the advent of ultra-rapid genome sequencing, it is now possible to sequence one almost complete microbial genome in few hours (Andersson et al. 2008; Eid et al. 2009). The new generation of DNA sequencers will enable sequencing of more than a dozen prokaryotic genomes in less than an hour, possibly making it cheaper and faster to sequence a whole genome than several genes for MLSA. In the future, MLSA might be used simply as a rapid screen methodology (Mahenthiralingam et al. 2006).

Currently, there are 24,788 prokaryotic genomes and 510 *Vibrionaceae* genomes sequenced (National Center for Biotechnology Information, NCBI, http://www.ncbi.nlm.nih.gov/sites/genome). With the increase of sequenced *Vibrionaceae* genomes, it is possible to establish the Vibrio taxonomy on the basis of complete genomes. Recently, Thompson et al. (2009) utilized genome sequences from 43 Vibrios strains, including 14 species, to define what constitutes a *Vibrio* species based on a set of specific genomic criteria, including MLSA, AAI, Karlin's signature, codon usage bias, proteome identity, and core and pan-genome analysis. Overall there was a significant correlation between the different methods (*Table 36.2*), but some methods had a stronger evolutionary signal and different taxonomic resolution than others. For instance, AAI and supertree showed the closest correlation with MLSA. All methods, except 16S rRNA and codon usage, provided significant ($P < 0.001$; T test) taxonomic resolution for differentiation of species and genera of vibrios. In general, the taxonomic resolution of 16S rRNA and codon usage was restricted to differentiation of genera. The AAI and the proteome matrix correlated well. Because the Karlin's genomic signature dissimilarity indexes genome-wide variation, its phylogenetic resolution is distinct of individual genetic marker genes. In addition, this signature considers variation in both coding and noncoding genomic regions. This may explain why

Table 36.2
Pearson correlation coefficient (expressed as percentage) between different methods (Extracted from Thompson et al. 2009)

	1	2	3	4	5	6
1. I6S rRNA gene identity	100					
2. Identity in MLSA	86.5	100				
3. Identity in supertree analysis	91.1	98.4	100			
4. Average aminoacid identity (AAI)	85.9	97.7	96.9	100		
5. Karlin genome signature dissimilarity	71.5	85.3	82.3	84.9	100	
6. BLAST proteome identity	77.1	89.0	86.4	92.5	85.5	100

Fig. 36.4 (continued) value next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The analysis involved 86 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 4,486 positions in the final dataset. Evolutionary analyses were conducted in MEGA5

Supplementary Table 36.2
Phenotypich characterization of *Vibrionaceae*

Species	Cellular			Colonial characteristics				Growth with NaCl (%)						Growth at temperature (°C)								
	Flagella	Straight rods	Motility	Swarming	Luminiscence	Pigment	Green coloies on TCBS	0	2.5	6	8	10	12	4	20	30	35	40	Oxidase	Nitrate reduction	Indole production	Voges-Proskauer
E. norvegicus	+		+		–		+		+						+				+	–	+	–
E. coralii			+				+	–	+	+	–	–	–	–	+	+		–	+	+	–	
G. hollisae			–	–	–	–	+	–	+	v	–	–	–				+		+	+	+	–
P. angustum				–	–			–	–	–	–	–		+	+	+	+	–	v	v	–	v
P. aplysiae	+		+						+						+	+			+	+	–	
P. damselae					–		+	–	+	+	–	–	–				+		+	+	–	+
P. frigidiphilum	+		+		–				+					6	+				+	+	?	
P. iliopiscarium	–					–						–		+			–			+		
P. leiognathi				–	–				–	–	–	–		–	–	+	–	–	v	–	–	–
P. lipoliticum	+		+					–	+	+	–			+		+	–		+	+	+	
P. phosphoreum	–			–	+	–		–	+	v	–			d	+	+	–	–	–	+	–	–
P. profundum	+		+		–			–	+	–				+	10	–	–	–	+	+	+	
P. rosenbergii		+	+				–	–	+	+	–	–	–	–	+	+		–	+	+	–	
S. costicola	+	–	+		–				+	+	+	+	+	+	+	+	+	+		–	–	+
V. aerogenes	+		+			+		–	+	+	+	–		–	+	+	+	–	–	+	+	–
V. aestuarianus	–	–	–	–	–	–		–	+	V	+	–		d	+	d	+	–	+	d	–	–
V. agarivorans	+		+	–	–	–	+	–	+	+				–	+	+	+	–	+	+	–	–
V. alginolyticus	+	+	+	+	–	–	–	–	+	+	+	+	+	–	+	+	+	+	+	+	+	+
V. anguillarum	–	d	+	–	–	–	+	d	d	+	d	–		–	–	d	+	–	+	+	+	d
V. brasiliensis	+		+		–		+	–	+		–			–		+			+	+	+	+
V. calviensis	+		+		–	+	+	–	+	+	+	+	+	+	+	+	+	–	+	+	–	–
V. campbellii	+	+	+	–	–	–	+	–	d	+	v	–		–	+	–	+	–	+	+	–	–
V. chagasii	+		+	–			+	–								+						
V. cholerae	–	d	+	–	v	–	–	+	+	v	–	–	–	–	+	+	+	+	+	+	d	+
V. cincinnatiensis	–	+	–	+	–	–	–	–	+	+	d	v	–	–		d	+	d	+	+	–	d
V. coralliilyticus	+		+		–		–		+		–					+						
V. crassostreae	+		+	–			–	–			–			+					+	+		
V. cyclitrophicus	+		+		–				+	+	+	+		+	+	+	+	–				
V. diabolicus	+	+	+	+	–	–			+							+	+	+	+	+	+	
V. diazotrophicus	+	–	+	–	–	–		–	+	+	+	v	–	–		–	+	v	+	+	d	–
V. ezurae	–	+	–	–	–	–	–		+	+				–	+	+		–	+	+	+	
V. fischeri	+	+	+	–	+	+		–	+	+	–	–		–	–	+	v	–	+	d	–	–
V. fluvialis	d	d		–	–	–	–	v	+	+	d	d	–	–	–	+	+	+	+	+	+	–
V. fortis	+		+	–			v	–	+	+	+			+	+	+	+		+		+	
V. furnissii	+	–	+	–	–	–	–	d	+	+	d	–	–	–	–	+	+	d	+	+	d	–

Supplementary Table 36.2 (continued)

Species	Cellular			Colonial characteristics				Growth with NaCl (%)						Growth at temperature (°C)								
	Flagella	Straight rods	Motility	Swarming	Luminiscence	Pigment	Green colies on TCBS	0	2.5	6	8	10	12	4	20	30	35	40	Oxidase	Nitrate reduction	Indole production	Voges-Proskauer
V. gallicus	–		–		–			–	+						+	+			+	+	+	
V. gazogenes	+	–	d	–	–	+		–	+	+	+	d	v	–	–	+	+	+	–	–	d	d
V. halioticoli	–		–	–	–	–			+	–				–	+	+	–	–	+	+	+	
V. harveyi	+	+	+	–	v	–	v	–	+	+	+	d		–	+	+	+	v	+	+	+	–
V. hepatarius	+		+	–			–	+	+	+	+			+	+	+	+		+	+	+	
V. hispanicus	+		+	–	–		–	+	+	+	+	+	–	+		+	+	+	+		+	–
V. ichthyoenteri	+		+					–	+	+	–			–	+	+	–	–	+	+	–	–
V. kanaloae	+		+	–			–	–						+								
V. lentus	+	+	+	–	–	–	+	–		+				v	+	+	+	–	+	+	v	–
V. logei	+	+	+	–	+	+		–	+	–	–	–		+	–	–	–	–	+	+	–	–
V. mediterranei	–	+	–	–	v	+		d	+	+	v	–		–	–	+	+	–	+	+	–	–
V. metschnikovii	+	d	d	–	–	–	–	d	+	+	v	d	–	–	+	+	+	d	–	–	d	+
V. mimicus		–	+	–	–	–	+	+	+	v	–	–	–	–		+	+	v	+	d	d	–
V. mytili	+		+	–	–	–	–	–	+	+	+	+	–	–	+	+	+	–	+	+	–	–
V. natriegens	–	+		–	–	–		–	+	+	v	–		–	+	+	+	v	+	+	–	–
V. navarrensis	+		+	–	–	–	–	–	+	+		–				+	+	+	+	+	+	–
V. neonatus	–	+	–	–	–	–	+	–	+	–				–	+	+		–	+	+	+	
V. neptunius	+		+	–	–		–	–	+		–			–		+		–	+	+	+	+
V. nereis	–	+		–	–	–		–	v	+	v	v	–	d	+	+	+	v	+	+	+	–
V. nigripulchritudo	–	+	+	–	–	+		v	+	d	v	–		–	+	+	–	–	+	+	d	–
V. ordalii	–	d		–	–	–		–	+	+	–	–		–	+	+	–	–	+	–	–	–
V. orientalis	–	–		–	+	–		–	–	–	–	–		+	–	+	+	–	+	d	–	–
V. pacinii	+		+	–	–		–	–	+	+	+	–	–	+	+	+	v	–	+	+	–	V
V. parahaemolyticus	+	+	+	v	–	–	+	–	+	+	+	–		–	+	+	+	+	+	+	+	–
V. pectenicida	+		+	+	–				+	+	–	–			+				+	+	–	–
V. pelagius biovar I	–	+		–	–	–		–	+	+	+	–		v	+	+	+	–	+	+	–	–
V. pelagius biovar II	–	–		–	–	–		–	+	+	v	–		v	+	+	+	–	+	+	–	–
V. penaeicida					–		ng	–	+	–	–			–	+	+	–	–	+	+	–	–
V. pomeroyi	+		+	–			–	+						+								
V. ponticus		+			–		–	–	–	–	–	+	+	–	+	+	+	–	+	+	+	–
V. proteolyticus	+	+	+	+	–	–		d	+	+	+	d	+	–	+	+	+	+	+	+	d	+
V. rotiferianus	+		+	–	–	–	–		+	+	–	–		–		+	+	+	+		+	–
V. ruber	+		+	–	–	+			+	+	+	+		–	+	+	+	+	–	+	–	
V. rumoiensis	–		–			–			+	+				+	+	+			+	+	–	–
V. salmonicida	+	–	+	–	–			–	v	–	–	–		d	–	–	–	–	+	–	–	–
V. scophthalmi			+		–		–	–	+	–	–	–		–	+	+	+	–	+	+	–	–

Supplementary Table 36.2 (continued)

Species	Cellular			Colonial characteristics				Growth with NaCl (%)						Growth at temperature (°C)								
	Flagella	Straight rods	Motility	Swarming	Luminiscence	Pigment	Green coloies on TCBS	0	2.5	6	8	10	12	4	20	30	35	40	Oxidase	Nitrate reduction	Indole production	Voges-Proskauer
V. splendidus biovar I	–	–	–	–	+	–		–	+	v	v	–		d	+	+	v	–	+	d	–	–
V. splendidus biovar II	d	d	+	–	–	–		–	+	v	v	–		–	+	+	–	–	+	d	v	–
V. succinogenes	+		+			+										+	+			+		
V. superstes	–		–		–	–				–					+	+		–	+	+	–	
V. tapetis	+		+	–		–	+	–	+	–	–	–		+	+	–	–		+	+	+	–
V. tasmaniensis	+		+	–			+	–	+	+	+	–		+	+	+	+		+	+	+	
V. tubiashii	+	–	+	–	–	–		–		v	v	–		–		+	+	–	+	+	+	–
V. vulnificus biovar I	–	–	d	–	–	–	+	–	d	d	–	–		–	–	–	+	d	+	d	–	–
V. vulnificus biovar II					–		+	–	–	+	–	–		–	–	–	+		+	–	–	–
V. wodanis	+	–	+	–	–				+	d					+				+		+	–
V. xuii	+		+	–	–		–	–	+	+	+	+		–		+			+	+		+

Species	Gas from D-glucose	Susceptibility		Amino acid utilization				Enzyme production					Utilization as sole source of carbon (Biolog GN2)									
		0/129 (10μg)	0/129 (150 μg)	Arginine dihydrolase	Lysine decarboxylase	Ornithine decarboxylase	Tyrosine	Gelatinase	Lipase	Amylase	Alginase	Chitinase	Ac sucrose	Util propionate	2, 3-butanediol	2-amino ethanol	Acetic acid	Adonitol	Alaninamide	Alpha-cyclodextrin	Alpha-D-glucose	Alpha-D-lactose lactulose
E. norvegicus			–	+				–						–			+				+	
E. coralii			–	+	–	–		–	–				+		–	–	+	–	+		+	+
G. hollisae	–	v		–	–	–	–	–	–				–									
P. angustum	–	–	–	–	v	–							v									
P. aplysiae				+				+	+													
P. damselae	–	+		+	–	–		–	–				–	–								
P. frigidiphilum		+	+	+					+												+	
P. iliopiscarium	+		+	+				–														
P. leiognathi	–	–	–	+	v	–							–								+	
P. lipoliticum	–			–	–		–	–		+			+				+					
P. phosphoreum	+	–	–	+	+	–		–	–	–			–								+	
P. profundum	–	–	–	+	–	–			+									–			+	–
P. rosenbergii			–	+	–	–		–	+						–	–	+	–	+	(+)	+	–
S. costicola		–		+	–	–	–	+	v				+	+				–				

Supplementary Table 36.2 (continued)

Species	Gas from D-glucose	Susceptibility		Amino acid utilization				Enzyme production					Utilization as sole source of carbon (Biolog GN2)									
		0/129 (10μg)	0/129 (150 μg)	Arginine dihydrolase	Lysine decarboxylase	Ornithine decarboxylase	Tyrosine	Gelatinase	Lipase	Amylase	Alginase	Chitinase	Ac sucrose	Util propionate	2, 3-butanediol	2-amino ethanol	Acetic acid	Adonitol	Alaninamide	Alpha-cyclodextrin	Alpha-D-glucose	Alpha-D-lactose lactulose
V. aerogenes	+	–	–	+	–	–		+	+	+			+				–	–				
V. aestuarianus	–	–	–	+	v	–		+		–			+									
V. agarivorans	–		+	–	–	–		–		–						–		–			+	
V. alginolyticus	–	d	d	–	+	+	+	+	+	+	–	+	+	+			+				+	
V. anguillarum	–	+	d	+	–	–	–	+	+	+	–	+	+	+			+				+	
V. brasiliensis		–	–	+	–	–		+					+		–	–		–	–		+	–
V. calviensis	–	+	+	–				+		–												
V. campbellii	–	d	+	–	+	–	d	+	+	+	–	+	–	+			d				+	
V. chagasii																						–
V. cholerae	–	+	d	–	+	+	–	+	+	+	–	+	+	+			+				+	
V. cincinnatiensis	–	d	d	–	+	–	–	–	–	+	–	+	+				+				+	
V. coralliilyticus			+	+										+			+		+			
V. crassostreae		+		+				+										–				
V. cyclitrophicus	–			+				+	+		–			–			+				+	
V. diabolicus	+		+	–	+	+		+	+	+		+	+								+	
V. diazotrophicus	–	v	–	+	–	–		–	d	+	–	–	+	v			+				+	
V. ezurae	–		+	–	–	–	–	–	–	–		–					–					
V. fischeri	–	+	–	–	+	–	–	–	+	–	–	d	–	–			–				d	
V. fluvialis	d	+	d	+	–	–	+	+	+	+	d	+	+	+			+				+	
V. fortis				–	–	–									–	–		–			+	
V. furnissii	+	d	d	+	–	–		+	+	+	–	+	+	+			d				+	
V. gallicus	–			+	–	–	–	–			+	–					–				+	
V. gazogenes	+	+	+	–	–	–	–	+	+	+	–	–	+	+			+				+	
V. halioticoli	–		+	–	–	–	–	–	–	–	+	–	–	–			–				+	
V. harveyi	–	v	d	–	+	+	d	+	+	+	v	+	v	+			+				+	
V. hepatarius					–	–									–	–	–	–	–		+	–
V. hispanicus	–	+	+	+	–	–	–	–							–	–	–	–	–		+	–
V. ichthyoenteri	–			–	–	–		–	–	–	–	–						–			+	
V. kanaloae				+				+					+									
V. lentus	–		–		–	–		+		+	v										+	
V. logei	–	v	d	–	+	+	–	–	d	–	–	+	v	–			–				+	
V. mediterranei	–			d	v	–		–	+			–	+	–								
V. metschnikovii	–	d	d	–	v	–	–	+	+	+	–	+	+	–			+				+	
V. mimicus	–	v	d	–	+	+		+	+				v	–			d				+	
V. mytili	+		+	+	–	–	+	–	+	–			+									
V. natriegens	–	–	v	–	–	–	d	+	+	d	–	–	+	+			+				+	
V. navarrensis	–		+	–	–	–		+		+			+	–		–		–			+	

Supplementary Table 36.2 (continued)

Species	Gas from D-glucose	Susceptibility		Amino acid utilization				Enzyme production					Utilization as sole source of carbon (Biolog GN2)									
		0/129 (10μg)	0/129 (150 μg)	Arginine dihydrolase	Lysine decarboxylase	Ornithine decarboxylase	Tyrosine	Gelatinase	Lipase	Amylase	Alginase	Chitinase	Ac sucrose	Util propionate	2, 3-butanediol	2-amino ethanol	Acetic acid	Adonitol	Alaninamide	Alpha-cyclodextrin	Alpha-D-glucose	Alpha-D-lactose lactulose
V. neonatus	–		+	–	–	–	–	–	–	–	+	–	–				–					
V. neptunius		–	–	+	–	–		+					+		–						+	
V. nereis	–	–	+	+	–	–	d	v	–	–	–	v	+	+			d				+	
V. nigripulchritudo	–	–	+	–	–	–	–	+	+	+	–	+	–	+			+				+	
V. ordalii	–	+		–	–	–	–	+	–	–	–	d	+	–			+					
V. orientalis	–			+			–	+	+	+	–	+	+	–			+					
V. pacinii		–	+	+	–	–		v							–	–	–	–	–		+	–
V. parahaemolyticus	–	–	+	–	+	+	+	+	+	+	–	+	–	+			+				+	
V. pectenicida	–		+	–	–	–		+		+	+		–	–								
V. pelagius biovar I	–	+	+	–	–	–	d	–	+	–	+	v	+	+			+				+	
V. pelagius biovar II	–	+	+	–	–	–	–	+	+	+	+	+	+	+			+				+	
V. penaeicida	–	+		–	–	–		+	+	+	+	+	–	–				–			+	
V. pomeroyi				+					–													–
V. ponticus					+	–		–			+							–				
V. proteolyticus	–	d	+	+	+	–	–	+	+	+	–	+	–	–			–				+	
V. rotiferianus	–	+	+	–	+	+		+							–	–	–	–	+		+	–
V. ruber	+	–	–	–	–	–		+	+	+							+	–			+	
V. rumoiensis		+	+	–				–		+	–	+									+	
V. salmonicida	–		+	–				–	–	–		–	–								+	
V. scophthalmi		+	+	+	–	–		–		–			+			–		–		–		
V. splendidus biovar I	–	+	+	+	–	–	d	+	+	+	v	+	v	+			d				+	
V. splendidus biovar II	–	+	+	–	–	–	d	+	+	+	–	+	–	+			+				+	
V. succinogenes																					+	
V. superstes	–			–	–	–	–	–	–	–	+	–		+			+				+	
V. tapetis	–		+	–	–	–		+	+	+	–		–	+			–				+	
V. tasmaniensis		+	+	–	–	–		–							–	–	–	–	–		+	–
V. tubiashii	–	v	–	+	–	–		+	+	+	–	+	+	–			–		–			
V. vulnificus biovar I	–	+	+	–	+	+		+	+	+	–	+	–									
V. vulnificus biovar II	–	+		–	+	+		+	+				–	+								
V. wodanis	–			–	–	–		+		+											+	
V. xuii		–	–	+	–	–		–					+		+	–	+				+	

Supplementary Table 36.2 (continued)

Species	Utilization as sole source of carbon (Biolog GN2)																						
	Alpha-hydroxy butyric acid	Alpha-keto butyric acid	Alpha-keto glutaric acid	Alpha-keto valeric acid	Alpha-lactose	Beta-hydroxy butyric acid	Beta-methyl D-glucoside	Bromo succinic acid	Cellobiose	*Cis*-aconitic acid	Citric acid	D, L-alpha-glycerol phosphate	D, L-carnitine	D, L-lactic acid	D-alanine	D-arabitol	Dextrin	D-fructose	D-galactonic acid lactone	D-galactose	D-galacturonic acid	D-gluconic acid	D-glucosaminic acid
E. norvegicus						–					–			–				+					
E. coralii			+	–	+	–			+	+		–	–	+	+	–	+	+	–	+		+	
G. hollisae																							
P. angustum			–																				
P. aplysiae			w			–			+					+			+	+		w		w	
P. damselae																							
P. frigidiphilum						–								+	+		+	+		+			
P. iliopiscarium									–		–												
P. leiognathi			–		–											–							
P. lipoliticum									–									+		–			
P. phosphoreum			–		–				–		–					–							
P. profundum									–									–		+			
P. rosenbergii	v	+	+	–	(–)	v		+	+	+	+	+	–	+	+	–	+	+	–	+	v	(–)	(–)
S. costicola			–						–		–			+				–		–		+	–
V. aerogenes									+		+												
V. aestuarianus																							
V. agarivorans						–			+	–	–				–	–		+		+	–	–	+
V. alginolyticus	–		+		–	–			–		+			+	+	–		+		d	–	+	
V. anguillarum			v		–	–			+		+			+	+	–		+		d	–	+	
V. brasiliensis	–		–	–	–	+	+	+	+	–	–	–	–	–		–	+	+	–	+	–		–
V. calviensis									+		+							–		+		+	
V.campbellii			–		–	–			v		v			+	+	–		+		–	–	–	
V.chagasii			+		–	–	–		+														
V. cholerae	–		–		–	–			–		d			+	d	–		+		+	–	+	+
V.cincinnatiensis			+		–				+		+				+	–						+	
V. coralliilyticus		+	+			–	+		–	+	+									+		+	
V. crassostreae									+														
V. cyclitrophicus									+					+	+					+			
V. diabolicus									–		+							+		+		+	
V. diazotrophicus	v		+		–	–			+		+				+	–		+		+	+	+	+
V. ezurae			–						–		–							+		v		+	+
V.fischeri	–		–		d	–			+		d			–	–	–		d		+	–	–	–
V. fluvialis	d		+		–	d			d		+			+	–			+		+	+	+	+
V. fortis	–	–							+		–			+		–	+	+	–	+		+	
V. furnissii			–		–	+			–		+							+		+	+	+	+
V. gallicus			–		–	–			–	–	–							+		–		–	–
V. gazogenes			+		+	–			+		+			+	–			+		+		–	

Supplementary Table 36.2 (continued)

Species	Utilization as sole source of carbon (Biolog GN2)																						
	Alpha-hydroxy butyric acid	Alpha-keto butyric acid	Alpha-keto glutaric acid	Alpha-keto valeric acid	Alpha-lactose	Beta-hydroxy butyric acid	Beta-methyl D-glucoside	Bromo succinic acid	Cellobiose	*Cis*-aconitic acid	Citric acid	D, L-alpha-glycerol phosphate	D, L-carnitine	D, L-lactic acid	D-alanine	D-arabitol	Dextrin	D-fructose	D-galactonic acid lactone	D-galactose	D-galacturonic acid	D-gluconic acid	D-glucosaminic acid
V. halioticoli			–		–				–	–	–							+		–		–	+
V. harveyi			+		V	–			+		+			+	+			+		d	–	+	–
V. hepatarius	–	–	–		–			–	+	–	–	–	–		–	–	+	+	–	–	–	–	–
V. hispanicus	–	–	–	–		+	+	–	+	–	+	–	–	+	–	–	+	+	–	+	–	+	–
V. ichthyoenteri					–	–			–								–	+		–			
V. kanaloae			+			?			–				–		+								
V. lentus									+														
V. logei			–		–	–			+		–			–	–			+		+	–	+	
V. mediterranei					+				+					+						+		–	
V. metschnikovii			–		d	–			–		d			+	d	–		+		d	–	+	
V. mimicus			+		–	–			–		+					–		+			–	+	+
V. mytili									+		v			+	+	–				+	–		+
V. natriegens			+		–	+			v		+			+	+			+		+	d	+	
V. navarrensis					–		+								+	–		+		+	–	+	+
V. neonatus			–		–				–		–							+		–		–	+
V. neptunius						–	+		–		+		–							–		–	
V. nereis			+		–	+			–		+			+	+			+		–	–	+	
V. nigripulchritudo			+		+	+			+		+			+	+			+		+	–	v	+
V. ordalii			–		–	–			–		+			–	–					–	–	–	
V. orientalis			–			+			+		+			+	+					+		+	
V. pacinii	–	–	–	–	V	–		–	+	–	–	–	–		–	–	+	+	–		–	–	–
V. parahaemolyticus			+		–	–			–		+			+	+	–		+		+	–	+	+
V. pectenicida			–																				
V. pelagius biovar I			–		d	–			–		+			+	+			+		+	–	+	
V. pelagius biovar II			–		d	–			–		+			+	+			+		+	–	+	
V. penaeicida			–		–	+			+		+						+	+		–		+	
V. pomeroyi	–		–						+				–							+			
V. ponticus									–	–	+					–	+	+			–	–	+
V. proteolyticus			+		–	–			–		+			+	+		–	+		–	–	+	+
V. rotiferianus	–	–	–	–	–		+		+	–	–	–	–	+	–	–	+	+	–	+	–	+	–
V. ruber					+				+		+									+			
V. rumoiensis					+						+							+		+		+	
V. salmonicida				–	–				–									+		d		+	
V. scophthalmi											–		–		–			+					
V. splendidus biovar I			+		–	–			+		+			+	+	–		+		+	–	v	–
V. splendidus biovar II			+		–	–			v		+			+	+	–		+		–	–	–	–
V. succinogenes					+				+								+	+		+			

Supplementary Table 36.2 (continued)

Species	Utilization as sole source of carbon (Biolog GN2)																						
	Alpha-hydroxy butyric acid	Alpha-keto butyric acid	Alpha-keto glutaric acid	Alpha-keto valeric acid	Alpha-lactose	Beta-hydroxy butyric acid	Beta-methyl D-glucoside	Bromo succinic acid	Cellobiose	*Cis*-aconitic acid	Citric acid	D, L-alpha-glycerol phosphate	D, L-carnitine	D, L-lactic acid	D-alanine	D-arabitol	Dextrin	D-fructose	D-galactonic acid lactone	D-galactose	D-galacturonic acid	D-gluconic acid	D-glucosaminic acid
V. superstes					+	–			+	–	–							+		+		+	+
V. tapetis			–			–			–	–	–							+		–	–	–	
V. tasmaniensis	–	–	–	–	–	–	–	–	+	–	–	–	–		–	–	+	+	–	–	–	+	–
V. tubiashii			d		d	d			+	–	+				d	–		+		+	–	+	
V. vulnificus biovar I			+						+													+	
V. vulnificus biovar II																							
V. wodanis					–				–								+			+			
V. xuii		–	+			+	–		+						+					–		+	

Species	Utilization as sole source of carbon (Biolog GN2)																						
	D-glucuronic acid	D-mannitol	D-mannose	D-melibiose	D-raffinose	D-saccharic acid	D-serine	D-sorbitol	D-trehalose	Formic acid	Gamma-amino-butyric acid	Gamma-hydroxy butyric acid	Gentiobiose	Glucose-1-phosphate	Glucose-6-phosphate	Glucuronamide	Glycerole	Glycerol	Glycyl-L-aspartic acid	Glycyl-L-glutamic acid	Hydroxy L-proline	i-erythritol
E. norvegicus		–	+					–	+				–					–				
E. coralii		+	+	+	+	–	–	+	+	–	–	–		–	–	–	–	+					–
G. hollisae																							
P. angustum		–		–				–															
P. aplysiae		+	+						+						+		+	+	+	w			
P. damselae	–																						
P. frigidiphilum		+	+						+						+		+	+	+	+			
P. iliopiscarium		–	+																				
P. leiognathi		–		–				–															
P. lipoliticum			–						–														
P. phosphoreum		–	+	–				–															
P. profundum		+	+		–			–	+								+	+					
P. rosenbergii		+	+	+	v	–	–	v	+	+	–	–	(–)	+	+	(–)	+	+	+	+	–	(–)	
S. costicola	–	+	–	–	–				+								+						–
V. aerogenes		+	+	–																			
V. aestuarianus		+	+					d															
V. agarivorans	–	+	–	+	–	–		–	–				–				–						–
V. alginolyticus	–	+	+	–	–		+	–	+	+	–						+	+			+		–
V. anguillarum	–	+	+	–	–		+	+	+	–	–						+	+			+		–

Supplementary Table 36.2 (continued)

Species	Utilization as sole source of carbon (Biolog GN2)																					
	D-glucuronic acid	D-mannitol	D-mannose	D-melibiose	D-raffinose	D-saccharic acid	D-serine	D-sorbitol	D-trehalose	Formic acid	Gamma-amino-butyric acid	Gamma-hydroxy butyric acid	Gentiobiose	Glucose-1-phosphate	Glucose-6-phosphate	Glucuronamide	Glycerole	Glycerol	Glycyl-L-aspartic acid	Glycyl-L-glutamic acid	Hydroxy L-proline	i-erythritol
V. brasiliensis	–	+	+	–	–	–	+	–	+	–		–	+	–	–	–	+	+	+	+		–
V. calviensis		+	+	+					–				+					–				
V. campbellii	–	v	+	–				–	+		–						+				+	–
V. chagasii		+				–							–									
V. cholerae	–	+	+	–	–			–	+	–	–						+				–	–
V. cincinnatiensis	–	d	+					–	+		+							+			+	
V. coralliilyticus		+		–			+							+							+	
V. crassostreae			+		–												+	+				–
V. cyclitrophicus	+							–			–											
V. diabolicus		+	+						+				–				+	+				
V. diazotrophicus	+	+	–	–	–			–	+	+	d						–	+			–	–
V. ezurae		+	–	–				–			–						–					
V. fischeri	–	+	+	–	–			–	d	–	–						+	+			–	–
V. fluvialis	+	+	+	–				–	+	–	+						+	+			+	–
V. fortis	–	+	+			–		+	+	–			+	–			+	+	+	+	–	–
V. furnissii	–	+	+	–	–			–	+	–	+						+	+			–	–
V. gallicus	–	+	–	–				–	–		–											–
V. gazogenes	–	+	+					+			–						+					–
V. halioticoli	–	+	–	–				–	–								+					–
V. harveyi	+	+	+	–				–	+		–						+					–
V. hepatarius	–	+		–	–	–	–		+	–	–		–	–	–	–	+	–	–	–	–	–
V. hispanicus	+	+	+	–	+	+	–	–	–	–	–	–	+	–	–	–		–	–	–	–	–
V. ichthyoenteri		–	+	–	–			–	+								–	–				–
V. kanaloae		+		–		–		–													–	
V. lentus		+	+	v					+													
V. logei	–	+	+	–				–	–		–						+					–
V. mediterranei		–		+				–			–											
V. metschnikovii	d	+	d	–	–			d	+	–	–						+	+				–
V. mimicus	+	+	+	–	–			–	+	–	–						+	+			–	–
V. mytili	–	+	–	–	+			–	+				+				+					–
V. natriegens	v	+	d	v				–	+		+						+					–
V. navarrensis	–	+			–			–	+				–				+					–
V. neonatus		+	–	–				–	–		–						–					
V. neptunius		–	+				+	–			–		–	+	+			+			–	
V. nereis	–	–	–	–				–	+		+						+					–
V. nigripulchritudo	+	–	+	+	–			+	+	–	–						+	+			–	d
V. ordalii	–	–	–	–				–	–													
V. orientalis	–	+	+					–	+													

Supplementary Table 36.2 (continued)

Species	Utilization as sole source of carbon (Biolog GN2)																				
	D-glucuronic acid	D-mannitol	D-mannose	D-melibiose	D-raffinose	D-saccharic acid	D-serine	D-sorbitol	D-trehalose	Formic acid	Gamma-amino-butyric acid	Gamma-hydroxy butyric acid	Gentiobiose	Glucose-1-phosphate	Glucose-6-phosphate	Glucuronamide	Glycerole	Glycerol	Glycyl-L-aspartic acid	Glycyl-L-glutamic acid	Hydroxy L-proline	i-erythritol
V. pacinii	–	+	–	–	–	–	–	–	+	–	–	–	+	–	–	–	–			–	–	–
V. parahaemolyticus	d	+	+	–				–	+		–						+	+				
V. pectenicida	+		–														+					
V. pelagius biovar *I*	–	+	v	–				–	+		–						+					
V. pelagius biovar *II*	–	+	+	–				–	+		+						+					
V. penaeicida	–	+	+		–			–	+		–						–	+				
V. pomeroyi	–					–	–			–			–			–				+	–	
V. ponticus		+		–		–		–	+	–			–									
V. proteolyticus	–	+	+	–				+	+		–						+	+				–
V. rotiferianus	+	–	+	+	+	–	+	–	+	–	–	–	+	–	+	–	–	+	+	–	–	–
V. ruber		+	+	+					–													
V. rumoiensis		+	+														+					
V. salmonicida		+	d	–	–			–									+	–				
V. scophthalmi		–			–												–					–
V. splendidus biovar *I*	+	+	+	–	–			–	+	–	–						d	+			–	–
V. splendidus biovar *II*	–	+	–	–	–			–	+	–	–						+				–	–
V. succinogenes					+			+	+	+												
V. superstes	+	+	+	+				–	+		+						–					–
V. tapetis	–	–	+	–				–	+		–						–					
V. tasmaniensis	–	+	+	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–			–	–
V. tubiashii	–	+	+	d	–		–	–	+	–							–	+			–	–
V. vulnificus biovar *I*	+	+	+	–				–														
V. vulnificus biovar *II*	+	–						–														
V. wodanis			+	–				–	+								+					
V. xuii		+	+	–	–		–	+					–	–			+			+	–	

Species	Utilization as sole source of carbon (Biolog GN2)																				
	Inosine	Itaconic acid	L-alanine	L-alanyl-glycine	L-arabinose	L-asparagine	L-aspartic acid	L-fucose	L-glutamic acid	L-histidine	L-leucine	L-ornithine	L-phenyl alanine	L-proline	L-pyro glutamic acid	L-rhamnose	L-serine	L-threonine	Malonic acid	Maltose	Methyl pyruvate
E. norvegicus	–		–			–			–					–					–	+	
E. coralii	+	–	+	+	–	+	+		+	+	–		–				+		–	+	
G. hollisae																					
P. angustum					–																
P. aplysiae	+		+	+													+	+		+	+

Supplementary Table 36.2 (continued)

Species	Utilization as sole source of carbon (Biolog GN2)																			
	Inosine	Itaconic acid	L-alanine	L-alanyl-glycine	L-arabinose	L-asparagine	L-aspartic acid	L-fucose	L-glutamic acid	L-histidine	L-leucine	L-ornithine	L-phenyl alanine	L-proline	L-pyro glutamic acid	L-rhamnose	L-serine	L-threonine	Malonic acid	Maltose	Methyl pyruvate
P. damselae																					
P. frigidiphilum	+		+			+	+		+								+			+	
P. iliopiscarium	–																				
P. leiognathi					–																
P. lipoliticum																				+	+
P. phosphoreum	–				–																
P. profundum					–											–				+	
P. rosenbergii	+	–	+	+	–	+	+	(–)	+	+	–	+	(–)	(+)	(+)	v	+	+	–	+	+
S. costicola			+			+			–	+	–	+	–	+		–	+	+		–	
V. aerogenes	+		+				+		+			–	–					+			
V. aestuarianus					–																
V. agarivorans			–		–		+	–	+	–				+		–	+		+	+	–
V. alginolyticus	–		+		–		d		+	+	+	–	–	+		–	+	+	–	+	+
V. anguillarum	v		d		+		+		+	+	–	–	–	+		–	+	–		+	+
V. brasiliensis	+	–	+	+	–	+	+	–	+	–	–	+	–	+	–	–	+	+	–	+	+
V. calviensis					–															+	
V. campbellii	–		d		–		–		–	–	–	–		+		–	d	+	d	+	+
V. chagasii			+		–							–	–								
V. cholerae	–		–		–		d		+	d	–	+	–	+		–	d	d	–	+	+
V. cincinnatiensis			+		+		+		+		–	–		+							
V. coralliilyticus	+									+	+										+
V. crassostreae					–			+													
V. cyclitrophicus					–				+					+			+			v	+
V. diabolicus					–														+	+	
V. diazotrophicus	–		+		v				+		–		–	+		–	d		–		+
V. ezurae					–				v							–					
V. fischeri	–		–		–		d		d	–	–	–	–	+		–	–	d	–	+	–
V. fluvialis	–		+		+		+		+	d	–	+	–	+		–	+	+	+	+	+
V. fortis	+	–	+	+		+	+	–	+				–	+	–	–	+	+	–	+	+
V. furnissii	–				d						–	–	–			–			–	+	+
V. gallicus					–														–	+	–
V. gazogenes	–				+		+		+		–	–		+		–	+		+	+	+
V. halioticoli	–				–				–							–				+	–
V. harveyi	–		+		d		d		+	d	–	–		+		–	+	+	d	+	+
V. hepatarius	+	–			–		–	–		–	–	–	–		–	–		–	–	+	+
V. hispanicus	+	–	–	–		+	+	–	+	–	–	–	–	–	–	+	+	–	–	+	+
V. ichthyoenteri					–											–				+	

Supplementary Table 36.2 (continued)

Species	Utilization as sole source of carbon (Biolog GN2)																				
	Inosine	Itaconic acid	L-alanine	L-alanyl-glycine	L-arabinose	L-asparagine	L-aspartic acid	L-fucose	L-glutamic acid	L-histidine	L-leucine	L-ornithine	L-phenyl alanine	L-proline	L-pyro glutamic acid	L-rhamnose	L-serine	L-threonine	Malonic acid	Maltose	Methyl pyruvate
V. kanaloae			+		+							+	–	+			+	+			
V. lentus	–				–											–				+	
V. logei	–		–		–				+	–	–	–		+		–	d		–	+	–
V. mediterranei	+		+		–		+		+			–		+		–	+		+		
V. metschnikovii	d		+		–		+		+	d	–	+		+		–	+	+	+	+	+
V. mimicus					–				–		–		–			–				+	
V. mytili	–		+				+			–				+		–			–	+	
V. natriegens	d		+		+		d		+	+	d	+		+		+	+	+	+	+	+
V. navarrensis		–	+		–		+	–	+	–	v			+		–	+		+	+	
V. neonatus					–				v											+	
V. neptunius					–								–					+			
V. nereis	–		+		–		d		+	d	+	+		+		–	d	+	+	+	+
V. nigripulchritudo	+		+		–		+		+	+	–	–		+		–	+	+	+	+	+
V. ordalii	–		–		–		+		+	d	–	–		+		–	–		–		+
V. orientalis			+		–		+		+	+	–			+			d		+		+
V. pacinii	+	–			–	+		–	+	–	–	–	–	–	–	–	+	–	–	+	–
V. parahaemolyticus	–		d		v		+		+	+	+	–	–	+		–	+	+	+	+	+
V. pectenicida			+				+			+						+				+	+
V. pelagius biovar *I*	–		+		–		d		+	d	–	d		+		–	+	+	d	+	+
V. pelagius biovar *II*	–		+		–		+		+	d	–	+		+		–	+	+	–	+	+
V. penaeicida					–						–					–			+	+	
V. pomeroyi	+									–	–		–	–	–		+	+			
V. ponticus					–					–	–	–	–	–		–			–		
V. proteolyticus	–		+		–		+		+	+	–	+	–	+		–	+	+	+	+	+
V. rotiferianus	+	–	+	+	+	+	+	–		–	–	–	–	–	–	–	+		–	+	–
V. ruber							+		+										–		
V. rumoiensis					+															+	
V. salmonicida	–				–			–	–							–				+	
V. scophthalmi	+		–							–			–			–			–		
V. splendidus biovar *I*	–		+		–		+		+	d	–	–		+		–	+	+	+	+	+
V. splendidus biovar *II*	–		d		–		d		d	d	–	–		+		–	d	d	d	+	+
V. succinogenes					+														+	+	
V. superstes					–				+										–	+	–
V. tapetis	–				–		–		–	–	–	–		–		–	–	–		–	–
V. tasmaniensis	+	–	+	+	–			–	+	–	–	–	–	–	–	–	+		–	+	–
V. tubiashii	–				–					–	–					–	–			+	+
V. vulnificus biovar *I*					–						–										

Supplementary Table 36.2 (continued)

Species	Utilization as sole source of carbon (Biolog GN2)																			
	Inosine	Itaconic acid	L-alanine	L-alanyl-glycine	L-arabinose	L-asparagine	L-aspartic acid	L-fucose	L-glutamic acid	L-histidine	L-leucine	L-ornithine	L-phenyl alanine	L-proline	L-pyro glutamic acid	L-rhamnose	L-serine	L-threonine	Malonic acid	Maltose	Methyl pyruvate
V. vulnificus biovar *II*					–																
V. wodanis	–				–									+		–				+	
V. xuii					+									+				+			+

Species	Utilization as sole source of carbon (Biolog GN2)																				
	M-inosito	Mono methyl succinate	*N*-acetyl-D–*galactosamine*	*N*-acetyl-D-*glucosamine*	Phenyl ethylamine	*P-hydroxy phenylacetic acid*	Propionic acid	Psicose	Putrescine	Quinic acid	Sebacic acid	Succinamic acid	Succinic acid	Sucrose	Thymidine	Turanose	Tween 40	Tween 80	Uridine	Urocanic acid	Xylitol
E. norvegicus	–						–	+					–	–							
E. coralii				+	–	–		+	–		–		+	–	+	+	–	–	+	–	
G. hollisae																					
P. angustum	–													v							
P. aplysiae				+				+					w	+			w	w	+		
P. damselae																					
P. frigidiphilum	+		+	+			+						+	+		–	+	+			
P. iliopiscarium	–													–							
P. leiognathi	–													–							
P. lipoliticum																	+	+			
P. phosphoreum	–													–							
P. profundum	–		–	–										–		–	+	+			
P. rosenbergii	(+)	+	(–)	+	–	+	+	+	(–)	(–)	–	(+)	+	+	+	v	+	+	+	–	–
S. costicola	–			+		–				–			+	+				–			
V. aerogenes	+													+							
V. aestuarianus	–													–							
V. agarivorans	–			+					–	–			+	–							
V. alginolyticus	–			+			+		v				+	+							
V. anguillarum	v		+	+			–		–				v	+				–			
V. brasiliensis	–		–	+	–	–		+		–	–	–	+	+	+				+		–
V. calviensis				–										+		+		+			
V. campbellii	–			+			+		–				+	–							
V. chagasii								+	–		–						+	+			
V. cholerae	–			+			+		–				+	+				+			
V. cincinnatiensis	–					–			–					–		–	–	–			
V. coralliilyticus	+						+														
V. crassostreae	–			+										+							–

