MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, Sept. 2004, p. 403–431 Vol. 68, No. 3
1092-2172/04/$08.00+0 DOI: 10.1128/MMBR.68.3.403–431.2004

Biodiversity of Vibrios

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INTRODUCTION: HISTORICAL ASPECTS

In 1854, the Italian physician Filippo Pacini (1812 to 1883) discovered the first *Vibrio* species i.e., *V. cholerae*, the causative agent of cholera, while studying outbreaks of this disease in Florence. Records of a cholera-like disease may well be traced back to the times of Hippocrates (460 to 377 BC) (38). Pacini examined the intestinal mucosa of fatal victims of cholera by using a microscope and detected *V. cholerae* in all samples. He further pointed out that cholera was a contagious disease, but at that time most scientists and physicians believed in the miasmatic theory of disease (the theory of disease causation by bad or pestilential airs). In the same period, John Snow (1813 to 1858) studied the epidemiology of cholera in several cities of England including Birmingham, London, and Manchester (359; available online at http://www.ph.ucla.edu/epi/snow/snowbook.html). Cholera had killed tens of thousands of people in England between the 1830s and 1850s. According to Snow, cholera was propagated by a "morbid poison entering the alimentary canal." The poison was in (polluted) drinking water. He recommended the provision of pure tap water, free of contamination by sewers and house drains, as an effective means of containing the dissemination of the disease.

Nearly 30 years latter, Robert Koch (1843 to 1910) obtained pure cultures of the deadly *V. cholerae* on gelatin plates. In August 1883, Koch and his team went to Egypt, where cholera had broken out and caused about 100,000 casualties. In Alex-

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andria, they examined a number of fatal cases and always found a characteristic bacterium in the tissue of the intestine, but they were not able to grow the organism. Subsequently, Koch and his team went to India, and by the end of 1883 they had obtained pure cultures of *V. cholerae*. They also described some properties of the organism: "It is a little bent resembling a comma or a spiral. It is highly motile and swarms on gelatine plates" and concluded that this organism was indeed the causative agent of cholera (47). In 1893, an outbreak of cholera occurred in Hamburg, Germany, with about 8,000 fatal cases. Koch was requested to study means of providing improved hygiene in that region. He proposed that water supply systems should incorporate filtration of drinking water in order to remove the bacteria. At that time, Koch and his team also realised that vibrios were ubiquitous in aquatic settings and that many "forms" of vibrios were non-pathogenic for humans (47). The first nonpathogenic *Vibrio* species, i.e., *V. fischeri*, *V. splendidus*, and *Photobacterium phosphoreum* isolated from the aquatic environment, were discovered by the Dutch microbiologist Martinus Beijerinck (1851 to 1931) in the late 1880s.

OCCURRENCE AND IMPORTANCE

According to *Bergey's Manual of Determinative Bacteriology* (1994) and *Bergey's Manual of Systematic Bacteriology* (in press), vibrios (*Vibrionaceae* strains) belong to the *Gammaproteobacteria*, are gram negative, usually motile rods, are mesophilic and chemoorganotrophic, have a facultative fermentative metabolism, and are found in aquatic habitats and in association with eukaryotes. They are generally able to grow on marine agar and on the selective medium thiosulfate-citrate-bile salt-sucrose agar (TCBS) and are mostly oxidase positive.

Vibrios are highly abundant in aquatic environments, including estuaries, marine coastal waters and sediments, and aquaculture settings worldwide (20, 95, 164, 297, 298, 321, 399, 446). Several cultivation-dependent and independent studies have showed that vibrios appear at particularly high densities in and/or on marine organisms, e.g., corals (331), fish (6, 146, 179, 325), molluscs (345), seagrass, sponges, shrimp (133, 404–406) and zooplankton (163, 194, 365, 408, 409).

Photobacterium leiognathi and *P. phosphoreum* are found in symbiotic associations with fish, and *P. leiognathi*, *V. logei*, and *V. fischeri* are found in symbiotic associations with squid. These bacteria colonize the light organs of the host and play a role (via emission of light) in communication, prey attraction, and predator avoidance (120, 126, 339). In the light organs of the squid *Sepiolla* spp., the abundance of vibrios can be as high as 10^{11} cells/organ (120, 285). Newly hatched squid excrete a mucus matrix from the pores of the light organs whereby *V. fischeri* cells present in sea water are caught (94, 289, 290). Subsequently, *V. fischeri* migrates into the organ and colonizes the crypt epithelium. Obviously, the flagella of *V. fischeri* play a crucial role in the colonization of the light organs, but hyperflagellated *V. fischeri* cells containing up to 16 flagella are defective in normal colonization (265). *V. fischeri*, *V. logei*, and *P. leiognathi* are apparently the only three organisms colonizing the light organs of squid, but this seemingly specific partnership remains to be confirmed. *V. fischeri* cells entrapped in the light organs of squid can sense the density of conspecific cells by signaling molecules or pheromones (e.g., *N*-acyl homoserine lactones) and thereby regulate bioluminescence (436). This cell-to-cell communication, or "quorum sensing," may play a role in the evolution of symbiotic bacteria (373). It has also been documented for the pathogens *V. anguillarum* (268), *V. cholerae* (53, 155, 451), *V. harveyi* (237, 252), *V. parahaemolyticus* (165), and *V. vulnificus* (259). These bacteria monitor cell density and regulate the expression of virulence genes by means of quorum sensing. Luminescence and virulence seem to be coregulated in *V. harveyi*, and therefore the infections caused by this organism in shrimp could be controlled by signaling antagonists produced by the alga *Delisea pulchra* (252).

Large numbers of *Vibrio* and *Photobacterium* (4.3×10^6/mm^2) attached to the external surface of zooplankton have also been reported (163). It has been suggested that a close partnership occurs between these bacteria and zooplankton. The biofilm formation by *Vibrio* spp. on the exoskeletons of these crustaceans and other marine organisms may in fact constitute a strategy to survive during starvation and/or other environmental stresses (238, 416). In biofilms these bacteria can use trapped and absorbed nutrients, resist antibiotics, and establish favorable partnerships with other bacteria or hosts. Copepods may, in turn, feed on these bacteria. *V. cholerae* moves along and attaches to surfaces with the aid of the flagellum and pili, which may act as adhesins. In as little as 15 min, *V. cholerae* forms microcolonies on surfaces; subsequently it produces exopolysacharides, which stabilize the pillars of the biofilm. A 15-μm-thick biofilm, with pillars of cells and water channels, is formed within 72 h (273, 423–425). According to these authors, the strong ability of *V. cholerae* E1 Tor to form densely packed biofilms in the environment gives a survival advantage to this organism, which is the predominant cause of cholera. Because *V. cholerae* is closely associated with plankton, it is assumed that cholera outbreaks are linked with planktonic blooms and the sea surface temperature, and so such outbreaks may be predicted by monitoring these parameters by e.g., remote sensing (238). The wide ecological relationships and ability to cope with global climate changes may be a reflection of the high genome plasticity of vibrios (238). Recently, a number of reports have highlighted the pathogenic potential of vibrios toward humans and marine animals (e.g. corals, gorgonians, and shrimp), which may be coupled with rising of sea water temperature due to global warming (215, 253, 331, 350).

Human Pathogens

V. cholerae, *V. parahaemolyticus*, and *V. vulnificus* are serious human pathogens (59a, 122, 415). Cholera is a severe disease mainly in developing countries as a result of poor water supplies and sanitation. The main route of contamination is via water and food. Cholera vectors include zooplankton (e.g., copepods), chironomid insects, and cyanobacteria (186, 238). The last three annual reports (2000 to 2002) of the World Health Organization noticed at least 11,399 deaths worldwide (96% of which occurred in Africa) due to cholera (Table 1) (http://www.who.int/wer) (439, 440). In 2003 and 2004, the World Health Organization reported at least 15,000 new cases of cholera in Africa alone, mainly in Mali, Mozambique, and Zambia.

A *Vibrio* surveillance system maintained by the Centers for

TABLE 1. World statistics of cholera cases and deaths in the last 3 years[a]

Location	2000		2001		2002[b]	
	No. of cases	No. of deaths	No. of cases	No. of deaths	No. of cases	No. of deaths
Africa	118,932	4,610	173,359	2,590	121,568	3,753
America[c]	3,101 (715)	40 (17)	535 (7)	0 (0)	3	0
Asia	11,246	232	10,340	138	2,408	10
Europe	35	0	58	0	5	0
Oceania	3,757	26	19	0	2	0
Total	137,071	4,908	184,311	2,728	123,983	3,763

[a] Data from World Health Organization (http://www.who.int/en/).
[b] Data for the year 2002 are incomplete.
[c] Numbers in parentheses indicate the numbers of cases and deaths in Brazil.

Disease Control and Prevention reported 296 cases of infection caused by vibrios in the United States in 2000 (http://www.cdc.gov/). Most strains ($n = 268$) were isolated from stool, wound, and blood samples. From this collection, 137, 64, and 27 isolates were identified as *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae*, respectively. Most patients ($n = 22$) who died were infected by *V. vulnificus*. The Centers for Disease Control and Prevention report also stated that most cases occur during the summer months and that seafood, e.g., oysters, shrimp, and fish, had been consumed by the patients. *V. cholerae* enters the human host via contaminated food and/or water (415). In the intestine, this bacterium adheres to the epithelium and produces an enterotoxin, cholera toxin (CT) (320). This toxin causes an intense watery diarrhea that may lead to death, but it appears to play no role when *V. cholerae* is in the environment (320). Several virulence genes ($n = 30$ to 40) within the ToxR regulon are involved in cholera disease (37, 84, 445). In addition to the essential role of CT in cholera, the toxin-coregulated pilus (TCP), encoded by the *tcpA to tcpF* genes, is pivotal for the colonization of the intestine epithelium. TCP helps in microcolony formation on the epithelial surface. Other colonization factors include mannose-fucose hemagglutinin, regulatory proteins (e.g., ToxR/ToxS and ToxT), outer membrane porins, biotin and purine biosynthetic genes, iron-regulated outer membrane proteins (e.g., IrgA), the O antigen of the lipopolysaccharide, and accessory colonization factors (115, 116, 320). Motility and chemotaxis also play a role in virulence (51, 424, 425). Studies of gene expression in vivo have shown that about 200 genes encoding flagellin synthesis, ribosomal proteins, iron transport, and anaerobic metabolism, as well as more than 700 genes of unknow function, are induced during cholera infection (37, 447).

V. parahaemolyticus causes gastroenteritis in which the hemolysins, thermostable direct hemolysin (TDH) and/or TDH-related hemolysin (TRH), have been considered to play a crucial role (171, 195, 284, 371). It has been suggested that *V. parahaemolyticus* acquired the genes encoding these hemolysins via horizontal gene transfer (284, 303). Raimondi et al. (317) have proposed that TDH acts as a porin in the enterocyte's plasma membrane and allows the influx of multiple ionic species, e.g., Ca^{2+}, Na^{+}, and Mn^{2+}. A high concentration of TDH increases the number of porin channels, and this, in turn, results in ionic influx, culminating in cell swelling and death due to osmotic imbalance (317).

Whole-genome sequencing of *V. parahaemolyticus* (251) revealed that the organism possesses two sets of genes for the type III secretion system (TTSS). TTSS is one of the bacterial protein secretion systems that secretes bacterial proteins into the extracellular environment, but it can also inject bacterial proteins directly into target host eukaryotic cells (175). TTSS is essential for the pathogenicity of bacterial pathogens such as *Salmonella*, *Shigella*, *Yersinia*, and plant pathogens, which cause disease by intimate interactions with eukaryotic cells. However, TTSS is absent from the genome of *V. cholerae* (162). Gene disruption experiments demonstrated that the two TTSSs of *V. parahaemolyticus* are functional and that they play a role in the pathogenicity of the bacterium (K. S. Park, T. Ono, M. Rokuda, M. H. Jang, K. Okada, T. Iida, and T. Honda, submitted for publication). Other toxins, proteases, cytolysins, and pili may also play a role as virulence factors in both *V. parahaemolyticus* and *V. vulnificus*. In the genome of *V. parahaemolyticus* (251), other genes that may be involved in pathogenicity have been identified. These genes include those used for bacterial adherence and biofilm formation, such as the genes for the biosynthesis of several pili and the *tad* genes (198) and for several toxin homologues including an RTX toxin. Certain strains of *V. parahaemolyticus*, probably derived from a common clonal ancestor, have recently caused a pandemic of gastroenteritis (58, 59, 69, 91).

V. vulnificus is an important etiologic agent of wound infections and septicemia in humans (59a, 122). This sort of septicemia occurs mainly in immunosuppressed people and in patients with high levels of serum iron (caused by genetic mutation, e.g., hemochromatosis, or by liver diseases, e.g., cirrhosis). Iron seems to enhance the virulence of vibrios. A capsular polysaccharide (CPS) is the primary virulence factor in *V. vulnificus* pathogenesis (270, 422, 441). The presence of this factor correlates with the opaque colony phenotype and is thought to play a inflammatory role within the human body. Smith and Siebeling (357) described four essential genes, i.e., *wcvA*, *wcvF*, *wcvI*, and *orf4*, responsible for the synthesis of CPS. They showed that mutation in any of these genes results in loss of capsule, which is typical of an avirulent translucent colony phenotype (441). Two lytic bacteriophages, i.e., CK-2 and 153A-5, have been successfully used to treat local and systemic infections caused by *V. vulnificus* in mice (64). A dose of 10^8 phage/mice significantly reduced the number of *V. vulnificus* organisms isolated from wounds and liver of mice. Estrogen seems to provide protection against *V. vulnificus* lipo-

polysaccharide-induced endotoxic shock in rats, halving the mortality rate of infected animals (263).

Other vibrios, e.g., *Grimontia hollisae*, *P. damselae*, *V. alginolyticus*, *V. cincinnatiensis*, *V. fluvialis*, *V. furnisii*, *V. harveyi*, *V. metschnikovii*, and *V. mimicus*, have been sporadically found in human infections (1, 46, 56, 89, 113, 114, 443). Apparently, they are less important as human pathogens (113, 114).