Supplementary Table 36.2 (continued)

Species	Utilization as sole source of carbon (Biolog GN2)																				
	M-inosito	Mono methyl succinate	*N*-acetyl-D—*galactosamine*	*N*-acetyl-D-*glucosamine*	Phenyl ethylamine	*P-hydroxy phenylacetic acid*	Propionic acid	Psicose	Putrescine	Quinic acid	Sebacic acid	Succinamic acid	Succinic acid	Sucrose	Thymidine	Turanose	Tween 40	Tween 80	Uridine	Urocanic acid	Xylitol
V. cyclitrophicus	–						+							–							
V. diabolicus				+										+							
V. diazotrophicus	–			+			d						+	+							
V. ezurae				+			–		–				+	–				+			
V. fischeri	–			+			–		–				+	–				–			
V. fluvialis	–			+			+		d				+	+							
V. fortis	–	+	+	+	–	–					–	–	+	+	+		+	+			–
V. furnissii	–			+			+						–	+							
V. gallicus							–		–				–	–				–			
V. gazogenes	–						+		–				+	+							
V. halioticoli	–			+			–		–				–	–							
V. harveyi	–			+			+		–				+	d							
V. hepatarius	–	–	–	+	–	–	–	+	–	–	–	–		+	–	–	–		–	–	–
V. hispanicus	–	–	–	+	–	–	–	+	–	–	–	–		+	+	–	–	–	+	–	–
V. ichthyoenteri	–													+							
V. kanaloae		+									–	–		+			+				
V. lentus	–													–				+			
V. logei	–			+			–		–				+	–							
V. mediterranei	–								+					d							
V. metschnikovii	d			+			–		–				+	+							
V. mimicus	+			+			–							–							
V. mytili	–			+										+							
V. natriegens	v			+			+		+				+	+							
V. navarrensis	–			+			–		–	–			+	+							–
V. neonatus				+			–		–				+	–				–			
V. neptunius		–	–			–			–					+				–			
V. nereis	–			+			+		+				+	d							
V. nigripulchritudo	+			+			+		–				+	–				+			
V. ordalii	–						–		–					+							
V. orientalis	–						–		+					+							
V. pacinii	–			+	–	–	–		–	–	–	–		+		–	–	–		–	–
V. parahaemolyticus	–			+			+		+				+	–							
V. pectenicida	–												+	–							
V. pelagius biovar *I*	–			+			+		+				+	+							
V. pelagius biovar *II*	–			+			+		+				+	d							
V. penaeicida	–						–		–					–							
V. pomeroyi		+							–	–	–	–		+	+				+		
V. ponticus				+					–		–		+	+		–	–	–		–	–

Supplementary Table 36.2 (continued)

Species	Utilization as sole source of carbon (Biolog GN2)																			
	M-inosito	Mono methyl succinate	*N*-acetyl-D–*galactosamine*	*N*-acetyl-D-*glucosamine*	Phenyl ethylamine	*P-hydroxy phenylacetic acid*	Propionic acid	Psicose	Putrescine	Quinic acid	Sebacic acid	Succinamic acid	Succinic acid	Sucrose	Thymidine	Turanose	Tween 40	Tween 80	Uridine	Urocanic acid	Xylitol
V. proteolyticus	–			+			–		+				+	–							
V. rotiferianus	–	–	–	+	–	–	–	+	–	–	–	–	–	+		–	–	–		–	–
V. ruber	–													+							
V. rumoiensis	+													+			+	+			
V. salmonicida	–													–							
V. scophthalmi	–			+					–		–							–			–
V. splendidus biovar *I*	–			+			+		–				+	+							
V. splendidus biovar *II*	–			+			+		–				+	–							
V. succinogenes														+							
V. superstes				+			+		–				+	+				–			
V. tapetis	–			+			+		–	–			+	–			+	–			
V. tasmaniensis	–	–	–	+	–	–	–	–	–	–	–	–		–		–	–	–		–	–
V. tubiashii	–			+			–		d					+				+			
V. vulnificus biovar *I*	–								–					–							
V. vulnificus biovar *II*	–													–							
V. wodanis				+														+			
V. xuii		+	+			+	–			–	–			+			+	+			

+ more than 90 % of the strains positive, (+) 75–89 % positive, (–) 25–11 % positive, – less than 10 % positive, *v* variable result, *d* dubious result, *ng* no growth

the correlation between the signature and the gene sequence-based methods obtained in this study is slightly lower. MLSA and supertree analyses resulted in a similar phylogenetic picture, with a clear distinction of four groups (Vibrio core group, *V. cholerae–V. mimicus*, *Aliivibrio* spp., and *Photobacterium* spp.). The authors suggested that a *Vibrio* species may be better defined based on whole-genome sequence analysis. **Based on the genomic taxonomy, a Vibrio species is defined as a group of strains that share >95 % DNA identity in MLSA and supertree analysis, >96 % AAI, ≤10 genome signature dissimilarity, and >61 % proteome identity** (*Fig. 36.5*). **Strains of the same species and species of the same genus will form monophyletic groups on the basis of MLSA and supertree. This definition may advance the field of Vibrio taxonomy.**

Haley et al. (2010), using the genomic taxonomy approach for vibrios, have described two new *Vibrio* species, *V. metecus* and *V. parilis*, which were phylogenetically closely related to *V. cholerae* and *V. mimicus*, respectively. Thompson et al. (2011) analyzed the taxonomic position of the genus *Listonella* based on a large number of genomic and phenotypic data and showed that the species of the genus *Listonella* were indeed nested within the genus *Vibrio*. The closest neighbor of *L. anguillarum* strains LMG 4437^T and ATCC 68554 was *Vibrio ordalii* LMG 13544^T, and *L. pelagius* was highly related to *V. splendidus*. The authors proposed to use the names *Vibrio anguillarum* and *Vibrio pelagius* rather than *Listonella anguillarum* and *Listonella pelagia*, respectively. Because the genus *Vibrio* was described first, the genus *Listonella* was to be considered a later heterotypic synonym of the genus *Vibrio*. Using the following set of genomic tools (MLSA, Karlin genomic signature, and AAI) *Vibrio* sp. PEL22A, a bacterioplanktonic and mixotrophic strain isolated in the Abrolhos Bank, and DS40M4 isolated from open ocean water were identified as *V. campbellii*. They shared >97 % DNA identity in MLSA, <6 in Karlin signature, and >96 % AAI with two strains of *V. campbellii* BAA-1116 and HY01 (Dias et al. 2012; Amaral et al. 2012).

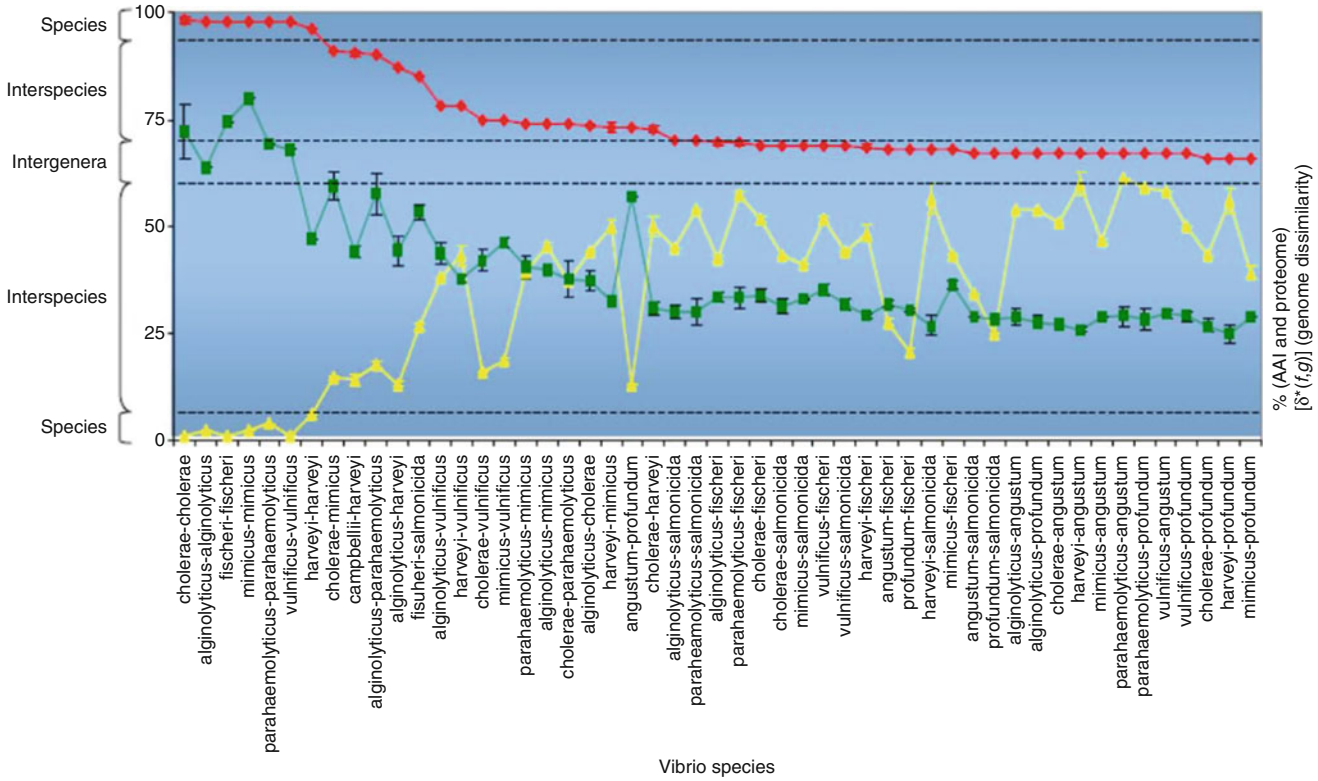

Fig. 36.5

***Taxonomic resolution of AAI, BLAST proteome and genome dissimilarity [δ*(f,g)] of vibrios*. Mean and standard deviations. *Red* AAI, *Green* proteome, *yellow* [δ*(*f*,*g*)]. The taxonomic resolution of AAI is down to the intergenera level, whereas [δ*(*f*,*g*)] has a resolution at interspecies level. The *dashed lines* delimit ($p < 0.001$) the different taxonomic levels for AAI and [δ*(*f*,*g*)] but not for the proteome. The proteome did not completely fit this figure (and *dashed lines* limits), showing some noise signal for *V. harveyi*-*V. harveyi* (Thompson et al. 2009)**

Molecular Analyses

Classical biochemical identification and dichotomous keys have been used for tentative identification of vibrios, despite their numerous limitations (Abbott et al. 1998; O'Hara et al. 2003). Dichotomous keys (Holt et al. 1994; Alsina and Blanch 1994a, b) are misleading for the identification of isolates of vibrios. Fatty acid methyl ester (FAME) profiling was evaluated for the differentiation of species (Lambert et al. 1983; Osterhout et al. 1991; Bertone et al. 1996). This technique is generally very useful as a chemotaxonomic marker, and apparently, differentiation at the genus level was possible. However, FAME profiles cannot be used to differentiate species (Lambert et al. 1983; Bertone et al. 1996). The ample phenotypic variability within species of vibrios urges classification and identification schemes to be based on genomic data.

An array of genomic techniques has become available for the identification of vibrios in the last three decades (Vandamme et al. 1996; Rademaker et al. 1998, 2000; Olive and Bean 1999; Savelkoul et al. 1999; Gurtler and Mayall 2001; Dijkshoorn et al. 2001). Ribotyping, amplified fragment length polymorphism (AFLP), repetitive extragenic palindromes (rep), but also multilocus enzyme electrophoresis (MLEE) and latter on multilocus sequence analysis (MLSA) have yielded the most valuable information and new insights into the population structure of some species of the *Vibrionaceae* and also provided means of identifying these organisms. Below we discuss the use of AFLP and rep-PCR and microarrays for identification purposes. Microarrays have only been used more recently. The use of other phenotypic and genotypic techniques for detection and identification of vibrios has been discussed in detail (Thompson et al. 2004b).

Amplified Fragment Length Polymorphism (AFLP)

The AFLP technique comprises the digestion of total genomic DNA with two restriction enzymes and subsequent ligation of the restriction half-site-specific adaptors to all restriction fragments and subsequent selective amplification of these fragments with two PCR primers that have corresponding adaptor and restriction site sequences as their target sites. The AFLP fragments are then separated and visualized on polyacrylamide gels and sequencers. During electrophoretic separation, there happens selective detection

of fragments which contain the fluorescently labelled primer (Vos et al. 1995; Huys and Swings 1999; Janssen 2001). AFLP patterns are amenable to computer-assisted numerical analysis.

AFLP was first developed with radioactive labelled primers (Vos et al. 1995), but now AFLP is mainly performed with fluorescently labelled primers. FAFLP indexes variation in the whole genome and thus is considered to give useful information on the short- and long-term evolution of bacterial strains (Larsen and Boesen 2001). Janssen and coworkers were the first group to use AFLP as a tool for bacterial taxonomy (Janssen et al. 1996). They examined 147 strains that had a broad G+C content (24–71 %) range focusing mainly on *Aeromonas* (n = 90) and *Xanthomonas* (n = 36). They also included three *V. anguillarum* and one *V. tubiashii* strain. The grouping obtained by AFLP corresponded well to that obtained by DNA–DNA similarity data. Janssen (Janssen et al. 1996) also reported that the complexity (i.e., number and size of the fragments) of the AFLP patterns could be tuned by using different restriction enzymes and selective primers, although in any case, the grouping of strains should be very similar. Because each bacterial species had a specific AFLP pattern, they concluded that AFLP could be used as an alternative to bacterial classification and identification. In the following years, AFLP was used to study various vibrios (Thompson et al. 2001), including *V. alginolyticus* (Vandenberghe et al. 1999), *V. cholerae* (Jiang et al. 2000a; Jiang et al. 2000b; Lan and Reeves 2002), *V. harveyi* and *V. campbellii* (Pedersen et al. 1998; Gomez-Gil et al. 2004a), *V. vulnificus* (Arias et al. 1997b), *V. wodanis* (Benediktsdottir et al. 2000), and *P. damselae* (Thyssen et al. 2000), but most of these studies did not include all the recognized *Vibrio* species. Examination of 80 *V. vulnificus* strains by several phenotypic (Biolog, API, SDS-PAGE, serotyping, ELISA) and genotypic (AFLP, ribotyping) methodologies (Arias et al. 1997a, b) showed that AFLP was the most suitable and discriminatory tool for epidemiological studies, even if AFLP was able to discriminate strains with identical ribotypes. Other AFLP analyses clearly pointed out that *V. carchariae* was a synonym of *V. harveyi* (Pedersen et al. 1998). Discrimination of pathogenic and probiotic *V. alginolyticus* strains using AFLP was achieved (Vandenberghe et al. 1999), and it was concluded that this technique can be used to authenticate probiotic cultures prior to their use. Also, this technique was useful for the differentiation of the two subspecies of *Photobacterium damselae*, i.e., *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida* (Thyssen et al. 2000).

AFLP has been validated as an alternative identification tool for vibrios (Thompson et al. 2001, 2004b). Strains sharing more than 70 % AFLP band pattern similarity will always share more than 70 % DNA–DNA similarity. In this case, strains may be considered to belong to the same species. We consider this an improvement in the current taxonomy of vibrios in that such type of data will aid researcher to sort out the taxonomic position of their isolates in a much faster and yet most reliable manner.

Repetitive Extragenic Palindrome (rep-PCR)

rep-PCR amplifies intervening sequences located between highly repetitive DNA motifs (Dijkshoorn et al. 2001). This technique has been used mainly with the aim of typing within the species *V. alginolyticus* (Sudheesh et al. 2002), *V. cholerae* (Rivera et al. 1995, 2001; Zo et al. 2002), *V. parahaemolyticus* (Wong and Lin 2001), and *V. vulnificus* (Warner and Oliver 1999; Sudheesh et al. 2002), and it is thus difficult to determine its taxonomic resolution and value for the whole family *Vibrionaceae*. *V. alginolyticus* and *V. parahaemolyticus* have different RAPD profiles and can be reliably separated by this fast screening methodology (Sudheesh et al. 2002). RAPD, rep-PCR, PFGE, and ribotyping were compared, and it was concluded that rep-PCR is the most discriminatory of the techniques (Wong and Lin 2001). Eighty-three *V. cholerae* strains were analyzed by rep-PCR and it found that toxigenic and nontoxigenic strains had different patterns, and it can be used in epidemiological studies (Rivera et al. 1995).

rep-PCR was used to identify presumptive *V. harveyi* isolates responsible for luminous vibriosis in aquatic organisms (Gomez-Gil et al. 2004a); all of the isolates analyzed had the main phenotypic features of the species *V. harveyi* (Farmer and Hickman-Brenner 1992; Holt et al. 1994; Alsina and Blanch 1994a, b). They grew on TCBS agar, were motile, fermented glucose, and were oxidase positive and sensitive to the vibriostatic agent 0/129 at 150 μg. Presumptive *V. harveyi* isolates were arginine dihydrolase negative and lysine and ornithine decarboxylase positive. Most isolates were luminescent and utilized D-gluconate, L-glutamate, D-glucuronate, heptanoate, D-galactose, and sucrose and grew at 40 °C, but they did not utilize L-histidine and L-arabinose. Most isolates (n = 31) clustered with the type strain of *V. campbellii* LMG 11216 T. Because the isolates assigned to *V. campbellii* and to *V. harveyi* were very heterogeneous, DNA–DNA hybridizations were performed with representative strains to check the robustness of the clusters based on rep-PCR. The DNA–DNA hybridization experiments clearly showed that the presumptive *V. harveyi* isolates belong to the species *V. campbellii*, having at least 71 % DNA similarity. In another study, rep-PCR was used to analyze the genomic diversity of vibrios isolated from the gut of abalones (*Haliotis* spp.) (Sawabe et al. 2002). rep-PCR patterns using the primer (GTG)5 pointed out that each abalone species has a particular population of vibrios which is related to *V. halioticoli*.

Microarrays

The sequences of the whole genome of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* strains have provided means for the development of modern molecular detection methods, e.g., microarrays and real-time PCR of pathogenic vibrios, e.g., *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. Microarray for the detection of pathogenic *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* strains has been designed (Panicker et al. 2004). Ten loci were spotted (*vvh* and *viuB* of *V. vulnificus*; *ompU*, *toxR*, *tcpI*, and *hlyA* of *V. cholerae*; and

tlh, *tdh*, *trh*, and open reading frame 8 of *V. parahaemolyticus*) on the slide. They reported a sensitivity of 1 CFU in 1 g in enrichment samples. *V. vulnificus* and *V. parahaemolyticus* were detected in wild oysters, but not *V. cholerae*. Microarrays have been used to identify the causative agents of vibriosis, *V. anguillarum*, and furunculoses, *Aeromonas salmonicida*, in fish (Gonzalez et al. 2004). A sensitivity and specificity of nearly 100 % of the technique for detecting the two fish pathogens was obtained. These studies highlight the usefulness of microarrays for detection and identification of vibrios and open up the possibility of applying this technique to all currently recognized species of vibrios.

MLSA: A Paradigm of *V. harveyi* Case in Species Identification

Identification and classification of *Vibrio* species have relied upon band pattern methods (e.g., AFLP) and DNA–DNA hybridization. Introduction of AFLP in vibrio taxonomy shows a great impact on unveiling the huge biodiversity of vibrios (Thompson et al. 2001, 2006). However, data generated by these methods cannot be used to build an online electronic taxonomy. In order to overcome these limitations, Thompson et al. (2007b) developed the first standard multilocus sequence scheme focused on the ubiquitous and pathogenic *V. harveyi* species group (i.e., *V. harveyi*, *V. campbellii*, *V. rotiferianus*, and a new as yet unnamed species). Because *V. campbellii* and *V. harveyi* share nearly 100 % 16S rRNA gene sequence similarity and around 70 % DNA–DNA hybridization (DDH) similarity, discriminating these species remains a hard task for taxonomy. Thompson et al. (2007b) examined a collection of 104 isolates from different geographical regions and hosts using segments of seven housekeeping genes. These two species formed separated clusters on the basis of *topA*, pyrH, *ftsA*, and *mreB* gene sequences, respectively. However, the phylogenetic picture obtained by *gyrB*, *recA*, and *gapA* was more complex though; *V. campbellii* appeared nested within *V. harveyi* in the *recA* tree, whereas *V. harveyi* formed a tight nested cluster within *V. campbellii* by *gapA*. The *gyrB* gene had no taxonomic resolution and grouped the two species together. In spite of this, the concatenated sequences provided evidence that the two species form two separated clusters. The open database resource (http://www.taxvibrio.lncc.br/) developed in this study is available for readily identification of *V. harveyi* and *V. campbellii* isolates. During the study, presence of some phylogenetically incohesive strains appeared from the *V. harveyi* global collection; finally based on a fine-scale polyphasic taxonomy of strains isolated from Australian Rock lobster and abalone, *V. jascicida* is recently proposed as a new member of *Vibrionaceae* (Yoshizawa et al. 2012). Recently, the MLSA also provide new information about misidentification of species belonging into *V. harveyi*, *V. campbellii*, *V. communis*, and *V. owensii* (Lin et al. 2010; Hoffmann et al. 2011; Can-Gomez et al. 2011); however, in some strains, DDH validation and/or genome comparison must be required for the final judgment. Therefore, in era that many MLSA schemes for *Vibrionaceae* are reported recently for not only species identification (Thompson et al. 2005b; 2008) but also population and/or evolutionary purposes (Sawabe et al. 2007a), we must use and/or develop a proper gene set showing clear cluster separation, e.g., *topA*, *pyrH*, *ftsA*, and *mreB* genes in the case of Harveyi clade species.

Genome Comparison

The complete whole-genome sequences are available from public databases, the three most important human pathogens *V. cholerae* El Tor N16961 (Heidelberg et al. 2000), *V. parahaemolyticus* RIMD2210633 (Makino et al. 2003), *V. vulnificus* YJ016 (Chen et al. 2003), CMCP6 (Kim et al. 2011), and MO6-24/O (Park et al. 2011) and the squid symbiont *V. fischeri* ES114 (Ruby et al. 2005) and MJ11 (Mandel et al. 2009), *V. furnissii* NCTC 11218 (Lux et al. 2011), *V. anguillarum* 775 (Naka et al. 2011), a bioluminescent strain *V. campbellii* ATCC BAA 1116 (Lin et al. 2010), *V.* '*splendidus*' LGP32, *A. salmonicida* (Hjerde et al. 2008), and *P. profundum* SS9 (Vezzi et al. 2005), EJY3, and Ex25 (*Table 36.3*). Furthermore, complete whole-genome sequences of at least additional seven *V. cholerae* strains and six *V. parahaemolyticus* strains are also determined. In addition with these completed strains, complete whole-genome sequences of *V. harveyi* S20 and *V.* '*tritonius*' AM2 are recently determined (Sawabe et al. unpublished data). Furthermore, at least 340 draft genome sequences within 52 species are also analyzed and/or underway (http://www.vibriobiology.net/) (*Table 36.1*). Totally nearly half of *Vibrionaceae* species are sequenced in their genome.

Since the first report of the presence of two unique chromosomes in three representative strains of *V. cholerae* (Trucksis et al. 1998), all species of the *Vibrionaceae*, of which genetic analysis and/or complete genome sequencing has been done, were confirmed to carry two unique circular chromosomes (Yamaichi et al. 1999; Okada et al. 2005) (*Table 36.3*). Based on the complete genome sequence data, large chromosomes (called chromosome 1: Chr. 1) range from 2.90 to 4.09 Mb and small chromosomes (chromosome 2: Chr. 2) from 1.07 Mb to 2.24 Mb (*Table 36.3*). Protein coding regions occupy up to 90 % of the vibrio chromosomes; 2,586–3,546 ORFs are found on Chr. 1 and 984–2,373 ORFs on Chr. 2 (*Table 36.3*). Five of the nine genomes sequenced also have a large plasmid (30–89 Kb).

Genes encoding for DNA replication and repair, transcription, translation, and cell wall biosynthesis and a variety of central catabolic and biosynthetic pathways (see central metabolism in Physiology section), but also genes encoding for bacterial pathogenicity (including region of CTX and TCP), are located on Chr. 1 of *V. cholerae* (Heidelberg et al. 2000). On the other hand, Chr. 2 contains 59 % of the hypothetical genes, 42 % of the genes with unknown function, and some potential toxin genes (*hap* and *hlyA*). It is noteworthy that partitioning hypothetical proteins on the *V. cholerae* chromosome 2 is highly localized in the integron islands.

Table 36.3
Representative genome constructions of *Vibrionaceae*

Chromosome accession numbers	Size (bp)	GC (%)	No. ORFs	Percent coding	rRNA operons	tRNAs
Photobacterium profundum SS9						
CR354531	4,085,301	42	3,425	82.38	14	145
CR354532	2,237,950	41.2	2,004	80.63	1	32
CR377818 (plasmid)	80,033	44	67	73.32	0	0
Vibrio cholerae El Tor N16961						
AE003852	2,961,151	47.7	2,770	88.6	8	94
AE003853	1,072,914	46.9	1,115	86.3	0	4
V. fischeri ES114						
NC006840	2,897,536	40	2,586		11	107
NC006841	1,330,333	37	1,174		1	11
NC006842 (plasmid)	45,849	38.4	57		0	0
V. parahaemolyticus RIMD2210633						
BA000031	3,288,558	45.4	3,262	86.9	10	112
BA000032	1,877,212	45.4	1,697	86.9	1	14
V. vulnificus YJ016						
BA000037	3,354,505	46.4	3,262	88.7	8	100
BA000038	1,857,073	47.2	1,697	90.3	1	12
AP005352 (pYJ016 plasmid)	48,508	44.9	69	89.8	0	0
V. furnisii NCTC 11218						
CP002377	3,294,546	50.7	3,013		7	95
CP002378	1,621,862	55.6	1,449		0	5
V. campbelii BAA 1116						
NC009783	3,765,351	45.6	3,546		9	105
NC009784	2,204,018	45.3	2,373		1	16
NC009777 (plasmid)	89,008	43.8	120		0	0
V. splendidus LGP32						
NC011753	3,299,302	44	2,946		7	97
NC011744	1,675,519	43.6	1,485		1	17
A. salmonicida LFI1238						
NC011312	3,325,165	39.2	2,820		11	92
NC011313	1,206,461	38.2	984		1	13
NC011311 (plasmid)	83,540	40.7	72		0	0
NC011314 (plasmid)	30,807	37.3	29		0	0
NC011315 (plasmid)	5,360	38.1	3		0	0
NC011316 (plasmid)	4,327	35.6	3		0	0

Most of the essential genes required for growth and viability, even *dsdA*, *thrS*, and genes encoding the ribosomal proteins L20 and L35, are located on Chr. 1 of *V. parahaemolyticus* (Makino et al. 2003). Comparative whole-genome analysis between *V. parahaemolyticus* and *V. cholerae* revealed that the type III secretion system (T3SS) is an important virulence factor of *V. parahaemolyticus*. T3SS may cause inflammatory diarrhea and septicemia (Makino et al. 2003). Genes coding for the T3SS are not found in the genome of *V. cholerae*. Genes responsible for the transcriptional regulation, solute transport, and lateral flagella are on the Chr. 2 of *V. parahaemolyticus*. This chromosome could have a role in tolerating changing environmental conditions (Makino et al. 2003). A large gene-capture system called superintegron is situated in Chr. 1 of *V. parahaemolyticus* and in Chr. 2 in *V. cholerae*. The gene encoding for the thermostable direct hemolysin (TDH) is located in a pathogenicity island of Chr. 2 of *V. parahaemolyticus*. T3SS2 encoded in pathogenicity island (Vp-PAI) is the main contributor to enterotoxicity. The expression of Vp-PAI encoded genes is regulated by two

Table 36.4

Properties of representative bacteriophages found in vibrios

Name	Original host	Genome size (kb)	Whole genome quence[a]	Properties	References
Inoviridae (filamentous, ssDNA)					
CTXΦ	*V. cholerae*	6.4	A	Encoding cholerae toxin	Waldor and Mekalanos (1996)
VSK	*V. cholerae* O139	7	N/A		Kar et al. (1996)
fs1	*V. cholerae* O139	6.3	A	Presence of zot gene homologue?	Honma et al. (1997), Ehara et al. (1997)
fs2	*V. cholerae* O139	8.7	A		Ikema and Honma (1998)
493	*V. cholerae* O139	9.3	N/A		Jouravleva et al. (1998)
f237	*V. parahaemolyticus* O3:K6	7.7	A	pO3K6 is RF form of f237	Nasu et al. (2000b)
VfO4K68	*V. parahaemolyticus* O4:K68	6.9	A		Chang et al. (2002)
KSF-1F	*V. cholerae*	7.5	A	RS1 packaging?	Faruque et al. (2003b)
VGJF	*V. cholerae*	7.5	A		Campos et al. (2003)
Myoviridae (icosahedral head with tail, dsDNA)					
K139	*V. cholerae* O139	33	A	Icosahedral head (40–50 nm) with tail (40–105 nm)	Kapfhammer et al. (2002)
FP15	*V. cholerae*	29	N/A	Icosahedral head (40–50 nm) with tail (40–105 nm)	Talledo et al. (2003)
KVP40	*V. parahaemolyticus*	244	A	Icosahedral head (70 × 140 nm) with tail	Miller et al. (2003)
VHML	*V. harveyi*	43	A		Oakey et al. (2002)
Siphoviridae (dsDNA) pVP-1	*V. parahaemolyticus*	111.5	A	Icosahedral head (40–50 nm) with tail (140–150 nm)	Kim et al. (2012)
Other type (dsDNA)					
VpV262	*V. parahaemolyticus*	46	A	Podoviridae, icosahedral head with sort tail Sipoviridae?	Hardies et al. (2003)
VP16(VP16T & VP16C)	*V. parahaemolyticus*	47–49	A	Icosahedral head (50–60 nm) with tail (80–100 nm). Virulence gene transfer?	Seguritan et al. (2003)
RS1	*V. cholerae*	2.5–2.7	A	Unclassified, unknown shape. Satellite phage genome associating with CTXF	Waldor and Mekalanos (1996), Davis et al. (2002)

[a]A available, N/A not available

transcriptional regulators, VtrA and VtrB. However, host-derived inducer for the Vp-PAI has not been fully elucidated yet. Recently, bile is identified as a host-derived inducer of T3SS2-related proteins via *vtrA*-mediated *vtrB* transcription (Gotoh et al. 2010).

The integron gene-capture system consists of an integrase (*intI*), a primary recombination site (*attI*), a multiple target-specific site (*attC*), and associated gene cassettes (Rowe-Magnus et al. 1999). In *V. vulnificus*, the superintegron spans 138 kbp of the large chromosome. A total of 188 *attC* sites have been found in the chromosome 1 of this organism (Chen et al. 2003). The genome of *V. cholerae* El Tor N16961 contains 175 *attC* sites accounting for 126 kb region of the chromosome 2 (Heidelberg et al. 2000). Recently, the regulations of integrase expression are identified to be involved in SOS responses by bacterial conjugation and transformation and new cyclic AMP protein (CRP)-dependent regulation (Baharoglu et al. 2012).

Virulence factors, e.g., cytolysin gene *vvhA*, metalloprotease gene *vvp*, and phospholipase gene *vpl*, are located on the small chromosome of *V. cholerae*.

Recently massive genome comparison was conducted by Thompson et al. (2009). This study showed that the vibrio pan- and core-genome contains 26,504 and 488 genes, respectively, corresponding to a vast reservoir of genetic diversity. The *V. cholerae* core genome and pan-genome consist of 1,520 and 6,923 genes, respectively. Pan-genomes might allow different strains of *V. cholerae* to niches.

Phages

At least 183 tailed and 10 filamentous vibriophages have been described (Kapfhammer et al. 2002). Tailed phages might belong to the *Caudoviridales* and filamentous phages to *Inoviridae*. Major original hosts of vibriophages are *V. cholerae* (O1, O139, and non-O1/O139), *V. parahaemolyticus* (including O3:K6 and O4:K68 serotypes), and *V. harveyi*. The size of filamentous vibriophage genomes is between 6 and 9 kbp of single-strand DNA and between 33 and 244 kbp of double-strand DNA in tailed phages (Table 36.4). The largest genome reported is of a broad host tailed phage KVP40 originally isolated from *V. parahaemolyticus* (Miller et al. 2003). Five whole-genome sequences of tailed phages and seven of filamentous phages have been described.

A CTX element encoding cholera toxin has been identified on the chromosome 1 of *V. cholerae* El Tor (Heidelberg et al. 2000). This element is capable of being transduced by a viruslike particle. Indeed, the CTX element itself is a filamentous Inovirus-like phage called CTXΦ (Waldor and Mekalanos 1996). The 6.4 kbp ssDNA of the CTX element consists of a 4.5 kbp core region encoding *cep, orfU, ace, zot* and *ctxAB* genes, and the remaining region contains repetitive sequences, called RS, which encode for *rstABCR, orfU,* and *zot* genes and may be involved in CTXΦ morphogenesis (Waldor and Mekalanos 1996). It is speculated that the RS regions have a function of satellite phage genome associating CTXΦ (Davis et al. 2002; Faruque and Mekalanos 2003). The pilus colonization factor (TCP) is necessary for the CTXΦ transduction, and the phage transduction is enhanced in vivo inside the mouse intestine environment. The genome construction of CTXΦ is similar to M13 (Waldor and Mekalanos 1996) and VJGΦ (Campos et al. 2003).

All types of known *Caudoviridales* and *Inoviridae* are found in *V. parahaemolyticus* as a host bacterial species (Table 36.4). Inovirus-like filamentous phages are isolated from *V. parahaemolyticus* O3:K6 (Nasu et al. 2000b) and O4:K68 (Chang et al. 2002) serotypes. O3:K6 serotype infected by the phage f237 constructs a circular plasmid called pO3K6 during the replication process (Nasu et al. 2000b). *Myoviridae*-, *Podoviridae*-, and *Siphoviridae*-like phages have been isolated from *V. parahaemolyticus*, among them, KVP40, a *Myoviridae*-like phage that has a larger head than other *Myoviridae*-like vibriophages, and which possess the biggest viral genome, 244 kbp (Miller et al. 2003). The TDH gene has never been detected in all the known phages infecting *V. parahaemolyticus*.

It is known that *V. harveyi* infects a wide variety of marine animals both vertebrates and invertebrates (Oakey and Owens 2000). A new *Myoviridae*-like bacteriophage called VHML (*V. harveyi* Myovirus-like) was isolated from a toxin-producing *V. harveyi* strain (Oakey and Owens 2000). The virus has a 40–50 nm icosahedral head with a 140–150 nm tail and a 43 kbp dsDNA packed in the head. A non-virulent strain of *V. harveyi* was transduced by experimental VHML infection and became virulent for shrimp larvae (Munro et al. 2003). Whole-genome sequence of the VHML suggests that transcriptional regulation of the viral N6-adenine methyltransferase gene (dam) may be associated with the virulence conversion of the virus (Oakey et al. 2002).

More recently, complete genome sequence of novel *Siphoviridae* phage from *V. parahaemolyticus* is reported (Kim et al. 2012); totally 48 vibrio phage genomes appeared in NCBI genome resource.

Phenotypic Analyses

Increasing knowledge about vibrio biodiversity shows versatile metabolism of this group, unique physiological findings in hydrogen production machinery, phototrophy, and a group behavior by swarming. In this section, recent findings for these physiological topics are described to emphasize the extreme vibrio diversity. The phenotypes of the representative species are shown in Supplementary Table 36.2.

Physiology

Central Metabolism: Fermentation and Gas Production

Extensive comparative studies of sugar and amino acid metabolisms have been done for differentiating *Vibrionaceae* and *Enterobacteriaceae* (Baumann and Schubert 1983). Few species of vibrio produce gas via hexose fermentation. Few species are oxidase negative (Baumann and Schubert 1983; Shieh et al. 2000), suggesting the existence of a different central metabolism (respiration and fermentation) than that of the *Enterobacteriaceae*.

Early biochemical studies revealed a D-glucose phosphotransferase system (PTS) in *V. cholerae* (Bag 1973) and *V. parahaemolyticus* (Matsumoto et al. 1974; Kubota et al. 1979), meaning that D-glucose is catabolized via a phosphoenolpyruvate. Whole-genome sequence analyses of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* confirmed the presence of at least six PTSs-related genes. Glucose-, trehalose-, and *N*-acetylglucosamine-specific PTSs are common in these *Vibrio*

species and are encoded on Chr. 1. Mannitol-, fructose-, and sucrose-specific PTSs are found in all Vibrio species, but these genes may be located on both chromosomes. Multiple copies of fructose-specific PTSs are found mainly in Chr. 1 of *V. cholerae* and in Chr. 2 of *V. parahaemolyticus* and *V. vulnificus*. *Vibrio* genomes also carry many putative and uncharacterized PTSs. *E. coli* lack the glucokinase gene that is an alternative path of glucose phosphorylation, suggesting that PTSs contribute greatly to the central metabolism of Vibrio species. Glucose-6-phosphate and fructose-6-phosphate are catabolized via a constitutive Embden–Meyerhof pathway (Baumann et al. 1973; Baumann and Schubert 1983), and the complete set of genes encoding for the enzymes of Embden–Meyerhof pathway are found in the Chr. 1 of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* (Heidelberg et al. 2000; Chen et al. 2003).

Early biochemical studies also revealed that D-gluconate is metabolized by means of an inducible Entner-Doudoroff pathway in *V. natriegens*, which was previously misidentified as an unusual pseudomonad, *Pseudomonas natriegens*, with a constitutive Embden–Meyerhof pathway (Eagon and Wang 1962; Baumann and Schubert 1983). *V. parahaemolyticus*, *V. alginolyticus*, *V. pelagius*, *V. nigripulchritudo*, and *V. nereis* also have an inducible gluconokinase (Baumann et al. 1973). Nowadays, D-gluconate assimilation is documented in most vibrios. Information from whole-genome sequences shows the ability of D-gluconate utilization, but not of D-xylose or L-arabinose, which is a precursor of ribose-5-phosphate (Baumann and Schubert 1983; Fraenkel 1996). The complete set of 6-phosphogluconate pathway genes for both the Entner-Doudoroff and pentose-6-phosphate pathways are found in *V. vulnificus*, but not in *V. cholerae* and *V. parahaemolyticus*.

Pyruvate generated through glycolysis is further aerobically metabolized by the TCA cycle. Most species of vibrio are reported to have the c type of cytochrome, which corresponds to oxidase-positive traits (Baumann et al. 1973; Baumann and Schubert 1983). Alternatively, pyruvate is anaerobically catabolized via fermentation; end products of glucose fermentation are formic, acetic, lactic, and succinic acid, as well as ethanol (Doudoroff 1942; Unger et al. 1961; Baumann and Schubert 1983; Sawabe et al. 2003). Three major fermentation end products of *Vibrio* species are formic–acetic acid (FA), lactic–formic–acetic acid (LFA), and lactic–acetic (LA). In fact, a set of lactate dehydrogenase, pyruvate formate lyase, phosphotransacetylase, and acetate kinase genes are found in *V. parahaemolyticus* and *V. vulnificus* which have LFA-type fermentation. Multiple copies and/or several types of alcohol dehydrogenase genes are also found in the three *Vibrio* species of which whole genomes are available.

Gas production of *E. coli* is suspected to occur in the large membrane protein architecture of the formate hydrogen lyase (FHL) complex encoded by the *hyp* and *hyc* operons (Böck and Sawers 1996). Formic acid is splitted into CO_2 and H_2; but genes responsible and related operons are not present in *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. Recently, new gas-producing vibrio (*V.* '*tritonius*') was isolated from wild rice root in India on the gut of sea hare *Aplysia kurodai* (Sawabe et al. unpublished data). Genome analysis of the species, in addition with known vibrio gas-producing vibrios (*V. ruber* and *V. porteresiae*), revealed the presence of gene clusters responsible to be FHL complex. Further analysis is underway to clarify the physiological characteristics of these gas-producing vibrios.

Phototrophy

Increasing attention on marine microbial diversity reveals that proteorhodopsins (PRs) are globally abundant photoproteins found in bacteria in the photic zone of the ocean. RPs are membrane embedded and light-driven proton pumps, which generate a chemiosmotic potential by translocating protons across an energy-transducing membrane (Gómez-Consarnau et al. 2010). Vibrios are well-known marine ubiquitous species and metabolically versatile heterotrophs; only a few researchers have expected to the findings of PRs possessing *Vibrionaceae* species. By whole-genome sequencing and analysis of *Vibrio* sp. AND4 isolated from surface water in the Andaman Sea in December 1966, PR-encoding gene is identified on the AND4 genome. Incongruence of topology in between PR amino acid sequence phylogeny and 16S rRNA gene phylogeny, it is concluded that PR genes might be acquired by horizontal gene transfer from possibly an alphaproteobacterium. In fact, transposable elements are found in a franking region of the PR gene. A gene cluster (*crtEIBY* and *blh*) responsible for chromophore retinal synthesis is also found at the same locus of the PR gene. The AND4 PR shares 87 % amino acid sequence similarity against that of *V. campbellii* BAA 1116, and both PRs are fine-tuned towards the green light due to conserved to possess L105. Growth experiments reveal no growth affect in AND4 in rich medium both in light and dark conditions, but reductive division of the strain in oligotrophic medium (sterile- and particle-free natural seawater supplemented with low concentration of organic and inorganic nutrients) is observed during couple of days. In 10 days of culture, the bacterial numbers under the light condition decrease but still higher than that under dark. PR phototrophy in vibrios is first demonstrated and may have a role to improve survival of this bacterium under starvation (Gómez-Consarnau et al. 2010).