Coral Pathogens

Corals consist of a coral host, e.g., the reef-building Scleractinia order (Anthozoa class) and symbiotic unicellular algae, the Zooxanthellae (341). Each coral colony may comprise thousands tiny coral polyps that are responsible for production of the calcium carbonate of the reefs. Coralline algae also play a role in the formation of coral reefs by cementing various corals together with compounds of calcium, while other organisms such as tube worms and molluscs donate their hard skeletons. In this partnership, various types of reefs are constructed by these organisms (83). Coral reefs are highly productive and very diverse ecosystems within coastal tropical environments, mainly in oligotrophic regions (83, 169). Brazilian coral reefs have an initial growth as a mushroom-like, relatively low diversity of coral fauna (mostly relics from the Tertiary), with an abundance of incrusting coralline algae and surrounding water rich in muddy siliciclastic sediment and nutrients (228).

Coral reefs are important sources of income for several countries via tourism and fishing, but they also represent protection to coastal areas and may be a source of biologically active compounds e.g., antimicrobials and antivirals. Tourism in the Caribbean generates nearly 90 billion dollars annually (169). Corals reefs may be used as clarifying agents in the sugar industry and as as building materials (228). Some 30 coral diseases (3 of them caused by microbial consortia) have been documented so far, but only 5 have fulfilled the Koch's postulates (370). Coral bleaching, i.e., the paling or the loss of color due to the disruption of symbiosis between the coral host and symbiotic Zooxanthellae, is one of the most serious diseases affecting corals worldwide (332, 370), although it is sometimes reversible in 3 to 6 months (48, 83, 308, 322, 323, 370).

Coral bleaching is thought to be linked to the increased seawater temperature due to recent global climate changes caused by greenhouse gas emissions, although other factors such as seawater eutrophication by sewage and aquaculture, sedimentation, light (UV radiation and photosynthetically active radiation), pollution by heavy metals, and reduction of salinity may also play a role (332). The strongest bleaching episodes have occurred during El Niño years, when surface seawater temperatures reach maxima higher than the summer maximum. The pivotal role of bacteria in coral bleaching and the effect of temperature in bacterial virulence have been studied by Rosenberg and collaborators (32, 33, 331, 333). *V. shilonii* (also known as *V. mediterranei*) and *V. coralliilyticus* have been proven to bleach corals, and their pathogenicity was shown to be temperature dependent.

V. shilonii was identified as an etiological agent of the bleaching of *Oculina patagonica*, and the main disease steps, i.e., adhesion, penetration, and multiplication (up to 10^9 CFU/cm^3 in 5 days) within the coral tissues have been described in detail (18, 19, 331). Within the coral tissues, most *V. shilonii* cells become viable but nonculturable (VBNC) but continue to be virulent. According to Sussman et al. (369), the fire worm *Hermodice carunculata* is a winter reservoir and summer vector of *V. shilonii*.

V. coralliilyticus, another temperature-dependent pathogen, was shown to cause patchy necrosis of tissues of *Pocillopora damicornis* when the coral was incubated at temperatures of 27°C or higher (33). Because tropical seawater temperatures have undergone warming in the past 100 years and increases of 1 to 2°C have been predicted by 2100 as a result of increased emission of greenhouse gases, it is expected that bleaching episodes will occur even more frequently (169). Infectious diseases may reduce the global diversity of corals (241).

Recent work on the diversity of vibrios associated with coral bleaching in Davies Reef and Magnetic Island (Great Barrier Reef, Australia) and in the Kaneohe Bay (Hawaii) indicated that different species, i.e., *Enterovibrio coralii*, *P. eurosenbergii*, *V. fortis*, *V. campbellii*, *V. harveyi*, *V. mediterranei*, and *V. rotiferianus*, may be involved in the process of coral bleaching (F. L. Thompson, D. Gevers, P. Dawyndt, C. C. Thompson, S. Naser, B. Hoste, and J. Swings, submitted for publication; F. L. Thompson, C. C. Thompson, S. Naser, B. Hoste, C. Munn, D. Bourne, and J. Swings, submitted for publication). *V. harveyi* has been implicated in the disease of a wide range of marine animals, including bleaching in *O. patagonica* and white band disease in *Acropora cervicornis* (14, 139, 370).

Nutrient Cycling

There are indications that vibrios play a role in nutrient cycling in aquatic environments by taking up dissolved organic matter (352, 353). Vibrios may provide essential polyunsatured fatty acids to the aquatic food web, which many aquatic organisms cannot produce de novo (88, 282). Vibrios are also able to break down chitin, a homopolymer of *N*-acetyl-D- glucosamine, which is one of the largest pools of amino sugars in the oceans (85–87, 324). *Vibrio harveyi*, for instance, excretes at least ten different chitin-degrading enzymes (367, 372). Accordingly, it was suggested that this ability may explain the ubiquitous occurrence of vibrios in aquatic settings (324). Some *Vibrio* species are able to degrade toxic polycyclic aromatic hydrocarbons within polluted marine sediments (161). Vibrios are important producers of antibiotics among marine bacteria (240). Inhibitory compounds produced by certain *Vibrio* isolates reduced the number of other community members, e.g., *Alfaproteobacteria* and *Alteromonas*. Long and Azam (240) supposed that this strategy accounts for the microscale variations in competing bacterial populations. Because vibrios are selectively grazed by flagellates, it has been suggested that they contribute to the cycling of organic matter in aquatic settings (29).

Role in Aquaculture

Vibrios are important bacterial pathogens for animals reared in aquaculture (14, 35, 168, 236). *V. anguillarum*, *V. salmonicida*, and *V. vulnificus* are among the main bacterial pathogens of several fish species (14), and *V. harveyi* is a major pathogen of shrimp, e.g., *Litopenaeus vannamei* and *Penaeus monodon* (15, 225, 227). Mortality caused by vibrios in reared fish and shellfish is very common during early larval stages and can

occur suddenly, leading sometimes to death of the entire population (42, 96, 157, 184, 185, 187, 217, 230, 283, 287, 295). Pathogenicity of *V. harveyi* is associated with the presence of a bacteriophage (15, 291). A bacteriological survey of cultures of sea bream (*Sparus aurata* L.) in Spain between 1997 and 2000 detected 25 outbreaks (450). Strains were isolated mainly from the spleen, liver, and kidney of diseased fish, and *Vibrio* was the dominant group, accounting for about 69% ($n = 71$) of the total number of isolated strains. It was striking that in different studies several *Vibrio* isolates had phenotypes different from those of known *Vibrio* species and thus remained identified only at the genus level (13, 276, 281, 406, 450).

The mode of infection in fish consists of three basic steps (14, 223, 224): (i) the bacterium penetrates the host tissues by means of chemotactic motility; (ii) within the host tissues the bacterium deploys iron-sequestering systems, e.g., siderophores, to "steal" iron from the host; and (iii) the bacterium eventually damages the fish by means of extracellular products, e.g., hemolysins and proteases. Grisez et al. (145) showed that infection of turbot (*Scophthalmus maximus*) larvae by *V. anguillarum* occurs in the intestinal epithelium, where the pathogen invades the bloodstream and spreads to different organs, culminating in death of the fish. More recently, Ringo et al. (326) detected bacterial endocytosis in the pyloric ceca and midgut of arctic charr (*Salvelinus alpinus* L.) adults and suggested that the whole gastrointestinal tract of fish may be subject to infection.

Internal symptoms of disease in fish caused by strains of vibrios include intestinal necrosis, anemia, ascitic fluid, petechial hemorrhages in the muscle wall, liquid in the air bladder, hemorrhaging and/or bloody exudate in the peritoneum, swollen intestine, hemorrhaging in or on internal organs, and pale mottled liver (14). External symptoms include sluggish behavior, twirling, spiral or erratic movement, lethargy, darkened pigment, eye damage/exophthalmia, hemorrhaging in the mouth, gill damage, white and/or dark nodules on the gills and/or skin, fin rot, hemorrhaging at the base of the fins, distended abdomen, hemorrhaging on the surfaces and muscles, ulcers, and hemorrhaging around the vent.

Using a very robust crustacean model organism, i.e., *Artemia* spp., and with the aid of transmission electron microscopy, Verschuere et al. (410) established the infection route of *V. proteolyticus* CW8T2. These investigators first infected *Artemia nauplii* by inoculating the pathogen in the rearing water. One day later, they detected bacteria that had penetrated "in" the gut epithelium, with subsequent tissue damage, qualified by the authors as "devastating," and had spread toward the host's body cavity. This study illustrates well the infectious capability of certain *Vibrio* strains and suggests that vibriosis in penaeid shrimp larva rearing systems would be even more devastating, taking into account the fragility of these larvae. Lavilla-Pitogo et al. (225) have reported massive losses in shrimp cultures in Philippines due to a so-called group of "luminous vibrios." According to these authors, there was a decrease of nearly 60% in the survival of reared shrimp between 1992 and 1994, associated with the presence of luminous vibrios in rearing water. Lavilla-Pitogo et al. (225) recommended to farmers that shrimp rearing should not start unless luminous vibrios were absent. The rationale that all luminous vibrios are invariably associated with disease outbreaks in shrimp rearing contrasts with the results obtained by Fidopiastis et al. (120, 121), McFall-Ngai (260, 261), Oxley et al. (301), and Ruby (339), among others, who have reported beneficial and/or harmless partnership between certain luminous vibrios e.g. *V. logei* and *V. fischeri* and host invertebrates. For instance, Oxley et al. (301) examined the bacterial flora of healthy wild and reared *Penaeus mergulensis* shrimp and found a high abundance of vibrios (including *V. logei* at ca. 10^4 to 10^5 CFU/g of gut). The authors also found that the bacterial floras of wild and reared penaeid shrimp are similar and suggested that shrimp may influence and/or select the composition of their gut microbiota. In the light of the current knowledge about the bacterial population structure of certain human pathogens, e.g., *Neisseria* spp. (256), it is more likely that under favorable conditions (e.g., high nutrient loads and high animal density) within rearing systems, a certain hypervirulent strain (or clone) dominates the bacterial community and causes disease in fish and shellfish rather than the disease being caused by the whole bacterial species. This view implies that only a minority of *Vibrio* strains are true pathogens and further underscores the idea that many *Vibrio* species are opportunistic pathogens.

The pathogenic effects of certain strains of vibrios are critical in aquaculture settings, where organisms, e.g., penaeid shrimps and salmonids, are reared at high densities under very artificial and unstable conditions (14, 35, 295). To maintain the productivity of such an intensive aquaculture, high inputs of fish protein originating from the sea have to be employed for feeding, together with high levels of water exchange and the massive use of antibiotics. It seems that the combination of these conditions favors the proliferation of vibrios and enhances their virulence and disease prevalence. This highly intensive aquaculture has disastrous effects for the environment (132, 279, 280, 431). According to Nailor et al. (279, 280) some of the most serious negative environmental impacts are (i) loss of wild fish (5 kg of wild fish has to be caught to feed 1 kg of carnivorous fish reared), (ii) loss of natural habitats (e.g., mangroves), (iii) use of wild seed to stock ponds, (iv) impact of foreign fish and shellfish introduced in the wild, and (v) effluent discharge and coral reef degradation. The spread of antibiotic resistance from aquaculture settings to natural environments has recently been shown (154, 178, 235, 419). About 70% ($n = 100$) of the vibrios isolated from aquaculture settings in Mexico are multiple-drug resistant (271, 330). Several *Vibrio* isolates have acquired resistance to the most commonly employed antibiotics (e.g., enrofloxacin, florfenicol, trimethoprim, and oxytetracycline) in shrimp rearing, suggesting that the recently initiated application of these antimicrobials has led to the generation of resistant strains of vibrios (271, 330). Ben-Haim et al. (34) have advanced the hypothesis that aquaculture settings serve as foci or reservoirs for pathogenic *Vibrio* strains: during certain periods of the year, pathogenic vibrios would endure adverse environmental conditions within aquaculture settings and when favorable environmental conditions are reestablished, vibrios would be able to cause disease in wild animals.

Alternatives involving more environmentally sound aquaculture have been proposed (35). Vaccination has been successfully used to control *V. anguillarum* and *V. vulnificus* infections in fish (49, 124). Because certain *Vibrio* strains may be potential probiotics and/or symbionts of commercially important

organisms such as penacid shrimp, salmonids, flatfish, oysters, and abalones, recent studies have suggested that such strains could act as biocontrol agents in aquaculture, diminishing the need for antibiotics and reducing effluent discharges (99, 327, 411). The normal bacterial community associated with *L. vannamei* has recently been examined in order to find potential probiotic organisms (133–135, 274, 404, 405). Planktonic and particle-associated vibrios seem to enhance the survival and growth of reared *L. vannamei*. Moss et al. (274) reported that *Vibrio* and *Aeromonas* compose up to 85% of the bacterial flora in the gut of this shrimp (about 10^9 CFU/g of gut tissue), whereas Gomez-Gil et al. (133) found a wealth of vibrios, i.e., 10^5 CFU/g and 10^4 CFU/ml, respectively, in the hepatopancreas and hemolymph of healthy *L. vannamei*.

Pujalte et al. (313) have reported a dominance of vibrios associated with cultured oysters: up to 6.5×10^5 CFU/g of oyster but only 10^2 CFU/ml in rearing seawater. Using fluorescence in situ hybridization (FISH), the same authors determined that vibrios accounted for up to 40% (156 cells/ml) of the heterotrophic culturable flora grown on marine agar. In a successful recirculating rearing system for rotifers, the abundance of *Vibrio* spp. was up to 1.7×10^5 CFU/ml, suggesting that these bacteria were playing a positive role in the health of the rotifers (365). These strains were later classified as a new species, *V. rotiferianus* (136).

Sawabe et al. (345) estimated the abundance of *V. halioticoli* strains in the gut of several abalone (*Haliotis*) species. They reportd that *V. halioticoli* is the dominant culturable bacterium, representing 40 to 64% of the total heterotrophic community, which varied from 10^3 to 10^7 CFU/g of gut. *V. halioticoli* strains were found to produce large amounts of acetic and formic acids (up to 68.1 mM), which may in turn be used as an energy source or precursor for protein synthesis by the abalones. The authors suggested that a mutual relationship may exist between *V. halioticoli* and abalones (173).

Because the use of probiotics for humans and domestic animals, e.g., pigs and chickens, has had a certain success (377), several researchers advocate that the use of probiotic bacterial strains or selected mixtures will have a positive impact on health management in marine organisms (10, 295, 411). A considerable difference between the culture of domestic and aquatic animals is that the latter are in constant and intimate contact with a wealth of microrganisms, e.g., viruses, protozoa, and fungi (352, 353). Unfortunately, studies of the use of "probiotic" bacteria have not looked at the interactions with the aquatic microbial food web (16, 352). The so-called probiotic bacterial strains could well be fueling the food web, giving rise to a high abundance of e.g., protozoan flagellates and ciliates, which in turn would be grazed by fish and/or shellfish larvae, improving their survival and growth (383).

ISOLATION AND MAINTENANCE

Vibrios are fairly easy to isolate from both clinical and environmental material, although some species may require growth factors and/or vitamins. There are several commercial media which may be used for the isolation of vibrios, but tryptone soy agar (Oxoid or Difco) supplemented with 1 to 2% NaCl and marine agar (Difco) generally allow the growth of very healthy colonies after 1 to 2 days of incubation at 28°C. Vibrios grow well at temperatures between 15 and 30°C, depending on the strain under analysis. Obviously, psychrophilic vibrios, i.e., *V. logei*, *V. wodanis*, and *V. salmonicida*, grow poorly at temperatures higher than 20°C. It is recommended to grow strains of these species at 15°C in Luria-Bertani broth (Difco) supplemented with 1 to 3% NaCl. Some *Vibrio* species, e.g., the *V. halioticoli* group and *V. agarivorans*, require addition of sodium alginate (0.5%) marine agar (343). Thiosulfate-citrate-bile salts agar (TCBS; Oxoid) is an ideal medium for the selective isolation and purification of vibrios. Strains which are able to use sucrose will form yellow colonies, while the others are green. So far this is the only proven selective medium for isolation of vibrios, although some isolates of *Staphylococcus*, *Flavobacterium*, *Pseudoalteromonas*, and *Shewanella* may present slight growth on it as well.

Most vibrios (except *V. ezurae*, *V. gallicus*, *V. pectenicida*, *V. penaeicida*, *V. salmonicida*, and *V. tapetis*) withstand the freeze-drying process very well. Coincidentally, 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 tryptone soy agar. Normally, these ampoules are filled with 0.01 g of bacterial culture previously suspended in 0.5 ml of cryoprotectant mix (horse serum–D- glucose–nutrient broth–MilliQ water, 3:0.3:0.3:1). Alternatively, strains may be kept viable in Microbank vials, which contain 10% glycerol and porous beads, at −80°C for at least 5 years.

GENOTYPIC IDENTIFICATION

An array of phenotypic and genomic techniques have become available for the identification of vibrios in the last three decades (97, 148, 296, 314, 315, 342, 402, 403). Ribotyping and PCR-based techniques, e.g., amplified fragment length polymorphism (AFLP), fluorescence in situ hybridization (FISH), amplified ribosomal DNA restriction analysis (ARDRA), random amplified polymorphic DNA (RAPD), repetitive extragenic palindromes (rep), and restriction fragment length polymorphism (RFLP), along with multilocus enzyme electrophoresis (MLEE) and multilocus sequence typing (MLST), have yielded the most valuable information about and new insights into the population structure of some species of the *Vibrionaceae* and have also provided a means of identifying these organisms. Below, we discuss some of the methods which have been most commonly used for the identification and typing of vibrios.

Amplified Fragment Length Polymorphism

The AFLP technique consists of three main steps: (i) digestion of total genomic DNA with two restriction enzymes and subsequent ligation of the restriction half-site-specific adaptors to all restriction fragments; (ii) selective amplification of these fragments with two PCR primers that have corresponding adaptor and restriction site sequences as their target sites; and (iii) electrophoretic separation of the PCR products on polyacrylamide gels with selective detection of fragments which contain the fluorescently labelled primer and computer-assisted numerical analysis of the band patterns (177, 193, 413). Originally, Vos et al. (413) used radioactively labeled primers,

but now AFLP is performed mainly with fluorescently labeled primers. AFLP measures the variation in the whole genome and thus is considered to give useful information about the short- and long-term evolution of bacterial strains (190). Janssen et al. (188) were the first to use AFLP as a tool in bacterial taxonomy. They examined 147 strains that had a broad range of G+C content (24 to 71%), and focused mainly on *Aeromonas* ($n = 90$) and *Xanthomonas* ($n = 36$). They also included three *V. anguillarum* strains and one *V. tubiashii* strain. The grouping obtained by AFLP corresponded well to that obtained by DNA-DNA similarity data. Janssen et al. (188) also reported that the complexity (i.e., the 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 (381), including *V. alginolyticus* (405), *V. cholerae* (191, 192, 221), *V. harveyi* (139, 307), *V. vulnificus* (7, 8), *V. wodanis* (31), and *P. damselae* (391), but most of these studies did not include all the recognized *Vibrio* species. Arias et al. (7, 8) examined 80 *V. vulnificus* strains by several phenotypic (Biolog, API, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, serotyping, enzyme-linked immunosorbent assay) and genotypic (AFLP and ribotyping) methods. With AFLP analysis, the authors were able to discriminate strains with identical ribotypes, and thus they concluded that AFLP is the most suitable and discriminatory tool for epidemiological studies of *V. vulnificus*. Other AFLP analyses of 94 *Vibrio* strains clearly pointed out that *V. carchariae* was a synonym of *V. harveyi* and also indicated that 34 isolates were different from known *Vibrio* species (307). Vandenberghe et al. (405) discriminated pathogenic and probiotic *V. alginolyticus* strains by using AFLP and concluded that this technique can be used to authenticate probiotic cultures prior to their use. Thyssen et al. (391) used AFLP to differentiate the two subspecies of *P. damselae*, i.e., *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida*.

Jiang et al. (191, 192) discriminated *V. cholerae* serogroups O1 and O139 by using AFLP with the ApaI and TaqI restriction enzymes. They found that the genetic backgrounds of environmental and clinical *V. cholerae* strains are quite similar and concluded that pathogenic strains may in fact arise from nontoxigenic strains within the aquatic environment. Jiang et al. (191) demonstrated by AFLP analysis that the population structure of *V. cholerae* undergoes seasonal shifts. Certain clones are abundant in winter, and others are abundant in summer. More recently, Lan and Reeves (221) examined 45 *V. cholerae* isolates from the seventh pandemic and partitioned these isolates into 38 AFLP profiles. They concluded that AFLP is the best tool for discriminating clones from the seventh pandemic and suggested the design of PCR primers which target particular AFLP bands that could be used for epidemiological analysis through multiplex PCR or microarays analyses.

Colony Hybridization by Species-Specific Probes

Detection of vibrios on selective media and subsequent colony hybridization with species-specific probes based on variable regions of the 16S rRNA has also been evaluated as a fast screening alternative tool for *V. anguillarum* (254), *V. halioticoli* (376), *V. harveyi* (158), *V. parahaemolyticus* (356), *V. proteolyticus* (277), *V. scophthalmi* (63), and *V. vulnificus* (61, 62). It was demonstrated that different "selective" media were not quite selective, and species-specific media are yet to be formulated. The specificity of certain probes, e.g., for detection of *V. anguillarum*, is not sufficient since they also hybridize with *V. ordallii*, *V. diazotrophicus*, and *V. navarrensis* (254). The probe for *V. scophthalmi* detection has not been evaluated against *V. ichthyoenteri*. The two species have nearly 100% 16S rRNA similarity, and there is thus a great chance of cross-hybridization. *V. scophthalmi* and *V. ichthyoenteri* have been isolated from similar fish hosts, i.e., turbot, but *V. scophthalmi* is argued to be probiotic while *V. ichthyoenteri* is a pathogen (60). Misleading conclusions could arise from ecological studies using this probe (63). The probe for *V. vulnificus* detection seemed to be very reliable (62).

Fluorescence In Situ Hybridization

The application of cultivation-independent techniques such as direct extraction of nucleic acids from environmental samples (e.g., water, gut tissue, and sediment) followed by clone library and 16S rRNA sequencing or alternatively FISH of filter-fixed cells with oligonucleotide probes targeting the 16S rRNA and subsequent visualization by epifluorescence microscopy has provided an efficient means of detecting, identifying, and quantifying marine bacteria, including vibrios (103, 104, 131, 318). These approaches have shed light on the distribution and ecology of vibrios in the marine environment and have overcome the problem of the great plate count anomaly, i.e., the difference of the order of 10^2 to 10^3 between direct cell counts by e.g., epifluorescence microscopy and the CFU counts on, e.g., marine agar plates (16). Vibrios may be in a dormant state (VBNC) or may not be able to grow on the selective media employed (82).

What do we know about the nonculturable vibrios? In 1982, Xu et al. showed that certain bacteria, e.g., *V. cholerae*, although metabolically active, were not able to grow on culture media. At that time, these authors already knew that environmental stresses (e.g., nutrient limitation or starvation and variations in pH, salinity, and temperature) could lead to such a state, for which they proposed the name "viable but nonculturable." Some researchers hypothesize that this is a "genetically programmed physiological response" to enable some bacteria to survive in the environment (258). Changes observed in VBNC bacteria include reduction of cell size, increase of cell wall thickness, decrease in the amount of RNA and DNA, and biofilm formation. Several *Vibrio* species, e.g., *V. cholerae*, *V. shillonii*, and *V. vulnificus*, can be VBNC and virulent. *V. shillonii* becomes VBNC when entering the cells of *O. patagonica*, but it remains metabolically active and multiples within its host (331, 333).

Several authors have recently shown that the most abundant prokaryotic groups, e.g., *Archaea*, cyanobacteria, the *Cytophaga-Flavobacterium* group, *Roseobacter*, SAR11, SAR86, SAR116, and SAR202 (α and/or β proteobacteria), found in the marine environment are not readily culturable and that vibrios are rarely found in clone libraries from environmental samples (88,

131, 318). Although a high abundance of vibrios occurs in euthrophic coastal waters and in association with eukaryotes, these authors showed that vibrios represent only a minor fraction of the total bacterioplankton.

Heidelberg et al. (164), in a study of the bacterioplankton in the Chesapeake Bay, showed that γ proteobacteria compose up to 10% (3.1×10^8 cells/liter) of the total bacteria, while *Vibrio* and *Photobacterium* compose up to 4% (2.1×10^8 cells/liter) of the total bacteria. In the North Sea, vibrios accounted only for 10^3 cells/ml (mainly particle associated) when genus-specific probes were used in FISH detection (103). The same authors found that by adding organic substrates (in micromolar concentrations) to the water, vibrios became dominant, reaching up to 65% (9.7×10^5 cells/ml) of the total bacteria in a few hours (104). Vibrios not only could rapidly respond to nutrient-enrichment experiments but also maintained viability for up to 50 days under starved conditions. These authors concluded the high rRNA content of vibrios provide the potential for such rapid responses, which allow them to grow rapidly, outcompeting other members of the bacterial community. The increase in nutrient concentration in the water could lead to an increase in the size of the cells of vibrios, which in turn would escape predation by protozoans. Beardsley et al. (29) have indeed suggested that the low abundance of vibrios observed during certain periods and in some places may be the effect of massive selective grazing by heterothrophic nanoflagellates, which are abundantly found in aquatic environments.

The low fluorescence intensity of marine bacteria is one of the main drawbacks of FISH technology (103, 104). This is not really a problem for vibrios since these organisms have a high content of ribosomes. On the other hand, because several *Vibrio* species (e.g., *V. harveyi*, *V. campbellii*, *V. rotiferianus*, and other closely phylogenetic neighbours) have very similar 16S rRNA sequences, it may be difficult to perform reliable species identification.

Microarrays

Microarrays have been successfully developed since the middle of the last decade (348). This method may be considered a refinement of Northern and Southern blot techniques and may be an alternative to DNA-DNA hybridizations currently performed in bacterial taxonomy (361, 432). Microarrays have been successfully used for the detection and quantification of bacteria in the environment (41). Dziejman et al. (101) constructed a microarray based on the genome sequence of *V. cholerae* N16961. They spotted about 3,600 open reading frames (ORFs), corresponding to about 93% of the bacterial genome, and showed that nine different representative strains of *V. cholerae* have only a 1% difference in gene content. It was also shown that the seventh-pandemic clone of *V. cholerae* contains two islands, i.e., VSP-I and VSP-II, that were probably acquired via horizontal gene transfer.

Cho and Tiedje (68) successfully designed a microarray, containing up to 96 genomic fragments (about 1 kb long), for the identification of *Pseudomonas* species. The DNA chip designed showed good correlation with DNA-DNA homology measrurments. It was suggested that a chip containing 100,000 genomic fragments would allow the identification of most gram-negative bacteria (68).