Use of *V. campbellii* BAA 1116 strain shows a different picture from the physiological case of AND4 strain on phototrophy (Wang et al. 2012). Δ*pR* mutant experiments demonstrate PR proton pumping is active in *V. campbellii* BAA 1116 cells. Comparative photoinduction assay reveals distinct upregulation of *pR* expression. These results indicate that PR-mediated photophosphorylation in wild-type *V. campbellii* cells occurred. Surprisingly, however, the effect of continuous light on BAA 1116 under the experimental conditions results in mortality and dominates any measurable contribution by PR for the cellular yield enhancement. The number of *pR* transcripts peaks early stationary phase in the strain. RpoS1 positively regulates *pR* expression and PR holoprotein pigmentation. It is possible to elucidate that

PR function in *V. campbellii* BAA 1116 strains is in protection against deleterious effects of photooxidative stress by scavenging harmful reactive oxygen species (Wang et al. 2012). As *pR* and retinal biosynthesis genes are also found in 25 % of *V. campbellii* isolates, roles of PRs in vibrios should be extensively studied. In fact, draft genome sequence of PR gene cluster containing *V. campbellii* PEL22A isolated from open ocean water in the Abrolhos Bank is recently reported (Amaral et al. 2012).

Motility and Chemotaxis

Most vibrios are motile (Baumann et al. 1973; Baumann and Schubert 1983). *Vibrios* present both polar and peritrichous flagella that provide motility. In *E. coli*, about 2 % of the total energy in the cell metabolism is spent with construction and movement (Macnab 1996). Synthesis and rotation of the flagellar system (basal body, hook, and flagellar filament) are meticulously controlled by sensing chemicals (chemotaxis) and physiological signals. There are many excellent studies on the flagellar super-architecture and regulation of peritrichous flagellar systems in *E. coli* and *Salmonella* (Macnab 1996), polar flagellar systems in *Pseudomonas aeruginosa*, pleomorphic flagellar system in *Caulobacter crescens*, and dual flagellar system (composed of sheathed and unsheathed polar flagella) in *V. parahaemolyticus* and *V. alginolyticus* (McCarter 2001; Stewart and McCarter 2003). Flagellar filaments are made of self-assembling flagellin proteins and bundled inside a helix-hollow tube (McCarter 2001). The flagellin subunits in the polar flagella of *V. cholerae*, *V. parahaemolyticus*, and *V. anguillarum* comprise complex subunits, not a single homologous subunit such as that of *E. coli* and Salmonella. At least six flagellin (*flaFBA* and *flaCDE*) genes are located on two loci in the *V. cholerae* and *V. parahaemolyticus* genomes (Heidelberg et al. 2000; Makino et al. 2003). Major subunits in wild-type *V. parahaemolyticus* are FlaA, FlaB, and FlaD (McCarter 2001). The peritrichous (lateral) flagella of *V. parahaemolyticus* consist of a single flagellin subunit encoded by *lafA* (Stewart and McCarter 2003), and the genes responsible for the Laf system are found only on the small chromosome (Makino et al. 2003).

Many *Vibrio* and *Photobacterium* species can swim liquid environments as fast as 60 μm/s (McCarter 2001; Larsen and Boesen 2001; Magariyama et al. 2001). Forward swimming speed (53 μm/s) of *V. alginolyticus* is higher than backward swimming (37 μm/s) in a buffered saline supplemented with glucose at room temperature (Magariyama et al. 2001). Both forward and backward speeds of a long-haired *V. alginolyticus* mutant are almost the same as those of the wild type (Magariyama et al. 2001). The flagellar filament acts as a propeller turned by a reversible rotary motor embedded in the membrane. The rotary motor is an important component of the basal body (Macnab 1996; McCarter 2001; Okabe et al. 2002). The energy to power flagellar rotation is derived from the transmembrane electrochemical potential of specific ions (H^+ and Na^+) (McCarter 2001; Stewart and McCarter 2003). *V. parahaemolyticus* and *V. alginolyticus* have unique polar and lateral flagellar systems. Sodium channel-blocking drugs, such as amiloride, specifically inhibit sodium-driven motility and have been used to prove motor function in *Bacillus* (Sugiyama et al. 1988; McCarter 2001). Sodium also drives the rotation of the polar flagella, and the proton motive force may power the rotation of the lateral flagella in *V. alginolyticus* and *V. parahaemolyticus* (Kojima et al. 1997, 1999a; Yorimitsu and Homma 2001); the single polar flagellum of *V. cholerae* is also sodium driven (Yorimitsu and Homma 2001).

Polar flagella rotation averages 1,100 rps, but a maximum of 1,700 rps can be achieved by *V. alginolyticus*. The structure of the sodium-driven torque generating apparatus was elucidated by Homma and coworkers (Okabe et al. 2002; Yorimitsu et al. 2003, 2004). Two cytoplasmic proteins, MotA and MotB (or PomA and PomB in *V. alginolyticus* and *V. cholerae* (Kojima et al. 2000), form the force-generating unit through which the protons are channeled (Yorimitsu et al. 2004). The stator complex MotA/MotB (or PomA/PomB) and rotor protein FliG interact to generate the torque for rotating flagella; torque is transmitted from the MotA–MotB complex to the flagellar basal body. Thus, torque generator acts as a stator to transmit force to the rotor. Critical electrostatic interactions between MotA and FliG have been demonstrated (Yorimitsu et al. 2003). Entire basic structure of the Na^+-driven polar flagellum of *V. alginolyticus* is unveiling using molecular biological experiments and electron microscopy observation; the structure is very similar to that of H^+-driven flagella of *Salmonella* and *E. coli*, but one of the differences is the presence of the T ring structure. The components of T ring are MotX and MotY, which are fist identified as motility proteins. The function of the T ring may be the incorporation or the stabilization of the stator units that surround the motor (Li et al. 2011). Furthermore, there are many progresses in the flagella structure and regulations: (1) FlhFG involve polar localization of the Na^+-driven flagellum, (2) MotX, MotY, PomA, and PomB involve the Na^+-driven startor complex interacting to the T ring, and (3) the dynamics of assembly (Li et al. 2011).

Bacteria respond to signals in the environment by modulating the direction of flagellar rotation; chemo-attractants trigger bacterial flagella counterclockwise to run the cells. Flagellar motility is also a virulence factor of *V. cholerae* (Guentzel and Berry 1975; Richardson 1991) and of *V. vulnificus* (Kim and Rhee 2003) and a mechanism for mutualistic colonization in *V. fischeri* (Millikan and Ruby 2003). In addition to its role as a propulsive organelle, the polar flagellum appears to act also as a sensor. Growth on surfaces or in viscous environments induces differentiation to a swarmer phenotype where cell division ceases, cells become transiently elongated (30 μm), and the lateral flagellar system is induced. The polar flagellum is produced constitutively, irrespective of liquid- or surface-associated growth (McCarter 2001; Stewart and McCarter 2003). Nowadays, transcriptional profiling using microarray

technology can reveal genes functioning in free swimming and in chitin-attached swarmer cells, as well as *V. cholerae* in the intestinal tract (ScottMerrell et al. 2002).

Many chemoreceptors or potential chemoreceptors have been found in *Vibrio* genomes. However, there are few studies on the identification of chemo-attractants (McCarter 2001). Chemotaxis has been thoroughly studied in *V. furnissii*, which shows chemotaxis towards a variety of chitin derivatives (Bassler et al. 1989, 1991; Yu et al. 1993; Keyhani and Roseman 1999). Recent studies in *V. fischeri* revealed chemotaxis towards serine, nucleosides, nucleotides, *N*-acetylneuraminic acid, and several sugars. *N*-acetylneuraminic acid is a component of the light-organ mucus of the Hawaiian squid *Euprymna scolopes*, which could contribute to the initiation of the *V. fischeri*–squid mutual partnership (DeLoney-Marino et al. 2003). *V. fischeri* needs the appropriate motility for the successful colonization of the light organ. After settlement, the motility of *V. fischeri* strains is lost. The sigma54-dependent transcriptional activator FlrA has recently been cloned, demonstrating its essential role for symbiosis (Millikan and Ruby 2003). *V. halioticoli*, *V. neonatus*, *V. ezurae*, *V. superstes*, *V. gallicus*, and *V. rumoiensis* do not have flagella and are nonmotile. Recently draft genome sequences of *V. halioticoli*, *V. neonates*, and *V. gallicus* are obtained, and no flagellar genes have been elucidated based on the draft genome (Sawabe unpublished data).

Swarming is a particular type of motility dependent on flagella to allow moving cells rapidly over and between surfaces and through viscous environments. Little has been known about the physiological aspects of swarming. Recently, however, the dynamics and mechanism of the swarming cells have been extensively studied in *E. coli* as well as vibrios (McCarter 2010), and various kinds of new findings on global gene expression controls linked to quorum sensing (QS) using swarming-proficient and their derivative strains of *V. parahaemolyticus* (Gode-Potrats and MacCarter 2011).

Under the presence of low cell density (low autoinducer (AI) molecule concentration), LuxO is phosphorylated by histidine kinase, and resultant LuxO-P induces small quorum regulatory RNAs (Qrrs). The Qrrs destabilized mRNA for the central output regulator of the system. At the high cell density (high AI concentration), the histidine kinase becomes LuxO phosphatases. Due to the LuxO inactivated, Qrrs are no longer transcribed, and the mRNA for the central output regulator is then translated. Vibrios share similar backbone in the QS system, but the numbers and kinds of AI, sensor kinases, Qrr genes, and output regulon are different (Gode-Potrats and MacCarter 2011). OpaR is an ortholog of LuxR, which is a central terminal output regulator of QS pathway. Comparison of the global gene expressions and ectopic expression among the opaque (OP) (*opaR*), translucent (TR) (opaR mutation), and the opaR + swarming-proficient TR mutant of *V. parahaemolyticus*, alternative LuxO (LuxO*), is identified to affect the expression of Qrrs and the activity of OpaR in swarming repair. In addition to the OpaR effects on the Laf gene system, Δ*opaR* transcriptome experiments reveal the extended role for OpaR as master negative regulator of the Laf gene system, controlling the entire surface-sensing regulon including *scrABC* operon, T6SS, and competence. Genes encoding T3SS1 are redepressed in Δ*opaR* strain, and these OpaR-silenced strains show higher cytotoxic effects on Chinese hamster ovary (CHO) cells than those of OpaR + strains (Gode-Potrats and MacCarter 2011). It is concluded that *V. parahaemolyticus* displays on/off phase switching with respect to the archetypal pathway of quorum sensing in *Vibrionaceae*.

New aspects of group activity controls in vibrios have been further revealed recentrly. S-signal is a pheromone that can be communicated between cells in coculture to regulate surface colonization, in a swarming-proficient and virulence strain of *V. parahaemolyticus* (Trimble and McCarter 2011). The signal harvested from cell-free culture supernatant can stimulate swarming gene expression at low cell density. The S-signal is generated by ScrABC; ScrA is an aminotransferase, ScrB is a periplasmic binding signal receptor, and ScrC is a cytoplasmic GGDEF–EAL domain-containing protein that has the ability to form and degrade the second messenger bis-(3′-5′) cyclic dimeric GMP (c-di-GMP). ScrA in neighboring cells can alter the activity of ScrC in ScrB-dependent way, and the transformed ScrC affects the swarming behavior in *V. parahaemolyticus* population; ScrA and ScrBC are responsible for signal generation and for the detection, respectively, and the S-signaling activates the Laf genes expression in dose-dependent manner and represses the expression of the capsular polysaccharide biosynthetic locus. As the *V. parahaemolyticus* strain is silenced for the vibrio archetypal quorum sensing pathway, the second messenger can stimulate swarming of the strain in the alternative quorum sensing pathway, of which the finding can expand the lexicon and language of cell–cell communication (Trimble and McCarter 2011).

Isolation, Enrichment, and Maintenance Procedures

Vibrios are fairly easy to isolate from both clinical and environmental materials, though some species may require growth factors and/or vitamins. Of special mention is the need of NaCl for most of the species of *Vibrio*, although some species can grow with minimum NaCl concentrations, e.g., *V. cholerae*, *V. mimicus*, *V. hispanicus*, and some strains of *V. fluvialis*, *V. furnissii*, and *V. metschnikovii* (Alsina and Blanch 1994b; Gomez-Gil et al. 2004b). Optimal Na^+ concentration for many marine bacteria is between 70 and 300 mM (Reichelt and Baumann 1974). Different strategies may be used in order to isolate specific *Vibrio* species from environmental and clinical samples. For instance, a protocol for specific isolation of virulent strains of *V. vulnificus* using a selective agar medium was developed (Sanjuan and Amaro 2004). The most common media used for the isolation and cultivation of vibrios are listed directly beneath.

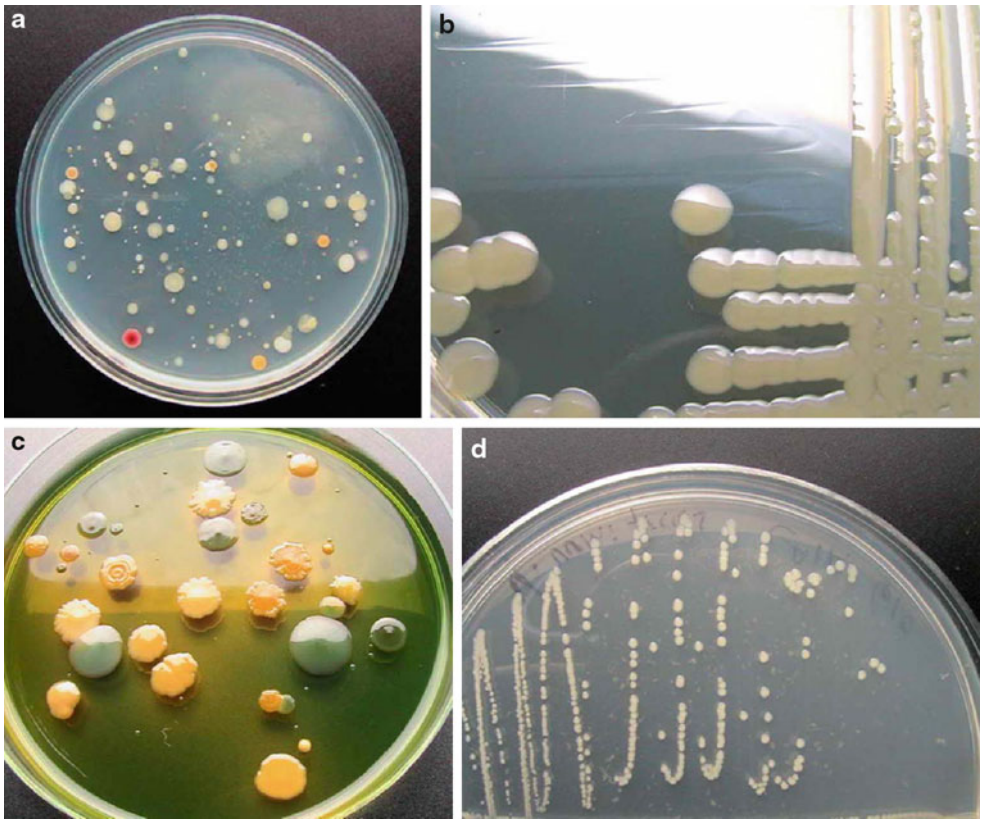

Fig. 36.6
Colonial morphology of vibrios in different media. (a) Vibrios and other bacteria on Marine agar, (b) vibrios on TSA agar, (c) vibrios on TCBS agar, and (d) a vibrio on marine agar

Culture Media

TCBS Agar

Thiosulfate–citrate–bile salts agar (TCBS, Oxoid, Difco, Merck) is an ideal medium for the selective isolation and purification of vibrios. Although this medium was originally designed for the isolation of *V. cholerae* and *V. parahaemolyticus* (Nakanishi 1963) and latter modified (Kobayashi 1963), most vibrios form healthy medium to large colonies with many different colonial morphologies (*Fig. 36.6c*). Gram positive and coliforms are strongly inhibited due to the presence of bile salts. Vibrios which are able to use sucrose will form yellow colonies, while sucrose-negative strains will grow as green colonies. Precaution has to be observed since in older cultures (more than 48 h), refrigerated plates, or in heavily grown plates, color of the colonies may change; therefore, colonial color has to be registered only in recent and well-isolated colonies. Since TCBS is composed of many ingredients not easily acquired, we recommend buying it from commercial sources. It is not necessary to add salt (NaCl) to the TCBS medium.

Until now this is the best selective medium for isolation of vibrios, though some strains of *Staphylococcus*, *Flavobacterium*, *Pseudoalteromonas*, and *Shewanella* may present slight growth on it as well, but usually, these colonies can be observed as very small and poorly developed. Some species of *Vibrio* do not grow on TCBS, namely, *V. penaeicida*, or very poorly as *V. cincinnatiensis*, *V. metschnikovii*, and *G. hollisae*.

Trypticase Soy Medium

TSA and TSB (for the agar and broth, respectively) are perhaps the most useful media for after-sample analysis of marine bacteria, provided an adequate concentration of salt (NaCl) is added. It is necessary to obtain a final concentration of NaCl between 1.5 % and 2.5 %. It is important to notice that TSA and TSB already have 0.5 % NaCl. Vibrios grow as big creamy colonies (*Fig. 36.6b*) after just 24 h at temperatures between 15 °C and 30 °C, depending on the strain under analysis.

Marine Agar

Marine agar generally permits the growth of very healthy colonies after 1–2 days, although some strains may require up to a week (*Fig. 36.6a* and *d*). A simplified ZoBell medium has

also been used by many laboratories with acceptable results, although some precipitation may occur after autoclaving (see below). Marine agar is not recommended for further analysis of bacteria (purification, identification, antibiotic susceptibility, etc.) because high concentration of ions may alter results.

Simplified ZoBell Agar (ZoBell 1941)

Bactopeptone or Polypeptone	5.0 g
Yeast extract	1.0 g
Ferric chloride ($FeCl_2$ 1.2 g/l H_2O)	1.0 ml
Distilled water	250 ml
Aged filtered seawater	750 ml
Bacteriological agar	15.0 g

pH has to be adjusted to 7.5 ± 0.1 before adding the agar. Autoclave at 121 °C for 20 min. Do not add the agar if you want a broth. Aged seawater: filter through 0.45 μm and keep in a dark container for more than a month. Precipitates can be sometimes observed in the medium after autoclaving.

Alkaline Peptone Water (APW)

This is the preferred enrichment medium for vibrios, especially devised for *V. cholerae*, but also used for *V. parahaemolyticus* and other species. The high pH of the medium (pH close to 9) and NaCl concentration inhibits many other bacteria and favors vibrios. Peptone concentration can range between 1 % and 2 %, the latest concentration more appropriate for marine species. NaCl can be omitted to favor the growth of *V. cholerae* and *V. mimicus*.

Alkaline peptone water

Peptone or Bactopeptone	10.0 g
NaCl	5.0 g
Distilled water	1,000 ml

pH has to be adjusted to 9.0 ± 0.1 with NaOH 1 N. Autoclave at 121 °C for 20 min.

Other Media

Other media have been developed for the selective isolation and/or differentiation of a specific group or species of vibrios, focusing primarily on human pathogenic vibrios.

Selective or differential media for *V. cholerae* include taurocholate tellurite gelatin (TTG) (Monsur 1961; O'Brien and Colwell 1985), sucrose teepol tellurite (STT) (Chatterjee et al. 1977), cellobiose-polymyxin B-colistin (Massad and Oliver 1987), and polymyxin mannose tellurite (PMT) (Shimada et al. 1990) (Tamura et al. 1971; De et al. 1977; Ozsan and Mercangoz 1980; Adzhieva 2000). For enrichment of *V. parahaemolyticus*, the most widely used medium and the one recommended by the FDA (DePaola and Kaysner 2004) is alkaline peptone water (APW); other enrichment media are glucose salt teepol broth (GSTB) (Sakazaki 1986) and salt polymyxin broth (SPB) (Hara-Kudo et al. 2001). Agar media used for *V. parahaemolyticus* differentiation are MT agar (Vanderzant and Nickelson 1972), trypticase soy agar + triphenyltetrazolium chloride (TSAT) (Kourany 1983), modified taurocholate tellurite gelatin agar (mTTG) (O'Brien and Colwell 1985), and chromogenic agar medium (CHROMagar Vibrio, CV agar) (Hara-Kudo et al. 2001). Media devised for *V. vulnificus* include VV agar (Brayton et al. 1983), sodium dodecyl sulfate-polymyxin B-sucrose medium (SPS) (Kitaura et al. 1983), cellobiose-polymyxin B-colistin agar (Massad and Oliver 1987), direct plating medium (VVE medium) (Miceli et al. 1993), cellobiose colistin (CC) agar (Hoi et al. 1998), peptone-NaCl-cellobiose colistin methanesulfonate (PNCC) (Hsu et al. 1998), and *Vibrio vulnificus* medium (VVM) (Cerda-Cuellar et al. 2000). A medium for *V. fluvialis* enrichment is FEM (Nishibuchi et al. 1983).

Media for isolation of aquatic animal pathogens include *Vibrio harveyi* agar (VHA) (Harris et al. 1996), *Vibrio anguillarum* medium (VAM) (Alsina et al. 1994), and a differential medium for *V. proteolyticus* (VP8) (Muniesa-Perez et al. 1996).

Isolation from Clinical Samples

Isolation of clinical samples is best accomplished if the stools are plated immediately after collection, preferably within the first 24 h of the onset of the symptoms (diarrhea) and prior to any antimicrobial treatment. Rectal swabs or stool specimens can be plated onto TCBS agar. If it is not possible to plate the sample immediately, the stool sample can be transported for a short period of time in a closed container as vibrios are susceptible to desiccation. For longer periods, inoculation in alkaline peptone water (APW) or in Cary and Blair transport medium is recommended. Blood agar is also a suitable medium for stool vibrios where hemolysis of some vibrios can be observed. Other enteric plating media should not be employed as vibrios grow poorly in these media (MacConkey). There are no special procedures for collecting vibrios from extraintestinal specimens, e.g., blood, wound, and tissue.

Isolation from Contaminated Food Products

The most important food products that can be contaminated with pathogenic vibrios are raw or undercooked shellfish, especially during the summer months. Cheese contamination has been recently reported (Feurer et al. 2004). *V. parahaemolyticus* is one of the leading causes of diarrhea associated with the consumption of these products, but other species may also be involved. Processing of shellfish samples can be done by following the methodologies of the Bacteriological Analytical Manual published by the US Food and Drug Administration, which has an online version (http://www.cfsan.fda.gov/~ebam/bam-toc.html).

Sample should be cooled immediately after collection (7–10 °C) but direct contact with ice should be avoided as vibrios are susceptible to extreme temperatures. Samples could be cut or pooled and blended at high speed for up to 2 min under sterile conditions in a buffer (PBS) or APW. Serial dilutions can be made in PBS or APW and plated onto TCBS. An overnight enrichment in APW at 35 ± 2 °C is preferable; after that, a loopful of the surface pellicle could be streaked in TCBS to obtain separate colonies. Typical colonies of *V. cholerae* are large (2–3 mm), smooth, yellow, and slightly flattened with opaque centers and translucent borders. *V. parahaemolyticus* colonies are large (2–3 mm), round, opaque, green or bluish colonies. *V. vulnificus* colonies are mostly green, although some could be yellow.

Isolation from Environmental Samples

There are several commercial media which may be used for the isolation of vibrios from marine environments, but ZoBell 2216E agar (ZoBell 1941), of which the commercial name is Bacto Marine Agar 2216 (MA; Difco 0979), is considered the best medium for primary isolation and quantification of marine heterotrophic bacteria. Some *Vibrio* species, e.g., the *V. halioticoli* group and *V. agarivorans*, require supplementation of sodium alginate (0.5 %) (Sawabe et al. 1995). TCBS agar is also an excellent medium for selective isolation of vibrios from environmental sources, but differences in media formulation between manufacturers occur which might be reflected in the enumeration of vibrios.

If the bacterial density of the sample is high, serial dilution in sterile saline solution (SSS, distilled water with 2.5 % NaCl) is an easy, reliable, and economic method to dilute the sample. In order to appreciate differences in colony morphology, especially in TCBS agar, it is crucial that colonies grow apart, preferably less than 100 colonies per plate.

For purification and after-sample culture, Tryptone Soy Agar or broth (TSA, TSB; Oxoid or Difco) supplemented with NaCl to achieve a final concentration of 1.5–2.5 % is more than adequate. Other general media can be used provided NaCl is added. Psychrophilic vibrios, i.e., *V. logei*, *V. wodanis*, and *V. salmonicida*, will grow poorly at temperatures higher than 20 °C; therefore, it is recommended to grow these strains in Luria–Bertani (Difco) supplemented with 1–3 % NaCl at 15 °C.

Preservation

Most vibrios (except *V. ezurae*, *V. gallicus*, *V. pectenicida*, *V. penaeicida*, *V. salmonicida*, and *V. tapetis*) stand very well the freeze-drying process. Coincidently, these species are also difficult to grow on any culture media. Ampoules containing freeze-dried cultures prepared nearly 30 years ago have yielded viable and healthy colonies on TSA. Normally, these ampoules are filled with 0.01 g of bacterial culture previously suspended in 0.5 ml cryoprotectant mix (horse serum/day-glucose/nutrient broth/MilliQ water 3:0.3:0.3:1) or 0.5 ml skim milk. Alternatively, strains may be maintained viable in cryopreservation at −70 °C to −80 °C for years. The cryopreservation methodology described by Gherna (1994) has been routinely used in our laboratories for more than 10 years without noticeable reduction in viability. A fresh bacterial culture is mixed with a cryoprotectant, usually 15 % glycerol to make a dense suspension. A cryovial with glass beads is filled with the suspension, thoroughly shaken and left standing for some minutes; the suspension is removed from the vial with a Pasteur pipette leaving only the soaked beads. The vial can be immediately put into the freezer. To recover the strain, a bead is removed from the vial and placed in TSB + 2.0 % NaCl or streaked directly onto TSA + 2.0 % NaCl or Marine agar and incubated.

Strains can be distributed grown in a small test tube with TSA + 2.0 % NaCl and covered with sterile mineral oil. Vibrios can withstand in this temporary preservation method for a few weeks.

Many culture collections have strains of vibrios. The most complete is the LMG Bacteria Collection Ghent University, Belgium, where all the type strains and hundreds of isolates are preserved either freeze-dried or cryopreserved at −80 °C (www.belspo.be/bccm/index.htm). The Collection of Aquatic Important Microorganism (CAIM) also houses the majority of type strains of the *Vibrionaceae* and more than a thousand strains, mainly from cultured aquatic organisms and aquaculture systems (www.ciad.mx/caim).

Ecology

The vibrios are ubiquitous in aquatic environment-free or in association with aquatic organisms. Recent developments of group- or species-specific identification methods have been providing new insights into the ecology of *Vibrionaceae* in which the number of members has still been expanded. The most notable ecological interaction of the vibrios appears to be the host–microbe interaction (pathogenicity and symbiosis). MLSA and WGS also provide a new insight into an ecological cohesive group concept in bacterial taxonomy as vibrios as a model (Preheim et al. 2011).

Habitat

Vibrios occur in a wide range of aquatic environments found, including estuaries, marine coastal waters and sediments, and aquaculture settings worldwide (Barbieri et al. 1999; Urakawa et al. 2000; Suantika et al. 2001; Thompson et al. 2001; Heidelberg et al. 2002a, b; Vandenberghe et al. 2003; Venter et al. 2004). Several cultivation-dependent and independent studies have shown that vibrios appear particularly in high densities in and/or on marine organisms, e.g., corals (Rosenberg and Ben Haim 2002), fish (Huys et al. 2001),

gorgonians (Martin et al. 2002), shellfish (Sawabe et al. 2003), sea grass (Weidner et al. 2000), sponges (Hentschel et al. 2001), shrimps (Gomez-Gil et al. 1998c), squids (Ruby 1996; Nishiguchi 2000), and zooplankton (Heidelberg et al. 2002). Halophilic vibrios can represent as much as 40 % of the total microbiota of subtropical coastal water (Chan et al. 1986). In the light organs of squids, for instance, there may be 10^{11} cells/organ (Fidopiastis et al. 1998; Nishiguchi 2000). Recent ecological studies have shown that seasonal changes in coastal water bodies, e.g., temperature, lead to the predominance of different populations of vibrios. *V. parahaemolyticus*-, *V. campbellii*- and *V. coralliilyticus* -related species increase during the summer months, whereas *V. splendidus*- and *V. pectenicida*-like occur year-round (Thompson et al. 2004c).

Group- or Species-Specific Identification Tools for Vibrios

Conventional phenotypic characterization and/or serological methods have been frequently used to study ecology and epidemiology of the *Vibrionaceae* (West and Colwell 1984; Alsina and Blanch 1994a, b). Species-specific identification using phenotypic characterization is still available for phenotypically distinct species with major modification on the use of commercially available kit (API 20E, Crystal E/NF, BIOLOG GN2, etc.) (O'Hara et al. 2003). PCR-based methodologies and extended database of gene sequences provide rapid and reliable methods to identify bacterial species based on rather stable genetic elements than phenotypic traits. Nowadays, various kinds of group-selective, group-specific, and/or species-specific identification systems have been developed and used to study ecology of the members of *Vibrionaceae* (Nishibuchi 2006). Recently, new methods to estimate intragenetic diversity have been used with many vibrios (see next section).

Ecology of Human Pathogenic Vibrios

Ecological studies among human pathogens have been done primarily with *V. cholerae*. Nowadays, *V. cholerae* is known to be an autochthonous to riverine, coastal, and estuarine ecosystem species (Colwell et al. 1977; Chakraborty et al. 2000; Faruque et al. 1998a). Heterogeneities found among *V. cholerae* strains make difficult to answer important ecological questions such as seasonal pattern of epidemics, presence of *V. cholerae* reservoirs, and new epidemic clones emerge (Faruque et al. 1998a; Rivera et al. 2001; Faruque and Mekalanos 2003). The excellent systematic surveillance of cholera (Chakraborty et al. 1997e) by means of conventional phenotypic characterization, serological identification, and molecular epidemiological methods (*Table 36.5*) of virulence genes has revealed many aspect of the *V. cholerae* ecology: (1) O1 and/or O139 biotypes are frequently found at the Ganges delta of Bangladesh and India but rarely outside the areas of epidemic infection (Kaper et al. 1979; Faruque et al. 1998a); (2) non-O1 and non-O139 are part of the normal free-living bacterial flora in riverine and estuarine systems (Colwell et al. 1977; Venkateswaran et al. 1989; Hervio-Heath et al. 2002; Heidelberg et al. 2002a); (3) *tcpA*-possessed and/or TCP-expressed *V. cholerae* non-O1 and non-O139 biotypes are frequently isolated (Chakraborty et al. 2000; Rivera et al. 2001; Singh et al. 2001; Faruque and Mekalanos 2003); (4) *V. cholerae* abundance is affected by environmental factors, such as temperature (above 19 °C) and salinity (ca. 2–14 ppt); the bacterium has never been isolated from anywhere below 10 °C (Colwell et al. 1977; Kaper et al. 1979; Venkateswaran et al. 1989); (5) *V. cholerae* is capable to attach to the chitin exoskeleton of live and/or dead copepods (Huq et al. 1983; Tamplin et al. 1990; Dumontet et al. 1996; Tarsi and Pruzzo 1999; Heidelberg et al. 2002b); and (6) genes encoding CT and TCP are horizontally acquired by phage infections and other mechanisms (Waldor and Mekalanos 1996; Faruque and Mekalanos 2003). However, the knowledge is not yet sufficient to control the cholera outbreak.

V. mimicus is a nonhalophilic species closely related to *V. cholerae* with the inability to ferment sucrose and negative to the Voges–Proskauer reaction (Davis et al. 1981b; Janda et al. 1988); both share a similar ecological niche (Chowdhury et al. 1989). Annual surveys of *V. mimicus* abundance have revealed maximum counts of 9.0 10^2 CFU/100 ml at Buriganga River and 1.3 10^2 CFU/100 ml at Dhanmondi Lake, both in Dhaka, Bangladesh, and 1.5 10^4 CFU/100 ml at the estuary of the Asahi River in Okayama, Japan (Chowdhury et al. 1989). *V. mimicus* is most abundant in brackish water at 4 ppt salinity and 20–23 °C and has also been detected in plankton samples collected at Okayama during May to September with maximum abundance of 6.0 10^4 CFU/100 g (Chowdhury et al. 1989). Species-specific differentiation systems based on 16S–23S rRNA intergenic spacer regions (Chun et al. 1999) or multilocus enzyme electrophoresis (Vieira et al. 2001) between *V. cholerae* and *V. mimicus* are available to investigate the epidemiology and ecology of *V. mimicus*.

Recently, intensive and long-term investigations of the ecology and epidemiology of *V. cholerae* and *V. mimicus* are in progress to understand how pandemic clones are generated and how to predict new cholera pandemics (Chakraborty et al. 2000; Jiang et al. 2000a; Rivera et al. 2001; Faruque and Mekalanos 2003; Louis et al. 2003; Constantin de Magny et al. 2008).

V. parahaemolyticus is also an important human pathogen. Ecology of *V. parahaemolyticus* has been thoroughly studied during the 60 years of history since its isolation as a pathogen. It is well known that pathogenic and not pathogenic strains of *V. parahaemolyticus* are frequently isolated from the marine environment. Early ecological studies for *V. parahaemolyticus* paid close attention to its habitat. The studies of Kaneko and Colwell in Chesapeake Bay, USA, revealed the annual life cycle of *V. parahaemolyticus*. *V. parahaemolyticus* is present in the sediment during winter when water temperatures fall below 10 °C; when the water starts to heat, it is moved to the surface by zooplankton and is abundant as free-living in the water column in coastal estuaries when the water temperature is

Table 36.5
Species of *Vibrionaceae* of medical importance

	Type of infections		
	Gastro-intestinal	External (wounds)	Systemic
Main pathogens			
Vibrio cholerae			
Serogroup O1	Strong gastroenteritis, Rice-water diarrhea		
Serogroup non-O1	Cholerae-like disease, mild diarrhea		Might cause septicemia
Serogroup O139	Similar to the O1		
V. parahaemolyticus	Mild to strong gastroenteritis	Infection of open wounds	–
V. vulnificus			
Biotype 1	Rarely	Infection of open wounds (necrotizing fasciitis)	Primary septicemia
Biotype 3	–	Infection of open wounds	–
Other pathogens			
V. alginolyticus	–	Wounds, ears, sometimes eyes	–
V. cincinnatiensis	–	–	Bacteremia, meningitis
V. fluvialis	"Cholera-like" diarrhea	–	–
V. furnissii	Diarrhea (?)		
V. metschinokovii	Diarrhea (?)	Foot ulcer	Bacteremia
V. mimicus	Similar to V. cholerae	–	–
Photobacterium damselae	–	Wound infections	Bacteremia
Grimontia hollisae	Diarrhea	–	Bacteremia

(?) doubtful

above 15 °C (Kaneko and Colwell 1973, 1974, 1975a, b; Janda et al. 1988; Chakraborty et al. 1997a). MLSA reveals diverse with a semiclonal population structure in *V. parahaemolyticus* populations and an epidemic structure similar to that of *Vibrio cholerae* (González-Escalona et al. 2008).

A third human pathogenic vibrio of which ecology and epidemiology have been well studied is *V. vulnificus*. *V. vulnificus* is responsible to cause two types of infection, primary septicemia and wound infection (Janda et al. 1988). Two biotypes are known among *V. vulnificus* strains: biotype 1 causes fatal human infection, but is avirulent for eels, and biotype 2 causes infection for both eels and human (Amaro and Biosca 1996). Both biotypes are serologically and genetically distinct, but phenotypically related with several differences (Biosca et al. 1997). *V. vulnificus* biotype 1 is responsible for 95 % of shellfish-related death in the United States (Coleman et al. 1996) especially during the summer months from oysters harvested from the Gulf Coast of the United States. *V. vulnificus* has been recovered from oysters at levels of 10^3–10^4 CFU/g during the summer months when the water temperature rises up to 26 °C; the salinity is between 5 and 25 ppt. It cannot be recovered during the winter season at a seawater temperature below 15 °C. *V. vulnificus* can also be recovered from the intestine of several fish species (DePaola et al. 1994). *V. vulnificus* biotype 2 is highly virulent to both eels and human; it is important that studies on the ecology of biotype 2 should pay attention to farmed-eel-mediated infection in humans (Marco-Noales et al. 1999). *V. vulnificus* biotype 2 is capable to survive for at least 3 years at salinities under 15 ppt and at 12 °C in an in vitro microcosm (Marco-Noales et al. 1999). Serological typing is the only method to differentiate between biotype 1 and 2 (Biosca et al. 1997; Marco-Noales et al. 1999). Species-specific identification systems have been developed to detect and enumerate *V. vulnificus* (Wright et al. 1993; Coleman et al. 1996; Campbell and Wright 2003) (*Table 36.5*).

Comparing to the intensive ecological and epidemiological studies of *V. cholerae*, *V. mimicus*, *V. parahaemolyticus*, and *V. vulnificus*, the ecology of *V. cincinnatiensis*, *V. metschnikovii*, *V. fluvialis*, and *V. furnissii* is not well understood (Janda et al. 1988; Chakraborty et al. 1997). As is the case of *V. cholerae* and *V. mimicus*, closely related species may pass between themselves genes responsible for virulence. Some strains of *V. alginolyticus* posses latent virulence factors and/or genes that can be a reservoir for its close species *V. parahaemolyticus*.

Ecology of Fish/Shellfish Pathogenic Vibrios

Vibrio spp. are frequently isolated in an association with marine animals. The facultative anaerobic metabolism of vibrios allows them to grow under limited oxygen environments, such as gastrointestinal tracts, muscle, and organs, some vibrios can infect fishes, shellfishes, and corals. The vibrios have been found very commonly in aquatic animal rearing with and/or without development of infections. Most studies have been focused on epizootiology of pathogens (Austin and Austin 1999). The ecology of pathogenic strains of vibrios has not been thoroughly studied and limited information exists (see further sections).

Ecology of Symbiotic Vibrios

The greatest paradigms of successful adaptations to an ecological niche occur, perhaps in genus Vibrio, particularly the associations of *V. fischeri*, *V. logei*, *V. halioticoli*, and recently described *V. halioticoli*-related species to diverse marine animals.

The light organs of temperate and tropical coastal benthic Monocentridae fishes and shallow bobtail sepiolid squids *Euprymna scolopes* are heavily colonized by *V. fischeri* (Ruby and Morin 1978; Haygood 1993; Ruby and Lee 1998) where it plays a major role in the emission of light. Before the observation that colonization of *V. fischeri* triggers light-organ morphogenesis of the newly hatched *E. scolopes* (McFall-Ngai and Ruby 1991; Foster et al. 2000), *V. fischeri* was studied just as a case of host-specific partnerships of light-organ symbiotic luminescent bacteria (Ruby and Lee 1998). *E. scolopes* is a small, nocturnal squid living in shallow-water reefs in Hawaii and is believed to use the light emitted by its bacterial symbionts as a camouflaging behavior called counter-illumination (Ruby and Lee 1998; Ruby 1999). Up to 10^{11} cells/cm^3 of *V. fischeri* have been recorded in the light organs of *E. scolopes*, which has three pores that connect its internal symbiont-containing crypts with the ambient seawater. Early ecological studies have been focused on the abundance of symbiotically competent *V. fischeri* cells in the squid water environment and the impact *V. fischeri* cells expulsion from the squid light organs (Lee and Ruby 1994; Ruby and Lee 1998). Studies by using a species-specific *lux* gene DNA probe for *V. fischeri* has revealed many aspects of the ecology of this bacterium, including the non-visible luminous (NVL) strains. The seawater in the habitat of *E. scolopes* in Hawaii contains approximately 200 CFU/l of the NVL cells. The sediment in the daytime habitat of *E. scolopes* also contains about 70-fold higher densities of *V. fischeri* cells. We can see a gradation of *V. fischeri* abundance (Wimpee et al. 1991; Lee and Ruby 1994; Ruby and Lee 1998). The *V. fischeri* ecology is strongly synchronized to the squid's life cycle, where daylight signals end the normal nocturnal activity of the squid and the squid buries itself in the sandy bottom of shallow-water habitats. *V. fischeri* is expelled from the squid light organ to the sediment once a day (Lee and Ruby 1994; Ruby and Lee 1998).

Another *Vibrio*, *V. logei*, has also been detected in a species-specific symbiotic association with the light organ of *Sepiola* squids, which dwell in the deep and cold Mediterranean and Atlantic waters (Fidopiastis et al. 1998). The light organ of *S. affinis* and *S. robusta* can contain mixed populations (10^7–10^8 CFU/light organs) of *V. logei* and *V. fischeri*, with up to 60–100 % of *V. logei* (Fidopiastis et al. 1998). *V. logei* colonization is possible but less effective in the light organ of *E. scolopes*, and differences might be affected by ambient water temperature surrounding these luminous *Vibrio* species.

V. halioticoli is found in gut of Japanese abalone *Haliotis discus hannai* as an alginolytic, nonmotile vibrio (Sawabe et al. 1995, 1998). Abundance of *V. halioticoli* in *H. discus hannai* ranges from 2.6 10^6 to 9.9 10^8 CFU/g of fresh organ, which accounts to 60 % of viable bacterial counts (Sawabe et al. 1995). A broad survey of *V. halioticoli*-like bacteria in abalone species collected from Australia, France, South Africa, and Japan, with the aid of a DNA probe (Tanaka et al. 2002a), DNA fingerprinting (Sawabe et al. 2002), and phenotypic characterization, revealed associations of *V. superstes* to the Australian abalones *H. laevigata* and *H. rubra* (Hayashi et al. 2003), *V. gallicus* to the French abalone *H. tuberculata* (Sawabe et al. 2004b), *V. halioticoli* to the South African abalone *H. midae* (Sawabe et al. 2003), *V. ezurae* to the Japanese abalones *H. diversicolor aquatilis* and *H. diversicolor diversicolor*, and *V. neonatus* to the Japanese abalone *H. discus discus* (Sawabe et al. 2002, 2004a). Three more novel species are isolated from Japanese and Californian abalones (Sawabe et al. 2007b). *V. breoganii*, which is phylogenetically related to *V. halioticoli* based on 16S rRNA phylogeny, is the first species that does not originate from the gut of abalone (Hidalgo et al. 2009). Motile and flagellated cells have never been observed in any species of *V. halioticoli*-like bacteria, and they have never been isolated from other seaweed-eating invertebrates (e.g., sea hare, sea urchin, and *Trochidae* and *Littoridae* shells), except from the *Turbo* shells (*Turbo cornutus*) (Sawabe et al. 2003, 2004a). Viable bacterial counts of *V. gallicus*, *V. ezurae*, and *V. neonatus* range from 10^4 to 10^7 CFU/g of fresh organs on each abalone host, and these species occupy up to 40 % of the bacterial flora present. On the other hand, *V. superstes* occupies no more than 10 % of bacterial flora of Australian abalones. *V. halioticoli* have also been found in environmental samples around abalone farms in Japan (Tanaka et al. 2002b), and the bacterium dominates the gut microflora and is influenced by changes in the feeding behavior of the abalones (Tanaka et al. 2003). Abalones are a marine herbivorous gastropod with preference for brown algae in which alginate is contained as a component of the cell wall matrix (Kloareg and Quatrano 1988). *V. halioticoli* is capable of fermenting alginate through the acetic acid/formic acid pathway; these volatile short chained fatty acids are probably involved as available energy sources or metabolic precursors for the abalone hosts (Sawabe et al. 2003).

V. porteresiae is isolated from disinfected root of wild rice (Rameshkumar et al. 2008). The species show nitrogen fixation activity based on acetylene reduction assay and possess

nifH gene. The draft genome analysis also reveals the presence of *nifHDK* in addition of almost genes responsible for nitrogenase processing proteins (Sawabe, unpublished data). Apparent nitrogenase activity is measured, so this may be the first case a vibrio might have a role for symbiotic interaction to plant host. Moreover, a closely related vibrio species, *V.* '*tritonius*', is recently isolated from the gut of sea hare *Aplysia kurodai*; similar *nif* gene cluster is found in the genome (Sawabe, unpublished data).

Ecology of *Photobacterium*

In the *Photobacterium* genus, *P. phosphoreum* and *P. leiognathi* are well-known symbionts of light organs, and the two subspecies of *P. damselae* are human and/or fish pathogens. Deep-sea and cold-water ecology of *P. phosphoreum*, ecology of the warm-water *P. leiognathi*, and the epidemiology of *P. damselae* have been intensely studied.

Ecology of *Photobacterium* spp. as Animal Symbionts

P. phosphoreum can be found in a variety of marine habitats, fish intestine, light organs, and seawater (Reichelt and Baumann 1973). The association of this bacterium with deep-sea fishes has been well studied (Ruby and Morin 1978; Ruby et al. 1980). *P. phosphoreum* has been isolated from the light organs of three species of the bathyal fish family *Macrouridae* (*Nezumia aequalis*, *N. stelgidolepis*, and *Sphagemacrurus hirundo*) dwelling from 600 to 1,260 m in the Atlantic and Pacific Oceans and from the midwater species *Opisthoproctus grimaldii* (family *Opisthoproctidae*, 200–600 m) of the Atlantic Ocean (Ruby and Morin 1978). The seawater temperature where these deep-sea fishes live varies between 2 °C and 10 °C. Nowadays, it is recognized that *P. phosphoreum* is a facultative light-organ symbiont of midwater or benthic fishes belonging to families *Opisthoproctidae*, *Chlorophthalmidae*, *Trachichthydae*, *Moridae*, *Macrouridae*, and *Steindachneriidae* which possess light-organ around the rectal or anal regions (Haygood 1993). The peak of abundance of *P. phosphoreum* is between 250 and 1,000 m in Puerto Rico Trench and in the North Atlantic Ocean; and the viable bacterial counts are <3 CFU/100 ml in Puerto Rico and >7 CFU/100 ml in the North Atlantic Ocean (Ruby et al. 1980). The *P. phosphoreum* population in the deep seas is likely to be stable through the years. Interestingly, *P. phosphoreum* was isolated from the skin of the Chum salmon *Oncorhynchus kisutch* (Budsberg et al. 2003). This salmon migrates up 1,228 km along the Yukon River (Alaska) which has a glacial water origin.

P. leiognathi is a light-organ symbiont of shallow water, tropical water ponyfishes belonging to families *Percichthyidae*, *Apogonidae*, and *Leiognathidae* of the Indo-West Pacific region (Dunlap and McFall-Ngai 1987; Haygood 1993). The light organ of these ponyfishes is an internal and circumesophageal ring tissue located just anterior to the stomach (esophagus). *P. leiognathi* strains can also be isolated from the surrounding water of these ponyfishes (Reichelt and Baumann 1973); however, high levels of luminescence of *P. leiognathi* are observed in the light organ. The high-level phenomenon could be explained because of a direct delivery of oxygen from the gas bladder which is located posteriorly (Dunlap and McFall-Ngai 1987) and osmotic controls of the luminescence/growth balance (Dunlap 1985). *P. leiognathi* cells in Leiognathid light organs are coccobacilli and lack storage granules and flagella, while the bacterial cells cultured in vitro are elongated and flagellated with large PHB granules (Dunlap and McFall-Ngai 1987). Average abundance of *P. leiognathi* in Leiognathid light organs from the Philippine Islands is 2.5×10^8 CUF/mL of light organ, which means $6\text{-}7 \times 10^9$ CFU/ml (Dunlap and McFall-Ngai 1987).

Ecology of *Photobacterium* spp. as Pathogen

Nonluminescent *P. damselae* includes two subspecies, subsp. *damselae* and subsp. *piscicida* (Gauthier et al. 1995). Both subspecies are well known as fish pathogens, and subsp. *damselae* also causes wound infections in humans (Love et al. 1981) that can even be fatal (Shin et al. 1996). Ecology of *P. damselae* has intensively been studied from an epidemiological point of view (Love et al. 1981; Romalde 2002).

P. damselae subsp. *damselae* (formerly *V. damsela*) was first isolated from naturally occurring skin ulcer on a temperate-water damselfish, the Blacksmith (*Chromis punctipinnis*). Laboratory infection of *P. damselae* subsp. *damselae* to the Blacksmith fulfilled Koch's postulates (Love et al. 1981). Long-term (10 years) survey of ulcerative fishes from Southern California revealed that *P. damselae* subsp. *damselae* infections are limited to *C. punctipinnis* and seasonal infectivity probably correlates with elevated seawater temperatures, at the time when the Blacksmith is nesting. *P. damselae* subsp. *damselae* strains are found in surrounding seawater and seaweeds adjacent to the Blacksmith's nests (Love et al. 1981). *P. damselae* subsp. *damselae* strains have also been isolated from a variety of fishes, molluscs, marine reptiles, and marine mammals (Fujioka et al. 1988, 1992). There are many reported cases of human wound infection caused by *P. damselae* subsp. *damselae*. This infections most probably occurred by laceration during swimming (Morris et al. 1982). *P. damselae* subsp. *damselae* can occur from 240 to 460 MPN/100 ml of coastal seawater at Florida, identified phenotypically with API 20E (Buck 1990).

P. damselae subsp. *piscicida* (formerly *Pasteurella piscicida*) is a fish pathogen causing pasteurellosis (Romalde 2002). This pathogen has a worldwide distribution with variety of marine fishes as natural hosts. The high degree of sequence similarity (>99 %) of rRNA genes (5S, 16S, and 23S) and the intergenic spacer region (ITS) between the two subspecies of *P. damselae* are unlikely to develop subspecies-specific

identification tools. However, *P. damselae* subsp. *damselae* harbors the *ureC* gene, which is responsible in urease activity. A multiplex PCR for 16S rRNA and *ureC* genes is a useful tool for diagnosis of pasteurellosis and/or for ecology (epidemiology) of these important bacteria.

There are limited studies on the ecology of *P. angustum* and *P. profundum*. Ecology of these species could be resolved by further intensive studies.

Ecology of the Genera *Grimontia*, *Salinivibrio*, and *Enterovibrio*

Three genera, *Grimontia*, *Salinivibrio*, and *Enterovibrio*, have been established with a single species. Except for *Grimontia*, a human pathogen, ecology of these species is not fully understood.

Grimontia hollisae (formerly *V. hollisae*) (Thompson et al. 2003) was first isolated from stool cultures of patients with diarrhea (Hickman et al. 1982). Clinical and environmental isolates of *G. hollisae* produce a thermostable direct hemolysin (TDH) related to the hemolysin of *V. parahaemolyticus*. Strains of *G. hollisae* have been isolated from the marine environments (Vuddhakul et al. 2000). A species-specific identification with selective PCR amplification of the toxR gene is available. This PCR method together with phenotypic characterization could be used to further understand the epidemiology and ecology of *G. hollisae* (Vuddhakul et al. 2000).

Salinivibrio costicola includes three subspecies, subsp. *costicola*, subsp. *vallismortis*, and subsp. *alcaliphilus*. All subspecies of *S. costicola* are probably free-living of saline or hypersaline environments or associated to salt crystals; there are no reports of associations with animal or plant hosts (Ventosa et al. 1982; Garcia et al. 1987; Mellado et al. 1996; Huang et al. 2000; Romano et al. 2005). *S. costicola* subsp. *costicola* can be isolated from solar saltern, salted foods, and brine as a moderate halophilic vibrio with 3–15 % salt for optimal growth (Ventosa et al. 1982; Garcia et al. 1987; Mellado et al. 1996). *S. costicola* subsp. *vallismortis* was isolated from sediments collected at the Death Valley, California (Huang et al. 2000), while *S. costicola* subsp. *alcaliphilus* was isolated from a saltish spring in the Campania region of Italy (Romano et al. 2005). Species-specific identification has been not fully established. However, unique signature sequences between the 178 and 219 nucleotide positions of the 16S rRNA gene sequence (*E. coli* positions) are probably a target for rapid *S. costicola* detection (Mellado et al. 1996; Huang et al. 2000).

Members of the *Enterovibrio* genus have been isolated from marine organisms, *E. norvegicus* from healthy turbot larvae at an aquaculture station in Norway (Thompson et al. 2002) and *E. coralii* from water extracted from a bleached coral (*Merulina ampliata*) (Thompson et al. 2005a). Ecology of both species of *Enterovibrio* is practically unknown. Together with *Grimontia* and *Salinivibrio*, it is desirable to seek new species belonging to these three genera and to further study the ecology of these genera.

Towards the Merging Ecological Information on Vibrio Taxonomy

As most of *Vibrionaceae* strains are isolated from environmental samples, incorporating fine-scale ecological information into taxonomic classification has remained difficult. However, the introduction of MLSA and/or massive whole-genome sequencing can open a way to merging taxonomy with ecological population prediction (Preheim et al. 2011). Recently, many efforts to delineate ecologically cohesive vibrio populations have been conducted using 3,400 isolates with different ecological origins and at different sampling date based on MLSA combined with a dynamic model to identify phylogenetically cohesive and ecologically distinct bacterial populations. Strains falling into 12 populations are recovered across the three studies. These results suggest that MLSA-based taxonomy may identify units akin to those of ecologically defined species. At least three unique ecologically defined units (F6, F10, and F11) are appeared to be evaluating DNA relatedness to known species.

Pathogenicity, Clinical Relevance

Diseases Caused by Vibrios in Humans

As many as 12 species of the family *Vibrionaceae* have been implicated in human diseases (*Table 36.5*), but *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* are the most important ones, with a worldwide distribution. Certain strains of the first two species cause diarrhea and the third extraintestinal infections. Vibrios have been isolated most commonly from stools and wounds, but also from blood, infected ears and eyes, spinal fluid, gall bladder, urine, and respiratory tract (Farmer and Hickman-Brenner 1992). Since there is no clear-cut criterion to establish the pathogenic potential of a vibrio isolate, it is safe to assume potential pathogenicity. With the advent of AIDS, other *Vibrio* species are expected to cause systemic infections. Vibrio pathogenic action to the human host can be classified into three broad forms: the systemic, the external, and the gastrointestinal.

Vibrio cholerae

Cholera has been one of the major plagues of mankind and remains a serious pathogen in Africa and Asia. It has caused millions of deaths and still is, perhaps, the most feared pathogen. *Vibrio cholerae*, the causative agent of cholera, is endemic in south Asia and particularly in the Ganges delta, where epidemics have been recorded since ancient times (Sack et al. 2004). Even more ancient records of diseases with similar characteristics are found dating from 500 BC. Descriptions of patients probably with cholera are found in Hippocrates works (Barua 1992). The first documented pandemic occurred in 1817 near Calcutta (India) and spread to almost all southeast and central Asian

countries, many African and Middle Eastern countries, and Russia. The severe winter of 1823–1824 prevented cholera of entering into Europe. Hundreds of thousands of people were affected during this first and furious cholera pandemic. During the second pandemic, beginning in 1826, the disease reached many major European cities by the early 1830s and reached the American continent in 1832 via Canada and the United States and spread to the rest of the continent. Five more pandemics have occurred and the seventh, which started as a pandemic in 1961, but originated in Indonesia during 1905 and firstly isolated from Indonesian pilgrims to Mecca in the Egyptian town of El Tor, involved almost the whole world and became endemic in many places. From 1991 to 1995, it caused an important epidemic in Latin America (Tauxe and Blake 1992; Wachsmuth et al. 1993; Salles et al. 1993). More than 1,200,000 cases were reported from 1991 to 2002 in Latin America, with 12,000 deaths (PAHO, 2004). All the strains involved in pandemics were assigned to the O1 serogroup, but in 1992, a non-O1 serogroup named O139 Bengal appeared in Madras, India, and spread rapidly to the rest of the country, into Bangladesh and neighboring countries. This serotype might be responsible for an eight pandemic. This new serotype is very similar to El Tor, and it was proposed that O139 is derived from El Tor, but molecular data from O139 strains suggest multiple progenitors among them, El Tor (Faruque et al. 2003).

The ability of emergence, reemergence, and the pandemic potential of *V. cholerae* pathogenic strains are probably consequences of a combination of bacterial genetic background, physiological conditions, and environmental factors such as plankton blooms. The viable but nonculturable state (VBNC) is a phenotype expressed by some bacterial species, including *V. cholerae*, that allows survival under nonfavorable conditions. The cells are viable but it is not possible to culture the organism on usual laboratory media. In this way, the organism can persist or can be introduced in a new region without being identified by standard bacteriological methods (Colwell 1993). Further changes in the metabolic state and/or environmental conditions can lead to the recovery of the bacteria from this stage. The beginning of the Latin American epidemic was striking in that various locations of Peru, separated by 1,200 km, started to report cholera cases within a few days (Seas et al. 2000) indicating a multiple introduction of the organisms from the sea.

Now, after more than 40 years into the seventh pandemic, it is still causing around 120,000 notified cases every year (WHO 2003). Cholera has been reported from around 50 countries during each year from 2000 to 2003 (WHO 2004). The vast majority of cases is occurring in Africa, which had, in 2003, 96 % of the total cases (WHO 2004). Countries with more than 20,000 cases in this period are Madagascar (2000), South Africa (2001), Malawi, the Democratic Republic of Congo and Mozambique (2002), and the Democratic Republic of Congo and Liberia (2003). The same African trend is valid for 2004, with a continued high number of cases in Mozambique, and important outbreaks in Cameroon, Chad, and Zambia. The vast majority of the cases are caused by *V. cholerae* O1, biotype El Tor.

There are two essential genetic elements involved in pathogenicity of *V. cholerae*: the CTX element, which is the genome of a bacteriophage (CTXΦ) and codes for the cholera enterotoxin (CT), and the vibrio pathogenicity island (VPI), which carries genes for the pilus colonization factor (TCP) in a 40 kb segment normally absent in non-epidemic strains (Karaolis et al. 1998; Sack et al. 2004). These virulence factors are regulated by the ToxR regulation factor, which is at the top of a regulatory cascade and under the control of environmental factors, such as temperature, pH, and NaCl (Skorupski and Taylor 1997). The VPI is also derived from a bacteriophage (VPI Φ) and can be transferred between *V. cholerae* strains (Karaolis and Kaper 1999). Production of CT is present in epidemic strains but absent in non-O1/O139 strains that can cause septic infections in individuals with predisposing medical conditions.

V. cholerae is found as a free-living organism in brackish and associated to the plankton (Huq et al. 1983), although the toxigenic strains are mostly isolated from environments exposed to fecal contamination and the nontoxigenic from less contaminated areas (Faruque et al. 1998b). The marine environment, including estuaries, is a reservoir of *V. cholerae*. Environmental factors, e.g., temperature and nutrients, have a role in the emergence of epidemics. The disease is transmitted primarily through the ingestion of contaminated food or contaminated water supplies by human feces. A direct person to person spread is uncommon. Infected persons normally do not develop a carrier state after the disease and only humans are known to be affected, and no animal reservoirs are known. It should be realized that *V. cholerae* makes an important and difficult transition in passing from an aquatic habitat to the human host. It has a dual life cycle, as an aquatic free-living organism, and as a human pathogen with epidemic/pandemic capacity, being able to survive the acidic environment of the stomach, to reach, and colonize the human intestine. Some global genetic studies are aimed at the study of this transition.

The incubation of *V. cholerae* in the gastrointestinal tract is between 18 h and 5 days. The classical clinical sign of "cholerae gravis" is an abrupt, abundant, painless watery diarrhea resembling rice water often accompanied with clear, watery vomiting and little desire to eat. Stools may be produced at a rate of up to 1 l/h, and therefore, signs of severe dehydration are observed promptly. Patients present sunken and dry eyes without tears, skin elasticity, acute renal failure, and lethargic or unconscious (Farmer et al. 2003; Sack et al. 2004). Severe muscle cramps of the extremities are common probably due to the electrolyte imbalance. Fatality rate can reach 50 % in untreated individuals and can happen very quickly after the signs of severe dehydration are detected.

Gastric acid is a nonspecific defense mechanism, as the cells are acid sensitive. As a consequence, the inoculum size is larger than for other enteric bacteria such as *S. typhimurium* or *Shigella*. It is taken that the inoculum size to cause disease in half of a group of people is 10^7 or 10^8 (Cash et al. 1974). The small intestine is the primary site of infection, and colonization depends on motility, to cross the mucous layer covering the cells, and adhesion of vibrios to the intestinal mucosal surface. Adhesion protects the cells from intestinal motility and helps

with the delivery of the cholera toxin to the nearby intestinal cells. Pathogenic *V. cholerae* is not invasive to the intestine, causing all the effect while adhered to the intestinal cells (Sprinz et al. 1962). Cholera toxin acts as described above, leading to a high concentration of cAMP and a consequent increase in chloride secretion in the crypt cells, inhibition of NaCl absorption, and osmotic transfer of water to the small intestinal lumen, resulting in severe secretory diarrhea.

Treatment with fluids is very effective. Replacement fluids should be administered as fast as they are lost and be composed of electrolyte solutions such as Ringer's lactate (Kaper et al. 1995). Besides fluid replacement, this intravenous fluid attends to the other problems such as metabolic acidosis and potassium deficiency. Later on, as the patient improves, this should be changed to oral rehydration solution (ORS), which has a higher concentration of potassium. A severe dehydrated individual might have lost 10 % of the body weight, and thus, this amount of fluids has to be administered in a 2–4-h period (Sack et al. 2004). Antibiotic treatment helps in reducing the illness and the need for rehydration fluids and hospital stay. Treatment with tetracyclines can reduce by half the duration of diarrhea, almost a third of the stool deposition, and greatly the percentage of therapeutic failure (Lindenbaum et al. 1967). Ciprofloxacin is the antibiotic of choice for adults proving significantly better clinically than other antibiotics (Khan et al. 1995, 1996). Doxycycline can also be used, except for pregnant women, but its efficacy can be significantly reduced (>50 %) if the strain is not susceptible to tetracyclines (Khan et al. 1996). An added advantage of these antibiotics is that only one dose is necessary, as opposed to tetracycline where several doses have to be administered. For children below 12 years, trimethoprim–sulfamethoxazole, erythromycin (Kabir et al. 1996), azithromycin (Khan et al. 2002), or tetracycline is recommended, whereas furazolidone is recommended for pregnant women (http://rehydrate.org/dd/su52.htm#page4). If resistance to these antibiotics is detected, erythromycin, norfloxacin, or chloramphenicol can be prescribed (Bopp et al. 1999).

Vibrio parahaemolyticus

V. parahaemolyticus has been implicated in diarrhea associated with seafood consumption in many parts of the world, mainly in (Raimondi et al. 2000) Japan, representing more than 70 % of the cases due to bacterial food poisoning (Okabe 1973). Disease outbreaks have also commonly been reported in Taiwan (Chiou et al. 2000), USA (Abbott et al. 1989; DePaola et al. 2000), Spain (Lozano-Leon et al. 2003), Vietnam (Tuyet et al. 2002), Chile (Cordova et al. 2002; Gonzalez-Escalona et al. 2005), Canada (Fyfe et al. 1997), Peru (Begue et al. 1995), and India (Lalitha et al. 1983; Okuda et al. 1997).

The most common cause of infection is by the consumption of raw or undercooked oysters, sea fishes, shrimps, and other shellfish. This species has many strains, but only those that produce a thermostable direct hemolysin (TDH) and/or the thermostable-related hemolysin (TRH) have the ability to cause gastroenteritis (Nishibuchi et al. 1992), and almost all the strains isolated from clinical samples have either or both of the genes (tdh and trh, respectively) that encode for the production of these enzymes. Environmental isolates often do not posses these genes. It has been estimated that only 1–2 % of strains isolated from nonclinical samples harbor TDH and/or TRH genes (DePaola et al. 2003; Cabrera-Garcia et al. 2004). The production of TDH is highly associated with the Kanagawa phenomenon on Wagatsuma agar where red blood cells are lysed (beta-type hemolysis) and a clear halo can be observed around TDH positive colonies (Miyamoto et al. 1969). More than 95 % of the strains isolated from human gastroenteritis produce the Kanagawa phenomenon, but only about 1 % of those of environmental origin do so. A strong correlation has also been observed between the presence of the *trh* gene and urease production, which is uncommon in *V. parahaemolyticus* (Okuda et al. 1997).

V. parahaemolyticus produces three major surface antigens, H (flagellar), heat stable O (somatic), and heat labile K (capsular) (Hsieh et al. 2003). All strains share a common H antigen, but 13 O and 71 K antigens have been identified, although many strains are untypable. Until 1996, infections were sporadic caused by multiple and diverse serotypes (Williams et al. 2004). After 1996, there appeared serotype O3:K6 which unleashed the first *V. parahaemolyticus* pandemic in India and which has also been reported in several Asian countries and in the United States (Chowdhury et al. 2000; Wong et al. 2000). Strains belonging to this serotype only possess the *tdh* gene, lacking the *trh*, and are urease negative (Okuda et al. 1997), and molecular typing suggests that the isolates from Korea, Japan, and Taiwan are clones of the original from India (Wong et al. 2000). The number of *V. parahaemolyticus* infections has increased considerably in many countries since 1996. The pandemic strains were originally defined as these belonging to the O3:K6 serovar, possessing the *tdh* gene, lacking the *trh* gene, and with a unique fingerprint pattern with RAPD analysis (Okuda et al. 1997). Two PCR methods were developed to identify strains belonging to the pandemic group. One of these was the method denominated GS-PCR (group specific), based on the sequence of the *toxRS* region (Matsumoto et al. 2000). The second was the detection of an orf8 from the filamentous phage f237 present in the pandemic clone (Nasu et al. 2000a). The O3:K6 clone has diversified into three or more serotypes, among these O4:K68, O1:K untypable (UT), and O1:K25, and there is active research for markers of the pandemic strains.

Clinical signs are gastroenteritis with nausea, watery diarrhea and less common, bloody diarrhea, vomiting, abdominal cramps, low-grade fever, and chills (Farmer et al. 2003). The disease is mild and self-limiting, but can be fatal especially in immunocompromised patients; onset of the symptoms can be from 4 h to 96 h (Wong et al. 2000) and can last for 3 days. Treatment is by rehydration and seldom there is a need for antibiotics because this illness is self-limiting.

Genomics and virulence mechanism studies in *V. parahaemolyticus* are hugely advancing currently, after the complete genome feature is solved in 2003 (Makino et al. 2003),

especially the findings of two sets of type III secretion system (T3SS), and the unexpectedly diversity and widely spreading traits in the other pathogenic vibrios are noteworthy (Okada et al. 2009, 2010).

On the basis of genome analysis, *Vibrio parahaemolyticus* is characterized by two type III secretion systems (T3SS), namely, T3SS1 and T3SS2. T3SS2 is indispensable for enterotoxicity but the effector(s) involved has been unknown. VopV is identified as a critical effector that is required to mediate *V. parahaemolyticus* T3SS2-dependent enterotoxicity. VopV also possesses multiple F-actin-binding domains, and the enterotoxicity caused by VopV correlates with its F-actin-binding activity. Interestingly, a T3SS2-related secretion system and a *vopV* homologous gene are also involved in the enterotoxicity of a non-O1/non-O139 *V. cholera* strain (Hiyoshi et al. 2011).

During the infection of mammalian cells, this pathogen exhibits cytotoxicity that is dependent on its type III secretion system (T3SS1). VepA, an effector protein secreted via the T3SS1, plays a major role in the T3SS1-dependent cytotoxicity of *V. parahaemolyticus*, and recently, the mechanism is elucidated as (1) protein transfection of VepA into HeLa cells resulted in cell death; (2) the ectopic expression of VepA in yeast *Saccharomyces cerevisiae* interferes with yeast growth, and VMA3 which encodes subunit c of the vacuolar H^+ −ATPase (V-ATPase) is the essential growth inhibitor on the basis of a yeast genome-wide screen; and (3) in HeLa cells, knockdown experiment of V-ATPase subunit c shows the decrease of VepA-mediated cytotoxicity, and lysosomal leakage observation reveals the bacterial effector VepA targets subunit c of V-ATPase and induces the rupture of host cell lysosomes and subsequent cell death (Matsuda et al. 2012).

Draft genome of *V. parahaemolyticus* strain 10329 of the O4:K12 serotype, which belongs to the main US West Coast clonal complex of *V. parahaemolyticus* (sequence type 36 [ST36]) causing oyster-associated human illness, is reported, and it contains the virulence determinants *tdh* and *trh* but appears to infect at much lower doses than *V. parahaemolyticus* strains with these same determinants from other areas, such as the US Gulf and Atlantic coasts (Gonzalez-Escalona et al. 2011).

Vibrio vulnificus

Three biotypes have been identified within *V. vulnificus*: biotype 1 typically associated with human infections; biotype 2 primarily pathogen of marine animals, although also an opportunistic pathogen of humans (Amaro and Biosca 1996a); and biotype 3 found only in Israel and causing wound infections and bacteremia (Bisharat et al. 1999b).

V. vulnificus can cause primary septicemia and wound infections (Blake et al. 1979) with an estimated mortality rate of up to 60 % in a fulminant in immunocompromised patients or with an iron overload due to a liver disease or hemochromatosis (Hlady et al. 1993; Gholami et al. 1998; Linkous and Oliver 1999). Infections due to this bacterium have been reported in the USA (Hlady et al. 1993), Denmark (Dalsgaard et al. 1996b), Japan (Matui et al. 2004), Taiwan (Chiang and Chuang 2003), Spain (Torres et al. 2002), Israel (Bisharat et al. 1999a), and many other countries. It is the leading cause of seafood-associated foodborne fatalities in the USA (Harwood et al. 2004).

Consumption of raw shellfish has been identified as the primary cause of septicemia, and especially the consumption of raw oysters (Hlady et al. 1993). *V. vulnificus* is a natural inhabitant of marine environments (Oliver et al. 1983; Harwood et al. 2004). It is commonly found when water temperature exceeds 18–20 °C during the summer months and salinity between 5 and 25 ppt (Motes et al. 1998; Pfeffer et al. 2003; Kinoshita et al. 2004). Below 10 °C, the bacterium enters a viable but nonculturable (VBNC) state (Oliver 1995), probably as a protection strategy to adverse environmental conditions. This stage is observed in many different bacteria (Colwell 2000). The VBNC cells can be brought back to activity in the lab, a process dubbed resuscitation, by an increase in temperature in special conditions (Oliver et al. 1995). An alternative method for recuperation of the active cells is in vivo inoculation and serial animal passages (Oliver and Bockian 1995).

Death can occur after 24 h of the onset of symptoms, which, in primary septicemia, include fever, chills, nausea, vomiting, and abdominal pain and at the extremities; mental changes can also be observed (Linkous and Oliver 1999; Strom and Paranjpye 2000). Hospitalization may be required for up to 43 days with a mean of 8 days (Hlady et al. 1993). Up to 60 % of the patients suffer septicemic shock with low systolic blood pressure (Strom and Paranjpye 2000). Secondary cutaneous lesions may appear at the limb extremities, e.g., edema, subcutaneous bleeding, and absence of superficial necrosis (Fujisawa et al. 1998). These lesions often become necrotic (necrotizing fasciitis) and require debridement or amputation (Strom and Paranjpye 2000).

Wounds may become infected with *V. vulnificus* when exposed to seawater, raw fish, or shellfish even in healthy individuals. Inflammation at the wound site can be observed with localized pain, edema, erythema, and necrosis of the surrounding tissue which leads to amputation or surgical debridement (Linkous and Oliver 1999; Strom and Paranjpye 2000). Infected patients may become septicemic presenting the same symptoms of primary septicemia. In these cases, the fatality rate is between 23 % and 30 % (Strom and Paranjpye 2000). Gastroenteritis could also be caused by *V. vulnificus*, but this is not clear since no screening for this pathogen has been conducted so far (Strom and Paranjpye 2000).

Antibiotic treatment should be performed as soon as possible due to the rapid evolution of the disease. Tetracycline has been recommended as the agent of choice, although a combination of cefotaxime and minocycline proved better in a mouse model (Chuang et al. 1998a), as well as other fluoroquinolones (evofloxacin, moxifloxacin, gatifloxacin, sparfloxacin, ciprofloxacin, lomefloxacin, and levofloxacin) (Chuang et al. 1998b; Tang et al. 2002).

The virulence of *V. vulnificus* is multifactorial and includes presence of cell surface factors, such as a polysaccharide

capsule (CPS), lipopolysaccharides (LPS), pili, exoenzymes, and iron acquisition systems (Wright et al. 1981; Johnson et al. 1984; Oliver et al. 1986; Morris et al. 1987; Linkous and Oliver 1999; Strom and Paranjpye 2000). Considerable variation has been found in the virulence of strains and a factor that distinguished between avirulent and virulent strains has been hard to identify. RAPD analysis of clinical and environmental strains has identified a 178–200 bp band present only in clinical isolates and in very few environmental isolates (Warner and Oliver 1999); this DNA fragment might represent a genetic marker of virulent strains.

V. vulnificus may express an extracellular acidic polysaccharide capsule (CPS) which is an important virulence determinant in many pathogenic bacteria (Strom and Paranjpye 2000). This capsule confers resistance to the action of macrophages and to the bactericidal action of sera (Johnson et al. 1984; Tamplin et al. 1985). Different capsule types have been identified in *V. vulnificus*, but no correlation has been established with virulence (Hayat et al. 1993). The presence and amount of capsule seem to correlate positively with virulence (Yoshida et al. 1985). The presence of the CPS confers an opaque colonial morphology to an isolate growing on a solid nutrient medium, whereas the translucent colony morphotypes lack the capsule (Warner and Oliver 1999).

Endotoxic activities of LPS molecules induce a pyrogenic response in the host which leads to inflammation, tissue damage, and bacterial septic shock, most probably synergistically with CPS (Linkous and Oliver 1999; Strom and Paranjpye 2000). Another important virulence factor is the pili or fimbriae, by which the bacterium attaches to the host cells and colonizes. Pili have been identified in clinical isolates which differ from environmental isolates in having a greater number of pili and a better adhesion to human epithelial cell lines (Gander and LaRocco 1989).

Production of exoenzymes, such as hemolysins (cytolysin and VIIY), proteases (metalloprotease), chitinase, and phospholipase, may contribute to the virulence of *V. vulnificus*, although some, like the chitinase, might be more related to colonization of crustaceans than to virulence in humans (Strom and Paranjpye 2000). Cytolysin has been shown to cause in animal models to lyse erythrocytes, induce vascular permeability, and cause extensive extracellular edema and damage to capillary endothelial cells (Gray and Kreger 1987), but no clear correlation has been established between hemolysin production and virulence (Oliver et al. 1986; Wright and Morris 1991). Similarly, phospholipases have not been associated with virulence as almost none of the other exoenzymes.

Iron is a very limiting factor for bacteria within the human body. *V. vulnificus* has the ability to produce sidephores to acquire iron from transferrin and hemoglobin (Morris et al. 1987). The reduction of sidephore production by the bacteria or high levels of transferring in the human host have been correlated with virulence (Wright et al. 1981; Morris et al. 1987). The low iron concentration inside the human host can be the signal to the bacterium to enhance the expression of virulence determinants; coordinate regulation of gene expression by iron depends on the regulatory gene fur (Litwin and Calderwood 1993b) and a fur homologue has been found in *V. vulnificus* (Litwin and Calderwood 1993a).

Other Pathogenic Vibrios

Certain strains of *V. alginolyticus*, *V. cincinnatiensis*, *V. fluvialis*, *V. furnissii*, *V. metschnikovii*, *P. damselae*, *V. mimicus*, and *G. hollisae* have been implicated in human infection (➤ *Table 36.10*), and two more have been isolated from diseased patients without a direct link proven.

Vibrio alginolyticus

Strains of *V. alginolyticus* have been isolated from soft tissue, wound, ear, and eye infections (Rubin and Tilton 1975; Pien et al. 1977; Mukherji et al. 2000; Gomez et al. 2003; Ardic and Ozyurt 2004; Feingold and Kumar 2004). It has been implicated in a case of acute enterocolitis (Hiratsuka et al. 1980) where a strain was isolated from rice-water stool and also from the fish eaten. Many of the infections occurred when seawater got into contact with open wounds or other trauma. *V. alginolyticus* is a common inhabitant of marine and estuarine environments with a worldwide distribution (Vandenberghe et al. 1999, 2003; Maugeri et al. 2000; Thompson et al. 2001).

Vibrio cincinnatiensis

Cases of infection due to this species are extremely rare. The first record of *Vibrio cincinnatiensis* was that of a 70-year-old male in Cincinnati Hospital, USA, with bacteremia and meningitis, showing signs of disorientation, lethargy, and altered mental status. Blood and cerebrospinal cultures showed the presence of only this bacterium (Brayton et al. 1986). Another case was of an immunocompromised elder patient suffering from enteritis from which an isolate was obtained from stools (Wuthe et al. 1993). It has been also isolated from wounds and from the ear (Farmer and Hickman-Brenner 1992). Environmental strains have been obtained from zooplankton (Heidelberg et al. 2002a), seawater (Heidelberg et al. 2002b), mussels (Ripabelli et al. 1999), shrimp (diseased), turbot, rotifers, and oysters (Thompson et al. 2001).

Vibrio fluvialis

V. fluvialis causes a "cholerae-like" diarrhea with vomiting, abdominal pain, dehydration, and fever (Huq et al. 1980; Tacket et al. 1982) and has been isolated primarily from infant, children, and young adult stools. Consumption of raw oysters, shrimp, and cooked fish (Janda et al. 1988; Klontz and Desenclos 1990) has been determined in some cases as the infection vector. Strains belonging to this species were also included in the "*Vibrio*-like group EF-6" or "Heiberg group III" (Huq et al. 1980) and in the "F group" (Furniss et al. 1977) until they were formally identified as *V. fluvialis* by Lee et al. (1981). Strains of *V. fluvialis* are able to produce an enterotoxigenic hemolysin (VFH) similar to the one produced by *V. cholerae* El-Tor (Kothary et al. 2003) and also a metalloprotease (Miyoshi et al. 2002).

Vibrio furnissii

This species was originally known as *V. fluvialis* biovar II or *V. fluvialis* aerogenic. DNA–DNA hybridization studies proposed to classify this biovar as a new species, *V. furnissii* (Brenner et al. 1983). This organism has been implicated in food-related gastroenteritis (Brenner et al. 1983; Hickman-Brenner et al. 1984a), but little clinical information is available to elucidate its true role as a pathogen. It has been isolated from humans with diarrheic stools (Hickman-Brenner et al. 1984b; Lam and Goi 1985; Farmer and Hickman-Brenner 1992; Dalsgaard et al. 1997) and animal feces (Brenner et al. 1983) and commonly found in aquatic environments, especially in estuaries (Lee et al. 1981), but also in river water (Thompson et al. 2001).

Vibrio metschnikovii

Human infections with *V. metschnikovii* are rare cases of diarrhea and have been reported in infants from Peru (Dalsgaard et al. 1996a), but the stools were only screened for vibrios, and no common source of infection was found. Other diarrhea cases have also been reported from patients with predisposing medical conditions in Japan (Miyake et al. 1988) and Belgium (Hansen et al. 1993). Only one study reports the presence of *V. metschnikovii* in patients with no underlying systemic illness (Magalhaes et al. 1996). Five cases of internal infection (blood and urine cultures) have been reported in elderly patients (Jean-Jacques et al. 1981; Farmer and Hickman-Brenner 1992; Hansen et al. 1993; Hardardottir et al. 1994). In one patient, a mixed bacteremia was encountered. *V. metschnikovii* has also been isolated from wound infections (Farmer and Hickman-Brenner 1992; Hansen et al. 1993; Linde et al. 2004). All the infections have occurred in elderly people or infants, which speaks of its opportunistic nature. It has been commonly isolated from fowl (Thompson et al. 2001), aquatic animals (shrimp, cockles, lobsters, crabs), rivers, and sewage (Lee et al. 1978; Martin and Bonnefont 1990; Farmer and Hickman-Brenner 1992; Monticelli and Wally 1993; Ivanova et al. 2001).

Vibrio mimicus

V. mimicus was first described by Davis (Davis et al. 1981b) when they found that atypical strains of *V. cholerae*, mainly sucrose negative, were found in human stools from several countries and also from environmental samples, oysters, and shrimp. Strains have been isolated in Bangladesh, Brazil, Canada, Guam, Japan, Mexico, New Zealand, Philippines, and the USA (Davis et al. 1981a; Sanyal et al. 1984; Chowdhury et al. 1989d; Vieira et al. 2001). *V. mimicus* is hardly found in the environment at salinities above 10 ppt, being 4 ppt the optimal.

Infections occur after ingestion of raw or undercooked seafood especially oysters (Shandera et al. 1983), sea turtle eggs (Campos et al. 1996), and raw fish (Kodama et al. 1991). Clinical signs are similar to cholera and include diarrhea, nausea, vomiting, and abdominal cramps, fever, headache, and sometimes bloody diarrhea (Shandera et al. 1983; Mitra et al. 1993). *V. mimicus* has also been isolated from ear infections (Davis et al. 1981b; Shandera et al. 1983). Virulence is less understood than that of *V. cholerae*, although it appears to be very similar. The complete lysogenic filamentous bacteriophage (CTXΦ) of *V. cholerae* has been also found in some strains of *V. mimicus* as well as the CTXΦ receptor, the filamentous phage VPIΦ (Boyd et al. 2000). In these DNA elements reside the genes for the cholera toxin (CT) and the toxin-coregulated pilus (TCP), both essential virulence factors. Other genes that encode virulence factors, such as thermostable direct hemolysin (tdh) and the heat-stable enterotoxin (st), are found only in some of the strains of *V. mimicus*. The hemolysin gene (vmh) is present in all *V. mimicus* strains either clinical or environmental (Shinoda et al. 2004).

Photobacterium damselae

Strains of *P. damselae* have been isolated from wound infections (Love et al. 1981; Morris et al. 1982) which can sometimes develop into a fulminant and fatal septicemia (Perez-Tirse et al. 1993) or necrotizing fasciitis (Yamane et al. 2004) (Yuen et al. 1993; Goodell et al. 2004). Fatal outcomes may arise in individuals with predisposing medical conditions (Clarridge and Zighelboim-Daum 1985b). In almost all cases, wounds were exposed to seawater or caused by marine animals (fish) (Morris et al. 1982).

Virulence factors identified in *P. damselae* virulent strains include hemolysins (Clarridge and Zighelboim-Daum 1985a), an extracellular heat-labile cytolysin (Kothary and Kreger 1985) identified as a phospholipase D (Kreger et al. 1987), the capacity to acquire iron from host tissues through sidephores (Fouz et al. 1997), and withstand the bactericidal effect of sera (Fouz et al. 1994).

Grimontia hollisae

G. hollisae was originally described as *Vibrio hollisae* from stools of patients with diarrhea (Hickman et al. 1982), but later it was reclassified as a new genus with the aid of molecular data (Thompson et al. 2003). *G. hollisae* has been correlated with diarrhea (Farmer and Hickman-Brenner 1992; Abbott and Janda 1994), but evidence as its true role as pathogen is still weak. Clinical signs include diarrhea or watery diarrhea, abdominal pain, and sometimes fever, vomiting, and elevated leukocyte count (Morris et al. 1982; Carnahan et al. 1994). Consumption of raw seafood has frequently been reported as the probable route of infection. Bacteremia has been diagnosed in patients, some with underlying medical problems (Morris et al. 1982; Lowry et al. 1986; Rank et al. 1988).

A thermostable direct hemolysin (Vh-tdh) has been found in *G. hollisae* strains isolated from clinical samples (Nishibuchi et al. 1985) and from fish (Nishibuchi et al. 1988). The *Vh-tdh* gene sequence of *G. hollisae* is homologous (>93 % similarity) to the hemolysin genes of *V. parahaemolyticus*, *V. cholerae* non-O1, and *V. mimicus* (Yamasaki et al. 1991). Sidephores (Okujo and Yamamoto 1994) and a heat-labile enterotoxin (Kothary and Richardson 1987) have also been found. Evidence suggests that the pathogenicity of *G. hollisae* is multifactorial (Miliotis et al. 1995).

Diseases Caused by Vibrios in Aquatic Animals

Several species of *Vibrio* and *Photobacterium* are responsible for disease in marine wild and reared organisms, including fish, molluscs, crustaceans, rotifers, and corals (*Table 36.6*). Vibriosis, the disease caused by several species of *Vibrio*, has been recognized in fish since the nineteenth century. *V. anguillarum* was isolated from diseased eels as early as 1883 (Canestrini 1883). The "red pest," probably equating vibriosis, was described already in 1718 by Bonaveri (Drouin de Bouville 1907). Most *Vibrio* species have been isolated from diseased aquatic organisms, but, obviously, this does not mean that all of them are pathogens. Therefore, only those species of *Vibrio* where the etiological agent has been proven or strong evidence exists are listed beneath.