Multilocus Enzyme Electrophoresis and Multilocus Sequence Typing

MLEE was first applied to bacterial systematics in the 1980s and has become the standard technique for studies of population genetics (57, 351) and identification (412). MLST was developed recently as an improved adaptation of MLEE and has been advocated as the most reliable molecular tool for epidemiology (250, 400). Both techniques index the variation in housekeeping genes. MLST assigns alleles directly from the nucleotide sequences, while MLEE compares the electrophoretic mobility of the enzymes encoded by the genes (118). Obviously, MLST has several advantages over MLEE, e.g., higher discriminatory power because it detects synonymous and nonsynonymous changes, accuracy and portability of the data, ease of performance, and reproducibility (250). MLEE analysis of 397 *V. cholerae* strains isolated from Mexico and Guatemala suggested that horizontal transfer and recombination are important processes in the evolution of clonal complexes of *V. cholerae* and indicated that successful clonal complexes may persist for decades (30). A high genetic diversity, as assessed by MLEE of 15 enzyme loci, was observed among 107 diverse *V. cholerae* isolates (109). These isolates displayed 99 different electrophoretic patterns and a large number of alleles (i.e., two to seven) per locus, but no significant clustering between serogroups, biotype, and country of isolation was observed (109). These authors applied MLST of six housekeeping-enzyme loci, i.e., *asd*, *cadA*, *idh*, *lap*, *mdh*, and *epd*, on a subset of 31 *V. cholerae* serogroup O139 strains and found four distinct groups of strains (110). They concluded that recombination has not occurred among these vibrios. Splits decomposition analysis of 10 representative *V. cholerae* strains of our collection indicated that *epd*, *asd*, and *lap* may have suffered recombination (unpublished data). The *lap*, *idh*, and *mdh* gene sequence similarity among the 10 *V. cholerae* strains was >98%, whereas for *asd* and *epd* the similarity was >96 and >93%, respectively. A more comprehensive study carried out by Garg et al. (128) analyzed the sequences of *dnaE*, *lap*, *recA*, *pgm*, *gyrB*, *cat*, *chi*, *rstR*, and *gmd* of 96 *V. cholerae* O139 strains isolated in India between 1992 and 2000. The strains were partitioned into 51 sequence types, whereas the most common alleles were present in at least 77% of the isolates. Overall, the gene sequence similarity for the different loci was higher that 98%, except for *recA*, for which it was around 97%. This study also indicated that conspecific homologous recombination may have occurred in different loci (e.g., *gmd*, *recA*, and *lap*). Such events would lead to cohesion of the species (128). MLST of four loci, i.e., *gyrB*, *recA*, *dnaE*, and *gnd*, applied to 81 isolates of *V. parahaemolyticus* revealed that pandemic strains are clonal (70). The intraspecies gene sequence similarity was >98% for *dnaE* and *gnd*, >97% for *gyrB*, and >96% for *recA*. Strains of a single serotype had different sequence types, suggesting that genes coding for different serotypes suffered lateral transfer in *V. parahaemolyticus*. Clearly, MLST data will be useful to delineate species in vibrios.

Random Amplified Polymorphic DNA and Repetitive Extragenic Palindrome PCR

RAPD involves PCR amplification of random fragments of genomic DNA by using arbitrary primers, while rep-PCR amplifies intervening sequences located between highly repetitive DNA motifs (97). This technique has been used mainly with the aim of typing within the species *V. alginolyticus* (366), *V. cholerae* (328, 329, 449), *V. parahaemolyticus* (437), and *V. vulnificus* (421), and it is thus difficult to determine its taxonomic resolution and value for the whole family *Vibrionaceae*. According to Sudheesh et al. (366), *V. alginolyticus* and *V. parahaemolyticus* have different RAPD profiles and can be reliably separated by this fast screening method. Wong and Lin (437) compared RAPD, rep-PCR, pulsed-field gel electrophoresis, and ribotyping and concluded that rep-PCR is the most discriminatory of the techniques. Rivera et al. (328) analyzed 83 *V. cholerae* strains by rep-PCR and found that toxigenic and nontoxigenic strains had different patterns. They concluded that this technique could be used in epidemiological studies.

rep-PCR was used to identify presumptive *V. harveyi* isolates responsible for luminous vibriosis in shrimp (139). These isolates had the major phenotypic features of the species *V. harveyi* (4, 5, 114, 170). They grew on TCBS agar, were motile, fermented glucose, were oxidase positive, and were 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 utilise L-histidine or 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 abalone gut (*Haliotis* spp.) (344). rep-PCR patterns using the primer GTG_5 showed that each abalone species has a particular population of vibrios which is related to *V. halioticoli*.

Real-Time PCR

Rapid-cycle real-time PCR is a high-throughput technique and is based on the kinetic reaction paradigm where multiple reactions, i.e., denaturation, annealing, and extension, may occur simultaneously (434). DNA extracted directely from water, food, or fecal samples is used as the template for PCR. PCR products are detected with a double-stranded DNA dye, e.g., SYBR Green I (127, 302), or probes (54, 242) and occurs in about 1 h. The sensitivity of this technique is high, and it can detect as few as 10 *V. cholerae* CFU per ml of water sample (242). Reliable identification of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* is apparent when a combination of 20 species-specific genes from different human pathogens are used (127). This technique promises to be very useful for rapid detection, identification, and quantification of vibrios, but this remains to be shown in more comprehensive studies including all currently known species.

Restriction Fragment Length Polymorphism

The PCR-RFLP technique consists of PCR amplification of certain genes, e.g., 16S rRNA, *gyrB*, and *rpoD*, and subsequent restriction of the PCR products with endonucleases to obtain band patterns (71, 72, 233, 375, 394, 395, 397). According to Urakawa et al. (394), who analyzed the restriction patterns of the 16S rRNA of 35 *Vibrionaceae* species, this technique is useful for the classification and identification of *Vibrionaceae* strains. A closer examination of the data presented by these authors reveals that all the *Vibrio* core group species (i.e., *V. alginolyticus*, *V. parahaemolyticus*, *V. proteolyticus*, *V. harveyi*, and *V. campbellii*) and *V. vulnificus* have the same band pattern and were thus indistinguishable. *P. iliopiscarius*, *P. leignathi* and *P. phophoreum* showed identical genotypes. This is quite striking since the 16S rRNA sequence similarity between these species is <96.5%, clearly proving the low discriminatory power of PCR-RFLP.

Ribotyping

Ribotyping consists of four main steps: (i) restriction of the bacterial chromosome with an endonuclease, (ii) gel electrophoresis of the resulting fragments, (iii) transfer of the fragments to a membrane, and (iv) hybridization of the gel with a labeled probe complementary to the 16S and 23S rRNAs (143). Ribotyping was one of the first fingerprinting techniques to be successfully used in the taxonomy of vibrios, and it has been particularly useful in the study of *V. cholerae* (143, 144, 218, 310, 311). A standardized ribotyping scheme was proposed as a reliable tool for epidemiological studies of *V. cholerae* (309). In this scheme, 214 *V. cholerae* O1 strains isolated from 35 countries were partitioned into 21 different ribotypes. The authors observed that the strains causing the previous fifth and sixth pandemics (from 1881 to 1923) and the current seventh pandemic belong to different ribotypes. They suggested that the wide circulation of different clones might favor the persistence of *V. cholerae* in the environment but demonstrated that certain clones were restricted to certain regions, e.g., ribotype 8 was restricted to central Africa and ribotype 5 was predominant in the Latin American epidemics. Using ribotyping to analyze the epidemiological relationships of *V. cholerae* isolates from Latin America, it was concluded that the cholera epidemic which started in Peru in the early 1990s was an extension of the seventh pandemic which started in 1970 in Africa (115). Other studies revealed that *V. cholerae* strains from different epidemics were clonal and that over the years there has been a continuous emergence of new pathogenic clones (75, 116). More recently, ribotyping has been used to assess the genomic diversity of environmental *Vibrio* strains associated with fish and oysters (13, 245, 313). According to Austin et al. (11), closely related *Vibrio* species, e.g., *V. anguillarum* and *V. ordalii*, can be differentiated on the basis of ribotyping. Macián et al. (245) analyzed 82 *V. splendidus*, 25 *V. harveyi*, and 10 *V. tubiashii* strains isolated in a 1-year period and found 64, 17, and 9 different ribotypes, respectively. Cer-

tain *V. splendidus* ribotypes were typical for isolates found in summer, while others were typical for isolates found winter.

PHENOTYPIC IDENTIFICATION: THE PITFALLS OF CLASSICAL BIOCHEMICAL IDENTIFICATION AND DICHOTOMOUS KEYS

Classical phenotypic identification techniques, including tests for arginine dihydrolase and lysine and ornithine decarboxylases, were among the most extensively used techniques to screen the diversity of *Vibrio* strains associated with marine animals and their habitat, and these tests have been proposed as reliable species identification schemes (4, 5, 12, 13, 39, 167, 222, 244, 297, 298). Variable results, e.g., for arginine dihydrolase of some species, have been reported, making their identification on this basis difficult (312). Biolog has been one of the most widely used phenotypic techniques for the identification of *Vibrionaceae* strains in the last decade (11, 13, 209, 266, 406). Bacterial strains are inoculated onto 95 different compounds which may serve as carbon sources. The identification of strains is based on the pattern of utilization of the 95 carbon sources. A very important diagnostic phenotypic feature for the identification of *Vibrio* species has always been the presence of flagella and thus motility (3). Nonmotile *Vibrio* species, e.g., the *V. halioticoli* group, have described (344, 346, 347), suggesting that the presence of flagella is not an essential diagnostic feature. Likewise, oxidase-negative *V. metschnikovii* and *V. gazogenes* strains have been documented, as have *Vibrio* strains that fail to grow on TCBS (4, 5). Colony variation is also a very common feature among *Vibrio* species, including the newly described species (12, 166, 425).

Fatty acids methyl ester (FAME) profiling was evaluated for the differentiation of *Vibrionaceae* species (36, 216, 299). FAME profiling is generally very useful as a chemotaxonomic marker, and apparently, differentiation at the genus level was possible. The similarity of FAME profiles among the different species examined was very striking, and the authors thus concluded that this technique could be used only as an additional phenotypic feature (36, 216). It became clear that the ample phenotypic variability within *Vibrionaceae* species pointed to the use of classification and identification scheme based on genomic data.

The phenotypic identification of genera and species of the *Vibrionaceae* is problematic. The main reason is the great variability of diagnostic phenotypic features, e.g., arginine dihydrolase and lysine and ornithine decarboxylases, susceptibility to the vibriostatic agent 0/139, flagellation, indole production, growth at different salinities and temperatures, and carbon utilization as revealed by Biolog (9, 13). Traditionally used as clear-cut tests for identification of species, the latter should thus be interpreted with greatest care. Dichotomous keys (see, e.g., references 4, 5,and 170) are misleading for the identification of *Vibrionaceae* isolates.

A comparison between a consensus molecular identification, including AFLP, DNA-DNA hybridization, and 16S rRNA sequences, on the one hand, and phenotypic identification (Biolog), on the other, shows that different *Vibrio* species appear within the same Biolog group. For instance, strains misidentified as *V. harveyi* by Biolog were later correctly identified as *V. campbellii* or classified as *V. rotiferianus* by AFLP and DNA-DNA hybridizations. Indeed, *V. campbellii*, *V. harveyi*, and *V. rotiferianus* have nearly indistinguishable phenotypes (136, 139). Strains misidentified by Biolog as *V. campbellii* turned out to be *V. chagasii*, while strains supposed to be *V. splendidus* were classified as *V. kanaloaei*. It was also remarked that many strains identified by AFLP as, e.g., *V. cincinnatiensis*, *V. splendidus*, and *V. tubiashii*, corresponded to multiple Biolog groups. Comparing AFLP and Biolog data, (i) a single genotype may correspond to a single phenotype (e.g., A8; *V. brasiliensis*), (ii) a single genotype may correspond to multiple phenotypes (e.g., A9; *V. fortis*), or (iii) multiple genotypes may be found in a single phenotype (e.g., A1, A2, A3, A4, and A5; *V. coralliilyticus* and *V. neptunius*) (388). It is nearly impossible to distinguish many of the currently known *Vibrio* species, e.g., *V. splendidus*-related species, solely on the basis of the phenotype (Table 2).

NUMERICAL AND POLYPHASIC TAXONOMY

Today, taxonomy includes the phylogeny, classification, nomenclature, and identification of bacterial isolates (140, 403). One of the main aims of taxonomy is to provide useful classification schemes to be used for various scientific and practical purposes. Ideally, such classifications should be stable, predictive, objective, and highly informative (140).

The genera *Vibrio* and *Photobacterium* are among the oldest known bacterial genera (100, 201). The beginning of the taxonomy of vibrios can be traced back to the work of Pacini. Until the middle of the 1900s, the taxonomy of vibrios 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. These studies led to the description of many new *Vibrio* species. In the seventh edition of *Bergey's Manual of Determinative Bacteriology* (45), the genus *Vibrio* belonged to the family *Spirillaceae* and consisted of 34 species, which, with the exception of *V. cholerae* (listed as *V. comma*) and *V. metschnikovii*, were later reclassified into other genera, e.g., *Campylobacter* (*C. fetus*, *C. jejuni*, and *C. sputorum*), *Comamonas* (*C. terrigena*), or *Pseudomonas* (*P. fluorescens*) or no longer accepted as validly described species according to the *Approved List of Bacterial Names* (355). The genus *Photobacterium*, on the other hand, harbored one species, i.e., *P. phosphoreum*, and was allocated into the genus *Bacterium* of the family *Bacteriaceae* (45).

The heterogeneity within the genus *Vibrio* was highlighted by Davis and Park (90, 305). By examining morphological and biochemical features of most species of the genus *Vibrio*, they showed that it was quite artificial and concluded that at least three genera existed among the species examined. The foundation of modern *Vibrio* taxonomy was laid by a number of numerical (phenetic) and/or polyphasic taxonomic studies (17, 21–28, 74, 81, 125, 152, 229, 319, 393, 427, 438). Most of these studies clustered large collections of strains on the basis of their ability to utilize different (ca. 50 to 150) compounds as sources of carbon and/or energy, enzyme activity (e.g., gelatinase, chitinase, and 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 orni-

TABLE 2. Some important phenotypic and chemotaxonomic features of *V. splendidus*-related species[a]

Characteristic	*V. splendidus*	*V. kanaloaei*	*V. pomeroyi*	*V. chagasii*	*V. tasmaniensis*	*V. lentus*	*V. cyclitrophicus*	*V. crassostreae*
Arginine	V[b]	V	V	V	−	V	V	+
Lysine	−	−	−	−	−	−	−	−
Ornithine	−	−	−	−	−	−	−	−
Growth at/on:								
4°C	V	+	+	V	+	V	+	+
8% (wt/vol) NaCl	V	V	+	V	−	V	+	−
Nitrate reduction	+	+	+	+	+	+	−	+
Fermentation of:								
Sucrose	V	+	V	−	−	−	+	+
Arabinose	−	+	−	−	−	−	−	−
Utilization of cellobiose	V	−	+	+	+	+	+	+
β-Galactosidase activity	V	−	+	−	−	+	−	−
Indole production	+	+	+	+	+	V	−	+
Fatty acids (%)								
Summed feature 3[c]	35	39.2	32.9	38.4	35.4	41.7	35.3	39.4
16:0	29.6	25.6	29.2	22.4	28.4	24.7	30.7	17.3
14:0	9	5.0	10.5	7.2	11.0	8.8	7.96	5.4
12:0	8.7	4.2	8.9	3.8	6.6	5.3	7.7	5.5
18:1 ω7*c*	8.2	10.2	7.6	9.7	7.6	8.7	7.5	7.0
12:0 3-OH	3.41	3.4	3.9	2.7	2.8	2.5	2.1	3.3

[a] Data from references 117, 161, 247, and 384–386. *V. splendidus*-related species have intermediate DNA-DNA similarity levels (40 to 60%) and high 16S rRNA similarity (97 to 100%).
[b] V, variable.
[c] Summed feature 3 comprises 16:1 ω7*c* and/or 15 iso 2-OH.

thine decarboxylases). The clusters defined by phenotypic features were further refined and validated by DNA-DNA hybridization experiments, and phenotypic clusters with about 80% similarity were found to correspond to DNA-DNA homology clusters with more than 80% similarity (24, 28). This suggests that for *Vibrionaceae* taxonomy, one should use 80% DNA-DNA similarity as the limit for species definition instead of the canonical 70% proposed by Wayne et al. (426).