Vibriosis in Fish

Vibrio species implicated in vibriosis that affect marine fishes are *V. alginolyticus*, *V. anguillarum*, *V. harveyi*, *V. ordalii*, *V. salmonicida*, *V. splendidus*, and *V. vulnificus*. *V. tapetis* and *V. furnissii* have also been isolated from moribund fish (Jensen et al. 2003) and eels (Esteve 1995), respectively, but the true pathogenicity of these species for fish has still to be clearly established. Pathogenic vibrios are associated with acute bacterial septicemias or chronic focal lesions (Hjeltnes and Roberts 1993). *V. anguillarum* and *V. salmonicida* appear to be primary pathogens, whereas the other species may harbor certain virulent strains that affect organisms under stressing conditions, e.g., crowded and polluted environments.

Vibrio alginolyticus

This species has been implicated as the causal agent of vibriosis or gas gut disease of groupers (Lee 1995), gilt-head sea bream (Sparus aurata) (Balebona et al. 1998a), and many marine aquaria fishes (Stoskopf 1993). Symptoms observed in sea bream are septicemia, hemorrhaging, dark skin, and sometimes ulcers on the skin surfaces. Internally, peritoneal accumulation of fluid and gas and hemorrhagic livers are found. Penetration of this bacterium into the fish occurs when the mucus layer is removed and the skin is damaged (Balebona et al. 1998a). Although, some strains of *V. alginolyticus* have chemotactic and adhesion abilities towards mucus from the intestine, gills, and skin of the sea bream (Bordas et al. 1998) and can even survive with mucus as sole source of nutrients (Balebona et al. 1998a). Primary control methods include reduction of stress factors, as this pathogen is an opportunist. Chloramphenicol, tetracyclines, nitrofurazone, and gentamicin have been reported as effective means to control *V. alginolyticus* although the diagnoses have not been clearly confirmed.

Vibrio anguillarum

V. anguillarum is the main agent of vibriosis. These organisms have a worldwide distribution and may cause disease in marine, estuarine, and freshwater fish (Egidius 1987; Actis et al. 1999). *V. anguillarum* is not an obligate parasite, but it is a primary pathogen of fishes, and its presence in fish will sooner or later result in disease (Post 1987). Warm weather, particularly when stocking densities, whether in farmed or wild fish, are high, and when salinities and organic loads are also high, usually leads to an acute hemorrhagic condition known as "red pest." The occurrence of other stressors may well precipitate the condition, but with many strains, inherent pathogenicity is such that often no predisposing cause can be found (Hjeltnes and Roberts 1993). Incubation period varies with strain and temperature (Hjeltnes and Roberts 1993) and may be as short as 3 days after exposure to the bacteria, depending on virulence of the pathogen and susceptibility of the fish (Post 1987).

V. anguillarum presents some properties which allow it first to colonize and invade the host tissues, then to survive and proliferate. Its iron-sequestering system partly explains its pathogenicity (Hjeltnes and Roberts 1993). It appears that most infections with *V. anguillarum* begin with the colonization of the fish gastrointestinal tract, probably through the posterior region or rectum (Ransom 1978). These bacteria are strongly attracted to intestinal mucus, and once they have colonized the fish intestine, they penetrate the intestinal wall and cause a systemic infection resulting in disease and death (Post 1987). Bacteria become septicemic after invasion and can be demonstrated in blood, kidney, liver, and other organs. Bacteria may be transmitted to water in feces at this time. Dead fishes become a source of infection to other fishes. Close crowding of fish in rearing systems makes exposure to a high population of the pathogen likely and transmission under these conditions will be more effective. Another route of transmission in fish culture has been through the feeding of infected fishes or fish viscera to hatchery fishes. This practice constitutes a direct transmission cycle and should be avoided (Post 1987). Among the 10 serotypes described for *V. anguillarum*, only serotypes 01, 02, and 03 have been associated with mortality in a great variety of farmed and feral fish worldwide (Toranzo et al. 1997). In young fish, mortalities can be 50 % or higher and lower in older fish, but infected fish do not feed or grow (Hjeltnes and Roberts 1993).

Table 36.6
Species of *Vibrio* that affect diverse marine organisms

Species	Host	References
V. alginolyticus	Aquarium fishes	Stoskopf (1993a)
	Groupers (*E. malabaricus*)	Lee (1995)
	Sea bream (*S. aurata*)	Balebona et al. (1998a), Bordas et al. (1998)
	Scallops (*Argopecten ventricosus and Nodipecten subnodosus*), Penshell (*Atrina maura*), Pacific oysters (*Crassostrea gigas*)	Luna-Gonzalez et al. (2002)
	Scallop (*Argopecten purpuratus*)	Riquelme et al. (1996)
V. anguillarum	Eels (*Anguilla anguilla*)	Canestrini (1883)
	Many species of fish	Toranzo et al. (1983), Egidius (1987)
V. harveyi	Penaeid shrimp	Sunaryanto and Mariam (1986) Lavilla-Pitogo et al. (1990a), Karunasagar et al. (1994), Groumellec and Haffner (1995), Vaseeharan and Ramasamy (2003)
	Packhorse rock lobster (*Jasus verreauxi*) larvae	Diggles et al. (2000b)
	Brine shrimp (*Artemia* sp.)	Soto-Rodríguez et al. (2003)
	Abalone (*Haliotis* spp.)	Nishimori et al. (1998), Nicholas et al. (2002), Sawabe et al. (2007c)
V. harveyi (prev. *V. carchariae*)	Sharks (*Carcharhinus plumbeus*, *Negaprion brevirostris*, *Squalus acanthias*)	Grimes et al. (1984b), Grimes et al. (1985), Stoskopf (1993a)
V. lentus	Octopus (*Octopus vulgaris*)	Farto et al. (2003)
V. ordallii	Salmonids	Schiewe et al. (1981)
V. parahaemolyticus	Penaeid shrimp	Roque et al. (1998), Roque et al. (2000)
	Brine shrimp (*Artemia* sp.)	Gomez-Gil et al. (1998a)
	Blue crab (*Callinectes sapidus*)	Johnson (1983), Muroga et al. (1991)
V. penaeicida	Penaeid shrimp	Ishimaru et al. 1995
V. pectenicida	Scallop (*Pecten maximus*)	Lambert et al. (1998)
V. proteolyticus	Brine shrimp (*Artemia* sp.)	Verschuere et al. (1999)
V. salmonicida	Atlantic salmon (*Salmo salar*)	Egidius et al. (1986)
	Rainbow trout (*Salmo garderi*)	Wiik et al. (1989)
	Cod (*Gadus morhua*)	Jorgensen et al. (1989)
V. splendidus	Oyster (*Crassostrea gigas*)	Sugumar et al. (1998)
	Scallop (*P. maximus*)	Nicolas et al. (1996)
	Rainbow trout fingerlings (*Oncorhynchus mykiss*)	Pazos et al. (1993)
	Flounder (*Paralichthys adspersus*)	Miranda and Rojas (1996)
	Gilt-head sea bream (*Sparus aurata*)	Balebona et al. (1998b)
	Turbot larvae (*Scophthalmus maximus*)	Gatesoupe et al. (1999), Farto et al. (1999)
	Corkwing wrasse (*Symphodus melops*)	Jensen et al. (2003)
V. tapetis	Manila clam (*Ruditapes philippinarum*)	Paillard et al. (1989), Borrego et al. (1996a)
	Fine clam (R. *decussates*)	Novoa et al. (1998)
V. tubiashii	Hard-clam (*Mercenaria mercenaria*)	Tubiash et al. (1965)
	American oyster (*Crassostrea virginica*)	Tubiash et al. (1970)
	Pacific oyster (*P. gigas*)	Takahashi et al. (2000c)
	Flat oyster (*Ostrea edukis*)	Lodeiros et al. (1987)
V. vulnificus	Eels (*A. anguilla* and *A. japonica*)	Muroga et al. (1976)

Vibrio harveyi

V. carchariae is a later synonym of *V. harveyi* (Pedersen et al. 1998); therefore, in this section, the disease caused by this species in sharks will be attributed to *V. harveyi*. Shark meningitis or vibriosis was first observed in brown sharks (*Carcharhinus plumbeus*) held in captivity (Grimes et al. 1984b). Later on, *V. harveyi* has been proven to infect other species of sharks experimentally, such as lemon sharks (*Negaprion brevirostris*) and spiny dogfish (*Squalus acanthias*) (Grimes et al. 1984a, 1985). Infections have also been detected in wild sand tiger sharks (Stoskopf 1993).

Infected sharks demonstrate lethargy, inappetence, and disinterest at first, later anorexia with progressive disorientation; if left untreated, convulsions, coma, and death follow. Sometimes, skin lesions with purulent brown exudates are observed. Meningitis is a distinctive feature, and *V. harveyi* can be isolated from the cerebrospinal fluid. Affected organs are the spleen, liver, and specially the kidney (Grimes et al. 1984b; Stoskopf 1993). Susceptibility to this pathogen varies with the species of shark affected, although few data are available; the spiny dogfish is very susceptible, while the lemon shark is more resistant (Grimes et al. 1985). Apparently, one mode of transmission might be through trematodes infesting shark, since *V. harveyi* has been isolated from these flukes.

Vibrio ordalii

V. ordalii was first described as *V. anguillarum* biovar II, but later it was recognized as a different species (Schiewe et al. 1981). It has been isolated from salmonids, especially from different geographic locations in the Pacific. This bacterium induces a pathogenesis similar to that of *V. anguillarum* but usually less severe (Hjeltnes and Roberts 1993). Focal muscle lesions rather than generalized hemorrhagic septicemia as seen in *V. anguillarum* infections are observed (Roberts and Shepherd 1986; Egidius 1987). Although, both species are capable to resist the bactericidal activity of the fish serum and have an iron-sequestering system that allows them to proliferate in iron poor environments (Trust et al. 1981), as those found inside the fish tissues.

Vibrio salmonicida

Vibrio salmonicida is specifically responsible for the condition known as cold-water vibriosis, hemorrhagic syndrome, or Hitra disease in Atlantic salmon. This disease took its name from the Island of Hitra in Norway, where heavy losses were recorded since the 1970s (Egidius et al. 1981, 1986; Hjeltnes and Roberts 1993). *V. salmonicida* was first discovered in North America in 1989 (O'Halloran and Henry 1993). The term cold-water disease is employed generically to describe any heavy losses during the winter months (Hjeltnes and Roberts 1993). The disease is observed only at low water temperature and normally not above 10° (Colquhoun and Sorum 2001). This disease affects almost all internal organs but especially swim bladder, spleen, and liver. External clinical signs are lesions on the fin, rectum, ventral abdominal wall, and the operculum. Internally, hemorrhage and severe damage should be found on the swim bladder, change of color on the spleen and liver, and severe heart and muscle changes (Egidius et al. 1981; Hjeltnes and Roberts 1993). General septicemia occurs with high numbers of bacteria in the blood of moribund fish (Egidius et al. 1986).

Vibrio salmonicida infects the Atlantic salmon (*Salmo salar*) (Egidius et al. 1986), the Arcto-Norwegian cod (*Gadus morhua*) (Jorgensen et al. 1989; Sorum et al. 1990; Schroder et al. 1992), and the rainbow trout (*Salmo gairdneri*) (Wiik et al. 1989; Farmer and Hickman-Brenner 1992). Two distinct serotypes of *V. salmonicida* exist, one of which is more prevalent among non-salmonid species such as cod, but both serotypes can be found in the different species. All serotypes are more pathogenic in salmon than in other fishes (Egidius et al. 1986; Hjeltnes and Roberts 1993).

As with other bacterial septicemias, control of vibriosis is best achieved by maintenance of water quality, good husbandry, and low stocking densities. Since this is not always possible, whenever an outbreak occurs, the treatment with an oral antibiotic is the only option.

Resistance to antibiotics is a serious problem with the vibrios. There are many strains of *V. salmonicida* resistant to the commonly used oxytetracycline and multiresistant to oxytetracycline and sulfonamides. Oxolinic acid resistance is growing in areas where salmon culture is intensive (Hjeltnes and Roberts 1993). Disease control by vaccination is widely used in European salmonid aquaculture against cold-water vibriosis and has proven effective especially when administered by injection (Press and Lillehaug 1995).

Vibrio splendidus

Vibrio splendidus was regarded as an environmental organisms with no pathogenic importance (Myhr et al. 1991), but it has been later implicated in infections in the gilt-head sea bream (*Sparus aurata*) (Balebona et al. 1998b), turbot larvae (*Scophthalmus maximus*) (Gatesoupe et al. 1999; Farto et al. 1999), turbot and brill (*Colistium nudipinnis* and *C. guntherik*, respectively) (Diggles et al. 2000a), rainbow trout fingerlings (*Oncorhynchus mykiss*) (Pazos et al. 1993), flounder (*Paralichthys adspersus*) (Miranda and Rojas 1996), and latter in the corkwing wrasse (*Symphodus melops*) (Jensen et al. 2003). Pathogenic and nonpathogenic strains of *V. splendidus* and *V. pelagius* have also been designated as *V. anguillarum*-like or *V. anguillarum*-related (VAR)because of the great biochemical similarity (Pazos et al. 1993).

There is not a description of the disease caused by *V. splendidus* in fish, but the clinical signs may be similar to those caused by *V. anguillarum*, including abdominal distension, hemorrhaging at the base of the fins, and focal hemorrhagic lesion on the skin, kidney, and liver (Diggles et al. 2000a). *V. splendidus* was recovered from kidney samples as a dominant colony (Jensen et al. 2003). Pathogenicity challenges

with turbot larvae have produced significantly higher mortalities of up to 100 %, depending on the strains injected into the fish (Gatesoupe et al. 1999).

Vibrio vulnificus

V. vulnificus serovar E (formerly biovar 2) is a primary pathogen for eels (*Anguilla anguilla*) (Amaro et al. 1992). It is the main infectious disease affecting farmed eels (Marco-Noales et al. 2001); it has been primarily isolated in Asia, although it is also found in Europe. The first isolates were obtained from Anguilla japonica in Japan between 1975 and 1977 (Muroga et al. 1976). Affected organisms show signs of hemorrhagic septicemia and redness of the body flanks and tail. The bacterium may also be recovered from internal organs (Amaro et al. 1992).

Vibriosis in Crustaceans

Most of the knowledge about vibriosis affecting crustaceans comes from experiences in the shrimp farming industry. Some differences have been observed in vibriosis affecting larval stages and juveniles and bloodstocks. During outbreaks in larval and postlarval shrimp rearing, luminescent *V. harveyi*, *V. campbellii*, and probably *V. splendidus* have been isolated. During the grow-out part of the culture, many species have been reported as responsible for vibriosis, but only a few have actually been proven to be pathogens; all others are only members of the normal microbiota of the shrimp and the environment. Species where some strains have been proven to be pathogenic for shrimps are *V. parahaemolyticus*, *V. penaeicida*, and probably *V. harveyi*. Vibrios are considered opportunistic pathogens, but evidence suggests that some strains can be regarded as primary pathogens, especially in the case of *V. penaeicida*. *Artemia* spp. can also be susceptible to infection by vibrios, as is the case of *V. proteolyticus* and strains of *V. parahaemolyticus* and *V. campbellii*.

Vibriosis affects almost all cultured or captive crustaceans (*Table 36.12*), including penaeid shrimps, lobsters, crabs, brine shrimp, and crayfish. Perhaps every crustacean may be susceptible to infection by vibrios.

Vibrio harveyi

Vibrio harveyi primarily infects larval stages of penaeid shrimps. Because many strains of this species are luminescent and have been associated with disease outbreaks, the disease has been named "luminescent vibriosis." *V. harveyi* also affects, at least in laboratory conditions, *Artemia nauplii* (Soto-Rodríguez et al. 2003) and rock lobster (*Jasus verreauxi*) larvae (Diggles et al. 2000b). *V. harveyi* has a worldwide distribution and can be isolated from most aquatic culture system. The first reports of luminescent vibriosis in Indonesian and Philippine penaeid larvae hatcheries during the late 1980s (Lavilla-Pitogo et al. 1990a) reported mortalities as high as 100 % with year-round epizootics, but most outbreaks occurred during the rainy season (Sunaryanto and Mariam 1986).

Infected larvae become luminescent because of the massive bacterial colonization, which can be seen by the naked eye. Weak and moribund larvae become opaque white in the thoracic region and settle in the bottom of the tanks showing very weak swimming movements (Lavilla-Pitogo et al. 1990a). Massive colonization of the appendages and foregut, followed by infection of the midgut, hepatopancreas, and a terminal septicemia (Lavilla-Pitogo et al. 1990a; Lightner 1993), is observed in moribund larvae. Necrosis of the appendages is often seen. Affected larvae show heavy bacterial colonization on the cuticle of mouthparts and appendages and on the cuticular lining of the esophagus and components of the stomach. Rounding up and sloughing of hepatopancreatic tubule and midgut epithelial cells into their lumen ("Bolitas blancas" = little white balls) are also commonly found. In the final stages of the infection, cuticular colonization with systemic infections is observed (Lightner 1996). Significant mortalities can occur after 48 h when luminescent vibrios reach an excess of 10^2 cell/ml (Lavilla-Pitogo et al. 1990b). Weak infected larvae can be cannibalized by healthier larvae that, in turn, became infected (Diggles et al. 2000a). Virulence seems to be enhanced by exposing the pathogenic strain to low salinities (Prayitno and Latchford 1995), which might explain why during the rainy seasons, outbreaks are more abundant. Sequencing of a bacteriophage present in a pathogenic strain of *P. monodon* larvae revealed a putative toxin that might have a neurotoxic effect (Oakey et al. 2002) further supported by experimental challenges with this toxin that produced weakness and intermittent swimming motion in larvae (Harris and Owens 1999). *V. harveyi* has been found in seawater samples close to the larval rearing facilities (Lavilla-Pitogo et al. 1990a) and in infected larvae (Groumellec and Haffner 1995). It is therefore suggested that one route of infection might be through the seawater and where *V. harveyi* is a natural inhabitant. Other studies did not detect antibiotic-resistant luminous *V. harveyi* (responsible for larval mortalities) in the intake of seawater (Karunasagar et al. 1994).

Recent molecular analyses of strains phenotypically identified as *V. harveyi* (Soto-Rodríguez et al. 2003) have revealed that they actually belong to the closely related species *V. campbellii* (Gomez-Gil et al. 2004a). *V. campbellii*-like strains have been reported as affecting *Penaeus indicus* larvae (Hameed et al. 1996). The identification tests did show clear-cut results, hampering the allocation of the isolates into either species. In fact, the phenotypic identification of *V. campbellii* and *V. harveyi* is nearly impossible with currently available tests (Gomez-Gil et al. 2004a). The pathogenic status of *V. campbellii* will now have to change because of the evidence provided, and a reexamination of *V. harveyi* should be performed.

"All in–all out" batch culture and thorough facility disinfection and drying have been proven to reduce the *V. harveyi* infections (Lightner 1993). Separation of the eggs from the broodstock and feces should be done as soon as

possible (Lavilla-Pitogo et al. 1990a). Chemical control in shrimp hatcheries may have limited effectiveness because of the rapid development of resistant strains and the limited tolerance of the larvae to the drugs (Lavilla-Pitogo et al. 1990a; Karunasagar et al. 1994).

Vibrio parahaemolyticus

Vibrio parahaemolyticus is a common inhabitant of estuarine and marine environments (Colwell et al. 1973; Ruangpan and Kitao 1991) and of healthy shrimp (Gomez-Gil et al. 1998c). It has also been isolated from diseased shrimp (Mohney et al. 1994) and proven to be pathogenic under controlled conditions (Roque et al. 1998, 2000; Gomez-Gil et al. 1998a). Evidently, it is an opportunistic pathogen that can infect immunodeficient crustaceans. *V. parahaemolyticus* has also been recorded to infect the brine shrimp *Artemia franciscana* (Gomez-Gil et al. 1998a) and the blue crab *Callinectes sapidus* (Johnson 1983; Muroga et al. 1991).

Clinical signs observed in affected shrimp are anorexia; lethargy, disoriented shrimp swimming weakly, gathering along the edges of the pond; generalized opacity or cloudiness of the muscle and/or red discoloration of the appendages, dorsal flexure at the third abdominal segment with slight rigidity, birds eating the weakened shrimp; and generalized septicemia (Brock and Leamaster 1992). Heavy losses have been recorded in the culture of shrimps (Sano and Fukuda 1987). Internally, multifocal necrosis, hemocytic inflammation with nodule and granuloma formation in the lymphoid organ, heart, gills, hepatopancreas, antennal gland, cuticular epidermis and subcutis, and other connective tissues, can be observed. Some hemocyte nodules contain Gram-negative bacteria within intracytoplasmic vacuoles or in the nodules and granulomas (Mohney et al. 1994). Hemocytic nodules can be melanized, with septic centers. Apparently, pathogenicity of *Vibrio parahaemolyticus* depends on the strain (Gomez-Gil et al. 1998a), and no data has been published where it was proven to be a primary pathogen. Because of the opportunistic nature of this pathogen, reducing stressing factors can prevent the infection. Special importance is to be paid to the water and sediment quality of the pond.

Several antibiotics have been used to control *V. parahaemolyticus* infection. Enrofloxacin is the agent of choice since no resistance has been found among vibrios, whereas 43 % of the strains tested were found resistant to the commonly used oxytetracycline (Roque et al. 2001). The MIC of enrofloxacin is 0.45 µg/ml (1.50–0.188 µg/ml).

Vibrio penaeicida

The disease caused by this bacterium (*Vibrio* sp. PJ) was first observed affecting Kuruma shrimp (*Penaeus japonicus*) during 1980 in Japan, but was also isolated from apparently healthy shrimp and from seawater (Ishimaru et al. 1995). Latter, this species was implicated in the *Penaeus stylirostris* disease called "Syndrome 93" in New Caledonia where high mortalities occurred (Costa et al. 1998). This bacterium seems to be restricted to shrimps in grow-out ponds and broodstock and does not cause mortalities in hatcheries or nurseries (Goarant et al. 1998). Affected shrimp displays similar symptoms of other vibriosis, cloudiness of the abdominal muscle, especially in the sixth segment, erratic swimming, lethargy, and weakness (Costa et al. 1998; Goarant et al. 1998). Melanized nodules are observed in lymphoid organ, gills, heart, hepatopancreas, and gonads. High numbers of bacteria are recovered from the hemolymph of disease shrimp (Goarant et al. 1998). Experiments suggest that the main route of entry is through the gastrointestinal tract, although wounds in the cuticle can also be portals of entry (De la Peña et al. 1998).

Disease outbreaks occur during the transition months between hot and cold seasons in New Caledonia (Goarant et al. 1998) and during summer and fall in Japan (De la Peña et al. 1997).

As with all bacterial diseases, good husbandry significantly reduces or eliminates the problem, and antibiotics should be employed with care.

Vibrio proteolyticus

V. proteolyticus has only been proven to be pathogenic to the brine shrimp *Artemia* sp. (Verschuere et al. 1999). Total mortalities have been recorded in axenic *Artemia* sp. cultures after 48 h, regardless of the inoculated density of bacteria (Verschuere et al. 2000). Bacteria penetrated the *Artemia nauplii* through the gut epithelium and invaded the body cavity, devastating this epithelium and the underlying cells and tissues (Verschuere et al. 2000). Axenic *Artemia nauplii* have been proven to be very sensitive to the pathogenic action of vibrios (Rico-Mora and Voltolina 1995; Gomez-Gil et al. 1998a), but when a bacterial flora is well established, this action is significantly reduced (Gomez-Gil 1998; Verschuere et al. 2000).

Vibriosis in Molluscs

Molluscs, as many other marine animals, are also subject to infection by members of the *Vibrio* genus (Elston and Leibovitz 1980). Species associated with disease outbreaks are *V. alginolyticus*, *V. pectinicida*, *V. splendidus*, *V. tapetis*, and *V. tubiashii*; *V. lentus* has been proven to cause infection experimentally, but no outbreaks have been registered in cultured organisms.

Species of molluscs principally affected are *Crassostrea gigas*, *C. virginica*, *Mercenaria mercenaria*, *Ostrea edulis*, *Pecten maximus*, *Tapes philippinarum*, *T. decussates*, *Argopecten purpuratus*, *A. ventricosus*, *Nodipecten subnodosus*, and *Atrina maura*, but probably others are also susceptible to infection.

Vibrio alginolyticus

Experimental infections with this species have shown mortalities in scallops (*Argopecten ventricosus* and *Nodipecten subnodosus*), pen shell (*Atrina maura*), Pacific oysters (*Crassostrea gigas*) (Luna-Gonzalez et al. 2002), and red abalone (Anguiano-Beltran et al. 1998). Diseased larvae showed signs common to bacillary necrosis: bacteria swarming around the velum, diminished swimming, ciliary paralysis, empty stomachs, and necrosis in several tissues (Luna-Gonzalez et al. 2002). Outbreaks were *V. alginolyticus* has been implicated have been reported in the culture of *Argopecten purpuratus* in Chile, where massive larval mortalities were observed (Riquelme et al. 1996) usually during the summer months when an increase in organic matter and temperature (above 18 °C) is observed. No evidence of invading bacteria could be observed in dying larvae, but the production of an exotoxin, as responsible for the mortalities detected, is suggested (Riquelme et al. 1996).

Vibrio anguillarum

This species has been reported to be associated bivalve larvae mortality episodes; species affected are the Japanese oyster (*Crassostrea gigas*), *Ostrea edulis*, and scallop (*Argopecten purpuratus*) (DiSalvo et al. 1978; Riquelme et al. 1995). Infected veliger larvae cannot stay in the water column and progressively settle in the bottom of the tanks. Attachment of bacteria to the periostracum and invasion of the mantle and other soft tissues are characteristic (Birkbeck et al. 1987). Experimental challenges have reported mortalities of 39 % after 24 h of inoculation and swimming inhibition in 96.6 % of the larvae (Riquelme et al. 1995). Cell-free supernatant also produced mortalities when added to larval rearing water, suggesting a toxic activity of a *V. anguillarum*-related (VAR) strain (Riquelme et al. 1995). Attachment of certain strains of *V. anguillarum* to gill tissue of various bivalve larvae has been observed, but specially to larvae of *C. gigas* and *O. edulis* (Birkbeck et al. 1987).

Vibrio lentus

V. lentus has been associated with diseased wild octopuses (*Octopus vulgaris*) in Galicia, Spain (Farto et al. 2003). Octopuses showed round hard skin lesions in the arm of head mantle with exposed muscle in advanced cases. Vibrios can be isolated from internal organs, nephridia, and gill hearts. Mortalities in healthy organisms rose to 50 % after 72 h of being exposed to the pathogen by bath challenge, and the symptoms observed were similar to those in diseased wild animals (Farto et al. 2003).

Vibrio pectenicida

V. pectinicida has been recently identified as responsible for scallop (*Pecten maximus*) larvae mortalities in France (Lambert et al. 1998). Outbreaks occur early in the culture cycle but with no particular symptom observed (Lambert et al. 1999); adult scallops are very resistant to infection. Apparently, a small molecule (<3 kDa) produced by the bacterium has a toxic effect in the larvae (Lambert et al. 1999).

Vibrio splendidus

V. splendidus biovar II has been reported to affect larvae of the Japanese oyster *Crassostrea gigas* (Sugumar et al. 1998; Le Roux et al. 2002) and of scallops *Pecten maximus* (Nicolas et al. 1996; Lambert et al. 1999). This species has been implicated in the disease called "summer mortality" (Lacoste et al. 2001; Le Roux et al. 2002) that affects 6- to 12-month-old oysters in France when the water temperature exceeds 16 °C.

The signs and course of the disease resemble bacillary necrosis (Sugumar et al. 1998). Experimentally, *V. splendidus* biovar II can cause high mortalities in Tapes philippinarum and *P. maximus* larvae, but results with *C. gigas* larvae are variable, suggesting pathogenic differences between strains. It has been suggested that the broodstock could be the source and route of transmission of this pathogen, since it has been detected, most preponderantly, in the gonad (Sugumar et al. 1998).

Vibrio tapetis

V. tapetis causes the disease named brown ring disease (BRD) that affects the Manila clam (*Ruditapes philippinarum*) and less frequently the carpet shell or fine clam (*R. decussates*). This disease was first observed in introduced clams (*R. philippinarum*) in the Atlantic French Coast (Paillard et al. 1989; Borrego et al. 1996a; Allam et al. 2002) but has also been observed in Spain, Portugal (Castro et al. 1997), Italy (Paillard et al. 1994), and England (Allam et al. 2002), but not in the Indo-Pacific or North America where it is also cultivated (Paillard et al. 1994). It can be classified as a cold-water disease (Paillard et al. 2004) with an increased incidence in summer or autumn when the water temperature is the highest (Martinez-Manzanares et al. 1998). Heavy economical losses have been reported in affected areas (Novoa et al. 1998).

The characteristic symptom is the appearance of brown conchiolin deposits in the inner shell, hence the name. *V. tapetis* adheres to and disrupts the production of periostracal lamina, causing the anomalous deposition of periostracum (Allam et al. 2002).

Most probable route of transmission is by direct contact with infected clams (Martinez-Manzanares et al. 1998). Artificial inoculation of *V. tapetis* into the extrapallial space of clams (*R. philippinarum*) has produced up to 100 % mortality, but complete resistance was observed in *R. decussatus* (Allam et al. 2002). Virulence variation has been observed among different strains of *V. tapetis* (Novoa et al. 1998); *V. tapetis* has also been isolated from healthy clams, suggesting

an opportunistic pathogen (Castro et al. 1997). Extracellular virulence factors alone were not capable of killing clams, but the intact bacterial cell is needed to cause mortalities (Allam et al. 2002); factors such as hemolysins, cytotoxins, exotoxins, and plasmids have been reported to occur in *V. tapetis* (Borrego et al. 1996b). Treatment with antibiotics has been proposed for infected clams prior to seeding (Poppe and Breck 1997).

Vibrio tubiashii

Bacillary necrosis or larval vibriosis is a disease caused by *V. tubiashii* (Hada et al. 1984) that affects larvae and juveniles of bivalve molluscs (Tubiash et al. 1965; Tubiash et al. 1970). Mortalities often exceed 90 %, and it is considered the most serious disease of hatchery-reared larvae (Takahashi et al. 2000). Affected species are *Crassostrea virginica* (Tubiash et al. 1970), *C. gigas* (Takahashi et al. 2000), *Mercenaria mercenaria* (Tubiash et al. 1965), *Fulvia mutica*, and *Ostrea edulis* (Lodeiros et al. 1987) from the United States, Japan, Spain, and the United Kingdom.

Symptoms can develop in 24 h and include reduced motility of larvae, increased larval quiescence, and extensive soft tissue necrosis (Kothary et al. 2001). Experimentally infected larvae can show these signs as early as 4–5 h after inoculation (Tubiash et al. 1965). Mass mortalities are observed during the summer months when the water temperature is high (Lodeiros et al. 1987).

Antibiotic treatment with chloramphenicol has been advised when crisis situations are present (Tubiash et al. 1965; Lodeiros et al. 1987).

Vibrio spp.

Juvenile oyster disease (JOD) has been reported since the late 1980s principally in the northeastern United States affecting juvenile *Crassostrea virginica* less than 30 mm in size and grown at salinities above 25 ppt (Lewis et al. 1996). Similar cases have been observed in larvae of *Mercenaria mercenaria* (Brown and Tettelbach 1988).

Mortalities can exceed 90 % in 2 weeks after experimental inoculation, or 4–6 weeks after deployment of seed at enzootic sites.

Conchiolin deposits in the inner shell are the characteristic symptoms of this disease, but lesion on the mantle, reduced growth, development of fragile and uneven shell margins, and cupping of the left valve are also observed (Boettcher et al. 2000). Mantle recession can be observed as the disease progresses (Lewis et al. 1996). The characteristics of JOD are very similar to those of BRD.

The etiology of this disease has not been clearly established, but some evidence suggests that a strain similar to *V. anguillarum* or *V. alginolyticus* might be associated with perhaps other strains in consortia (Brown and Tettelbach 1988; Lee and Yii 1996; Paillard et al. 1996). Members of the *Roseobacter* group (alpha-proteobacteria) could also be implicated (Boettcher et al. 2000). Antibiotic treatment of infected oysters reduced mortalities, significantly supporting the bacterial origin of the disease (Boettcher et al. 1999). Experimental inoculation of strains has failed to reproduce typical symptoms of JOD, but high mortalities have been achieved.

Photobacterium

Photobacterium damselae subsp. *piscicida*

This disease is known as pasteurellosis, because the previous name of the pathogens was *Pasteurella piscicida*. This disease is responsible for major outbreaks among cultured marine fishes. Pasteurellosis was first observed on the east coast of the United States in 1963 affecting the white perch (*Roccus americanus*) and the striped bass (*Morone saxatilis*) (Snieszko et al. 1964). Later it was diagnosed in Japan, where it caused severe losses in young yellowtail (*Seriola quinqueradiata*) (Kusuda and Yamaoka 1972). In 1990, it appeared for the first time in the Mediterranean coast of Europe; it has been diagnosed in cultured population of sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) in France, Italy, Spain, Portugal, Malta, and Croatia, as well as in Turkey and Israel (Thyssen et al. 2000). This pathogen is not restricted to a few species of fish but has a wide number of hosts; at least eight have been reported in Japan alone (Kitao 1993).

This disease has also been named as bacterial pseudotuberculosis because granulomatous-like deposits may develop in the kidney and spleen of affected animals; greyish white nodules are often seen in the spleen, a classical sign of pasteurellosis (Kitao 1993). Histologically, these lesions are composed of bacterial masses, epithelial cells, and fibroblasts (Kubota et al. 1970) The parenchyma of the spleen may show acute necrotic changes and masses of bacteria lodged within the capillaries and interstitial spaces; accumulation of purulent material in the abdominal cavity might be present (Kitao 1993).

A rise in temperature (above 25 °C in Japan) and a reduction in salinity due to rain have led to disease outbreaks. This bacterium survives shortly (4–5 days) in estuarine waters (Toranzo et al. 1983) and up to 12 days in marine sediments (Magarinos et al. 1994), so a reservoir organism is most likely to occur (Kitao 1993).

Control of this pathogen has relied on the use of antimicrobial agents, but drug-resistant strains have been found associated with transferable R-plasmids since the mid-1980s (Aoki and Kitao 1985). As always, prevention can be achieved by avoiding overcrowding and good management practices. Vaccination has also been tried with good results in sea bass (*Dicentrarchus labrax*) when washed cells were enriched with extracellular products (Fabris et al. 1998). Bacteria that have been artificially mutated in order to make them nonpathogenic have been also tested as vaccines with very good results (Thune et al. 2003).

Antibiotic Resistance

Antibiotic Resistance Among Vibrios of Medical Importance

Resistance to antibiotics in pathogenic *V. cholerae* is a recent phenomenon. During the fourth cholera pandemic in Tanzania, 76 % of the strains examined become resistant (November 1977) to tetracycline within 5 months of antibiotic use (Mhalu et al. 1979). The resistance was encoded in a transferable plasmid which also harbors resistance genes for ampicillin, sulfonamides, chloramphenicol, kanamycin, and streptomycin (Towner et al. 1980). Plasmid encoded multiple resistance genes have been detected worldwide, e.g., Bangladesh (Glass et al. 1980), Cameroon (Garrigue et al. 1986), Kenya (Finch et al. 1988), India (Ramamurthy et al. 1992), Ukraine (Khaitovich et al. 1996), Vietnam (Dalsgaard et al. 1999), Indonesia (Tjaniadi et al. 2003), Argentina (Petroni et al. 2002), Albania, and Italy (Falbo et al. 1999). Resistance to fluoroquinolones (ciprofloxacin, norfloxacin, and nalidixic acid) has been reported for toxigenic and nontoxigenic *V. cholerae* strains isolated in India since 1995 (Mukhopadhyay et al. 1998; Garg et al. 2001). Resistance among *V. cholerae* strains is not a definitive trait, because when the antibiotic is no longer used, the strains may become again sensitive to that compound (Garg et al. 2000). At least four genetic determinants of resistance have been found in *V. cholerae*: conjugative plasmids, transposons, integrons, and an SXT constin (Goldstein et al. 1986; Waldor et al. 1996; Mazel et al. 1998; Hochhut et al. 2001b). These genetic determinants facilitate the movement of resistance genes intracellularly and also between cells.

Antibiotic Resistance in Vibrios from Environmental Sources

Vibrios represent a considerable fraction of the microbiota in rearing systems. The high density of animals and feeding loads applied favor a proliferation of vibrios in these settings. In addition, the use of antibiotics in the aquaculture industry is a well-known but yet regrettable practice that probably increases the abundance of resistant strains of vibrios. In many developing countries, where the majority of aquaculture practices take place, there is no regulation, or where regulations exist, they are not enforced (Alderman and Hastings 1998). This type of antibiotic abuse has led to the development of resistance, especially in microenvironments (Walsh 2003).

Antibiotic resistance has been reported from many vibrios, but especially from isolates obtained from the aquaculture industry. Resistance to various antibiotics has been found in vibrios isolated from the marine environment (Pradeep and Lakshmanaperumalsamy 1985; Molitoris et al. 1985), Artemia nauplii (Hameed and Balasubramanian 2000), penaeid shrimps (Bhattacharya et al. 2000; Roque et al. 2001; Molina-Aja et al. 2002), fish (Austin et al. 1982; Sanjeev and Stephen 1992; Miranda and Rojas 1996; Li-Jun et al. 1999), and molluscs (Tubiash et al. 1965; Martinez-Manzanares et al. 1998). The spread of antibiotic resistance among vibrios has been documented to occur by transfer of plasmids that carry antibiotic resistance determinants (e.g., TEM or *tet* genes) between species or genera by conjugation (Aoki et al. 1984; Li-Jun et al. 1999; Molina-Aja et al. 2002). We discuss below some of the most commonly used antibiotics in aquaculture as well as the problems related to resistance to these compounds.

β-Lactams

Resistance to β-lactams antibiotics, penicillins (*Table 36.7*) and cephalosporins (*Table 36.8*), is frequently accomplished by the production of a β-lactamase enzyme that cleaves the β-lactam ring rendering the antibiotic ineffective. Other methods involve the alteration of the targets (penicillin-binding proteins, PBPs) for these antibiotics or by a reduction in the ability of the antibiotic to access its target (Dowson and Coffey 1998). Resistance to penicillins is widely distributed and not uncommon that all isolates analyzed are resistant (Pradeep and Lakshmanaperumalsamy 1985; Rosily et al. 1987; Sanjeev and Stephen 1992; Liu et al. 1997; Bhattacharya et al. 2000). Resistance to ampicillin, carbenicillin, and cefuroxime has been found also very disseminated and with high percentages of resistant strains (Sanjeev and Stephen 1992; Li-Jun et al. 1999; Bhattacharya et al. 2000; Zanetti et al. 2001; Roque et al. 2001; Molina-Aja et al. 2002). The ample presence and worldwide distribution of resistant strains in vibrios and other genera to β-lactam antibiotics (Alderman and Hastings 1998) and the uncommon use of these antibiotics (at least in many aquaculture environments) support the idea that the resistance of vibrios might not have been as a consequence of antibiotic selection pressure imposed by aquacultural practices but by a response to natural selection (Teo et al. 2000). Penicillin-resistant genes can be found in the chromosome and in plasmids.

Tetracyclines

Tetracycline was one of the first antibiotics employed against many Gram-negative pathogens (Inglis 2000). Oxytetracycline has become a very popular antibiotic employed in many aquaculture practices (Alderman and Hastings 1998; Nonaka and Suzuki 2002; Furushita et al. 2003). Resistance to oxytetracycline and tetracycline has also been detected among many strains of vibrios (*Table 36.9*). The emergence of tetracycline resistance is a modern event that has followed the introduction of these agents for clinical, veterinary, agricultural, (Chopra and Roberts 2001), and aquacultural use (Schmidt et al. 2001). Oxytetracycline is approved for the treatment of fish in several countries (Alderman and Hastings 1998; Furushita et al. 2003). Resistance to these antibiotics is obtained by acquisition of *tet* genes (Chopra and Roberts 2001),

Table 36.7
Resistance of vibrios from environmental sources to various penicillin β-lactam antibiotics

Antibiotic	Percentage of resistant isolates (total isolates)	Source	References
Ampicillin	85.5 (505)	Seawater and seafood (shrimp, clam, and squid)	Molitoris et al. (1985)
	75.3 (20)	Seawater and penaeid (*P. monodon*) larvae	Baticados et al. (1990)
	82.1 (84)	Finfish and shellfish	Sanjeev and Stephen (1992)
	100 (4)	Penaeid shrimp (*P. monodon* and *P. japonicus*)	Liu et al. (1997)
	44.4 (36)	Cockles (*Anadara granosa*)	Radu et al. (1998)
	58.8 (51)	Sea bream (*Sparus sarba*)	Li-Jun et al. (1999)
	62.5 (8)	Penaeid shrimp (*P. monodon*)	Bhattacharya et al. (2000)
	68.0 (144)	Shrimp culture systems	Roque et al. (2001)
	88.9 (54)	Water	Zanetti et al. (2001)
	70.0 (22)	Penaeid shrimp	Molina-Aja et al. (2002)
Carbenicillin	33.3 (36)	Cockles (*Anadara granosa*)	Radu et al. (1998)
	69.0 (144)	Shrimp culture systems	Roque et al. (2001)
	73.3 (22)	Penaeid shrimp	Molina-Aja et al. (2002)
Penicillin	60 (5)	Bivalve mollusks	Tubiash et al. (1965)
	100 (120)	Water, sediment, plankton, fish, and prawn	Pradeep and Lakshmanaperumalsamy (1985)
	100 (203)	Penaeid larval culture (*P. indicus*)	Rosily et al. (1987)
	100 (25)	Seawater and penaeid (*P. monodon*) larvae	Baticados et al. (1990)
	100 (84)	Finfish and shellfish	Sanjeev and Stephen (1992)
	100 (4)	Penaeid shrimp (*P. monodon* and *P. japonicus*)	Liu et al. (1997)
	58.3 (36)	Cockles (*Anadara granosa*)	Radu et al. (1998)
	100 (8)	Penaeid shrimp (*P. monodon*)	Bhattacharya et al. (2000)
	96.6 (505)	Seawater and seafood (shrimp, clam, and squid)	Molitoris et al. (1985)
Methicilllin	78.0 (505)	Seawater and seafood (shrimp, lamc, and squid)	Molitoris et al. (1985)

as might have been the case in some marine bacteria (Andersen and Sandaa 1994; Schmidt et al. 2001; Furushita et al. 2003). *tet* genes confer resistance in two basic ways, by coding for efflux proteins that pump the antibiotic out of the cell and for ribosomal protection proteins (Roberts 1998).