In the eighth edition of *Bergey's Manual of Determinative Bacteriology* (50), the family *Vibrionaceae*, which was proposed by Véron, comprised *Vibrio* and *Photobacterium* along with *Beneckea*, *Aeromonas*, *Plesiomonas*, and *Lucibacterium*. The combination of *Vibrio* (*V. anguillarum*, *V. cholerae*, *V. costicola*, *V. fischeri*, and *V. parahaemolyticus*) and *Photobacterium* (*P. mandapamensis* [*P. leiognathi*] and *P. phosphoreum*) in a single family was an improvement in the taxonomy of these two related genera, which were thought for a long time to be only distantly related. Baumann et al. (22) 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 et al. (24–26) proposed that *Beneckea* species and *Lucibacterium harveyi* should be reallocated to the genus *Vibrio*, *Aeromonas* and *Plesiomonas* should be placed into other families, and *V. costicola* should be placed in another genus. These authors also suspected that the evolution of *Vibrio* and *Photobacterium* species was driven mainly by vertical processes (mutations) rather than horizontal gene transfer. The DNA-DNA relatedness studies among *Vibrio* and *Photobacterium* species underpinned the taxonomy of these groups (26, 27, 319). These studies disclosed a core group of related vibrios, i.e., the *V. 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 to 74% DNA-DNA similarity, while *V. parahaemolyticus* and *V. alginolyticus* had 61 to 67% similarity. Reichelt et al. (319) also proposed biotypes I and II for *V. splendidus* and *V. pelagius*, but they suspected that these biotypes could be different species. Biotypes I and II of *V. splendidus* and *V. pelagius* showed a maximum of 61 and 58% DNA-DNA similarity, respectively. Additionally, the biotypes of both species were clearly distinguishable by phenotypic features. Nevertheless, researchers are still using the biotype designation today (368). Arias et al. (7, 8, 111) have suggested that the two biotypes of *V. vulnificus* should be abolished. These biotypes should be considered as different species according to the current species definition (361).

Baumann et al. (24–26) compared the amino acid sequence differences of glutamine synthetase, superoxide dismutase, and alkaline phosphatase to distinguish *Vibrionaceae* species. Because the determination of amino acid sequences was very time- consuming and cumbersome at that time, Baumann and colleagues applied a technique called microcomplement fixation, which is based on the immunological reaction of antigens and antisera of the target proteins. On the basis of this analysis,

they concluded that *Beneckea* species, *P. fischeri*, and *P. logei* should be transferred to the genus *Vibrio* (25) (see also the first edition of *Bergey's Manual of Systematic Bacteriology* [210]). They also mentioned that they applied a certain "subjective judgement" about the limits of the genus *Vibrio* because they found that 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.

PHYLOGENY OF THE VIBRIOS

In the last two decades, bacterial phylogeny has been enriched with chronometers, e.g., rRNAs (5S, 16S, and 23S), to reconstruct bacterial phylogenies but also to be used as taxonomic markers for identification. In many cases, the phylogenies obtained by 16S rRNA sequencing pointed out the inadequacy of grouping bacteria by the classical criteria, e.g., morphology and biochemical features. The close relationship of *Vibrio* and *Photobacterium* was confirmed by this approach, and both genera were placed within the purple bacteria, a diverse group of gram-negative phototrophic and heterotrophic bacteria (435), later renamed *Proteobacteria* (360). More recently, *Proteobacteria* has been elevated to the rank of phylum (206; see *The Prokaryotes* online at http://link.springer-ny.com/link/service/books/10125/index.htm or http://141.150.157.117:8080/prokPUB/index.htm). *Proteobacteria* is the largest group within *Bacteria*, having about 1,600 species partitioned in five classes, i.e., *Alfaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, and *Epsilonproteobacteria*. These classes are phenotypically indistinguishable. More detailed phylogenetic analyses within the family *Vibrionaceae*, using 5S and 16S rRNA sequences, were performed in the following years, leading to a refinement of this group (98, 208, 243, 312, 340).

Application of 5S rRNA as a First Phylogenetic Attempt

MacDonell and Colwell (243), analyzing the 5S rRNA of superfamily I (*Vibrionaceae* plus *Enterobacteriaceae*), correctly concluded that *V. marinus* (now *Moritella marina* in the family *Alteromonadaceae*), *V. psychroerythrus* (now *Colwellia psychoerythrus* in the family *Alteromonadaceae*), and *Aeromonas* spp. (now in the family *Aeromonadaceae*) were not authentic members of the *Vibrionaceae* and should be placed into other families. These authors also proposed the creation of two new genera, *Listonella* and *Shewanella*. The genus *Shewanella* is currently found within the family *Alteromonadaceae*, but the genus *Listonella*, which would encompass the species *V. anguillarum*, *V. pelagius*, and *P. damselae*, has not been recognized by many expert taxonomists in the field (11, 246). Further phenotypic and phylogenetic studies clearly showed that these three species should be retained in their original genera (98, 208, 358). The use of 5S rRNA to reconstruct the phylogeny is clearly of limited use (312), probably due to its small size (ca. 120 bp [435]).

Phylogenetic Picture Obtained by the 16S rRNA Chronometer

The 16S rRNA molecule (about 1,500 bp in length) consists of highly conserved regions which may reveal deep-branching (e.g., classes, phyla) relationships but may also demonstrate variable regions which may discriminate species within the same genus. This feature has prompted researchers to use 16S rRNA both as a phylogenetic marker and as an identification tool (428). The Ribosomal Database Project II (http://rdp.cme.msu.edu/html/ [249]) consists of over 75,000 16S rRNA sequences. These entries can be easily queried using publicly available software, e.g., BLAST and FASTA (306).

Dorsch et al. (98) determined the almost complete 16S rRNA sequences of 10 *Vibrio* species and obtained results for the *Vibrio* core group which were in agreement with previous DNA-DNA homology data obtained by Baumann et al. (24). Dorsch et al. (98) also indicated that *V. hollisae* should be allocated into a new genus. Kita-Tsukamoto et al. (208) presented a comprehensive phylogenetic study of the *Vibrionaceae*. Although this study was based on partial 16S rRNA sequences, Kita-Tsukamoto et al. selected a broad collection of 50 species, including the type species of the family *Vibrionaceae*, *V. cholerae*, most *Vibrio* species, 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), genus (95 to 96%), and family (90 to 91%) borders within the *Vibrionaceae* and (ii) the delineation of seven main groups of *Vibrionaceae* species that would correspond to different genera or families. The suggestions of reclassification proposed by Kita-Tsukamoto et al. (208) were further addressed by Mellado et al. (262), who transferred *V. costicola* into *Salinivibrio costicola*, and Urakawa et al. (396, 398), who transferred *V. marinus* into *Moritella marina* and *V. iliopiscarius* into *Photobacterium iliopiscarius*. According to Kita-Tsukamoto et al. (208), *V. cholerae* and *V. mimicus* would correspond to a genus on their own. *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*, *V. mimicus*, and the *V. fischeri*-related group are to be elevated to the genus level, one might argue for the revival of *Beneckea* to encompass all other remaining vibrios, an idea which was originally supported by Baumann et al. (22).

New Phylogenetic Insights Obtained by Other Chronometers

Any phylogenetic marker has weaknesses and strengthes because no single gene is completely resistant to lateral transfer, hidden paralogy, and changes in the evolutionary clock among related species (130, 193). Alternative phylogenetic markers should fulfill several criteria as put forward by Zeigler (448): (i) the genes must be widely distributed among genomes, (ii) the genes must be single copy within a given genome, (iii) the individual gene sequences must be long enough to contain sufficient information but short enough to allow sequencing in a convenient way (900 to 2,250 nucleotides), and (iv) the sequences must predict whole-genome relationships with acceptable precision and accuracy that correlates well

with the 16S rRNA and with whole-genome similarity measured by, e.g., DNA-DNA hybridizations. *recA*, 23S rRNA, elongation factors G and Tu/1α, the catalytic subunit of the proton-translocating ATPase, the hsp60 and hsp70 heat shock proteins, glyceraldehyde-3-phosphate dehydrogenase (*gap*), *rpoB*, and *gyrB* are among the most promising alternative markers (52, 117, 247, 248, 272, 414). A comprehensive list containing the so-called bacterial core genes, which may be useful for further phylogenetic analyses, was proposed by Gevers et al. (130).

Zeigler (448) examined the whole-genome sequences of 49 bacterial species from different phyla, including *Bacillus* spp., *Escherichia* sp., *Mycobacterium* spp., and *Streptococcus* spp., with the aim of finding genes which are phylogenetically informative and may predict genome (taxonomic) similarity. He found high correlation between genes, e.g., *recA*, *thdF*, *rpoA*, *ligA*, and *dnaX*, and whole-genome sequences. The genome similarities calculated from whole-genome sequence alignments were very compatible with the values obtained by standard DNA-DNA hybridization experiments. He concluded that the sequences of three or four protein-coding genes can be used to precisely assign new isolates to species. *recA*- and *rpoA*-based phylogenies of the vibrios correlate very well with the one based on the 16S rRNA, but *recA* gene sequences seem to be much more discriminatory (378; Thompson, Gevers, et al., submitted). For 16S rRNA values above 98%, there was a wide range of *recA* similarities, varying from 83 to 99%. *G. hollisae* was distantly related to *Vibrio* (66.3% *recA* sequence similarity) and *Photobacterium* (70.5% *recA* sequence similarity). *rpoA* and *recA* gene sequences suggest that *V. fischeri*-related species are closely related to *Photobacterium*.

DEFINING TAXA WITHIN THE VIBRIOS

The Families *Vibrionaceae*, *Enterovibrionaceae*, *Photobacteriaceae*, and *Salinivibrionaceae*

16S rRNA sequencing is considered the most reliable tool for the allocation of genera, species, and strains into the family *Vibrionaceae*. Following this approach, the outline of *Bergey's Manual of Systematic Bacteriology*, 2nd ed., 2004 (see http://dx.doi.org/10.1007/bergeysoutline200310 [129]) lists eight genera, i.e., *Allomonas* (1 species), *Catenococcus* (1 species), *Enterovibrio* (1 species), *Grimontia* (1 species), *Listonella* (2 species), *Photobacterium* (7 species), *Salinivibrio* (1 species), and *Vibrio* (51 species) within the family *Vibrionaceae*. The genera *Allomonas* (199) and *Enhydrobacter* (363) were tentatively allocated to the family *Vibrionaceae* based mainly on phenotypic features.

Phylogenetic analyses of concatenated 16S rRNA, *recA*, and *rpoA* gene sequences clearly show that vibrios are distributed in four different families, i.e., *Salinivibrionaceae* (comprising the genus *Salinivibrio*), *Enterovibrionaceae* (comprising the genera *Enterovibrio* and *Grimontia*), *Photobacteriaceae* (comprising the genus *Photobacterium*) and *Vibrionaceae* (comprising all the *Vibrio* species expect the *V. fischeri* group) (Fig. 1; Table 3).

The proposal to split the family *Vibrionaceae* into three new families was put forward by Thompson (388) on the basis of 16S rRNA and phenotypic features (Table 3) and was intended to facilitate further studies of vibrios. The creation of these three new families resulted in a more compact *Vibrionaceae* family. Allocation of strains into different families and genera is done on the basis of 16S rRNA (Fig. 1) and phenotypic analyses, while allocation of strains into species is currently best achieved by using AFLP, rep-PCR, or *rpoA*, *atpA*, and *recA* gene sequences.

Genera within the *Vibrionaceae*

The newly proposed family *Vibrionaceae* comprises only the genus *Vibrio*, with 63 species (Table 4) (388). The number of *Vibrio* species increased from 20 in 1981 to 47 in 2002 (106; http://www.bacterio.cict.fr/). New species isolated from the marine environment have been described each year, with *V. crassostreae* (117), *Enterovibrio coralii*, and *P. eurosenbergii* (Thompson, Thompson, et al., submitted) being the most recent ones. We have recently described 21 new species of vibrios (Fig. 1). *V. fischeri*-related species appear together at the outskirts of this diverse taxon, which we proposed to name *Vibrionaceae* (Fig. 1). Ruimy et al. (340) compared the 16S rRNA sequences of several *Aeromonas*, *Grimontia*, *Photobacterium*, *Salinivibrio*, *Vibrio*, and *Plesiomonas* representatives and concluded that the genus *Vibrio* is a distinct phylogenetic group which deserved elevation to the family rank. Overall, the analyses of several chronometers, e.g., 16S rRNA (208), 23S rRNA (247, 248), *recA* (378), and *gyrB*, and phenotypic features (26) indicate that *V. cholerae* and *V. mimicus* should be given genus rank. *V. cholerae* and *V. mimicus* are the bona fide members of the genus *Vibrio*. Whether all the other named *Vibrio* species should be retained in the same taxon as *V. cholerae* and *V. mimicus* or whether *Vibrio* should be split into different genera remains to be determined by future studies. Many species of the genus *Vibrio*, particularly those of the *V. harveyi* group, do not readily show conspicuous phenotypic features to warrant their elevation to the genus rank on this basis.

If *V. cholerae* and *V. mimicus* are to be considered the only current members of the genus *Vibrio*, then one might argue the revival of *Beneckea* to encompass all other remaining vibrios, an idea which was originally supported by Baumann et al. (22). It is clear that certain *Vibrio* species, i.e., *V. aerogenes*, *V. ruber*, *V. gazogenes*, *V. rumoiensis*, and *V. halioticoli*-related species, form distinct clades, most probably different genera, within the emended *Vibrionaceae*, whereas *V. fischeri*-related species represent a genus on its own, between *Vibrionaceae* and *Photobacteriaceae*. Striking phenotypic and ecological features of some of these *Vibrio* species illustrate clearly the biodiversity within this group of vibrios. For instance, *V. fischeri* and *V. logei* cells have a yellow pigment and several polar flagella but are generally unable to grow at 35 to 40°C (17, 113, 114). Glutamine synthetase and superoxide dismutase sequence divergence analyses also suggested that *V. fischeri* and *V. logei* are separate from the genus *Vibrio* (25). Also, these two species form a unique symbiotic association with squid (339). Clearly, the analyses of whole-genome sequences of *Photobacterium* and *Vibrio* spp. will shed light on the taxonomic position of *V. fischeri* and related species. *V. gazogenes* and *V. ruber* have a red pigment, are indole and oxidase negative, but grow in 10% NaCl and at 40°C. The further expansion of the current databases of, e.g., *atpA*, *obg*, *pyrH*, *serS*, *pheS*, and *ligA* sequences of

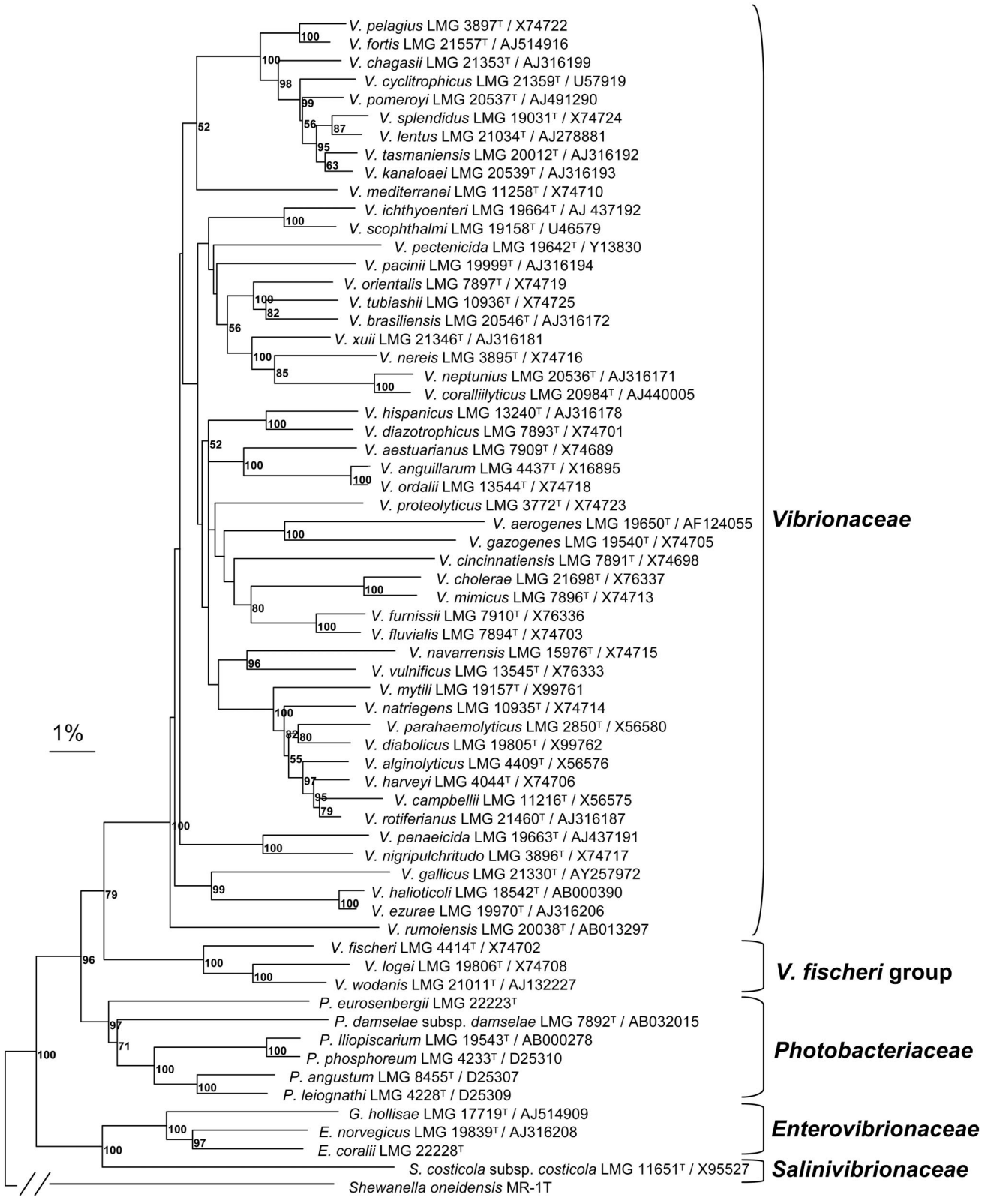

FIG. 1. Phylogenetic tree based on the neighbor-joining method, using the 16S rRNA, *recA* and *rpoA* concatenated gene sequences (2,898 bp), showing the different families of vibrios. Distance estimations were obtained by the model of Jukes and Cantor (196). Bootstrap percentages after 1,000 simulations are shown. Bar, 1% estimated sequence divergence. Sequences originated from the type strains of each species. The *rpoA* and *recA* gene sequences are from our own databases.

TABLE 3. Phenotypic differences among *Vibrionaceae*-related families

Characteristic	*Enterovibrionaceae*	*Photobacteriaceae*	*Salinivibrionaceae*	*Vibrionaceae*
D-mannitol fermentation	−	−	V	V
PHB accumulation	−	+	−	V
Growth in/at:				
20% NaCl	−	−	+	−
45°C	−	−	+	−
Voges-Proskauer	−	V	+	V
Indole	V	−	−	V
ADH[a]	V	+	−	V
ODH[a]	−	−	−	V
Presence of fatty acids:				
16:1 ω9*c*	+	−	+	V
18:1 ω9*c*	+	−	−[b]	V

[a] ADH, arginine dihydrolase; ODH, ornithine decarboxylase.
[b] Trace amounts (<1%) occur in *Salinivibrio* strains.

vibrios and the search for alternative phylogenetic markers will yield a much better view of the heterogeneity within this group of marine organisms. Based on *recA* and *rpoA* analyses, *V. halioticoli*-, *V. harveyi*-, and *V. splendidus*-related species formed compact separate groups while *V. tubiashii*-related species may be scattered within the genus *Vibrio*. *Vibrio* strains have at least 84 and 73% *rpoA* and *recA* gene sequence similarity, respectively.

Allomonas enterica is almost identical to *V. fluvialis* on the basis of 16S rRNA, DNA-DNA hybridization and phenotypic features (112). The Subcommittee on the Taxonomy of *Vibrionaceae* had already concluded that *A. enterica* is a junior synonym of *V. fluvialis* (112). *Enhydrobacter aerosaccus* is located within the family *Moraxellaceae* and is closely related to *Moraxella osloensis* (~100% 16S rRNA similarity) (388).

Species within the *Vibrionaceae*

Bacterial species can be thought of as "condensed nodes in a cloudy and confluent taxonomic space" (403). This view signifies that "classification is a frame for the condensed nodes where some isolated internodal strains (unclustered strains) also must get a place and name" (403). According to Stackebrandt et al. (361), a species is "a category that circumscribes a genomically coherent group of isolates sharing a high degree of similarity in many independent features." In this case, DNA-DNA similarity remains the "gold" standard for species delineation. Strains from the same species have at least 70% DNA-DNA similarity under stringent conditions. Other authors suggest a more relaxed limit, i.e., 50 to 70% similarity (334; T. Coenye, D. Gevers, Y. Van de Peer, P. Vandamme, and J. Swings, submitted for publication). Young (444) has pointed out that the phenotypic circumscription of the bacterial species boundaries is the most important criterion for species definition. Other researchers have developed a species definition based only on the 16S rRNA sequences to delineate marine bacterioplankton species (153). These authors accept that species would be entities with at least 97% 16S rRNA similarity. It is our opinion that bacterial taxonomy is moving toward a genomically based concept in which phenotypic data may not have a clear standing.

In this respect, AFLP analysis proved to be valuable for the discrimination of phylogenetically and phenotypically related *Vibrio* species, e.g., the pairs *V. coralliilyticus* plus *V. neptunius* and *V. harveyi* plus *V. rotiferianus* (139). For classification and identification purposes, AFLP offers an alternative to DNA-DNA hybridizations by adopting the AFLP pattern similarity levels of 60 to 70%, which correspond to a DNA-DNA similarity higher than 70%. AFLP is one of the most reliable genomic fingerprinting identification tools in the taxonomy of the *Vibrionaceae* to date. Aside from its high discriminatory power and value for species circumscription, AFLP is easy and fast to perform and is amenable to automation. AFLP data can also be accumulated in databases.

The discrimination of AFLP is indeed very high and is based on a survey of the entire bacterial genome (190). In our experiments with AFLP (381), the average number of fragments obtained for the 506 strains analyzed with the enzyme combination HindIII plus TaqI and with the primer combination H01 (a adenine as selective base) plus T03 (a guanine as selective base) was 107 ± 23 (minimum of 46 and maximum of 164 fragments). The number of fragments obtained corresponded well to the expected number of fragments, i.e., 76 (assuming a average genome size of 5 Mb, a probability-of-cutting frequency of 0.0002 [$1/4^6$], and a factor of 0.0625 [1/16, due to the selective bases in each primer]). AFLP proved to be a most valuable tool for the circumscription of new species. With few exceptions (Table 3), strains from the same species were found in a single AFLP cluster. Indeed, AFLP screens a large number of point mutations. For instance, the estimated number of point mutations surveyed among 5 *V. rotiferianus* and 12 *V. harveyi* strains is 23,052 (average number of fragments of 113 × 12 nucleotides [6 plus 4 of the restriction sites plus the two selective bases] × number of strains). AFLP has been validated as an alternative to DNA-DNA hybridizations for a number of bacterial genera, including *Aeromonas* (176), *Agrobacterium* (275), *Acinetobacter* (189, 190), *Bradyrhizobium* (429), *Burkholderia* (76), *Stenotrophomonas* (159), and *Xanthomonas* (315). We compared the AFLP band pairwise similarities found in the study by Thompson et al. (381) with all subsequent DNA-DNA homology values obtained in the