Quinolones

One of the most widely used groups of antibiotics in aquaculture is the quinolones, comprising old 4-quinilones, such as oxolinic acid and nalidixic acid, and newer fluoroquinolones such as enrofloxacin, norfloxacin, florfenicol, and sarafloxacin. Quinolones act by blocking DNA replication and repair and by interacting with the topoisomerases. The resistance occurs by mutations in the genes coding for these topoisomerases (Walsh 2003). Some resistance has been observed among vibrios to the older quinolones and norfloxacin (*Table 36.10*), but, in general, the sensitivity to the fluoroquinolones is high (Morris et al. 1985; Ruangpan et al. 1997; Li-Jun et al. 1999; Bhattacharya et al. 2000; Roque et al. 2001).

Aminoglycosides

Aminoglycoside antibiotics interfere with the protein synthesis at the 16S rRNA, and resistance is acquired if the coupling of the antibiotic and the 16S rRNA is blocked. Their use to control vibrios has also been extensive, and resistance has been observed in Asia (Ruangpan et al. 1997; Inglis et al. 1997), and Latin America (Molina-Aja et al. 2002) (*Table 36.11*).

Table 36.8
Resistance of vibrios to cephalosporin β-lactam antibiotics

Antibiotic	Percentage of resistant isolates (total isolates)	Source	References
Cefuroxime	66.7 (51)	Sea bream (*Sparus sarba*)	Li-Jun et al. (1999)
Cephalothin	36.7 (22)	Penaeid shrimp	Molina-Aja et al. (2002)
	6.0 (36)	Salmon	Alderman and Hastings (1998)
	19.8 (121)	*Artemia* nauplii	Hameed and Balasubramanian (2000)
	50–80	Salmon	Inglis (2000)
	43.8 (98)	Shrimp culture systems	Roque et al. (2001)
	15.5 (197)	Water, sediment and shrimp (*P. monodon*)	Tendencia and De la Peña (2001)

Table 36.9
Resistance of vibrios to tetracyclines

Antibiotic	Percentage of resistant isolates (total isolates)	Source	References
Tetracycline	40 (5)	Bivalve mollusks	Tubiash et al. (1965)
	9.1 (505)	Seawater and seafood (shrimp, clam, and squid)	Molitoris et al. (1985)
	76.2 (84)	Finfish and shellfish	Sanjeev and Stephen (1992)
	12.4 (121)	*Artemia* nauplii	Hameed and Balasubramanian (2000)
Oxytetracycline	82.0 (27)	Seawater and penaeid (*P. monodon*) larvae flounder	Baticados et al. (1990)
	12.5 (8)	(*Paralichthys adspersus*)	Miranda and Rojas (1996)
	45.9 (135)	Shrimp pond sediment	Ruangpan et al. (1997)
	23.8 (231)	Fish, shrimp, and water	Inglis et al. (1997)
	20.0 (22)	Penaeid shrimp	Molina-Aja et al. (2002)

Horizontal Gene Transfer

Horizontal gene transfer may launch environmental strains into new pathogenic lifestyles. Antibiotic multiresistance R conjugative plasmids have been detected in *V. cholerae*, mostly in environmental strains (Amaro et al. 1988). They can be transferred by conjugation to other strains of the same species or even to other genera of bacteria (Kruse and Sorum 1994). There are also relevant reports of outbreaks of cholera with multiresistant strains carrying plasmids (Falbo et al. 1999; Dalsgaard et al. 2000). The SXT element is a conjugative self-transmissible, chromosomally integrated element that encodes resistance to various antibiotics, including streptomycin, chloramphenicol, sulfamethoxazole, and trimethoprim. The 100 kb SXT was initially found in the O139 strains, but now it is also found frequently in many strains of the Indian region (Beaber et al. 2002). SXT may mobilize other plasmids for transfer, as well as the chromosome itself, in a manner similar to an Hfr conjugation (Hochhut et al. 2001a). This mechanism as such is an important agent of horizontal gene transfer. An instigating recent finding is that the SOS response to DNA damage increases the transfer of SXT (Beaber et al. 2004).

Vibrio cholerae can exchange DNA through conjugation and transduction. Transformation of *V. cholerae* is not normally used as a gene transfer tool, as the cells produce nucleases that probably degrade the DNA and hamper transformation (Marcus et al. 1990; Focareta and Manning 1991). Nevertheless, electroporation systems have been developed and are widely used (Hamashima et al. 1995). There was resurgence in the theme of phages and phage-mediated transduction in *V. cholerae*, once it was discovered that the *ctx* genes for the cholera toxin are part of the filamentous lysogenic phage genome CTXΦ (Waldor and Mekalanos 1996). The TCP

Table 36.10
Resistance of vibrios to various antibiotics

Antibiotic	Percentage of resistant isolates (total isolates)	Source	References
Chloramphenicol	0.2 (505)	Seawater and seafood (shrimp, clam, and squid)	Molitoris et al. (1985)
	6.7 (120)	Water, sediment, plankton, fish, and prawn	Pradeep and Lakshmanaperumalsamy (1985)
	15.8 (203)	Penaeid larval culture (*P. indicus*)	Rosily et al. (1987)
	45.5 (27)	Seawater and penaeid (*P. monodon*) larvae	Baticados et al. (1990)
	14.8 (135)	Shrimp pond sediment	Ruangpan et al. (1997)
	5.7 (245)	Fish, shrimp, and water	Inglis et al. (1997)
	2.0 (51)	Sea bream (*Sparus sarba*)	Li-Jun et al. (1999)
	14.0 (121)	*Artemia* nauplii	Hameed and Balasubramanian (2000)
	1.0 (22)	Penaeid shrimp	Molina-Aja et al. (2002)
Oxolinic acid	6.3 (223)	Fish, shrimp, and water	Inglis et al. (1997)
	11.1 (135)	Shrimp pond sediment	Ruangpan et al. (1997)
Nalidixic acid	Majority (226)	ayu (*Plecoglossus altivelis*)	Aoki et al. (1984)
	19.4 (36)	Cockles (*Anadara granosa*)	Radu et al. (1998)
Erythromycin	3.0 (505)	Seawater and seafood (shrimp, clam, and squid)	Molitoris et al. (1985)
	61.1 (36)	Cockles (*Anadara granosa*)	Radu et al. (1998)
	20.3 (133)	Fish, shrimp, and water	Inglis et al. (1997)
	18.2 (121)	*Artemia nauplii*	Hameed and Balasubramanian (2000)

pilus is the receptor for the phage, but recent reports show that other viruses, such as VGJΦ and CP-T1 (Boyd and Waldor 1999; Campos et al. 2003), can transduce CTXΦ with the use of different receptors, as the mannose-sensitive hemagglutinin for VGJΦ. This implies that the TCP pilus is not absolutely required for CTXΦ acquisition. A generalized transduction system is known for *V. cholerae*, with the use of the phage CP-T1 (Ogg et al. 1981; Hava and Camilli 2001; O´Shea and Boyd 2002).

The most used procedure in strain construction for *V. cholerae* is the use of conjugation with R6K-derived plasmids (Miller and Mekalanos 1988). The gene, or region of interest, is cloned into such a plasmid and desired modifications are done in vitro. The resulting clone is maintained in *Escherichia coli* that carries a *pir* gene, allowing for the replication of the plasmid. These plasmids also carry a plasmid RP4-derived mobilization region. They are helped to transfer, by conjugation, with the use of a helper plasmid, generally pRK2013, which expresses RP4 mobilization factors. Some particular strains, like SM10, carry the RP4 mobilization functions in the chromosome, and conjugation can be achieved directly from these strains without a helper plasmid (Simon et al. 1983). The plasmid is then transferred into *V. cholerae*, where it behaves as a suicide plasmid, in the absence of the *pir* gene. Selection for the plasmid marker, usually resistance to an antibiotic, leads to selection of bacteria with an insertion of the plasmid into the chromosome. Homologous recombination may lead to insertion in the corresponding gene to that cloned previously in the plasmid, in a first step of an "allele exchange." A second recombination will conduct to plasmid excision, either leaving in the chromosome the original allele present there or exchanging the allele for the one carried in the incoming plasmid.

Virulence Genes

The main virulence factor in *V. cholerae* is the cholera enterotoxin (CT) (Dutta et al. 1959; Finkelstein 1992). The CT genes (*ctx*) are arranged as an operon present in the CTX Φ bacteriophage genome, which also contains the zot and ace toxin genes (Waldor and Mekalanos 1996). Some virulent strains may carry several copies of the CTX element. Zonula occludens toxin (Zot) alters the permeability of the mucosa of the small intestine by opening intercellular tight junctions (Di Pierro et al. 2001). The third toxin gene in the *V. cholerae* virulence cassette is ace. There are few studies concerning the action of this toxin, but some results indicate that Ace increases transcellular ion transport, which contribute to diarrhea in cholera (Trucksis et al. 1997).

Table 36.11
Resistance of vibrios to aminoglycosides

Antibiotic	Percentage of resistant isolates (total isolates)	Source
Amikacin	16.7 (22)	Penaeid shrimp
Gentamycin	4.3 (505)	Seawater and seafood (shrimp, clam, and squid)
	100? (22)	Manila clam (*Tapes philippinarum*)
Kanamycin	31.7 (505)	Seawater and seafood (shrimp, clam, and squid)
	100 (25)	Seawater and penaeid (*P. monodon*) larvae
	74.1 (135)	Shrimp pond sediment
	8.3 (36)	Cockles (*Anadara granosa*)
Streptomycin	66.9 (505)	Seawater and seafood (shrimp, clam, and squid)
	100 (25)	Seawater and penaeid (*P. monodon*) larvae
	25.0 (4)	Penaeid shrimp (*P. monodon* and *P. japonicus*)
	58.9 (180)	Fish, shrimp, and water
	36.1 (36)	Cockles (*Anadara granosa*)

The cholera toxin CT is a member of a family of A–B toxins, consisting of an active enzymatic subunit A and a receptor-binding subunit B. It is a thermolabile toxin and is similar to the *E. coli* LT. The crystal structure of CT has been determined (Zhang et al. 1995). The CT-A subunit has an MW of 27,500 Da and is composed of two domains, A1 and A2. The A1 subunit will reach the cytosol, being responsible for the intoxication. The B subunits have an MW of 11,800 Da, and they form a pentamer ring (Spangler 1992). As a consequence of the action of CT, adenylate cyclase is activated, leading to fluid loss and diarrhea (de Haan and Hirst 2004).

CT assembly and secretion have been reviewed (Hirst 1991). The A and B subunits are transported separately to the periplasmic space using the Sec-dependent pathway. There they fold and assemble into the holotoxin. The terminal branch of the general secretion pathway is responsible for the transport of the holotoxin through the outer membrane (Sandkvist et al. 1997; Sandkvist et al. 2000).

CT-B subunit is a lectin that binds, with high affinity, to GM1 ganglioside receptors, present in eukaryotic cells such as enterocytes. After binding to GM1, CT is internalized in the polarized intestinal epithelial cells, using endocytic vesicles that are targeted to the trans-Golgi network and the endoplasmic reticulum. CT enters the cells in vesicles carrying the early endosomal marker Rab5 (Sugimoto et al. 2001). From the TGN, CT appears to enter a retrograde pathway to the ER, probably using COPI-coated vesicles (Nambiar et al. 1993; Lencer et al. 1993; Cosson and Letourneur 1997). Once in the ER, the A1 domain is released to the cytosol, after disulfide bond reduction and proteolytic cleavage of the A subunit (Mekalanos et al. 1979; Kassis et al. 1982). The proteolytic cleavage can occur via a Zn metalloprotease (Booth et al. 1984; Naka et al. 1998) or another endogenous protease present in epithelial cell endosomes (Lencer et al. 1997). A1 remains attached to A2 by a single disulfide bond. This disulfide bond is reduced by the protein disulfide isomerase (PDI) (Orlandi 1997; Tsai et al. 2001), before A1 exits the ER, possibly through a channel (Hazes and Read 1997). In the cytosol, A1 has an ADP–ribosyltransferase activity, transferring ADP from NAD resulting in the permanent ribosylation of G proteins (Moss and Vaughan 1977; Cassel and Selinger 1977; Gill and Meren 1978; Gill and Coburn 1987). One particular target is the Gs protein, involved in adenyl cyclase activation (Sharp and Hynie 1971). The ribosylated Gs loses its GTPase activity and continuously activates the basolateral membrane adenyl cyclase, resulting in a high concentration of cAMP, an important cellular messenger (Schafer et al. 1970). This leads to an increase in chloride secretion, inhibition of Na^+ absorption, and osmotic transfer of water to the intestinal lumen, resulting in severe secretory diarrhea (Field 1981; Peterson and Ochoa 1989; Cheng et al. 1991; Sears and Kaper 1996). In some severe cases, the patient can lose 10–20 l of fluid in a day. The isolation of *V. cholerae* strains lacking the CTX phage from patients with cholera-like diarrheal disease and the remaining capacity to cause diarrhea in CT-mutant strains suggest the existence of other virulence factors in *V. cholerae* (Kaper et al. 1995).

An RTX toxin cluster was identified (Lin et al. 1999), which encodes a protein with cytotoxic activity in CT-producing and CT strains. This toxin causes a loss of the barrier function of the paracellular tight junction due to covalent cross-linking of actin monomers leading to depolymerization of actin in El Tor strains (Fullner and Mekalanos 2000). Interestingly, the classical strains present a deletion that overlaps the *rtxA*, *rtxC*, and *rtxB* genes.

It has been shown that *V. cholerae* of both biotypes produce a metalloprotease, hemagglutinin/protease (Hap), encoded by *hapA*, which belongs to the thermolysin family of metalloproteases. Hap has cytotoxic and mucinolytic activity. Hap plays an important role in cholera pathogenesis, facilitating the attachment to the intestinal surface by digestion of the intestinal mucosa (Silva et al. 2003).

A heat-stable toxin (ST) gene homologous to *E. coli* ST gene has been found mainly in *V. cholerae* non-O1 and *V. mimicus* strains (Vicente et al. 1997). This toxin has been shown to produce diarrhea in volunteers (Morris et al. 1990). The ST gene isolated from *V. cholerae* or *V. mimicus* is flanked by a 123 bp repeat denominated VCR and present in many copies in the *V. cholerae* superintegron.

V. cholerae hemolysin HlyA (or VCC cytolysin) is a pore-forming, membrane-damaging toxin, recognized as a virulence factor because of ample evidence for its cytotoxic and enterotoxic activities (McCardell et al. 1985). The purified protein causes fluid accumulation in ligated rabbit ileal loops (Ichinose et al. 1987). It induces lysis of erythrocytes and other mammalian cells. At low concentrations, it has been shown to cause extensive vacuolation of some cell lines and is found in the vacuoles, inside the cell (Coelho et al. 2000; Moschioni et al. 2002). This secreted protein is first synthesized as a preprohemolysin, processed in its transfer to the periplasmic space and finally processed to 65kDa monomers after secretion outside the cell. Monomers oligomerize on target eukaryotic cell membranes, forming anion-selective pores of approximately 1.5 nm. HlyA oligomerizes on lipid vesicles, particularly in the presence of cholesterol and sphingolipids (Zitzer et al. 1999). It has been shown that HlyA oligomerizes on cholesterol microcrystals (Harris et al. 2002), further supporting the role of cholesterol in the interaction of HlyA and membranes. HlyA production is widespread in environmental strains and was one of the phenotypes associated with the early El Tor strains, but not later strains of El Tor, even though these still carry the whole *hlyA* gene (Barrett and Blake 1981). Classical strains have a small internal deletion in this gene. Regulation of the *hlyA* gene deserves study, as the amount of the toxin synthesized during the infection could be relevant as reinforcement of symptoms of the cholera disease.

Colonization

A crucial step for the successful enteropathogen's action is the colonization of the intestinal epithelium. The factors promoting *V. cholerae* adherence to the intestine are not completely understood, but the major role of the toxin-coregulated pilus (TCP) in colonization by classical El Tor and Bengal strains has been recognized. The TCP is a type 4 pilus that is expressed by epidemic strains of *Vibrio cholerae*. The TCP structure is assembled as a polymer of repeating subunits of pilin, the major structural pilus protein encoded by the *tcpA* gene, that form long fibers, which laterally associate into bundles. In vitro and in vivo analyses of the *tcpA* mutants revealed that TCP mediates bacterial interaction through direct pilus–pilus contact required for microcolony formation and productive intestinal colonization (Kirn et al. 2000).

The genes for TCP biosynthesis and assembly are clustered with genes of the *Vibrio cholerae* accessory colonization factor (ACF) forming the VPI (vibrio pathogenicity island) which presents the int gene at the 3′ end. This gene encodes a protein related to the integrase family of site-specific recombinases (Kovach et al. 1996).

The nucleotide sequences of the TCP cluster from El Tor and classical strains show only minor differences corresponding to the major regulatory regions and in *tcpA*. The *tcpA* gene is only 77 % homologous between these two biotypes, with most of the amino acid changes at the C-terminus. These differences are thought to account for the alternate conditions required for expression of TCP by the two biotypes and the antigenic variation and lack of cross-protection (Jonson et al. 1991). The Bengal O139 isolate, which, as several analyses suggest, originated from an El Tor strain, carries *tcpA* with a sequence identical to El Tor isolates.

The presence of VPI has been detected in some non-O1 strains as well as in *V. mimicus*. In some of the isolates, new *tcpA* alleles are present (Novais et al. 1999).

The ToxR-Regulon, Quorum Sensing, and Biofilms

Virulence genes of *V. cholerae* and their regulation started to be studied early on for their medical importance. Regulation has been studied in vitro, in different conditions, and also in animal models. The use of more realistic in vivo models is a necessity and is being dealt with now, with the use of the more recent global techniques, such as RIVET (Camilli and Mekalanos 1995; Lee et al. 1999) and microarray analysis.

One major regulon, the ToxR regulon, has been described in this species and has been shown to be a prototype of regulons present in other *Vibrio* species. The ToxR–ToxS and TcpP–TcpQ proteins are outer membrane proteins, interacting with the environment on the outside of the cell, and with the DNA and other proteins in the inside. They positively regulate a number of important genes, several of them involved with virulence. The *toxT* gene is a key element of the circuit, as ToxT itself is a regulator of many other genes. The ToxR regulon is thus considered to have two branches, one for genes regulated by the primary regulators and the other including genes regulated by ToxT (Champion et al. 1997). Positive regulation by ToxT affects major virulence genes such as the *ctx* genes that are responsible for the synthesis of CT and *tcpA*, the pilin gene for the TCP pilus.

A noticeable aspect of the ToxR regulon is the interconnection of various regulatory factors into a complex network of interaction and expression of various genes. An early estimate suggested that about a dozen genes were regulated by the ToxR regulon. A more recent analysis with the use of microarrays extends this number to approximately 50, with various positively and negatively regulated genes (Bina et al. 2003). Moreover, an increasing number of general or specific regulators of this regulon are described, including HNS, cAMP/CRP, AphaA, AphaB, and HapR.

Genetic studies in many bacteria have turned their focus also to interactions between bacteria and their collective behavior. In some cases, the sheer number of the cells is capable of effects that a single cell would not be. The topics of quorum sensing, biofilm development, and virulence all fit into a description of collective behavior. In fact, as more regulators are studied, it becomes clear that all these processes are interconnected. In *V. cholerae*, quorum sensing regulates biofilm production and negatively

regulates the expression of virulence genes of the ToxR regulon. Virulence gene regulation is achieved through HapR (hemagglutinin/protease regulatory protein), which represses tcpP.

Quorum sensing is the ability of cells to communicate by signaling molecules (pheromones or autoinducers), responding to the actual population density. *V. cholerae* cells react to quorum sensing with a variety of regulatory responses. A parallel has been drawn between the two-channel quorum sensing system of *V. harveyi* and that of *V. cholerae*. In *V. harveyi*, the existence of two different autoinducers is well established. Autoinducers are small molecules used for chemical signaling between cells. At low cell density, in *V. harveyi*, the sensors Lux N (system 1) and LuxQ (system 2) act as kinases and lead to a phosphorylation cascade that results in the activation of *luxO* and repression of the light genes from the luciferase structural operon *luxCDABE*. At high cell density, LuxN and LuxQ/LuxP interact with their cognate inducers and act as phosphatases, leading to LuxO inactivation and light production. In the case of *V. cholerae*, a system similar to the LuxQ/LuxP system 2 has been detected by genome comparison. Recently, a system based on the *cqsS*/*cqsA* genes has been reported, as the system corresponding to system 1 of *V. harveyi*, but with a different autoinducer. Both of these systems lead to the activation/inactivation of LuxO in conditions of low/high numbers of cells. When LuxO is active, HapR is not expressed, and when LuxO is inactive, HapR is expressed (Miller et al. 2002). HapR is a repressor of virulence genes, so it is expected that at high cell densities, the virulence genes are not expressed. Repressed HapR results in the production of the virulence regulator TcpP. A third regulatory system was proposed for *V. cholerae* (Miller et al. 2002), acting directly on LuxO, so *V. cholerae* has a complex regulatory network regulation of HapR.

In aquatic environments, many bacteria do not stay in a planktonic form as single cells. Instead, they form biofilms on biotic and abiotic surfaces. *V. cholerae* has been isolated from zooplankton, insect eggs, plants, filamentous green algae, and crustaceans (Tamplin et al. 1990; Halpern et al. 2004). *V. cholerae* is known to survive on the degradation of chitin (Nalin et al. 1979). It was natural to investigate biofilm development by this species. *V. cholerae* forms biofilms, with cell pillars and water channels to bring nutrients in and take away waste products of the cells. A group of vps genes is responsible for exopolysaccharide (EPS or VPS) production. Biofilm formation occurs in two stages, the first one during the first 4 h or 5 h of initial cell contact to the surface and then an active phase of EPS expression, in which the biofilm characteristic structures are formed. Motility is important for this phenotype, as is the production of the MSHA (mannose-sensitive hemagglutinin type IV pilus). Quorum sensing activates biofilm formation (Zhu et al. 2002). Other environmental regulators of biofilm formation are surface contact and the nutrient composition, with glucose and other carbohydrates increasing biofilm deposition. Important regulators of biofilm development have been recently described, with VpsR increasing expression of vps genes (Yildiz et al. 2001) and CytR regulating negatively their expression (Haugo and Watnick 2002). The rugose variant of *V. cholerae* produces an increased amount of EPS and thicker biofilms.

VSP-1 Function

Seventh pandemic El Tor strains are genetically distinguishable from classical strains by the presence of two genomic islands, Vibrio 7, the pandemic island-1 (VSP-1) and VSP-2. Recently, the function of the VSP-1 in cholera pathogenesis has been elucidated using chromatin immunoprecipitation sequencing (ChIP-Seq) and RNA sequencing (RNA-Seq) in El Tor C6706 (1991-Peru isolate) (Davies et al. 2012). All six ToxT-ChIP peaks are concentrated on the TCP island and CTX prophage. One of this, a newly found ToxT-ChIP peak, is identified as ToxT-activated sRNA B (TarB). Comparison of the expression between wild-type and Δ*tarB* mutants identifies that one of the most significantly affected target gene is VC0177, which is on the VSP-1 island. Based on protein structure prediction, transposon experiments, and ChIP, VC0177 has a role as transcriptional repressor of four genes (VC0176, VC0178, VC0179, and VC0180) on VSP-1 island, finally named the gene product as the *V. cholerae* 7th pandemic regulator (VspR). The TarB-VspR regulatory circuit is apparently involved in *V. cholera* pathogenesis using *V. cholerae* intestinal colonization experiment of the infant mouse model; in more detail, the target gene is VC0179 for the intestinal colonization defect. VC0179 product shows dinucleotide cyclase activity to generate preferentially c-di-AMP rather than c-di-GMP from homogeneous nucleotide substrate, more surprisingly to be capable of generating hybrid c-AMP-GMP from mixed nucleotides substrates. VC0179 is finally designated as a novel class of dinucleotide cyclase (DncV/dinucleotide cyclase Vibrio). The c-AMP-GMP is a dominant product in vivo. DncV expression is significantly affected on downregulations of chemotactic genes as well as efficient intestinal colonization of the infant mouse. As these results, VSP-1 is defined as a pathogenicity island in *V. cholera* to have the roles in benefit for host adaptation through the production of a regulatory cyclic dinucleotide.

Secretion and Ion Motive Force

V. cholerae secretes various proteins, including many of the known virulence factors. The general secretion pathway (GSP) based on the Sec proteins (Driessen et al. 1998; de Keyzer et al. 2003) is used for the transfer from the cytoplasm to the periplasmic space. Type II secretion mechanisms, or the main terminal branch of the GSP, are used in various Proteobacteria to secrete proteins from the periplasm to the outside medium. In *V. cholerae*, a complete type II secretion system has been described, with 14 eps (extracellular protein secretion) genes and VcpD/PilD (Russel 1998; Sandkvist 2001; Robien et al. 2003). Twelve of these genes are arranged as a cluster.

The proteins assemble as a structure spanning the cell envelope, with proteins located in the outer membrane, others in the periplasmic space, others in the cytoplasmic membrane, and some in the cytoplasm, attached to the internal part of the membrane. An EpsD oligomer is supposed to be a pore in the outer membrane, through which folded proteins can pass. The whole cholera toxin (subunit A and five subunits B) is secreted in this way (Hirst and Holmgren 1987). It was found that the EpsD protein is additionally used in phage CTXΦ extrusion of the cell (Davis et al. 2000). One particularly interesting finding is the polar localization of the secretion proteins, in the old pole of a dividing cell (Scott et al. 2001). This could be a mechanism of concentrated delivery at one point. A second system for traversing the outer membrane is the type IV pilus biogenesis system. It has been looked at, with a focus on resemblances to the type II secretion system. The TCP pilus of *V. cholerae* is a type IV pilus, forming a bundle of pili outside the cell (Taylor 1991). Several of the type II proteins are similar to proteins required for the type IV pilus biogenesis, and the TcpF protein, a soluble colonization factor, is secreted to the outside of the cell using the TCP system (Kirn et al. 2003).

V. cholerae utilizes both the H^+ and the Na^+ cycles of energy, establishing a sodium motive force (smf) as well as a proton motive force (pmf) (Hase and Barquera 2001). Examination of the energy requirements for the type II secretion mechanism of the Hap protease indicated that an smf is required, as well as a pmf, for the translocation of the membrane (Hase 2003). The polar flagellum of *V. cholerae* has been shown to be driven by the electrochemical gradient of Na^+ (Kojima et al. 1999b), contrary to what happens in *E. coli*, in which the pmf is used. On the other hand, the F1FO-type ATPase of *V. cholerae* transports protons, not Na^+ ions (Dzioba et al. 2003), emphasizing the importance of both energy cycles for this species.

Polar Flagella, Lateral Flagella, and Chemotaxis

Many *Vibrio* species have a single polar flagellum, sheathed by a membrane, continuous to the cell outer membrane. This flagellum propels the bacterium in liquid medium. Energy for flagellum movement comes from sodium motive force. The polar flagellum of *V. parahaemolyticus*, in particular, is being intensively studied (McCarter 1995; Kim and McCarter 2000). The flagellum is arranged with flagellin subunits in a helix forming a hollow tube (Namba and Vomierviszt 1997). In *V. parahaemolyticus*, there are six flagellins, all with some degree of similarity to one another. Five of these flagellins require sigma 28 for expression. The flagellin genes are disposed in two chromosomal locations, as is the case for *V. cholerae*. The flagellum is linked to the membrane at the basal body, consisting of several membrane rings (Ferris et al. 1984; Engelhardt et al. 1993). A flexible hook links the flagellum to the basal body or motor, embedded in the cytoplasmic membrane. The *V. parahaemolyticus* polar flagellum system involves at least 60 genes (Kim and McCarter 2000), disposed in five chromosomal regions, and is very similar to the flagellum system of *V. cholerae*, even though in *V. cholerae*, there is an insertion carrying non-flagellar genes within the region (Heidelberg et al. 2000). The polar flagellar system is continually expressed but, with all these genes, is well regulated, and with a chain of regulators, allowing or not the expression of downstream genes. The regulation in *V. parahaemolyticus* and *V. cholerae* is very similar. Three regulatory levels are recognized as early, middle, and late genes. The early master genes, potentially interacting with sigma 54, are *flaK* and *flaM* (*flrA* and *flrC*, respectively, in *V. cholerae*). The middle genes are sigma54 dependent and the late genes sigma 28 dependent. Sigma 28 is made from one of the middle genes. The sodium-driven motor is dependent on the expression of four genes, *motA*, *mot*B, *motX*, and *motY*. MotA and MotB interact, catalyze the Na^+ flux, and can be isolated as a complex. The role of *motX* and *motY* is still not defined, but mutants in these genes are nonmotile.

In addition to this polar flagellum, some species, such as *V. parahaemolyticus* and *V. alginolyticus*, present a phenotype of many unsheathed lateral flagella when plated on agar or on other solid surfaces (Allen and Baumann 1971). This allows for swarming of the cells on agar plates (Shinoda and Okamoto 1977). In contrast to the polar flagellum, these peritrichous flagella are driven by proton motive force. It has been shown that the impairment of the free rotation of the main flagellum and increase in viscosity are factors in the induction of the lateral flagella (McCarter and Silverman 1990). The two flagellar systems appear to be separate. The lateral flagella laf genes are organized in two separate regions of the chromosome, with a total of 38 genes, leading to the production of the LafA flagellin, and positively regulated by the Laf K gene. Swarming and laf gene expression are σ54 dependent, as is the case for *V. cholerae* (Klose and Mekalanos 1998; Stewart and McCarter 2003). In addition to sigma 54, some laf genes such as *lafA* need an additional sigma factor, a sigma 28, encoded in one of the *laf* regions.

The presence of two flagellum systems allows locomotion in different conditions and may reflect the habitats of *V. parahaemolyticus* as free-living cells in planktonic environments or on surfaces or in biofilms. Flagella also play important roles in cell attachment and biofilm formation.

There is an interesting connection between the flagellar systems and chemotaxis, the capacity to swim in the direction of an attractant. Some important chemotaxis genes, such as *cheA* and *cheB*, are present in polar flagellar operons. Mutations in these two genes affect not only the swimming movement, effected by the polar flagellum, but also the motility on agar plates by the lateral flagella (Sar et al. 1990). Chemotaxis will be an important topic for Vibrio, as many more genes seem to be involved for these species than in *E. coli*. In fact 43 methyl-accepting chemotaxis genes have been described for *V. cholerae* (Heidelberg et al. 2000), in contrast to 5 in *E. coli*.

Stress and Stress Response

The survival of vibrio in different environments is followed by responses to specific conditions found. This can be different energy sources, a change in pH, and a temperature variation among many other possibilities, including starvation and oxidative stress.

Sigma S is the product of the *rpoS* gene and is a regulator for many stationary phase expressed genes. In addition to this role in stationary phase gene expression, RpoS is also important in stress response and pathogenesis of many bacteria (Hengge-Aronis 2000). In *V. cholerae*, *rpoS* is involved in stress response and host colonization (Yildiz and Schoolnik 1998; Merrell et al. 2000). An *rpoS V. vulnificus* mutant had a decreased ability to survive when exposed to many different stresses such as exposure to hydrogen peroxide, hyperosmolarity, and acidic conditions (Hülsmann et al. 2003). *rpoS* is important for adaptation to environmental changes and may have a role in virulence. Several exoenzymatic activities were not present in the mutant. Albuminase, caseinase, and elastase were absent in the mutant, and collagenase and gelatinase were reduced. In addition to the effect in macromolecule-degrading enzymes, motility of the *rpoS* mutant was severely decreased. In another *rpoS* mutant, there was impairment to survival under oxidative stress, nutrient starvation, UV irradiation, and acidic conditions (Park et al. 2004).

These results confirm a role for *rpoS* in survival in stressful conditions that could be correlated with the various niches for *V. vulnificus*, including seawater, oyster guts, eels, and humans.

Changes in the external acidity are one of the most common environmental stresses that bacteria encounter. Acid conditions usually found inside humans and also in the presence of food addictives added to raw seafood. A *cadBA* operon has been described in *V. vulnificus*, with a role in acid tolerance. The protein encoded by *cadB* is proposed to be a lysine/cadaverine antiporter and that of *cadA* is a lysine decarboxylase (Rhee et al. 2002). The excretion of cadaverine results from the combined action of lysine decarboxylase and cadaverine/lysine antiporter, and this is a system for neutralization of low extracellular pH.

Global Gene Expression Studies

The development of tools to study many genes at the same time was an important goal for integrated studies of gene expression. Studies with the RIVET system of *V. cholerae*, in which gene expression leads to recombination of a tetracycline gene cassette, allowed in vivo studies aiming at a description of virulence gene expression (Lee et al. 2001).

Gene expression is being addressed at a global level now with the use of microarrays in *V. cholerae* (ScottMerrell et al. 2002). They described two "expression stages" of the bacteria, with one of these occurring after human infection. mRNA was extracted directly from stools of cholerae patients. Another study used a rabbit "in vivo" model for comparative analysis of gene expression of El Tor strain N16961 "in vivo" and in LB culture medium (Xu et al. 2003). The metabolic differences found indicate that the cells are subject to iron limitation, anaerobiosis, and nutrient limitation in the rabbit upper intestine. Several genes from CHr. 2 were induced in "in vivo" conditions. Applied microarrays for an extensive description of the ToxR regulon (Bina et al. 2003) have been done.

The counterpart to mRNA studies is the proteome study, with a view of the proteins present at different growth conditions. Abundant proteins from *V. cholerae* have been used in strain characterization in MLEE studies (Salles and Momen 1991). Gene regulation studies at the protein level started with the heat shock response (Sahu et al. 1994) and the ToxR regulon. Convalescent human antisera have been recently employed to detect proteins expressed during the disease (Hang et al. 2003). Protein analysis has been recently described for a classical strain, comparing mild acid and neutral pH conditions (Hommais et al. 2002). A more extensive proteome analysis of the El Tor strain N16961 is now available (Coelho et al. 2004). These are first steps towards a comprehensive gene expression analysis of *V. cholerae* under different conditions.

Application

Probiotics

The term probiotic has been defined as "a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance" (Fuller 1992). Many groups of bacteria have been used as probiotics in human and farm animals, but most of the research has been focused mainly on *Lactobacillus* spp., *Bifidobacterium* spp., and in a lesser degree on *Streptococcus salivarius*, *Enterococcus faecium*, *Lactococcus lactis*, *Leuconostoc* sp., *Pediococcus*, and *Bacillus* sp. (Gomez-Gil et al. 1998b).

Potential probiotic bacteria have been used to combat vibrios that affect marine cultured organisms and also vibrios as probionts against other pathogenic vibrios (Gomez-Gil et al. 2000; Hjelm Mette et al. 2004). Only vibrios that have probiotic characteristics will be discussed in more detail beneath (*Table 36.12*).

Vibrio alginolyticus

V. alginolyticus is probably the species most studied as a potential probiont. This organism has been found as the dominant species in healthy cultures of rotifers and turbot (*Scophthalmus maximus*) larvae (Gatesoupe 1990) and as a growth promoter of rotifers (*Brachionus plicatilis*) (Bogaert et al. 1993). *V. alginolyticus* has been found to be more abundant in the intestine of healthy fish larvae than in those where mortality outbreaks have been observed (Tanasomwang and Muroga 1988;

Table 36.12
Potential probiotic *Vibrio* spp.

Probiotic species	Host	Pathogen
V. alginolyticus	Shrimp (*Penaeus monodon*)	*Aeromonas salmonicida*, *V. anguillarum*, and *V. ordalii*
	Pacific white shrimp (*Litopenaeus vannamei*)	*V. parahaemolyticus*
	Brine shrimp (*Artemia* spp.)	*V. parahaemolyticus*
	Brine shrimp (*Artemia* spp.)	*V. proteolyticus*
V. alginolyticus-like	Turbot larvae (*Scophthalmus maximus*)	*V. splendidus*
V. pelagius	Turbot larvae (*Scophthalmus maximus*)	*A. caviae*
Vibrio sp.	Chilean scallop (*Argopecten purpuratus*)	*V. anguillarum*-related

Verdonck et al. 1997; Grisez et al. 1997), suggesting that this species protects fish larvae against the colonization of potential pathogens. The application of a strain identified as *V. alginolyticus* to Atlantic salmon (*Salmo salar*) 7 days prior to the addition of pathogenic bacteria reduced the mortalities to up to 100 % when the fish were challenged with *Aeromonas salmonicida*, *V. anguillarum*, and *V. ordalii* (Austin et al. 1995). A strain of *V. alginolyticus*-like, introduced via the rotifer (*Brachionus plicatilis*) into turbot (*Scophthalmus maximus*) larvae, reduced the mortality of experimentally infected fish to up to 80 %, (Gatesoupe 1997). *V. alginolyticus* has been employed in the culture of Pacific white shrimp (*Litopenaeus vannamei*) larvae in Ecuador, but no challenge tests against a pathogen were carried out so far. Improvements in survival were evaluated (Garriques and Arevalo 1995), but no experimental design and statistical analyses were performed, making it difficult to evaluate the validity of this work. *L. vannamei* exposed to a probiotic strain were observed more active and larger than those not exposed (Garriques and Wyban 1993), and juveniles coming from the probiotic treatment had higher survival in the nursery systems. This strain was capable of out-competing a luminescent *Vibrio* sp. The introduction of probiotics in penaeid shrimp hatcheries in Ecuador in 1992 permitted an increment of production volumes by 35 % and a very significant reduction of antimicrobials (Daniels 1993). No hard scientific data is available to support all these claims.

A strain identified as *V. alginolyticus* (C14) was tested as a potential probiont for *Artemia* spp. (Gomez-Gil 1998). A higher survival was observed when the nauplii were exposed to the probiont prior to the challenge with the pathogen *V. parahaemolyticus* HL58 as compared with nauplii exposed only to the pathogen. In another study, two strains presumptively identified as *V. alginolyticus* were tested as probionts against the pathogen *V. proteolyticus* CW8T2 in *Artemia* spp. (Verschuere et al. 2000). The probiotic strain LVS8 totally protected the nauplii even after 4 days of the infection. Only 23 % of the nauplii treated with the probiont died, compared to 93 % of the untreated nauplii after 48 h postinfection. The growth of the pathogen was slowed down in the culture medium in the presence of the probiotic strain. Sterile filtrates of the probiont did not protect the nauplii, suggesting that the live bacteria, and not extracellular compounds, are involved in the protective mechanism.

Vibrio pelagius

A strain of *Vibrio pelagius* was tested as a probiont for turbot larvae. According to the authors, this strain colonized the gut of the fish larvae and prevented mortalities caused by a strain of *Aeromonas caviae* (Ringo and Vadstein 1998). On the other hand, Gatesoupe (Gatesoupe 1999) reported that six *V. pelagius* strains were pathogenic for turbot larvae. It is obvious that the same *Vibrio* species may harbor harmless, probiotic, and pathogenic strains.

Other Vibrios

Vibrio mediterranei Q40 (Huys et al. 2001) inoculated into 1 l glass beaker containing turbot larvae had a distinctive positive and reproducible effect on survival compared to controls where no bacteria were added. Since no challenge test was done with a pathogenic strain, it can only be speculated that strain Q40 could play a role as first colonizer of the gut and consequently protect the larvae from harmful bacteria. Other unidentified *Vibrio* species (strain 11) isolated from microalgae have been proven to protect to veliger larvae of the Chilean scallop *Argopecten purpuratus* when the mollusc is challenged with a pathogenic *Vibrio anguillarum*-related (VAR) strain (Riquelme et al. 1997). The larvae were "preconditioned" with the probiotic strain 1 h prior to the inoculation with the VAR strain; after 24 h, the percent mortality was 2.7; in the control (not preconditioned) mortality was 89.5 %. In another study (Riquelme et al. 2000), it was shown that *A. purpuratus* could efficiently ingest strain 11 in 6 h when exposed at a density of 10^6 cell ml^{-1}.

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37 The Unclassified Genera of Gammaproteobacteria: *Alkalimonas*, *Arenicella*, *Chromatocurvus*, *Congregibacter*, *Gallaecimonas*, *Halioglobus*, *Marinicella*, *Methylohalomonas*, *Methylonatrum*, *Orbus*, *Plasticicumulans*, *Porticoccus*, *Sedimenticola*, *Simiduia*, *Solimonas*

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Abstract
This chapter compiles 15 recently described genera placed in Gammaproteobacteria according to 16S rRNA sequences that cannot be placed in any known order and family of this class. These genera comprise strains isolated from diverse environments, with wide range of phenotypic and physiological properties. We present detailed information about *Alkalimonas*, *Arenicella*, *Chromatocurvus*, *Congregibacter*, *Gallaecimonas*, *Halioglobus*, *Marinicella*, *Methylohalomonas*, *Methylonatrum*, *Orbus*, *Plasticicumulans*, *Porticoccus*, *Sedimenticola*, *Simiduia*, and *Solimonas*.

Taxonomy: Historical and Current

According to *Bergey's Manual of Systematic Bacteriology*, the class Gammaproteobacteria has 165 genera accommodated in 26

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families and 14 orders (Garrity et al. 2005). Gammaproteobacteria is defined as the class of bacteria having 16S rRNA gene sequences related to those of the members of the order Pseudomonadales, which is the type order of the class (Garrity et al. 2005).

Recently, 15 new genera that cannot be included in any known family and order were placed in Gammaproteobacteria according to the 16S rRNA sequence similarity (Fig. 37.1). These are the unclassified genera of Gammaproteobacteria that will be presented in detail in this chapter. The genera are *Alkalimonas*, *Arenicella*, *Chromatocurvus*, *Congregibacter*, *Gallaecimonas*, *Halioglobus*, *Marinicella*, *Methylohalomonas*, *Methylonatrum*, *Orbus*, *Plasticicumulans*, *Porticoccus*, *Sedimenticola*, *Simiduia*, and *Solimonas*.