TABLE 4. List of species of vibrios

Species name	Type strain	Place and date of isolation[a]	Source
Enterovibrio coralii	LMG 22228^T	Magnetic Island (Australia) 2002	Water extract of bleached coral (*Merulina ampliata*)
Enterovibrio norvegicus	LMG 19839^T	AARS Austevoll (Norway), 1997	Gut of turbot larvae (*Scophthalmus maximus*)
Grimontia hollisae	LMG 17719^T	Maryland (United States)	Human feces
Photobacterium angustum	LMG 8455^T	Hawaii (United States)	Sea water
P. damselae subsp. *damselae*	LMG 7892^T	United States	Diseased damsel fish (*Chromis punctipinnis*)
P. eurosenbergii	LMG 22223^T	Magnetic Island (Australia), 2003	Tissue extract of bleached coral (*Pachyseris speciosa*)
P. iliopiscarius	LMG 19543^T	Norway	Gut of fish
P. leiognathi	LMG 4228^T	Thailand	Leiognathidae fish (*Family Leiognathidae*)
P. phosphoreum	LMG 4233^T	Hawaii (United States)	Seawater
P. profundum	LMG 19446^T	Ryukyu Trench (Japan)	Sediment
Salinivibrio costicola subsp. *costicola*	LMG 11651^T	Australia	Bacon-curing brine
Vibrio aerogenes	LMG 19650^T	Seagrass bed in Nanwan bay (Taiwan)	Sediment
V. aestuarianus	LMG 7909^T	Oregon (United States)	Oyster
V. agarivorans	LMG 21449^T	Valencia (Spain)	Seawater
V. alginolyticus	LMG 4409^T	Japan	Spoiled horse mackerel (*Trachurus trachurus*)
V. anguillarum	LMG 4437^T	Norway	Diseased cod (*Gadus morhua*)
V. brasiliensis	LMG 20546^T	LCMM Florianópolis (Brazil), 1999	Bivalve larvae (*Nodipecten nodosus*)
V. calviensis	LMG 21294^T	Bay of Calvi (Mediterranean), France	Seawater
V. campbellii	LMG 11216^T	Hawaii (United States)	Seawater
V. chagasii	LMG 21353^T	AARS Austevoll (Norway), 1997	Gut of turbot larvae (*Scophthalmus maximus*)
V. cholerae	LMG 21698^T	Asia	Clinical
V. cincinnatiensis	LMG 7891^T	Ohio (United States)	Human blood and cerebrospinal fluid
V. coralliilyticus	LMG 20984^T	Indian Ocean near Zanzibar, 1999	Diseased *Pocillopora damicornis*
V. crassostreae	LMG 22240^T	IFREMER La tremblade (France)	Hemolymph of diseased reared oysters (*Crassostera gigas*)
V. cyclitrophicus	LMG 21359^T	Washington (United States)	Creosote-contaminated sediment
V. diabolicus	LMG 19805^T	East Pacific Rise, 1991	Dorsal integument of polychaete (*Alvinella pompejana*)
V. diazotrophicus	LMG 7893^T	Nova Scotia (Canada)	Sea urchin (*Strongylocentrotus*)
V. ezurae	LMG 19970^T	Kanagawa (Japan), 1999	Gut of abalone (*Haliotis diversicolor supertexta*)
V. fischeri	LMG 4414^T	Massachusetts (United States), 1933	Dead squid
V. fluvialis	LMG 7894^T	Bangladesh	Human feces
V. fortis	LMG 21557^T	Ecuador, 1996	*Litopenaeus vannamei* larvae
V. furnissii	LMG 7910^T	Japan	Human feces
V. gallicus	LMG 21330^T	Brest (France), 2001	French abalone *Haliotis tuberculata*
V. gazogenes	LMG 19540^T	Massachusetts (United States)	Mud from saltmarsh
V. halioticoli	LMG 18542^T	Kumaishi (Japan); 1991	Gut of abalone (*Haliotis discus hanai*)
V. harveyi	LMG 4044^T	Massachusettes (United States), 1935	Dead amphipod (*Talorchestia* sp.)
V. hepatarius	LMG 20362^T	CENAIM (Ecuador), 2000	Digestive gland of white shrimp (*Litopenaeus vannamei*)
V. hispanicus	LMG 13240^T	Barcelona (Spain), 1990	Culture water
V. ichthyoenteri	LMG 19664^T	Hiroshima (Japan)	Gut of diseased Japanase flounder (*Paralichtys olivaceus*)
V. kanaloaei	LMG 20539^T	IFREMER (France), 1998	Diseased oyster larvae (*Ostrea edulis*)
V. lentus	LMG 21034^T	Mediterranean coast, Valencia (Spain)	Oysters
V. logei	LMG 19806^T	United States	Gut of Arctic scallop
V. mediterranei	LMG 11258^T	Valencia (Spain)	Coastal seawater
V. metschnikovii	LMG 11664^T	Asia	Diseased fowl
V. mimicus	LMG 7896^T	North Carolina (United States)	Infected human ear
V. mytili	LMG 19157^T	Valencia (Spain)	Bivalve (*Mytilus edulis*)
V. natriegens	LMG 10935^T	Sapelo Island (United States)	Salt marsh mud
V. navarrensis	LMG 15976^T	Villa Franca Navarra (Spain), 1982	Sewage
V. neonatus	LMG 19972^T	Kanagawa (Japan), 1999	Gut of abalone (*Haliotis discus discus*)
V. neptunius	LMG 20536^T	LCMM Florianópolis (Brazil), 1998	Bivalve larvae (*Nodipecten nodosus*)
V. nereis	LMG 3895^T	Hawaii (United States)	Seawater
V. nigripulchritudo	LMG 3896^T	Hawaii (United States)	Seawater
V. ordalii	LMG 13544^T	Washington (United States), 1973	Diseased coho salmon (*Oncorhynchus rhoddurus*)
V. orientalis	LMG 7897^T	Yellow Sea (China)	Seawater
V. pacinii	LMG 19999^T	Dahua (China), 1996	Healthy shrimp larvae (*Penacus chinensis*)
V. parahaemolyticus	LMG 2850^T	Japan	Diseased human
V. pectenicida	LMG 19642^T	Brittany (France), 1991	Diseased bivalve larvae (*Pecten maximus*)
V. pelagius	LMG 3897^T	Hawaii (United States)	Seawater
V. penaeicida	LMG 19663^T	Kagoshima (Japan)	Diseased kuruma prawn (*Penaeus japonicus*)
V. pomeroyi	LMG 20537^T	LCMM Florianópolis (Brazil), 1998	Healthy bivalve larvae (*Nodipecten nodosus*)
V. proteolyticus	LMG 3772^T	United States	Intestine of isopod (*Limnoria tipunctala*)
V. rotiferianus	LMG 21460^T	ARC Gent (Belgium), 1999	Rotifer in recirculation system (*Brachionus plicatilis*)
V. ruber	LMG 21676^T	Keelung (Taiwan)	Seawater
V. rumoiensis	LMG 20038^T	Japan	Drain pool of a fish-processing plant
V. salmonicida	LMG 14010^T	Norway	Diseased Atlantic salmon (*Salmo salar*)
V. scophthalmi	LMG 19158^T	Spain	Turbot juvenile (*Scophthalmus maximus*)
V. splendidus	LMG 19031^T	North Sea	Marine fish
V. superstes	LMG 21323^T	Australian Coast	Gut of abalone (*Haliotis laevigata* and *H. rubra*)
V. tapetis	LMG 19706^T	Landeda (France)	Clam (*Tapes philippinarum*)
V. tasmanienis	LMG 20012^T	MPL (Tasmania)	Atlantic salmon (*Salmo salar*)
V. tubiashii	LMG 10936^T	Milford, Conn. (United States)	Hard clam (*Mercenaria mercenaria*)
V. vulnificus	LMG 13545^T	U.S.A.	Human wound infection
V. wodanis	LMG 21011^T	Norway, 1988	Salmon with winter ulcer (*Salmo salar*)
V. xuii	LMG 21346^T	Dahua (China), 1995	Shrimp culture water

[a] AARS, Austevoll, Aquaculture Research Station of Austevoll, Austevoll, Norway; LCMM Florianópolis, Laboratory for Culture of Marine and Molluscs, Florianópolis, Brazil; CENAIM, National Center for Marine and Aquaculture Research, Guayaquil, Ecuador; IFREMER, French Institute for Exploration of the Sea, Brittany, France; MPL, Mount Pleasant Laboratories, Tasmania, Australia.

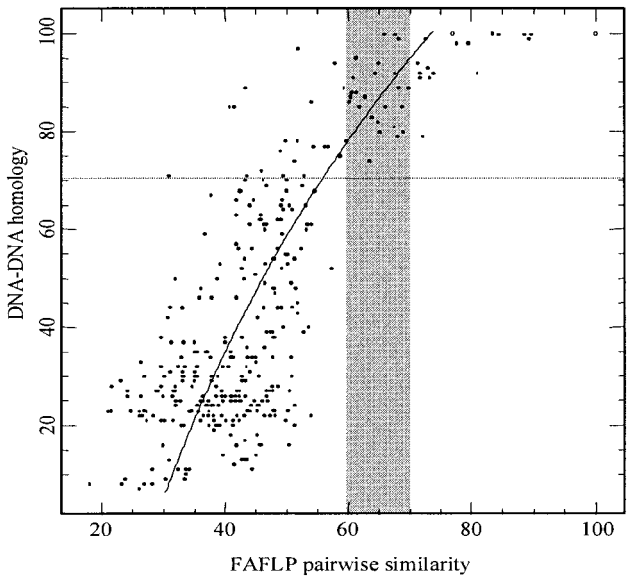

FIG. 2. Polynomial regression (second degree) of FAFLP versus DNA-DNA homology data. FAFLP pattern pairwise similarities were calculated with the Dice coefficient, and 0.2% band position tolerance was used to allow technical errors. The diagonal (i.e., 100% theoretical values) of the DNA-DNA hybridization matrices was not included, in order to give a more realistic view of the relationship between the two techniques. The dashed line indicates the threshold (70% DNA-DNA similarity) for species-level circumscription.

course of the new species descriptions (388). In total, we included 234 values in the regression and correlation analyses. Using Pearson's product-moment correlation and Kendall's tau coefficients, the correlation of FAFLP and DNA-DNA data was found to be high, i.e., 0.80 and 0.50, respectively. The data fit well in a polynomial regression of second degree ($r = 0.8$) (Fig. 2). The data depicted in Fig. 2 clearly show a close relationship between FAFLP and DNA-DNA homology data. In fact, DNA-DNA homology values can be predicted from the FAFLP similarities; FAFLP band pairwise similarities of about 60% corresponded to DNA-DNA homologies of about 75 to 95%, while FAFLP similarities of about 70% corresponded to DNA- DNA homologies of about 80 to 100%. This level of correlation is very similar to that obtained in *Xanthomonas* by Rademaker et al. (315).

Currently, the gold standard DNA-DNA hybridization experiments for delineating new species are performed mainly by the method described by Ezaki et al. (107, 108). Experiments are run in microplates in which DNA is noncovalently adsorbed and subsequently hybridized with photobiotin-labeled probe DNA. This method, which was thoroughly described by Willems et al. (430), is much faster than the classic DNA-DNA hybridization techniques (e.g., initial renaturation, hydroxyapatite, and S1 nuclease), can be performed in quadruplicate and with reciprocal reactions simultaneously, and has high correlation with classic techniques (142). To evaluate the reproducibility of this technique for *Vibrionaceae*, DNA-DNA hybridizations between various strains were repeated over a certain time period. It was found that a mean variation of 5% ($\pm$3.5% [standard deviation]; $n = 24$) between pairs of strains hybridized up to four times consecutively. This value is in agreement with the error of 7% estimated for this DNA hybridization method previously (142, 430).

Stackebrandt (362) has listed the main shortcomings of DNA-DNA hybridization techniques in the study of bacterial taxonomy: (i) few laboratories can execute this technique; (ii) the method is the slowest and most problematic step in the species description; and (iii) DNA-DNA data are not cumulative, and each new experiment requires the inclusion of reference strains. Finally, Stackebrandt (362) concluded that the method cannot be improved and that researchers should therefore seek new alternatives and new species proposals based on new approaches.

Applying MLST and the principles of bacterial population genetics (255, 256), bacterial taxonomists think that they will formulate an evolutionarily sound species concept (361, 362). A recent ecological and evolutionary theory has for the first time provided a species concept—the ecotype—based on the population genetics principles applied to MLST data (78, 79). According to this concept, an ecotype is a clonal complex, i.e., a cluster of strains that have five or six identical 500-bp gene fragments out of seven housekeeping genes. For nonclonal populations, e.g., *Neisseria* spp., an ecotype has four identical loci out of seven (250). Cohan (78, 79) also argues that most of the currently recognized species thus harbor several different ecotypes, each of which occupies a different ecological niche. This author concluded that actually recognized species could be regarded as genera. This view has been supported by other population geneticists, who propose a species genome definition based on phylogenetic and genetic analyses of housekeeping genes (219, 220). Because gene loss and acquisition play a role in speciation, some authors have proposed the species genome concept; i.e., strains from the same species would have at least 95% of their genes in common (219, 220). Venter et al. (407) advanced the concept of genomic species as biological unities having >94% sequence similarity in their housekeeping (protein-coding) genes. The idea that a bacterial species concept should be shaped by both evolutionary and ecological grounds has also been argued by other microbiologists (420). According to Ward, each ecologically unique population is in fact a unique species.

Maynard Smith et al. (256) see the *Neisseria* species as clusters, partially characteristic but having some identity to other clusters through horizontal gene transfer, and consequently they concluded that "there are no such entities as species in these pathogenic bacteria." Maynard Smith et al. (256) also recommended that "a study of the genetic and phenotypic variation in a (diverse) taxon such as *Neisseria* should be compulsory for all philosophers who believe in the existence of natural kinds, for all cladists who believe in the universal validity of phylogenetic classification, and for all pheneticists, whatever they believe. In the end, we are forced to adopt a pragmatic approach, and view the *Neisseria* genus as a kind of commonwealth of phenetic and genetic clusters (which do not quite correspond to each other), each in turn partially characteristic, but also sharing some common identity with other clusters through horizontal gene transfer." Whether the thoughtful perception of Maynard Smith et al. (256) about *Neisseria* species holds for other or perhaps all currently circumscribed bacterial species is yet to be proven. Accordingly, in the next few years bacterial taxonomists will probably be

faced with the goal of identifying clones and clonal complexes if they intend to verify the hypothesis of Maynard Smith and colleagues.

The advantage of these ideas lies in the fact that microevolution in the nucleotides of the strain level and phylogeny can finally be merged into one consensus classification. The nomenclature can be of only secondary importance and might even be completely revised. A highly accessible and applicable prokaryotic genomic taxonomy will emerge from the analyses of the nucleotide sequences.

GENOMIC DIVERSITY AND PHYLOGENY AS REVEALED BY IDENTIFICATION METHODS: TOWARD A SYNTHESIS

Recent work on vibrios has provided an extended taxonomic framework for the identification and classification of *Vibrionaceae* isolates abundantly found in aquatic environments (388) (Table 4; Fig. 1). It is clear that these findings will have a positive impact on future studies of the taxonomy and ecology of these organisms. The descriptions of new *Vibrionaceae* species are in agreement with recommendations by the ad hoc committee for the reevaluation of the species definition in bacteriology (361). The new classification of vibrios is based on genomic data rather than on more traditional phenotypic features (33, 117, 136–139, 160, 346, 347, 378–388). FAFLP and to some extent rep-PCR have been applied to and validated for the classification of the new taxa. For identification purposes, FAFLP band pairwise similarities of about 70% will correspond to DNA-DNA homologies of about 80 to 100%, which equate to the recommended 70% DNA-DNA similarity.

The 16S rRNA gene sequences have elucidated the phylogenetic structure of the vibrios. Clearly, *Salinivibrionaceae*, *Enterovibrionaceae*, and *Photobacteriaceae* species are separate from one another and from the members of the family *Vibrionacae*. This family is in turn also heterogeneous and may require further splitting. Several *Vibrionaceae* species have nearly identical 16S rRNA gene sequences. In these cases, the only alternatives for identification are the genomic fingerprinting, e.g., FAFLP and rep-PCR, DNA-DNA hybridizations, or MLST. The genomic taxonomy of vibrios is in a phase of tremendous improvement as a result of the application of the MLST concepts. A more complete view of the genomic diversity of *Vibrionacae* is emerging with the analysis of the sequences of other housekeeping genes (Thompson, Gevers, et al., submitted). A consensus phylogenetic analysis based on concatenated *recA*, *rpoA*, and 16S rRNA gene sequences has revealed different groups, i.e., genera (e.g., the *V. splendidus*-, *V. harveyi*-, and *V. halioticoli*-related species) within the family *Vibrionaceae*.

It is our intention to identify and classify vibrios by their nucleotide sequences in protein-coding genes, using particularly the genes of the bacterial core genome (119, 130, 232, 448). Data gathered so far show that strains of the same species have >97% 16S rRNA, >99% *rpoA*, >98% *gap*, >97% *atpA*, >98% *obg*, >95% *recA*, >98% *lap*, >98% *idh*, >98% *mdh*, >96% *asd* and >93% *epd* gene sequence similarity. This type of data will aid in the development of an acceptable and coherent species concept, better reflecting the population structure and its (micro) evolution (79).