Among the unclassified genera of Gammaproteobacteria, only a genus had its name corrected and two species were relocated in a new genus. The genus *Chromatocurvus* was first named *Chromocurvus* (Csotonyi et al. 2011). However, the validation list n° 145 reports the correction according to Rule 61 (Euzéby 2012). The species *Solimonas flava* and *Solimonas variicoloris* firstly named *Sinobacter flavus* Zhou et al. 2008 and *Singularimonas variicoloris* Friedrich and Lipski 2008 were relocated in genus *Solimonas* (Sheu et al. 2011). The combined analysis of phylogenetic data, physiological and biochemical characteristics and the similarities in fatty acid contents, polar lipid profiles, respiratory quinones, and DNA G+C contents of strains supported the transfer of *Sinobacter flavus* and *Singularimonas variicoloris* to the genus *Solimonas* as *Solimonas flava* comb. nov. and *Solimonas variicoloris* comb. nov.

Phylogenetic Structure of Unclassified Gammaproteobacteria and Its Genera

The genera *Alkalimonas*, *Arenicella*, *Chromatocurvus*, *Congregibacter*, *Gallaecimonas*, *Halioglobus*, *Marinicella*, *Methylohalomonas*, *Methylonatrum*, *Orbus*, *Plasticicumulans*, *Porticoccus*, *Sedimenticola*, *Simiduia*, and *Solimonas* are related with other Gammaproteobacteria genera. However, the 16S rRNA sequences place these strains as independent, deep phylogenetic lineages (Fig. 37.1).

According to phylogenetic tree of 16S rRNA sequences, *Congregibacter litoralis* belongs to a branch comprising the recently described species *Haliea salexigens* and *Spongiibacter marinus* as well as sequences retrieved from uncultured marine bacteria (Spring et al. 2009). The closest relatives of *Halioglobus* spp. are *Pseudohaliea rubra* and *Congregibacter litoralis* (Park et al. 2012). Marinicella represents a separate branch within the Gammaproteobacteria, related to the genera *Reinekea*, *Kangiella*, *Spongiibacter*, *Alcanivorax*, and *Microbulbifer* (Romanenko et al. 2010b) and the genus *Porticoccus* forms an independent lineage which has the Gammaproteobacteria genus *Microbulbifer* as the closest classified relative (Oh et al. 2010).

Phenotypic Analyses

A variety of phenotypic and physiological properties and metabolisms will be found among the unclassified Gammaproteobacteria genera. It reflects the diverse origin of the microbial samples that includes costal marine environments to alkaline lakes, passing by gut of butterfly and boar, terrestrial soil, and bioreactors. Table 37.1 shows an overview of phenotypic characteristics that differentiate the unclassified genera of Gammaproteobacteria. Detailed information of each genus is further given.

The Genus *Alkalimonas* Ma et al. 2007

Alkalimonas (Al.ka.li.mo' nas. N.L. n. alkali from Arabic *al-qaliy* ashes of saltwort, Gr. fem. n. monas a unit, monad, N.L. fem. n. *Alkalimonas*, alkaline monad).

The members of the genus are Gram-negative, strictly aerobic, chemoorganotrophic rods, mesophilic, alkaliphilic, and slightly halophilic. Colonies are smooth, circular, and convex, with color varying from creamy white to pale brown (Kurata et al. 2007a; Ma et al. 2004). They are motile by means of a single polar flagellum and no endospores are formed. Na^+ is required for growth. Growth occurs at salinity of 0–10 % (w/v) NaCl and temperatures of 5–42 °C, with an optimum of around 33–37 °C. *Alkalimonas* species do not accumulate poly-β-hydroxybutyrate as an intracellular reserve product. Oxidase and catalase production are positive. Urease, indole, and Voges–Proskauer tests are negative. Amylase, lipase, gelatinase, protease, and collagenolytic activities are variable and negative for agarase and DNase. *Alkalimonas* utilizes glucose, mannose, maltose, and cellobiose, but acid production from substrates is variable. Reduction of nitrate to nitrite and formation of H_2S is also variable; hydrolyzes casein, gelatin, starch, and Tween 80. Tests were negative for formation of indole. Major quinone is Q8. The predominant cellular fatty acids are $C_{16:0}$, $C_{16:1}\omega7c$, and $C_{18:1}\omega7c$. Major polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylglycerol phosphate, and phosphatidylethanolamine. DNA G+C content is 49.3–55.4 mol%. The cells are susceptible to (μg per disc) streptomycin (10), erythromycin (15), and chloramphenicol (30) (Euzéby 2007; Kurata et al. 2007a; Ma et al. 2004).

The type species is *Alkalimonas amylolytica* (strain $N10^T$, which has been deposited as AS 1.3430 in the China General Microbiological Culture Collection Center), isolated from soda Lake Chahannor, China (Ma et al. 2004).

Remarks

Since the species of the genus present many differences, some detailed information about each species is presented in Table 37.2 and an electron micrograph of the type strain is shown in Fig. 37.2.

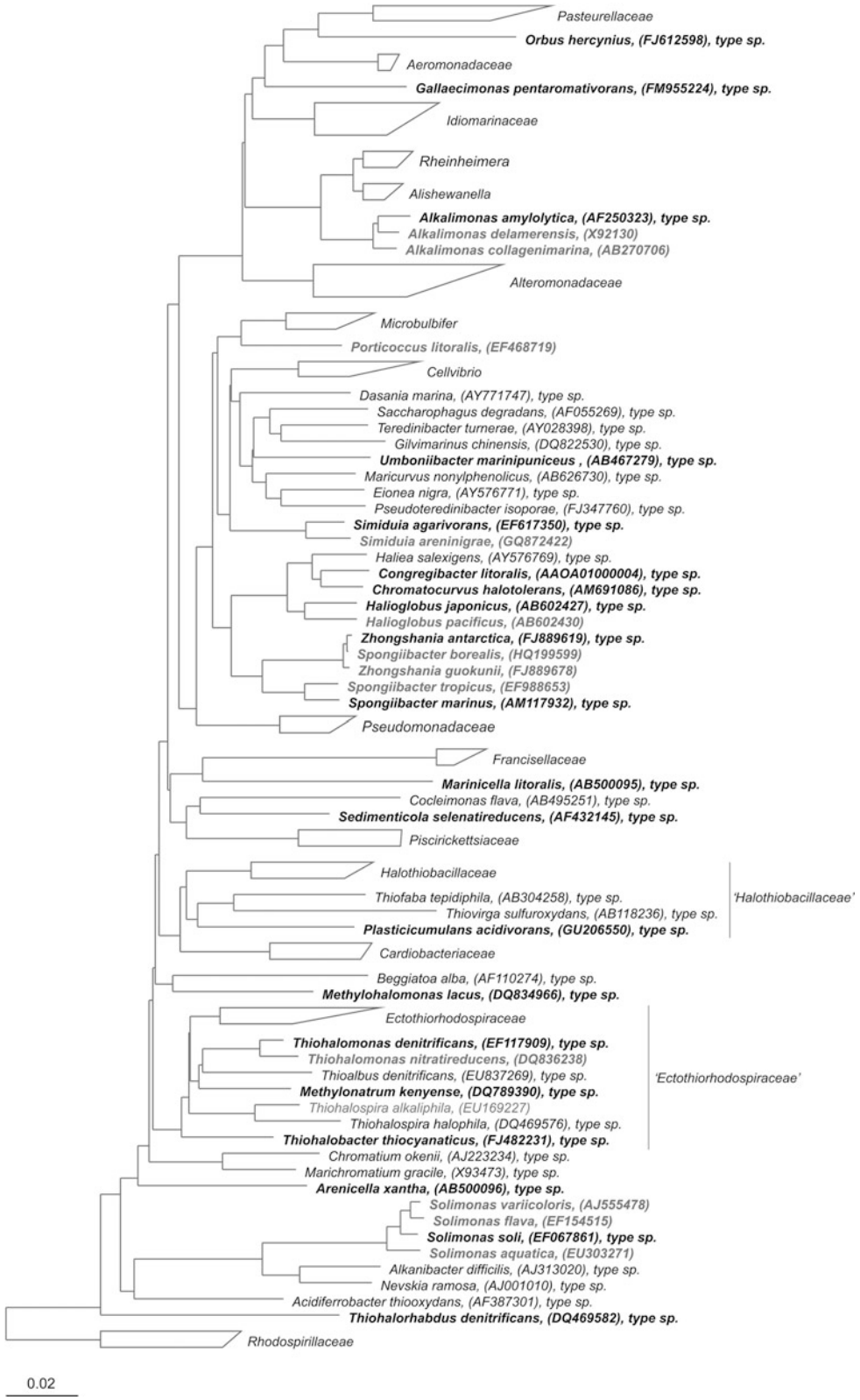

Fig. 37.1

Phylogenetic position of unclassified genera of Gammaproteobacteria based on 16S rRNA sequences. The sequence dataset and alignment were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree)

Table 37.1
Comparison among 15 unclassified genera of Gammaproteobacteria

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Cell length (μm)	1.1–4.0	2.1–4.0	1.5–3.0	0.5–4.5	1.4–4.1	–	1.8–4.5	1.0–3.0	1.0–1.2	1.0–1.5	3.0–5.0	0.4–0.9	1.5	1.4–5.0	0.3–2.6
Cell diameter (μm)	0.5–0.8	0.5–0.6	0.7	0.4–0.7	0.3–0.7	0.3–0.5	0.4–0.6	0.5–0.6	0.5–0.7	0.5–1.0	1.5–5.0	~0.6	0.5	0.4–0.7	0.2–0.8
Strictly aerobic	+	+	+	+	ND	+	–	+	+	–	+	+	–	–	V
Motility	+	–	+	+	+	–	–	–	–	+	–	–	–	V	V
Na+ required for growth	+	+	+	+	–	+	+	+	+	–	–	+	+	+	V
Growth in 20 % (w/v) NaCl	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–
Growth at 4 °C	–	+	–	–	–	–	–	ND	ND	+	–	–	ND	–	–
Growth at 37 °C	+	+	+	–	+	+	–	ND	ND	+	V	+	ND	+	V
Optimal temperature for growth (°C)	33–37	25–28	37	28	25–30	20–25	25–28	ND	ND	20–25	30–40	20–25	ND	25–35	25–30
Optimal pH for growth	8.5–10.5	8–9.5	7	7.5–8	6–8	7–8	8.5–9.5	7.5	10	7–8	6–8	7–8	ND	7–9	6–7.5
Utilization of glucose	+	+	–	–	+	+	–	–	–	+	V	+	ND	V	V
Acid from glucose	V	–	–	–	–	+	–	–	–	+	–	–	ND	–	V
Gelatin hydrolysis	+	+	–	+	+	+	+	ND	ND	–	–	–	ND	V	V
Starch hydrolysis	+	+	–	+	ND	–	–	ND	ND	ND	ND	ND	ND	+	V
Nitrate reduction	V	–	–	–	–	+	–	ND	ND	+	+	–	ND	+	–
Quinone	Q8	Q8	Q8	Q8	ND	Q8	Q8	ND	ND	Q8	Q8		ND	Q8	Q8
Major fatty acids	$C_{18:1}\omega$ 7c	$C_{16:1}\omega$7c	$C_{16:0}$	$C_{16:0}$	$C_{16:0}$	$C_{16:1}$ ω7c	iso-$C_{15:0}$	$C_{16:0}$	$C_{18:1}\omega$7,	$C_{18:1}\omega$7c	$C_{16:1}\omega$7c	ai-$C_{15:0}$	$C_{16:0}$	$C_{16:1}\omega$7c	$C_{18:1}\omega$7c
	$C_{16:0}$	iso-$C_{16:0}$	$C_{16:1}$	$C_{16:1}$	$C_{16:1}$ ω7c	$C_{18:1}$ ω7c	$C_{16:0}$	cyc $C_{17:0}$	$C_{16:0}$	$C_{16:0}$	$C_{16:0}$	ai-$C_{17:0}$	$C_{18:0}$	iso-$C_{15:0}$ 2-OH	$C_{16:0}$
	$C_{16:1}\omega$ 7c	iso-$C_{18:0}$	$C_{18:1}$	$C_{18:1}$	$C_{18:1}$ ω7c	$C_{17:1}$ ω8c	$C_{16:1}$ ω7c				$C_{18:1}\omega$7c		$C_{16:1}\omega$7c	$C_{17:1}$ ω8c	
		$C_{18:1}\omega$7c											$C_{18:1}\omega$7c		
G+C mol%	49.3–55.4	46.3–48.1	63.0	57.8	61.1–61.3	59.4–59.6	43.8	59.6	62.9	32.1–36.4	67.4–68.5	47.8	57	53.3–55.6	64.9–68.4

1 = *Alkalimonas*, 2 = *Arenicella*, 3 = *Chomatocurvus*, 4 = *Congregibacter*, 5 = *Gallaecimonas*, 6 = *Halioglobus*, 7 = *Marinicella*, 8 = *Methylohalomonas*, 9 = *Methylonatrum*, 10 = *Orbus*, 11 = *Plasticicumulans*, 12 = *Porticoccus*, 13 = *Sedimenticola*, 14 = *Simiduia*, 15 = *Solimonas*. + Positive, – Negative or absent, *V* variable between species, *ND* not determined

Table 37.2
Phenotypic characteristics of *Alkalimonas* species

	Alkalimonas amylolytica	*Alkalimonas delamerensis*	*Alkalimonas collagenimarina*
	Ma et al. 2007	Ma et al. 2007	Kurata et al. 2007
Type strain	N10^T = AS 1.3430	1E1$^{P, T}$ = CBS 391.94	AC40^T (=JCM14267^T = NCIMB 14266^T).
GenBank 16SrRNA accession numbers	AF250323	X92130	AB270706
Locality of origin	Lake Chahannor (39° 14′ N and 108° 04′ E), Inner Mongolia, China	Lake Elmenteita (0° 25′ S and 36° 15′ E), Kenya, East Africa	Deep-sea sediment, (30° 55.050′ N and 141° 48.980′ E), Torishima Island, Japan
Cell length (μm)	2.0–4.0	1.7–3.3	1.1–2.2
Cell diameter (μm)	0.5–0.7	0.5–0.7	0.6–0.8
Temperature °C for growth – range/ optimum	10–42/37	10–42/37	5–37/33
pH for growth – range/ optimum	7.5–11/10	8–11/10–10.5	7–10.5/8.5–10
NaCl (%, w/v) for growth – range/optimum	0–7/2–3	0–7/3	0–10/1
Reduction of nitrate	+	+	–
Production of H_2S	+	+	–
DNA G+C content (mol%)	52.5	55.4	49.3

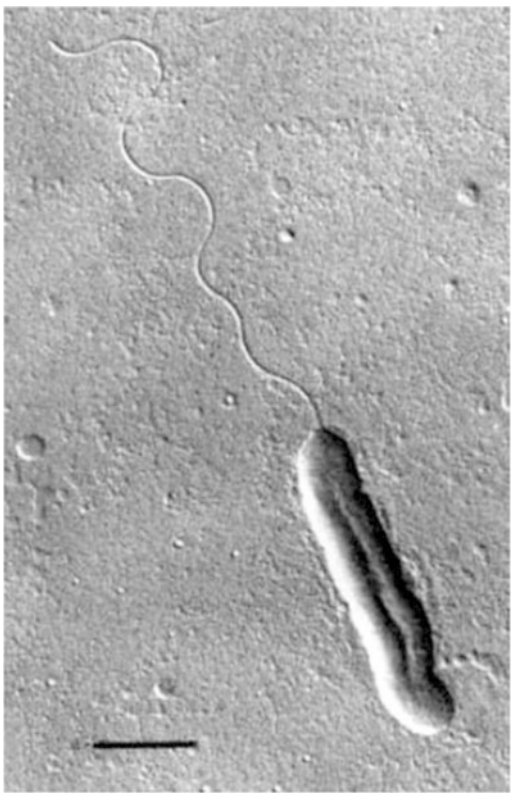

Fig. 37.2
Electron micrograph of strain N10. Platinum shadowed cell, showing single polar flagellum. Scale bar: 1.0 μm (From Ma et al. (2004), with permission)

The Genus *Arenicella* Romanenko et al. 2010

Arenicella (A.re.ni.cel'la. L. n. *arena* sand; L. fem. n. *cella* a chamber, a cell; N.L. fem. n. *Arenicella* a cell from sand).

The members of the genus are Gram-negative, strictly aerobic, nonmotile, chemoorganoheterotrophic, yellow-pigmented, rod-shaped bacteria. They produce deep-yellow-pigmented, smooth, shiny colonies with regular edges, 2–3 mm in diameter, on marine agar. Na^+ is required for growth. Growth occurs at salinity of 0.5–5 % (w/v) NaCl and temperatures of 4–38 °C, with an optimum of around 25–28 °C. The pH range for growth is 5.5–11 with an optimum at pH 8–9.5 (Nedashkovskaya et al. 2013; Romanenko et al. 2010a). *Arenicella* strains are positive for oxidase, catalase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, and b-glucosidase activities; hydrolysis of aesculin, casein, gelatin, starch, and Tween 40 and Tween 80; utilization of arabinose, glucose, and L-alanine. They are negative for nitrate reduction; hydrolysis of agar, urea, and DNA; acid production from L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, lactose, mannose, melibiose, raffinose, L-rhamnose, ribose, sorbose, sucrose, xylose, *N*-acetylglucosamine, glycerol, inositol, mannitol, sorbitol, and citrate; utilization of lactose, raffinose, sorbitol, *N*-acetylglucosamine, L-histidine, L-leucine, DL-methionine, L-phenylalanine, L-tryptophan, adipate, caprate, citrate, gluconate, malate, malonate, and phenylacetate; presence of lipase (C14), cystine arylamidase, α-galactosidase, β-glucuronidase, α-glucosidase, *N*-cetylglucosaminidase, α-mannosidase, and α-fucosidase activities; H_2S, indole, and acetoin

Table 37.3
Phenotypic characteristics of *Arenicella* species

	Arenicella chitinivorans Romanenko et al. 2010	*Arenicella xantha* Nedashkovskaya et al. 2013
Type strain	KMM 6208^T (=KCTC 12711^T = LMG 26983^T)	KMM 3895^T (=NRIC 0759^T = JCM 16153^T)
GenBank 16SrRNA accession numbers	KC136313	AB500096
Locality of origin	Sea urchin *Strongylocentrotus intermedius* collected at Troitza Bay, Gulf of Peter the Great, Sea of Japan	Sandy sediment offshore of the coast of Sea of Japan, Russia
Cell length (μm)	2.1–3.3	3.0–4.0
Cell diameter (μm)	0.5–0.6	0.5–0.6
Temperature °C for growth – range/optimum	4–38/25–28	5–35/25–28
pH for growth – range/optimum	5.5–10.5/8	6–11/8.5–9.5
NaCl (%, w/v) for growth – range/optimum	0.5–5/ 1.5–3	1–5/2–3
Hydrolysis of chitin	+	–
Hydrolysis of Tween 20	–	+
Utilization of galactose, maltose, mannose, rhamnose, sucrose	+	–
Utilization of melibiose	–	+
Utilization of inositol, mannitol	+	–
Activity of a-chymotrypsin, b-galactosidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase	+	–
Susceptibility to ampicillin, benzylpenicillin, carbenicillin, oxacillin, vancomycin	–	+
DNA G+C content (mol%)	46.3	48.1

production. The predominant isoprenoid quinone is Q-8. Major fatty acids are $C_{16:1}\omega7c$, iso-$C_{16:0}$, iso-$C_{18:0}$, and $C_{18:1}\omega7c$. Polar lipids include phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, and an aminophospholipid DNA G+C content is 46.3–48.1 mol%. The cells are susceptible (μg per disc) to cephalexin (30), chloramphenicol (30), erythromycin (15), gentamicin (10), nalidixic acid (30), neomycin (30), ofloxacin (5), oleandomycin (15), rifampicin (5), and streptomycin (30); resistance to cefazolin (30), doxycycline (10), kanamycin (30), lincomycin (15), polymyxin B (300 U), and tetracycline (5) (Nedashkovskaya et al. 2013; Romanenko et al. 2010a).

The type species is *Arenicella xantha* (strain KMM 3895^T (=NRIC 0759^T =JCM 16153^T)), isolated from a sandy sediment sample collected offshore from the Sea of Japan, Russia (Romanenko et al. 2010a).

Remarks

The genus *Arenicella* comprises the species *Arenicella xantha* Romanenko et al. 2010, isolated from a marine sandy sample collected offshore from the Sea of Japan and *Arenicella chitinivorans* Nedashkovskaya et al. 2013, isolated from the sea urchin *Strongylocentrotus intermedius*. According to 16S rDNA sequence similarity, those species constitute a genus, named *Arenicella*, and it belongs to the class Gammaproteobacteria. Physiological and biochemical properties support the phylogenetical position. Differences between species are presented in *Table 37.3*.

An uncultivated bacteria closely related with *A. chitinivorans* was isolated from the brown alga *Saccharina japonica*, which serves as food for this sea urchin species (Nedashkovskaya et al. 2013).

The Genus *Chromatocurvus* corrig. Csotonyi et al. 2012

Chromatocurvus (Chro.ma.to.cur'vus. Gr. n. chroma, -atos color; L. masc. adj. curvus curved, bent; N.L. masc. n. chromatocurvus the colored curved microorganism).

The genus is characterized by pleomorphic bacteria, ranging from short rod-shaped to bent and irregularly shaped cells (*Fig. 37.3*). The colonies are small (1–2 mm diameter), pale pinkish-purple tinged slightly with orange on the surface of agar media and bore an adhesive consistency (Csotonyi et al. 2011). Cells are Gram-negative, non-spore-forming. They are pink-purple in culture due to production of carotenoids and bacteriochlorophyll with an absorption peak at 877 nm. The strain presents motility and is strictly aerobic anoxygenic

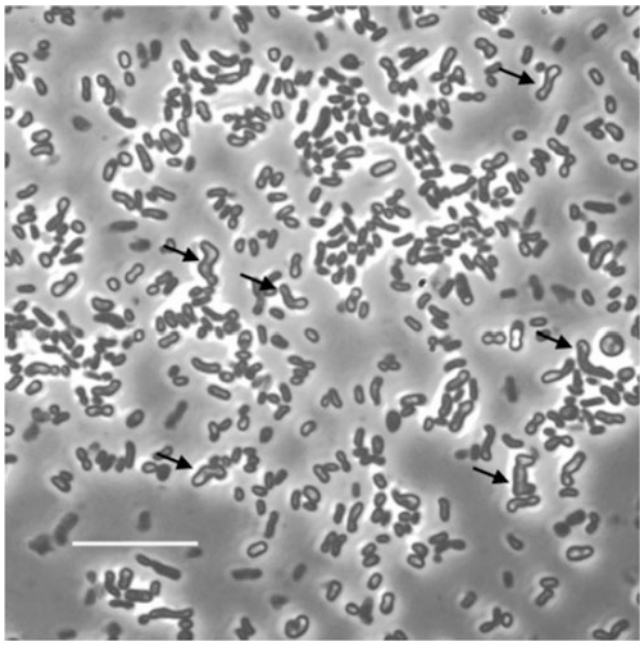

Fig. 37.3
Phase-contrast microscopy of pleomorphic strain EG19^T. *Arrows* indicate examples of bent and irregularly shaped cells. Scale bar: 10 μm (From Csotonyi et al. (2011), with permission)

photoheterotrophs. *Chromatocurvus* moderately halophilic, growing with 0–18 % NaCl, and demonstrating most rapid initial growth at 4 %, also tolerated a wide range of pH, between 7.0 and 12.0, with optimal pH being 7.0 and temperature, from 7 °C to 40 °C, showing near optimal biomass yield from 12 °C to 37 °C, with an optimum at 37 °C. Heterotrophic growth occurred in defined media amended with the organic compounds acetate, L-alanine, butyrate, fumarate, L-glutamate, glutathione, DL-3-hydroxybutyrate, L-isoleucine, DL- malate, oxaloacetate, L-proline, propionate, pyruvate, succinate, and L-threonine. Biomass yield was best on the complex carbon sources bactopeptone, casamino acids, and yeast extract. No fermentation of glucose, sucrose, or fructose was observed. *Chromatocurvus* cannot grow with L-arginine, butanol, citrate, ethanol, formate, D-fructose, D-glucose, glycerol, glycolate, D-L-lactate, methanol, 2- oxoglutarate, L-phenylalanine, L-serine, and sucrose. Thiosulfate does not stimulate growth. The genus possessed neither amylase nor gelatinase activity and was incapable of hydrolyzing Tween 60, indicating a lack of lipase activity. It produced catalase and cytochromes of the c-type are dominating in redox difference spectra (Spring et al. 2013); does not produce urease, arginine dihydrolase, tryptophanase, or aesculinase. Nitrate is not reduced to nitrite. Major cellular fatty acids are $C_{16:0}$, $C_{16:1}$, and $C_{18:1}$. The dominating hydroxy fatty acids are $C_{11:0}$ 3OH, $C_{12:0}$ 3OH, and $C_{12:1}$ 3OH (Spring et al. 2013). Phosphatidylglycerol, phosphatidylethanolamine, an unidentified phospholipid, and an unidentified aminophospholipid are the major polar lipids. Ubiquinone 8 represents the sole respiratory lipoquinone. The DNA G+C content of the type strain EG19^T was 63.0 mol%. It shows resistance to (μg per disc) ampicillin (2), imipenem (10), and streptomycin (10); was weakly resistant to kanamycin (30), but was sensitive to chloramphenicol (30), nalidixic acid (30), penicillin G (10 IU), and polymyxin B (300 IU). Habitat: hypersaline environment (Csotonyi et al. 2011; Spring et al. 2013).

The type species is *Chromatocurvus halotolerans* (strain is EG19^T (=DSM 23344^T, =VKMB-2659^T)), isolated from a runoff stream from the hypersaline (56.7–66.3 % total dissolved solids) brine spring system known as East German Creek, near Swan River, Manitoba, Canada (Csotonyi et al. 2011).

Remarks

The type species of *Chromatocurvus* genus is aerobic anoxygenic photoheterotrophic that use light as additional energy source for mixotrophic growth and play a significant role in the microbial ecology of marine environments. Aerobic anoxygenic photoheterotrophic belonging to the Alphaproteobacteria have been intensively studied, but those representatives belonging to the Gammaproteobacteria class have been described in the last years. The pigment composition of the photosynthetic apparatus in all obligatorily aerobic Gammaproteobacteria studied so far seems to be identical (Spring et al. 2013).

A particularity of the strain EG19^T is that deviation to higher salinity enhances bacteriochlorophyll a production. It opposes to that observed to aerobic anoxygenic phototrophs of Alphaproteobacteria class (*Thalassobacter stenotrophicus*, *Hoeflea phototrophica*, and *Citromicrobium bathyomarinum*) (Csotonyi et al. 2011).

The Genus *Congregibacter* Spring et al. 2009

Congregibacter (Con.gre.gi.bac'ter. L. adj. *congregus* -a -um, united in flocks; N.L. masc. n. *bacter*, a rod; N.L. masc. n. *Congregibacter* a rod that grows in flocks).

Cells are Gram-negative, non-spore-forming, multiply by binary fission, and are pleomorphic. Colonies can reach a size of 1–2 mm. They have a round shape with regular edges and are cream colored, thin, slightly convex, and soft (Spring et al. 2009). Depending on the growth conditions, they are either coccoid or irregular rod-shaped with rounded ends (*Fig. 37.4*). Motility is conferred by one or two polar to subpolar flagella. Aggregates are frequently formed in liquid medium under suboptimal growth conditions, especially carbon starvation. They are mesophilic and moderately halophilic, strictly aerobic, although high oxygen concentrations inhibit growth under oligotrophic conditions. Respiratory and heterotrophic metabolism occur. Growth was observed between 9 °C and 33 °C and at sea salts concentrations ranging from 1 % to 15 % (w/v). In media containing 10 mM $MgSO_4$, the range of suitable NaCl concentrations was 1–7 % (w/v) with an optimum at 2 % (w/v) NaCl. The requirement for salts is complex and sodium, chloride, and either magnesium or calcium ions were needed for growth. No growth occurs below pH 6.0 or above pH 9.5.

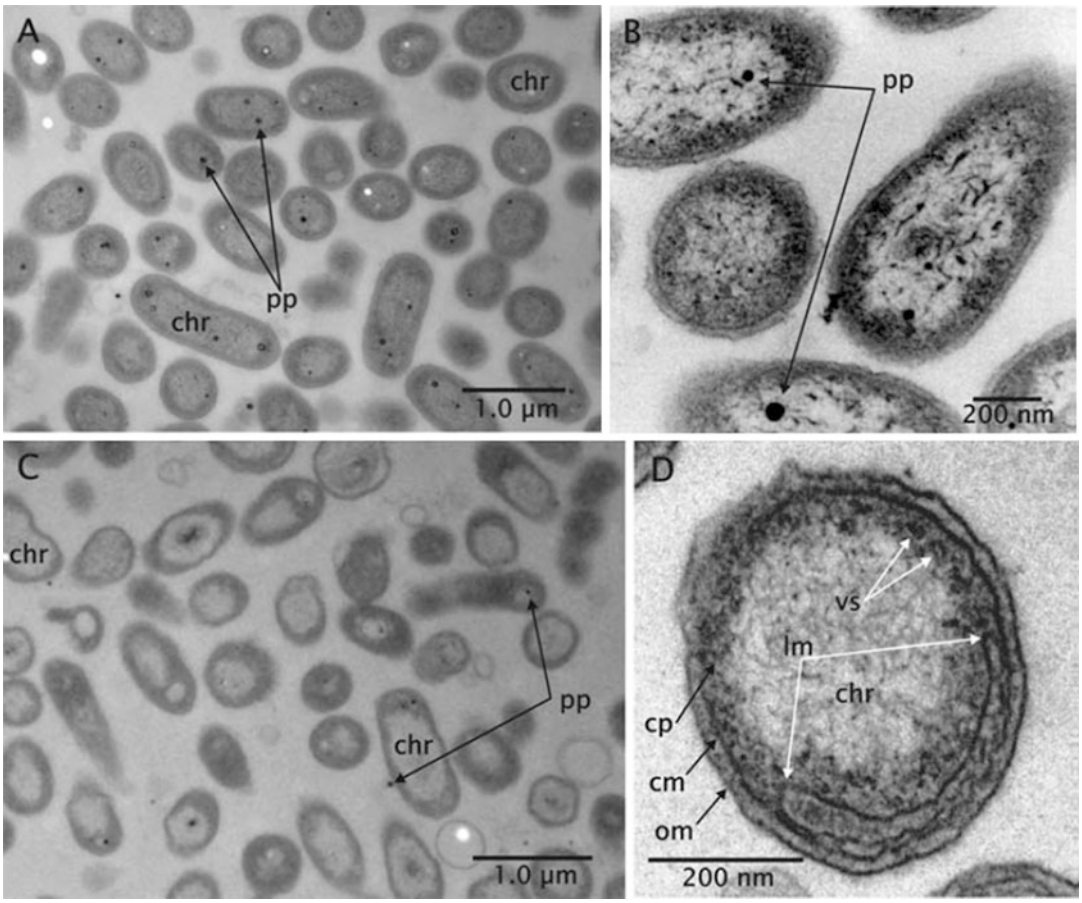

Fig. 37.4

Ultrastructure and intracytoplasmic membranes. (a, b) Chemoheterotrophic growth. (c, d) Photoheterotrophic growth. Abbreviations: *chr* chromosome, *pp* polyphosphate inclusion, *vs* vesicle, *om* outer membrane, *lm* lamellar membrane invagination, *cm* cytoplasmic membrane, *cp* cytoplasm. (a, c) 90 nm ultrathin sectioned cells after osmium and uranium pre- and lead citrate-uranylacetate poststaining. (b, d) Untreated 35 nm sectioned cells as reversed prints of HCl images (From Spring et al. (2009), with permission)

Optimal conditions for growth are 28 °C, a sea salts concentration of around 4 % (w/v), and a pH value between 7.5 and 8.0 (Spring et al. 2009). They present urease, tweenase, oxidase, and catalase activity and are negative for tryptophanase, arginine dihydrolase, and esculinase activity. Cytochromes of the c-type are dominating in redox difference spectra. Bacteriochlorophyll a and carotenoids of the spirilloxanthin series are produced in photosynthetically active cells. In the presence of photosynthetic pigments, light stimulates growth under semiaerobic conditions. The production of photosynthetic pigments is not repressed in aerobically growing cells by illumination with dim light, i.e., below 2,000 lx of incandescent light (equivalent to 40 mE m22 s21). Under certain incubation conditions, water-insoluble polar pigments with a pale yellow to orange-red color can be formed. Hydrolysis casein, gelatin, starch, cellulose, alginate, agar, and DNA does not occur. Utilizes alkanes: decane (weak), dodecane (weak), and octane (weak); alcohols: glycerol and propanol (weak); carboxylic acids: acetate (weak), butyrate (weak), dodecanoate, heptanoate, DL-3-hydroxybutyrate, hexanoate (weak), DL-malate, oleate, oxaloacetate, 2-oxoglutarate, palmitate, pentanoate, pyruvate, propionate (weak), and succinate; amino acids: D-alanine, L-alanine, D-arginine (weak), L-arginine, L-asparagine, D-aspartate (weak), L-aspartate, L-cysteine (weak), L-glutamate, glutathione, L-proline, and L-serine; carbohydrates: D-galactose and sucrose. But does not utilize: alkanes: hexane, hexadecane and tetradecane; alcohols: meso-erythritol, ethanol, myo-inositol, D-mannitol, methanol, and resorcinol; carboxylic acids: acrylate, 2-aminobenzoate, benzoate, citrate, decanoate, formate, glycolate, DL-lactate, and octanoate; amino acids: L-cysteate, DL-glycine, L-histidine, L-isoleucine, L-lysine, L-methionine, L-ornithine, L-phenylalanine, D-proline, D-serine, L-valine, and taurine; carbohydrates: L-arabinose, cellobiose, D-fructose, D-glucose, and melibiose. Negative for H_2S formation (Spring et al. 2009). Major cellular fatty acids are $C_{16:0}$, $C_{16:1}$, and $C_{18:1}$. The dominating hydroxy fatty acid is $C_{10:0}$ 3OH. Ubiquinone 8 represents the sole respiratory lipoquinone. Phosphatidylethanolamine, phosphatidylglycerol, and an unidentified phospholipid are the major polar lipids. Representatives can be found in seawater and the surface layer of littoral marine sediments. The DNA G+C content of the type strain is 57.8 mol% (Spring et al. 2009).

The type species is *Congregibacter litoralis* (strain KT71^T (=DSM 17192^T =NBRC 104960^T)), isolated from the water column (8 m depth) of the North Sea near Helgoland (Germany) (Spring et al. 2009).

Remarks

Congregibacter litoralis was the first cultured representative of marine aerobic anoxygenic phototrophic Gammaproteobacteria (Spring et al. 2009, 2013). Morphological differences can be observed in *Congregibacter* cells depending on the metabolism that is being used (Fig. 37.4).

The Genus *Gallaecimonas* Rodríguez-Blanco et al. 2010

Gallaecimonas (Gal.la.e.ci.mo'nas. L. n. *Gallaecia* of Galicia, a region of northwest Spain; L. fem. n. *monas* a monad, unit; N.L. fem. n. *Gallaecimonas* single microbe isolated from Galicia).

The members of the genus are Gram-negative, flagellated rods. Colonies are smooth, circular, convex, and gray-colored, with regular edges and diameter of 2–3 mm (Rodriguez-Blanco et al. 2010; Wang et al. 2013). It grows with 0–7 % NaCl with optimal concentration of 1–2 %, tolerated a wide range of pH, between 5 and 10, with optimal pH of 5–8 and temperature from 20 °C to 37 °C with an optimum at 30 °C. Oxidase activity is positive. Hydrolysis of aesculin and gelatin is variable between species. Positive for urease (weak) and utilization of capric acid, D-glucose, maltose, L-arabinose, malic acid, *N*-acetyl-D-glucosamine, and trisodium citrate, but negative for nitrate reduction, denitrification, indole production and utilization of adipic acid, D-mannitol, D-mannose, phenylacetic acid, and potassium gluconate. Positive for acid phosphatase, alkaline phosphatase, esterase lipase (C8), leucine aminopeptidase, lipase (C14), naphthol-AS-BI-phosphohydrolase, and valine arylamidase activities and weakly positive for cystine arylamidase activity but negative for trypsin, α-fucosidase, α-galactosidase, α-mannosidase, β-glucosidase, and β-glucuronidase activities. The major fatty acids are $C_{16:0}$, $C_{12:0}$ 3-OH, $C_{16:1}\omega6c$ and/or $C_{16:1}$ $\omega7c$, and $C_{18:1}$ $\omega6c$ and/or $C_{18:1}$ $\omega7c$.

Both strains were sensitive (μg per disc) to chloromycetin (30), ciprofloxacin (5), gentamicin (10), kanamycin (30), norfloxacin (10), ofloxacin (5), polymyxin B (30 IU), rifampicin (5), and streptomycin (10), but resistant to ampicillin (10), carbenicillin (100), cephalexin (30), cefazolin (30), cefobid (30), cephradin (30), clindamycin (2), lincomycin (2), metronidazole (5), minomycin (30), oxacillin (1), penicillin G (10), piperacillin (100), tetracycline (30), and vancomycin (30). The DNA G+C content of the type strain is 61.1–61.3 mol% (Rodriguez-Blanco et al. 2010; Wang et al. 2013).

The type species is *Gallaecimonas pentaromativorans* (strain CEE131^T (=DSM 21945^T =CECT 7479^T)), isolated from intertidal sediment of Corcubion Ria in Cee, A Coruña, Spain (Rodriguez-Blanco et al. 2010).

Remarks

Gallaecimonas has two described species: the type species *G. pentaromativorans* Rodriguez-Blanco et al. 2010 (strain CEE131^T (=DSM 21945^T =CECT 7479^T)) and *G. xiamenensis* Wang et al. 2013 (strain 3-C-1^T (=CCTCC AB 209060^T =LMG 25226^T =MCCC 1A01354^T)), isolated from a crude-oil degrading consortium produced by enrichment of a sample of surface seawater collected near the coast of Xiamen Island, China. They show many differences in physiological tests (for instance, enzyme activities, assimilation of different carbohydrates, and susceptibility to different antibiotics) and only *G. pentaromativorans* are able to degrade polycyclic aromatic hydrocarbons (PAHs) (Rodriguez-Blanco et al. 2010; Wang et al. 2013).

In the neighbor-joining phylogenetic tree based on 16S rRNA gene sequences, both species of *Gallaecimonas* formed an independent monophyletic cluster (Wang et al. 2013).

The Genus *Halioglobus* Park et al. 2012

Halioglobus (Ha.li.o.glo'bus. Gr. adj. halios belonging to the sea or marine; L. masc. n. globus a ball, sphere, globe; N.L. masc. n. *Halioglobus* a marine coccus).

Cells are Gram-negative coccoid shaped, nonmotile, and obligately aerobic; catalase-negative and oxidase-positive. NaCl is needed for growth. Temperature range for growth is 15–30 °C. Optimal temperature for growth is 20–25 °C. No growth occurs above 37 °C. pH range for growth is 6.0–10.0; optimum is pH 7.0–8.0. Optimum NaCl concentration for growth is 2 %; tolerates up to 4 % (w/v) NaCl. Production of acetoin and reduction of nitrate to N_2 are observed. Gelatin is hydrolyzed. Alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, and acid phosphatase are positive, but leucine arylamidase, β-glucosidase α-galactosidase, β-galactosidase, α-glucosidase, β-glucuronidase, α-mannosidase, and α-fucosidase are negative. Acid is produced from ribose, glucose, fructose, sucrose, and glycogen but not from galactose, *N*-acetylglucosamine, melibiose, mannose, turanose, 5-ketogluconate, D-arabinose, D-tagatose, xylitol, D-xylose, L-xylose, methyl β-D-xylopyranoside, rhamnose, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, salicin, cellobiose, lactose, melezitose, inulin, raffinose, gentiobiose, D-fucose, L-fucose, erythritol, adonitol, sorbose, dulcitol, inositol, mannitol, starch, sorbitol, D-arabitol, L-arabitol, gluconate, or 2-ketogluconate. The respiratory quinone is Q8. Predominant cellular fatty acids are $C_{16:1}\omega7c$, $C_{18:1}\omega7c$, $C_{17:1}\omega8c$, and $C_{11:0}$. The polar lipids present are phosphatidylglycerol, diphosphatidylglycerol, and an undefined phospholipid (Park et al. 2012).

The type species is *Halioglobus japonicus* (strain S1-36^T (=NBRC 107739^T =KCTC 23429^T)), isolated from the northwestern Pacific Ocean near Japan (30° 11′ N 145° 05′ E; depth: 100 m) (Park et al. 2012).

Remarks

Differences between the type species *Halioglobus japonicus* (strain S1-36^T (=NBRC 107739^T =KCTC 23429^T)) and *Halioglobus pacificus* (strain S1-72^T (=NBRC107742^T =KCTC 23430^T)), both isolated from the same locality, are not very wide. The pH maximum value tolerated by *H. japonicus* is 9, whereas *H. pacificus* tolerates pH 10. In addition to those characteristics given to the genus description, *H. japonicus* also hydrolyzes aesculin. Naphthol-ASBI-phosphohydrolase and *N*-acetyl-β-glucosaminidase activities are positive for *H. japonicus* and naphthol-AS-BI-phosphohydrolase; *N*-acetyl-β-glucosaminidase activities are also negative for *H. pacificus*. Acid is not produced from D-arabinose, maltose, arabinose, trehalose, and aesculin in *H. pacificus*. Finally, $C_{10:0}$ and $C_{17:0}$ are also major fatty acids for *H. japonicus* (Park et al. 2012).

The Genus *Marinicella* Romanenko et al. 2010

Marinicella (Ma.ri.ni.cel'la. L. adj. marinus of the sea; L. n. cella a chamber, a cell; N.L. fem. n. *Marinicella* a cell from the sea).

Cells are Gram-negative, aerobic, oxidase- and catalase-positive, rod-shaped, nonmotile, chemoorganoheterotrophic bacteria. Colonies are grayish-yellowish-pigmented, transparent, smooth and shiny, with regular edges, 2–3 mm in diameter, on marine agar; not capable of photoautotrophic growth with CO_2. The temperature range for growth is 5–35 °C with an optimum of 25–28 °C. No growth occurs at 4 °C or above 35 °C. The pH range for growth is 6.0–11.0, with an optimum at pH 8.5–9.5. NaCl is required and growth occurs at 1–5 % NaCl (w/v) and is optimal in 2–3 %. Positive for hydrolysis of gelatin, DNA, and Tween 20, Tween 40, and Tween 80; does not degrade CM-cellulose, agar, chitin, or starch. No acid production is observed from D-glucose, maltose, sucrose, lactose, D-galactose, D-mannose, cellobiose, D-xylose, L-arabinose, L-rhamnose, D-sorbitol, or D-mannitol under aerobic or anaerobic conditions. Negative for H_2S production, nitrate reduction, indole production, glucose acidification (under anaerobic conditions), arginine dihydrolase, urease production, aesculin hydrolysis, and assimilation of D-glucose, D-mannitol, maltose, L-arabinose, D-mannose, *N*-acetylglucosamine, D-gluconate, caprate, adipate, L-malate, citrate, and phenylacetate. Negative results for ONPG, acetoin production, trisodium citrate utilization, and oxidation and fermentation of D-glucose, D-mannitol, inositol, D-sorbitol, L-rhamnose, sucrose, melibiose, amygdalin, and D-arabinose. Does not grow on D-glucose, maltose, sucrose, D-xylose, D-galactose, *N*-acetylglucosamine, lactose, melibiose, raffinose, L-rhamnose, L-arabinose, D-ribose, D-mannose, cellobiose, glycerol, acetate, glutamic acid, DL-methionine, D-mannitol, citrate, L-alanine, L-asparagine, L-arginine, L-phenylalanine, L-valine, or L-lysine. Positive for alkaline phosphatase, esterase lipase (C8), leucine arylamidase, and naphthol-AS-BI-phosphohydrolase; weakly positive for trypsin and negative for esterase (C4), lipase (C14), valine arylamidase, cystine arylamidase, α-chymotrypsin, acid phosphatase, *N*-acetyl β-glucosaminidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucuronidase, β-glucosidase, α-mannosidase, α-fucosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, and tryptophan deaminase (Romanenko et al. 2010b).

Cells are susceptible to (μg per disc) ampicillin (10), benzylpenicillin (10 U), carbenicillin (100), gentamicin (10), lincomycin (15), oleandomycin (15), rifampicin (5), streptomycin (30), vancomycin (30), kanamycin (30), nalidixic acid (30), neomycin (30), ofloxacin (5), polymyxin (300 U), erythromycin (15), cephazolin (30), cephalexin (30), and chloramphenicol (30) and resistant to oxacillin (10) and tetracycline (30). The predominant isoprenoid quinone is Q-8. Polar lipids include phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, and unknown phospholipids. The major fatty acids are iso-$C_{15:0}$, $C_{16:0}$, and $C_{16:1}\omega7c$. The DNA G+C content of the type strain of the type species is 43.8 mol% (Romanenko et al. 2010b).

The type species is *Marinicella litoralis*, strain KMM 3900^T (=NRIC 0758^T =JCM 16154^T), isolated from a coastal seawater sample collected from the Sea of Japan, Russia (Romanenko et al. 2010b).

The Genus *Methylohalomonas* Sorokin et al. 2007

Methylohalomonas (Me.thy'lo.ha.lo.mo'nas. N.Gr. n. *methyl* from Gr. n. *methu* wine and Gr. n. *hulê* wood, the methyl radical; Gr. n. hals, halos salt; Gr. fem. n. monas a unit, monad; N.L. fem. n. *Methylohalomonas* salt (-tolerant), methyl-group-utilizing monad).

Cells are Gram-negative, nonmotile rods and are strictly aerobic, obligately methylotrophic, halophilic, and neutrophilic. They occur singly or in short chains and are covered with a layer of EPS-like material. They utilize methanol and methylamine as carbon and energy sources, and C1 compounds as carbon and energy sources using the serine cycle for carbon assimilation. With methanol, they grow at pH 6.5–8.2 (optimum pH 7.5). *Methylohalomonas* is extremely salt-tolerant, moderate halophile with a NaCl range for growth between 0.5 and 4 M and an optimum at 2 M; unable to grow autotrophically with H_2 or thiosulfate as the energy source. Ammonium serves as a nitrogen source. $C_{16:0}$, cyc $C_{17:0}$, and 10-methyl $C_{16:0}$ are the dominant cellular fatty acids. Habitat is hypersaline chloride-sulfate lakes. The G+C content in the DNA of the type strain is 59.6 mol% (Sorokin et al. 2007).

Methylohalomonas lacus is the type species (strain HMT 1^T (=DSM 15733^T =NCCB 100208^T =UNIQEM U237^T)), isolated from hypersaline inland lakes in southwestern Siberia (Altai, Russia) (Sorokin et al. 2007).

The Genus *Methylonatrum* Sorokin et al. 2007

Methylonatrum (Me.thy'lo.na.trum. N.Gr. n. methyl from Gr. n. *methu* wine and Gr. n. *hulê* wood) the methyl radical; N.Gr. n. *natron* arbitrarily derived from the Arabic n. *natrun* or *natron* soda; N.L. neut. n. *Methylonatrum* methyl-group-utilizing, soda-loving bacterium).

Cells are Gram-negative, short, coccoid, nonmotile rods and are obligately aerobic, restricted methylotrophs. Autotrophic Calvin–Benson cycle is used for carbon assimilation during methylotrophic growth. They are moderately salt-tolerant and obligately alkaliphilic; occur singly or in pairs; and utilize methanol, formate, ethanol, and acetate as carbon and energy sources. With methanol, they grow at pH 8.3–10.5 (optimum pH 10). They are extremely salt-tolerant, growing at salt contents between 0.3 and 4 M total Na^+ with an optimum at 0.5–1.0 M; cannot grow autotrophically with H_2 or thiosulfate as the energy source; and utilize ammonium and nitrate as nitrogen sources. $C_{18:1}\omega7$ is the dominant cellular fatty acid. Habitat is soda lakes. G+C content in the DNA is 62–62.9 mol% (Sorokin et al. 2007).

Methylonatrum kenyense is the type species (strain AMT 1^T (=DSM 15732^T =NCCB 100209^T =UNIQEM $U238^T$)), isolated from the soda lake Magadi in Kenya. The closely related strain AMT 3 (=NCCB 100206) originated from soda lakes in the Kulunda Steppe (Altai, Russia) (Sorokin et al. 2007).

The Genus *Orbus* Volkmann et al. 2010

Orbus (Or'bus. L. masc. n. orbus orphan).

Cells are coccoid or rod-shaped, Gram-negative, catalase-positive, mesophilic, psychrotolerant, chemoheterotrophic bacteria. Colonies of *Orbus hercynius* show a "bull's-eye" appearance and grow up to 1–2 mm in diameter on cefsulodin–irgasan–novobiocin agar (Volkmann et al. 2010). Colonies of *O. sasakiae* are opaque, ivory, smooth, circular, raised, and 1–2 mm in diameter on trypticase soy agar medium (Kim et al. 2013). Metabolism is aerobic and facultatively anaerobic. Flagella-independent motility alongside wet surfaces can be observed. Growth at 4–37 °C, in the presence of 0–5 % (w/v) NaCl and at pH 6.0–10.0. The optimal growth conditions are 20–25 °C, 1 % NaCl, and pH 7.0–8.0. Oxidase activity is species-dependent. Positive reactions for nitrate reduction, glucose fermentation, urease activity, and aesculin hydrolysis. Negative for arginine dihydrolase activity, indole production, gelatin hydrolysis, and β-galactosidase activity. No assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, gluconate, capric acid, adipic acid, malic acid, citrate, or phenylacetic acid occurs. The major polar lipids are phosphatidylethanolamine, phosphatidylglycerol, and two unidentified aminophospholipids. Major fatty acids are $C_{18:1}\omega7c$ and $C_{16:0}$. The G+C content of the DNA of the type strain is 36.4 mol% (Kim et al. 2013; Volkmann et al. 2010).

The type species is *Orbus hercynius* (strain $CN3^T$ (=DSM 22228^T =CCUG 57622^T)), isolated from feces of wild boar collected at the Halberstadt Zoo, Germany (Volkmann et al. 2010).

Remarks

The type strain of *Orbus* was obtained in an attempt to isolate enteropathogenic bacteria. However, the poor growth of the type strain $CN3^T$ at 37 °C and the lack of evidence for interaction with HeLa cells suggest it is not a potential pathogen of endotherms (Volkmann et al. 2010). Nevertheless, another related strain was isolated and described occurring in the gut of the butterfly *Sasakia charonda* in South Korea. This is the type strain of *Orbus sasakia* Kim et al. (2013) (strain C7T (=KACC 16544T =JCM 18050T)) (Kim et al. 2013).

The gene sequence showed highest similarity (90.3 %) with *Obesumbacterium proteus* DSM 2777^T, a member of the family *Enterobacteriaceae*, but the best phylogenetic position and relationships of *Orbus* remains to be determined (Kim et al. 2013).

The Genus *Plasticicumulans* Jiang et al. 2011

Plasticicumulans (Plas'ti.ci.cu'mu.lans. N.L. n. *plasticum* plastic; L. part. adj. *cumulans* accumulating; N.L. part. adj. used as a masc. n. *Plasticicumulans* accumulating plastic).

Cells are Gram-negative; shape varies from coccoid to ovoid; obligately aerobic and heterotrophic. The cells of *P. acidivorans* (the type species of the genus) can exhibit two colony types. One was similar to the original wild type, which is round, dome-like, white-yellowish colonies on mineral agar plates, and the other was smoother and flat. These two colony types also corresponded to different modes of growth in liquid culture; the dome-like colonies were mostly formed from aggregated coccoid cells, and the flat, smooth type were mostly formed from free elongated cells (Jiang et al. 2011). The cells of P. *lactativorans* form colonies after 2 months of incubation and reach a maximum of 1 mm in diameter, with hard consistency, and are pearl white with irregular edges (Jiang et al. 2014). *Plasticicumulans* grows at 20–45 °C (optimum 30 °C or 40 °C, depending on species) and pH 6–8. They are salt-sensitive; growth and respiration are inhibited in the presence of 150–00 mM NaCl or KCl; are mesophilic and neutrophilic; have high capacity to store polyhydroxyalkanoates (PHAs), in the form of large intracellular granules; and possess positive oxidase and catalase activities. Hydrolysis of aesculin and gelatin is negative. Nitrate is reduced to nitrite. It utilizes the following organic compounds as carbon and energy sources: acetate, propionate, butyrate, valerate, pyruvate, succinate, lactate, ethanol, and yeast extract. Utilization of D-glucose varies with species. Formate, methanol, L-arabinose, and H_2 are not utilized. Ammonium and nitrate serve as nitrogen sources. Identified membrane polar lipids include diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, unidentified phospholipids, and unidentified aminolipid.

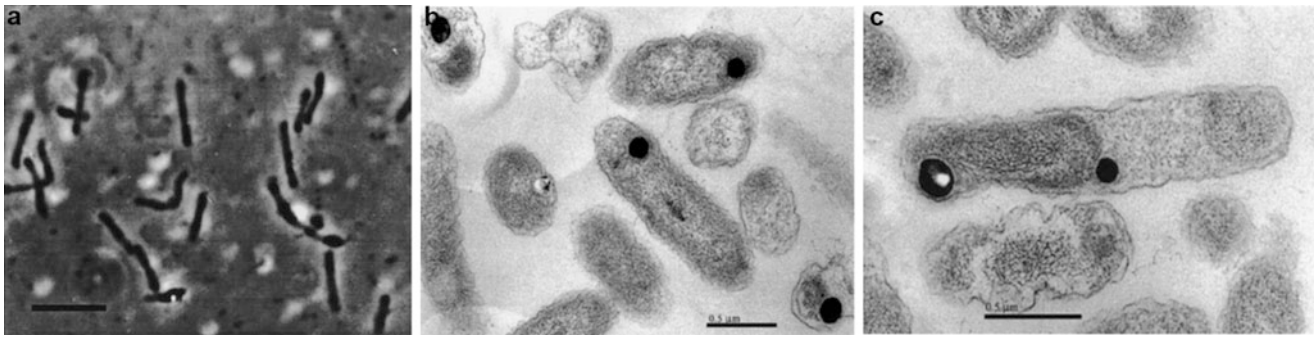

Fig. 37.5

Morphology of type strain of *Sedimenticola selenatireducens*. (a) Phase-contrast micrograph of a wetmount showing refractile granules. Scale bar indicates 5 μm. (b, c) are electron micrographs of thin sections showing dense selenium granules accumulated inside the cells (From Narasingarao and Haggblom (2006), with permission)

The major cellular fatty acids are $C_{16:1}\omega7c$, $C_{16:0}$, and $C_{18:1}\omega7c$. The major respiratory lipoquinones are Q-8 and Q-7 (~9: 1). The DNA G+C content is 67.4–68.5 mol% (Jiang et al. 2014, 2011).

The type species is *Plasticicumulans acidivorans* (strain TUD-YJ37^T =DSM 23606^T =CBS 122990^T), isolated from a sequencing-batch bioreactor fed with acetate (Jiang et al. 2011).

Remarks

The genus has two species. The species *Plasticicumulans lactativorans* (strain YD^T =DSM 25287^T =NCCB 100398^T) that was isolated from a sequencing-batch bioreactor fed with lactate and the type species *Plasticicumulans acidivorans* (strain TUD-YJ37^T =DSM 23606^T =CBS 122990^T), isolated from a sequencing-batch bioreactor fed with acetate. They differ from one another especially in wide capacity of utilization of sugars found in *P. lactativorans* (Jiang et al. 2014).

The Genus *Porticoccus* Oh et al. 2010

Porticoccus (Por.ti.coc'cus. L. n. *portus* a harbor, haven, port; N.L. masc. n. *coccus* a coccus; N.L. masc. n. *Porticoccus* a coccus isolated from a harbor).

Cells are cocci, Gram-negative, nonmotile, chemoheterotrophic, obligately aerobic. Colonies are circular, convex, entire, and opaque with 0.05 mm in diameter on marine agar. The color varies from beige to off-white; are oxidase-positive and catalase-negative; and require NaCl for growth. Growth occurs from a range of temperature of 15–42 °C (optimally at 20–25 °C), at pH 5–11 (optimally at pH 7–8) and 1.5–5.0 % NaCl (optimally 3.5 % NaCl). Tests are negative for arginine dihydrolase, urease, PNPG (β-galactosidase), alkaline phosphatase, lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase, indole production, hydrolysis of aesculin and gelatin, glucose fermentation, and nitrate reduction. Tests are positive for esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, and acid phosphatase.

Utilize methylamine, glycerol, D-ribose, D-fructose, D-mannose, D-maltose, D-xylitol, and L-lysine whereas methanol, ethanol, D,L-glyceraldehyde, D-arabinose, D-galactose, *N*-acetyl-D-glucosamine, D-glucosamine, α-D-glucose, rhamnose, cellobiose, lactose, melibiose, D-sucrose, D-trehalose, D-melezitose, D-raffinose, adonitol, arabitol, D-mannitol, myo-inositol, D-sorbitol, citric acid, gluconic acid, glucuronic acid, pyruvic acid, L-alanine, L-arginine, glycine, L-histidine, L-ornithine, L-proline, and L-serine are weakly positive. Carbon source tests for itaconic acid, malonic acid, propionic acid, succinic acid, L-glutamic acid, and L-leucine are negative (Oh et al. 2010).

Cells are susceptible to (μg per disc) erythromycin (15) and rifampicin (50); resistant to ampicillin (10), chloramphenicol (25), gentamicin (10), kanamycin (30), penicillin G (10), streptomycin (10), tetracycline (30), and vancomycin (30) (Oh et al. 2010).

Predominant cellular fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, and $C_{16:0}$. The DNA G+C content of the type species is 47.8 mol% (Oh et al. 2010).

The type species is *Porticoccus litoralis* (strain IMCC2115^T =KCCM 42369^T =NBRC 102686^T), isolated from coastal seawater of the Yellow Sea in Korea (37° 19′ N 126° 33′ E) (Oh et al. 2010).

The Genus *Sedimenticola* Narasingarao and Häggblom 2006

Sedimenticola (Se.di.men.ti' co.la. L. neut. n. sedimentum sediment, L. masc./fem. Suffix n. -cola dweller, inhabitant, N.L. masc. n. Sedimenticola, sediment dweller).

Cells are Gram-negative, nonmotile, and rod-shaped (*Fig. 37.5*); strictly anaerobic. In agar shake tubes, colonies are round, about 5 mm in diameter, and bright red colored due to elemental selenium formed during selenate reduction. Growth

occurs in minimal salts medium (1.1–2.3 % NaCl) using selenium or nitrogen oxyanions as electron acceptor. Selenate is reduced to selenite, coupled to 4-hydroxybenzoate utilization; utilize aromatic acids such as 3-hydroxybenzoate, 4-hydroxybenzoate, benzoate, and short chain fatty acids such as acetate, pyruvate, and lactate. The predominant cellular fatty acids are hexa- and octadecanoic acid and their corresponding monounsaturated fatty acids ($C_{16:0}$, $C_{18:0}$, $C_{16:1}\omega7$cis, and $C_{18:1}\omega7$cis). The G+C content of the DNA is 57 mol%. The type species is *Sedimenticola selenatireducens* (strain AK4OH1^T =ATCC BAA-1233^T), isolated from an estuarine sediment sample of the Hudson River estuary, NY, USA (Narasingarao and Haggblom 2006).

Remarks

The species *Sedimenticola selenatireducens* is the first described being able to couple an aromatic substrate with selenate respiration. It is a bacterium that dissimilatorily reduces selenate using 4-hydroxybenzoate as carbon and energy source. The elimination of the toxicity of selenite by conversion of selenite to elemental selenium is well known. However, this process was never coupled with growth (Narasingarao and Haggblom 2006). Currently, the dissimilatory selenate-reducing microbes use short chain fatty acids or H_2 as electron donor.

Among microorganisms that carry out this process is *Pseudomonas stutzeri* that aerobically transform up to 48 mM selenate or selenite to elemental selenium and *Stenotrophomonas maltophilia*, *Wolinella succinogenes*, and *Desulfovibrio desulfuricans* which cannot use selenium compounds as terminal electron acceptors for respiration, but they convert it to elemental selenium and may accumulate it inside or outside of the cell (Narasingarao and Haggblom 2006).

The Genus *Simiduia* Shieh et al. 2008

Simiduia (Si.mi'du.ia. N.L. fem. n. Simiduia named after Usio Simidu, a Japanese microbiologist, to honor his work in marine microbiology).

Cells are Gram-negative rods. Colonies of *Simiduia agarivorans* are circular, convex, and nonluminescent, surrounded by depressions when grown on marine agar and polypeptone/yeast extract (PY) plate medium for 3–7 days. Marine agar and PY plate medium were cream colored and off-white, respectively. Clear yellow haloes are formed around the colonies, in contrast to the purple-brown background when the agar plates are flooded with iodine/potassium iodide solution. It indicates diffusion of agarase out from the colonies and release of reducing compounds during agar hydrolysis (Shieh et al. 2008). Colonies of *S. areninigrae* are dark ivory in color with diameters of 1–3 mm, circular, and raised with entire margins on marine agar plates (Kim et al. 2012). Motility depends on species and are chemoorganotrophic; aerobic or anaerobic metabolism, depend on species; oxidase- and catalase-positive. *Simiduia* members have agarolytic activity. Growth occurs between 10 °C and 40 °C, with optimum growth at 25–35 °C; at NaCl concentrations of 0.5–7 % (w/v), with optimum growth at 2–3 % (w/v); no growth occurs without NaCl, pH range of 7–10, with optimum at pH 6.

Simiduia hydrolyzes alginate, aesculin, starch, casein, CMC, and DNA, but does not hydrolyze Tween 80 and urea. Hydrolysis of cellulose, chitin, and gelatin depends on species (Table 37.3). Positive results for leucine arylamidase, valine arylamidase, esterase (C4), esterase lipase (C8), *N*-acetyl-β-glucosaminidase, naphthol-AS-BI-phosphohydrolase, acid phosphatase, and alkaline phosphatase activities, whereas trypsin activity depends on species. Absent activities of arginine dihydrolase, urease, lipase (C14), α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase, and α-fucosidase. Nitrate is reduced to nitrite. Indole is not produced. *Simiduia* is negative for glucose fermentation. Both *Sumiduia* species assimilate cellobiose, maltose, sucrose, acetate, b-hydroxybutirate, L-alanine, L-arginine, L-glutamic acid, L-lysine, and tyrosine. But they do not assimilate L-arabinose, D-mannose, D-mannitol, maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid. The major isoprenoid quinone was Q8. The major polar lipid is phosphatidylethanolamine. Predominant fatty acids include $C_{16:1}\omega7$c and/or iso-$C_{15:0}$ 2-OH and $C_{17:1}\omega8$c (Kim et al. 2012; Shieh et al. 2008). Cells are susceptible to (μg per disc) ampicillin (10), carbenicillin (100), chloramphenicol (30), erythromycin (15), kanamycin (30), lincomycin (15), nalidixic acid (30), neomycin (30), sulfamethoxazole (50), tetracycline (30), trimethoprim (5), and penicillin G (10 U) (Kim et al. 2012; Shieh et al. 2008). The DNA G+C content is 53.3–55.6 mol%. The type species is *Simiduia agarivorans* (strain SA1^T =BCRC 17597^T =JCM 13881^T), isolated from shallow coastal water from Keelung, Taiwan (Shieh et al. 2008).

Remarks

The genus has two agarolytic species: S. *agarivorans* and *S. areninigrae*. Information about type strains and comparative features are presented on Table 37.4.

The Genus *Solimonas* Kim et al. 2007

Solimonas (So'li.mo'nas. L. n. *solum* soil; L. fem. n. *monas* a unit, monad; N.L. fem. n. *Solimonas* a monad from soil).

The members of *Solimonas* are Gram-negative, rod-shaped cells. Characteristics of the colonies vary according to the species. They have aerobic or facultatively anaerobic metabolism; do not form endospores. Motility by means of a single polar flagella can be found, depending on the species. Also, they are chemoorganotrophs and oxidase- and catalase-positive. The predominant cellular fatty acids are $C_{16:0}$ and $C_{18:1}\omega7$c. The predominant polar lipids include phosphatidylethanolamine,

Table 37.4
Phenotypic characteristics of *S. agarivorans* and *S. areninigrae*

	Simiduia agarivorans Shieh et al. 2008	*Simiduia areninigrae* Kim et al. 2012
Type strain	SA1^T(=BCRC 17597^T = JCM 13881$^{T)}$	M2-5^T(=KCTC 23293^T = NCAIM B02424^T)
GenBank 16SrRNA accession numbers	EF617350	GQ872422
Locality of origin	Seawater collected in the shallow coastal region of Keelung, Taiwan (33° 14′ 39″ N 126° 34′ 29″ E)	Black sand off the shore of Jeju Island, Republic of Korea
Cell length (μm)	2.0–5.0	1.4–1.8
Cell diameter (μm)	0.4–0.6	0.5–0.7
Motility by flagella	<1 % exhibit motility and monotrichous flagella	+
pH for growth range	7–10	7–9
Temperature °C for growth – range/optimum	15–40/30–35	10–37/25–30
NaCl (%, w/v) for growth – range/optimum	0.5–7/2–3	1–6/1–2
Aerobic growth	+	+
Anaerobic growth	By reducing nitrate as terminal electron acceptor	
Hydrolysis of cellulose, chitin, gelatin	+	−
Activity of trypsin	+	−
Assimilation of D-glucose and *N*-acetylglucosamine	+	−
Resistant to vancomycin	+	−
Major isoprenoid quinone	Q8, Q10, MK-9, MK-7	Q8
Major polar lipid fatty acids	Phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylserine	Phosphatidylethanolamine, phosphatidylglycerol, and three unknown lipids
Major cellular fatty acids	$C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH, $C_{17:1}\omega8c$, $C_{16:0}$, $C_{18:1}\omega7c$, $C_{17:0}$, $C_{14:0}$	$C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH, $C_{17:1}\omega8c$, $C_{17:0}$
DNA G+C content (mol%)	55.6	53.3

phosphatidylglycerol, diphosphatidylglycerol, and an uncharacterized aminophospholipid. The major respiratory quinone is Q8. The DNA G+C content is 64.9–68.4 mol% (Friedrich and Lipski 2008; Kim et al. 2007, 2014; Sheu et al. 2011; Zhou et al. 2008).

The type species is *Solimonas soli* (strain DCY12^T =KCTC 12834^T =LMG 24014^T), isolated from soil from a ginseng field in South Korea (Kim et al. 2007).

Remarks

The genus comprises five species: *S. solis* Kim et al. 2007 (type strain), *S. aquatica* Sheu et al. 2011, *Solimonas flava* (Zhou et al. 2008) Sheu et al. 2011, comb. nov., *Solimonas variicoloris* (Friedrich and Lipski 2008) Sheu et al. 2011, and comb. nov., *Solimonas terrae* Kim et al. 2014. Comparative physiological characteristics are presented in *Table 37.5*.

Ecology

Habitat

The habitat of strains can be quite different even between species of the same genus. Most genera have their members from aquatic environments, particularly from marine water column. *Chomatocurvus halotolerans* is from a hypersaline mat of a brine spring runoff stream in the west central region of the province of Manitoba, Canada; *Congregibacter litoralis* habits the water column of the North Sea near Helgoland; *Gallaecimonas xiamensis* is from the seawater enriched with crude oil, near Xiamen Island in China; both species of *Halioglobus* are from seawater in the northwestern Pacific Ocean, near Japan; *Marinicella litoralis* is from a coastal seawater sample collected from the Sea of Japan, Russia; *Porticoccus* is from coastal seawater of the Yellow Sea in Korea; and *Simiduia agarivorans* was isolated from a seawater sample collected in the shallow coastal region of Keelung,

Table 37.5
Phenotypic characteristics of *Solimonas* species

	S. solis Kim et al. 2007	*S. aquatica* Sheu et al. 2011	*S. flava* (Zhou et al. 2008) Sheu et al. 2011, comb. nov.	*S. variicoloris* (Friedrich and Lipski 2008) Sheu et al. 2011, comb. nov.	*S. terrae* Kim et al. 2014
Type strain	DCY12^T (=KCTC 12834T = LMG 24014T)	NAA16^T (=BCRC 17835T = LMG 24500T)	CW-KD 4^T (=DSM 18980T = KCTC 12881T = CCTCC AB 206145^T)	MN28^T (=DSM 15731^T = LMG 22844T)	KIS83-12^T (=KACC 16967^T = DSM 27281T)
GenBank accession numbers	EF067861	EU303271	EF154515	AJ555478	KF112046
Locality of origin	Ginseng field in South Korea	Freshwater spring in Kaohsiung County, Taiwan – 22°36′36.5″ N 120°22′32.8″ E	Surface layer of a polluted farmland soil from Nanjing Jiangsu Province, China	Hexane-treated, full-scale biofilter from an oil mill	Soil of Gaui island in Taean region, South Korea
Cell length (μm)	.3–0.5	1.0	2.4–2.6	ND	1.5–2.4
Cell diameter (μm)	0.2–0.4	0.3–0.8	0.3–0.4	ND	0.5–0.6
pH for growth – range/optimum	7–9/7	7–8/7.5	5–8/6–7	ND	5–8/7
Temperature °C for growth – range/optimum	20–42/30	20–40/25	15–42/30	20–42/30	15–33/28
NaCl (%, w/v) for growth – range/optimum	0–2/0.5	0–1/0.5	0–3/0–1	0–2/0	0/0
Colony pigmentation	Yellow	Semi-opaque	Yellow	Yellow	Light yellow
Cell morphology	Rod	Rod	Long rod	Long rod	Long rods
Motility	+	+	–	–	+
Anaerobic growth	–	–	+	+	–
Hydrolysis of DNA	+	–	–	–	–
Hydrolysis of aesculin	–	+	–	+	+
Hydrolysis of gelatin	–	+	+	+	+
Alkaline phosphatase activity	+	–	+	+	+
C4 esterase activity	+	–	+	+	+
C8 esterase lipase activity	+	–	+	+	+
Valine arylamidase activity	+	+	–	–	–
β-Glucosidase activity	–	–	–	+	–
Utilization of *N*-acetylglucosamine	+	–	+	+	–
Utilization of arabinose	+	–	+	+	–
Utilization of citric acid	–	–	+	–	–

Table 37.5 (continued)

	S. solis Kim et al. 2007	*S. aquatica* Sheu et al. 2011	*S. flava* (Zhou et al. 2008) Sheu et al. 2011, comb. nov.	*S. variicoloris* (Friedrich and Lipski 2008) Sheu et al. 2011, comb. nov.	*S. terrae* Kim et al. 2014
Utilization of dextrin	–	–	–	+	–
Utilization of glycerol	–	–	+	–	–
Utilization of D-L-lactic acid	–	–	+	–	–
Utilization of mannitol	+	–	+	+	–
Utilization of mannose	+	–	+	+	–
Utilization of L-rhamnose	–	–	+	–	–
Utilization of L-aspartic acid	–	+	–	–	–
Utilization of L-lysine	–	+	–	–	–
Utilization of L-methionine	–	+	–	–	–
Nitrate reduction	–	–	–	–	–
Characteristic polar lipid(s)	PDE, PL2, APL3, APL4	PL2	PDE, PL4, APL3	PDE, APL3, APL4, APL5	PE, PG, DPG
Major cellular fatty acids	$C_{18:1}\omega7c$	$C_{18:1}\omega7c$	$C_{18:1}\omega7c$	$C_{18:1}\omega7c$	$C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$
	$C_{16:0}$	$C_{16:1}\omega7c$ and/or $C_{16:1}$	$C_{16:0}$	$C_{16:0}$	$C_{16:0}$
	$C_{14:0}$ 3-OH	$C_{16:0}$	iso-$C_{16:0}$	$C_{14:0}$ 3-OH	$C_{16:1}\omega5c$
	$C_{16:1}\omega5c$	iso-$C_{16:0}$	$C_{14:0}$ 3-OH	$C_{12:0}$	iso-$C_{16:1}$ I and/or $C_{14:0}$ 3-OH
DNA G+C content (mol%)	68.4	66.2	65.1	64.9	67.9

Taiwan. Differently, *Solimonas aquatica* is from a freshwater spring in Kaohsiung County, Taiwan.

Other species come from marine sediment. *Alkalimonas collagenimarina* is from a deep-sea sediment off Tori-shima Island, Japan, at a depth of 4,026 m; *Arenicella xantha* is from a marine sandy sample collected offshore from the Sea of Japan; *Gallaecimonas pentaromativorans* habits intertidal sediment of Corcubion Ria in Cee, A Coruña, Spain; *Sedimenticola* is from an estuarine sediment sample of the Hudson River estuary; and *Simiduia areninigrae* was isolated from black sand off the shore of Jeju Island, Republic of Korea. Two species of *Solimonas* are from terrestrial soil: *Solimonas soli* is from a ginseng field in South Korea and *Solimonas flava* is from a polluted soil sample collected from Jiangsu Province, China.

Other species are from particular environments such as hypersaline inland lakes which are the habitat of two species of *Alkalimonas* (*A. amylolytica* is from Lake Chahannor in China and *A. delamerensis* is from Lake Elmenteita in East Africa) as well as the genus *Methylohalomonas* (southwestern Siberia (Altai, Russia)) and *Methylonatrum* (soda lake Magadi, in Kenya).

Arenicella chitinivorans habits the tissues of sea urchin *Strongylocentrotus intermedius*.

Orbus hercynius habits feces of wild boar from the Halberstadt Zoo, Germany, and *Orbus sasakiae* habits the gut of the butterfly *Sasakia charonda*.

Finally, there are those species that come from artificial habitats. *Plasticicumulans acidivorans* was isolated from a mixed-culture bioreactor by using a feast–famine regime; *Plasticicumulans lactativorans* was isolated from a sequencing-batch bioreactor fed with lactate; and *Solimonas variicoloris* was isolated from a hexane-treated, full-scale biofilter from an oil mill.

Metabolism

Aerobic anoxygenic photoheterotrophic bacteria form a functional group that includes representatives of *Roseobacter* genus of Alphaproteobacteria and unclassified genera of Gammaproteobacteria belonging to OM60/NOR5 clade (Yutin et al. 2007). They are highly abundant and because of that, they can play a significant role in the marine carbon cycle through bacteriochlorophyll a photophosphorylation (Jiao et al. 2010; Kolber et al. 2001). Aerobic anoxygenic photoheterotrophic Alphaproteobacteria present light-dependent growth, as well as light-induced production of pigment. Differently, aerobic anoxygenic photoheterotrophic Gammaproteobacteria (which includes *Congregibacter* and *Chromatocurvus*) exhibit a correlation between carbon source and production of photosynthetic pigments (Spring et al. 2009; Spring and Riedel 2013). The more reducing is the substrate, the less is the yield of production of photosynthetic pigments (Spring et al. 2009; Spring and Riedel 2013). It is due to the reductants (e.g., NADH) accumulated in cells fed with reducing carbon sources, which affect the intracellular redox state. The decrease of the intracellular redox state is indicative of a surplus of suitable carbon sources, making the photosynthetic apparatus to be redundant (Spring and Riedel 2013). Also, other characteristics shared by aerobic anoxygenic photoheterotrophic Gammaproteobacteria are that oxygen in limiting growth conditions also reduces the pigment expression and the effect of light varies among OM60/NOR5 members, but it does not correlate directly as it is observed for Alphaproteobacteria (Spring et al. 2009; Spring and Riedel 2013).

Genetics, Genomics, and Post-genomics

Genomes

The genome of *Gallaecimonas xiamenensis* (strain 3-C-1^T) was sequenced by Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China), using Solexa paired-end sequencing technology (Lai et al. 2012). More than 7 Mi paired-end reads were generated to reach a 267-fold depth of coverage with an Illumina/Solexa genome analyzer IIx (Illumina, San Diego, CA). The gaps among scaffolds were closed by specific PCR and Sanger sequencing, using the ABI 3730 system (Applied Biosystems). The genome of *G. xiamenensis* 3-C-1^T shows an average GC content of 60.58 %. The genome contains 3,798 candidate protein-encoding genes (with an average size of 970 bp), giving a coding intensity of 90.7 %. A total of 2,738 proteins could be assigned to a cluster of orthologous group (COG) families. Sixty-five tRNA genes for 18 amino acids (lacking Glu and Ile) were identified. Corroborating the lack of ability of *G. xiamenensis* 3-C-1^T to degrade the tested PAHs, no PAH-degrading related dioxygenase was found in the genome sequence, nor was there any AlkB-like monooxygenase (Lai et al. 2012).

The nearly complete genome sequence of *Congregibacter litoralis* strain KT71^T, isolated from North Sea surface water, revealed a complete photosynthesis superoperon, including genes for accessory pigments. The presence of two potential BLUF (blue light using flavin adenine dinucleotide sensors), one of which was found adjacent to the photosynthesis operon in the genome, indicates a light- and redox-dependent regulation of gene expression. Also, genomic evidence show KT71^T needs organic substrates like carboxylic acids, oligopeptides, or fatty acids for growth (Fuchs et al. 2006).

The genome of *Simiduia agarivorans* was sequenced and its size was estimated to be approximately 4.29 Mb (Lin et al. 2013). The genome is circular, has no plasmid, and contains more than five polysaccharide-hydrolyzing enzyme systems, with a total of 45 coding sequences involved in the hydrolysis of agar, alginate, cellulose, chitin, and xylan. Also, we find more than 47 coding sequences in the genome involved in the cell division process, including those for cell division regulation, murein and shape determination, chromosome partition, Z-ring formation, the membrane-embedding Tol–Pal system, and amidase (Lin et al. 2013).

Proteomics

The salinity and alkalinity tolerance of *Alkalimonas* are subject of interest in many aspects. A proteomic analysis of cellular membrane and cytoplasm of *Alkalimonas* in three different pH conditions revealed pH-dependent expression of many proteins. In higher pH levels, some membrane-expressed proteins were involved in iron transport, and most of differential proteins with increased or bell-shaped mode of pH dependence were involved in bioenergetic process and metabolism of carbohydrates, fatty acid, amino acids, and nucleotides (Wang et al. 2009).

Gene Analyses

The alkaline proteases (AcpI and II) were purified from *Alkalimonas collagenimarina* AC40^T. Optimal conditions of activities were determined, as well as inhibitors. AcpI favorably hydrolyzed gelatin, collagen, and casein. AcpI is a subtilisin-like serine protease belonging to subtilase family A. It consists of a prepropeptide, a catalytic domain, and a prepeptidase C-terminal domain like other serine proteases. DNA and amino acid sequence analyses and heterologous expression analysis suggest that both the prepropeptide and prepeptidase C-terminal domains are cleaved off to give the mature form (Kurata et al. 2007b). AcpII comprises a prepropeptide, a catalytic domain that includes a protease-associated domain (PA domain), and tandem repeat prepeptidase C-terminal domains. Genetically engineered proteins showed catalytic domains lacking the PA domain exhibit increased ability to degrade proteinaceous substrates including gelatin, casein, and collagen when compared with catalytic domains containing PA domain (Kurata et al. 2010).

It is known that membrane proteins are differentially expressed in haloalkaliphilic bacterium *Alkalimonas amylolytica* strain N10, and it indicates that ion transporters are important for adaptation to high pH and salt conditions. Indeed, K^+ transporters to have been shown to be important and the genes Aa-trkA and Aa-trkH are alkaline adaptable and partially halo-adaptable K^+ transporters (Guo et al. 2009). Besides, the mechanism of pH homeostasis requires cation/proton antiporters. Antiporters can exchange cytoplasmic cations for external protons to achieve a cytoplasmic pH, significantly less alkaline than the external pH. Four genes of antiporters have been detected and described so far, suggesting that soda lake alkaliphile uses $K^+(NH_4^+)/H^+$ antiport as part of its alkaline pH homeostasis mechanism and part of its capacity to reduce potentially toxic accumulation of cytoplasmic K^+ or NH_4^+, respectively, under conditions of high osmolarity or active amino acid catabolism (Liu et al. 2005; Wei et al. 2007).

Molecular and phenotypic data support the affiliation of the genera *Chromatocurvus*, *Congregibacter*, and *Halioglobus* as members of the clade OM60 (Rappé et al. 1997)/NOR5 (Eilers et al. 2001). The OM60/NOR5 clade is a coherent cluster of 16S rRNA gene sequence which was mainly retrieved by culture-independent methods from marine habitats around the world. The members of this clade have ecological importance in several marine ecosystems, especially the euphotic zone of coastal areas due to its abundance and widespread occurrence in saline environments and coastal waters (Spring et al. 2013). The OM60/NOR5 clade includes aerobic anoxygenic phototrophic Gammaproteobacteria (Park et al. 2012), which use light as additional energy source for mixotrophic growth. Genetic analysis of the photosynthetic reaction center gene sequences (*puf*L and *puf*M) belonging to several distinct groups of Gammaproteobacteria, Betaproteobacteria, and Alphaproteobacteria revealed that in Gammaproteobacteria, the photosynthetic reaction center genes are derived from a common ancestor, contrasting to apparently horizontal gene transfer of *puf*L and *puf*M genes detected among phototrophic members of Alphaproteobacteria and Betaproteobacteria (Spring et al. 2013). The pigment composition of the photosynthetic apparatus in all obligately aerobic Gammaproteobacteria seems to be identical and includes Bacteriochlorophyll *a*, and Spirilloxanthin, and finally cytochromes of the *c*-type are dominating in redox difference spectra (Spring et al. 2013).

Biological and Technological Relevance

Enzymes

Many studies have been investigating the alkaline α-amylase of *Alkalimonas* for its ability to confer tolerance to salinity and alkalinity. Alkaline α-amylase is known by its wide application in the food, textile, and pharmaceutical industries (Wang et al. 2006). The enzyme hydrolyzes starch under alkaline conditions in which it has high stability and catalytic efficiency. In the last years, many efforts have been made in order to produce mutant enzymes with different amino acid residues (Deng et al. 2014; Liu et al. 2014; Yang et al. 2012a, b, c) and fusion proteins (Han et al. 2013; Yang et al. 2013b, d) in order to enhance the enzyme performance in saline–alkali conditions for industrial purposes. Moreover, it was also determined which is the best heterologous host (Yang et al. 2013a), the involvement of genes with the stress tolerance (Xian et al. 2013), and the ability to confer stress tolerance in genetic engineered plants grown in saline–alkali soil (Zhong et al. 2012).

The species *Alkalimonas collagenimarina* is a collagenolytic enzyme-producing bacterium. Collagenolytic enzymes are widely used in several industries to hydrolyze collagen, which is produced in large quantities as a by-product in the livestock industry. Recent studies have shown that peptides derived from collagen possess physiological activities that are useful in food and medical products (Kurata et al. 2007a).

Gallaecimonas pentaromativorans are able to degrade polycyclic aromatic hydrocarbons (PAHs) (Rodriguez-Blanco et al. 2010; Wang et al. 2013). Diverse anthropogenic activities, such as the combustion of fossil fuels and oil spills, are responsible for its accumulation in natural environments. Many high-molecular-mass PAHs (i.e., containing from four to seven benzene rings) are mutagenic, carcinogenic, or teratogenic for a wide range of organisms including fish, amphibians, birds, and mammals. Thus, they are very interesting for bioremediation uses for the treatment of PAH-contaminated sediments.

Another interesting characteristic is found in the genus *Plasticicumulans*. The intracellular accumulation of polyhydroxyalkanoates (PHAs) is ecologically advantageous for bacteria, and it is a commercial interesting feature for biodegradable eco-biotechnology industry. PHA is the raw material for biodegradable plastic manufacturing. Bacteria of *Plasticicumulans* genus are very interesting not only because they produce PHAs but also because they accumulate 85–90 % (w/w) of dry cell weight of PHB in mixed cultures under nonsterile conditions, which is in the most cost-effective way (Jiang et al. 2011).

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