PLASTICITY OF VIBRIO GENOMES

Studies of the genome of vibrios, particularly *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, have improved our current knowledge of their ecology, pathogenicity, and epidemiology. The whole-genome sequences of three vibrios, i.e., *V. cholerae* (162), *V. parahaemolyticus* (251), and *V. vulnificus* (C. Chen et al., unpublished data; accession no. NC_004459 and NC_004460) are completed. The genome sequencing of a strain of *V. fischeri* http://ergointegratedgenomics.com/Genomes/VFI/), *V. lentus* (F. Le Roux and D. Mazel, unpublished data), and *P. profundum* (D. Bartlett et al., unpublished data) are under way. The paradigm of cholera epidemics held until recently was that the disease originated in a particular region of the globe and then spread to other places via human contact and/or contaminated material (269, 415). It has now been pointed out that the genetic backgrounds of environmental and clinical *V. cholerae* strains are quite similar and that pathogenic strains may arise from nontoxigenic strains within the aquatic environment (44, 65, 115, 234, 269, 350, 354). *V. cholerae*, "once a harmless environmental organism, has become pathogenic via multiple horizontal gene transfers" (162).

Compared with the genome of, for example, pathogenic *E. coli*, which shows numerous traces of horizontal gene transfer (294), vibrios seem to have fewer mobile genetic elements, e.g., transposons and phages, and DNA regions with a G+C content that differ from the whole-genome average, which are indicative of recent horizontal transfer (162, 251). Nevertheless, it has been demonstrated by studies in the last few years that horizontal gene transfer has contributed to several important characteristics of vibrios, such as pathogenicity and ecological niches (43, 203–205, 335–338, 417).

The genes for CT, the most important virulence factor of *V. cholerae*, have long been thought to be encoded in the chromosome of the bacterium. In 1996, Waldor and Mekalanos (417) reported that these genes are actually encoded in the genome of a newly identified bacteriophage, CTXϕ, and that the phage genome is integrated into the bacterial chromosome as a prophage. Unlike other toxin-converting phages, most of which are double-stranded DNA phages, CTXϕ is a filamentous phage with a single-stranded DNA genome, and it shares some features with the well-known filamentous phage, M13. Although the M13 phage does not integrate into the host chromosome, CTXϕ is able to do so. CTXϕ infects host cholera vibrios through pili, using the TCP as its receptor (417). Phylogenetically related filamentous phages have been identified for *V. cholerae* and other *Vibrio* species (55, 66, 172, 183, 202, 278). The genomes of these filamentous phages are composed of conserved regions and distinctive regions. The distinctive regions are considered to be foreign DNA sequences, often containing genes unique to each phage, e.g., the CT genes of CTXϕ. Thus, the phages can play a role in genetic transmission between bacterial strains. It has been revealed that this group of phages targets *dif*-like sites as their integration site (55, 174, 180). The *dif* site is located in the replication terminus region of bacterial chromosomes and plays a role in resolving dimeric chromosomes formed during replication.

One of the most striking characteristics of the genomes of vibrios is the presence of super-integrons (73). Classical integrons are natural cloning and expression systems that incorpo-

rate ORFs and convert them into functional genes. Integrons have been widely identified as the constituents of transferable elements responsible for the evolution of multidrug resistance, i.e., multiresistance integrons (335). The integron platform codes for an integrase (*intI*) that mediates recombination between a proximal primary recombination site (*attI*) and a target recombination sequence, called an *attC* site (59 base elements). The *attC* site is usually associated with a single ORF in a circular structure, termed a gene cassette. Insertion of the gene cassette at the *attI* site drives the expression of the encoded proteins. Super-integrons comprise a large ancestral integron which was first discovered in the *V. cholerae* genome (73, 257). The super-integron spanned 126 kb and harbored 179 cassettes of mainly unassigned function (162). To date, super-integrons have been identified in several *Vibrio* species, including *V. cholerae* (335), *V. parahaemolyticus* (374), and *V. vulnificus* (357). A comparison of the super-integrons of *V. cholerae* and *V. parahaemolyticus* revealed that the contents of the gene cassettes differed substantially between the two (251). This suggests that the genes making up the super-integron are highly diverse between species. Considering the high abundance of *Vibrionaceae* strains in aquatic environments, it can be said that super-integrons constitute a vast gene pool in these systems. Recently, it was reported that chromosomal super-integrons of vibrios may be a genetic source for the evolution of resistance to clinically relevant antibiotics through integron-mediated recombination events (335–338).

Pathogenicity islands (PAIs) were first described in the genomes of pathogenic *E. coli* (40). Subsequently, they were found in other pathogens, where they form specific entities associated with bacterial pathogenicity (147, 150). PAIs are DNA regions in bacterial genomes, generally 10 to 200 kb long, that have features characteristic of transferred elements, i.e., different G+C content and codon usage compared with the rest of the genome, and the presence of mobility genes, e.g., insertion sequences and parts of phages. PAIs often accommodate large clusters of genes contributing to a particular virulence phenotype, and they appear exclusively in pathogenic strains of a given species. It is now accepted that the generation of PAIs often starts with the integration of plasmids, phages, or conjugative transposons into specific target genes, preferentially on the chromosomes (149). These targets are often tRNA genes. After integration into the bacterial genome, the integrated elements experience multiple genetic events such as mutations, deletions, and insertions of genes under specific selective pressures (149). In *V. cholerae*, a cluster of genes for the biogenesis of TCP, which play an important role in human intestinal colonization by the pathogen, are located in a PAI designated VPI (205). The sequencing of the whole genome of *V. parahaemolyticus* RIMD2210633 revealed a PAI of ca. 80 kb on chromosome 2 (181, 182, 251, 303, 371). This PAI encodes the genes for hemolysins, toxins, enzymes, possible membrane proteins, and the TTSS (175). The involvement of these newly identified genes in the pathogenicity of *V. parahaemolyticus* is now under investigation.

Sequencing of entire bacterial genomes revealed that PAI-like DNA regions are much more widespread than previously thought and represent a paradigm of more general entities that are present in the genomes of many bacterial species, termed "genomic islands" (150). Functions encoded in genomic islands are not restricted to pathogenicity but include many aspects such as antibiotic resistance, symbiosis, metabolism, degradation, and secretion, which may increase the fitness of bacteria in certain environments. The evolutionary advantage of genomic islands over mutations and other smaller insertions is that a large number of genes (e.g., operons and gene clusters encoding related functions) may be transferred and incorporated en bloc into the recipient genome. This transfer may lead to dramatic changes in the behavior of the organism, resulting ultimately in "evolution in quantum leaps" (147).

Genome Configuration

Several *Vibrio* species, including *V. anguillarum*, *V. cholerae*, *V. fluvialis*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, and *V. fischeri*, contain two chromosomes (392, 442). Most *Vibrionaceae* species have this feature. How did the two- chromosome structure of vibrios develop? Heidelberg et al. (162) hypothesized that chromosome 2 of *V. cholerae* was a megaplasmid acquired by an ancestral vibrio. Several lines of evidence support this hypothesis, e.g., the presence of an integron (an element often found on plasmids) (257). In *V. parahaemolyticus* and *V. vulnificus*, an integron is present on the chromosome 1, suggesting that the position of integrons does not provide evidence for the megaplasmid hypothesis (67, 251, 374). Other researchers argue that the small chromosome may have arisen by excision from a single, large ancestral genome (418). The scenario has been reported for *Brucella* spp. (197). Egan and Waldor (102) identified the origins of replication (*ori*) of the two *V. cholerae* chromosomes. Their work revealed that the replication origin of chromosome 1 ($oriCI_{VC}$) largely conforms to the basic features of *E. coli oriC*, which has been thought to define chromosomal origins of replication in *Gammaproteobacteria*. Although the replication origin of chromosome 2 ($oriCII_{VC}$) shares certain features with *E. coli oriC*, several unusual features for a bacterial chromosome are also present. $oriCII_{VC}$-based replication displays several features that characterize certain plasmid replicons. The authors suggested that these similarities may support the proposal that chromosome 2 was originally acquired as a plasmid. In any case, since the two *Vibrio* chromosomes appear to have coexisted throughout the speciation of vibrios, the generation of the two-chromosome configuration must have occurred before the diversification of this group.

Why do vibrios have two chromosomes? Why has the small chromosome not been integrated into the large chromosome? Yamaichi et al. (442) suggested that the split of the genome into two replicons would be advantageous for the rapid DNA replication normally observed in *V. parahaemolyticus*, a species with a doubling time of only 8 to 9 min (2). Heidelberg et al. (162) hypothesized that under certain conditions, differences in the copy numbers of chromosome 1 and chromosome 2 might occur, potentially increasing the effective level of gene expression on the more numerous chromosome, to the organism's advantage. The extreme in chromosome copy number asymmetry is the loss of one chromosome, and nonreplicating, single-chromosome cells ("drone" cells), within a nutrient-stressed population of normal two-chromosomal cells, might contribute to the survival of the community by continuing the secretion of enzymes that break down molecules in the sur-

rounding microenvironment. Heidelberg et al. (162) proposed that the resulting nutrients might be used by normal members of the population, thereby promoting the survival of the species. Although there is no direct evidence for the change in stoichiometry of the two chromosomes, splitting the genome into two replicons might be advantageous for the vibrios that experience changing environmental conditions (80, 349).

The distribution of genes of known function between the two chromosomes of vibrios provides tantalizing clues about how this configuration may confer an evolutionary advantage to vibrios. Although chromosome 1 contains most of the genes that are required for growth (162, 251), chromosome 2 contains more genes for bacterial adaptation to environmental changes, indicating that the chromosomes play different roles (251). The global expression pattern of the genes on the two chromosomes of *V. cholerae* has been analyzed by using a whole-genome microarray (37, 231, 264, 447) and other methods (156). By using the rabbit ileal-loop model, Xu et al. (447) compared the global transcriptional pattern of in vivo-grown cells with that of cells grown to mid- exponential phase in rich medium under aerobic conditions. Under both conditions, the genes showing the highest levels of expression reside primarily on chromosome 1. During bacterial growth in the intestine, many more genes of the small chromosome are expressed.

A comparison of the genomes of *V. cholerae* and *V. parahaemolyticus* revealed that although chromosome 1 does not differ greatly in size between the two genomes (3.0 and 3.3 Mb respectively), chromosome 2 is much larger in *V. parahaemolyticus* than in *V. cholerae* (1.9 and 1.1 Mb, respectively) (251, 374). Examination of more species from the *Vibrionaceae* also demonstrated that while the sizes of chromosome 1 do not differ greatly, those of chromosome 2 are variable (T. Iida et al., unpublished data). The small chromosome seems to have higher proportions of genes unique to each *Vibrio* species (251). The size differences and the discrepancy in the number of unique genes suggest that the small chromosome of vibrios is more diverse in structure and gene content than is the large chromosome.

The relative position of the conserved genes in *V. cholerae* and *V. parahaemolyticus* demonstrates that extensive genome rearrangements have occurred within, and between, the two chromosomes (251, 374). Most of the intrachromosomal rearrangements, particularly in chromosome 1, may have occurred without changing the relative distance from the replication origin (251, 374). Such symmetrical rearrangements have been observed in other bacterial chromosomes (105, 239, 364). Of the 2,293 conserved genes on chromosome 1 of *V. cholerae*, 2,076 (90.5%) are also found on chromosome 1 in *V. parahaemolyticus;* and 539 (85.0%) of 634 conserved genes found on *V. cholerae* chromosome 2 are found on *V. parahaemolyticus* chromosome 2 (251). Despite extensive genome rearrangements, the locations of most of the conserved genes on both chromosomes are conserved between the two *Vibrio* species. Apparently, intrachromosomal rearrangements have occurred more often than interchromosomal rearrangements.

Driving Forces in the Evolution of Vibrios

Comparative genome analysis has revealed a variety of genomic events, including mutations, chromosomal rearrangements, loss of genes by decay or deletion, and gene acquisitions through duplication or horizontal transfer (200, 251). All of these events may be driving forces in evolution and speciation of vibrios (149–151, 292, 293). Horizontal gene transfer seems to be an efficient mechanism for introducing new phenotypes into the genome of these bacteria. Several researchers have claimed that this process can even make the delineation of bacterial species difficult (141, 226). Kurland et al. (211) and Daubin et al. (92), however, argued against this view, insisting that horizontal gene transfer events most often have little influence on organismal phylogeny. Cohan argues that mutation is one of the main driving forces in the evolution of bacteria (79). Although its influence on phylogenetic interpretation is controversial, the impact of horizontal gene transfer on the evolution of vibrios is apparent, and it can explain a number of unique genotypes e.g., the *V. cholerae* seventh-pandemic E1 Tor O1 strains (101, 116).

It has long been thought that the complex interactions between pathogens and the hosts they infect are the primary driving forces that determine the strategies used by bacteria to counter host defenses. However, new evidence suggests that the external environment, including other hosts, might play a greater role in the evolution of certain pathogens than was previously thought (433). In particular, acquisition of virulence traits through horizontal gene transfer might occur at high frequency through microbial contacts in the environment, either as mixed microbial soups in the guts of animals or as aquatic biofilms.

PERSPECTIVES AND EXPLOITATION

Research into many other molecular chronometers, e.g., *atpA*, *obg*, *pyrH*, *serS*, *pheS*, and *ligA* (130, 232, 448), is urgently needed to complete the phylogeny of the *Vibrionaceae*, as well as to refine the discrimination of closely related species. Analyses of the sequences of many different loci may also shed more light on the role of recombination and mutation in the evolution of vibrios. It is our conviction that the taxonomy and phylogeny of vibrios will be improved by MLST. The delineation of clonal complexes within each recognized *Vibrio* species by means of MLST may result in an unambiguous and direct method of identifying isolates in the future (119). According to the ecotype concept advanced by Cohan (79), each clonal complex is a new species and each currently recognized bacterial species represents in fact a genus. No more than 100 clonal complexes are expected to exist within a given species (79). The actual number of clonal complexes established for *Neisseria meningitidis*, *Campylobacter jejuni* and *Streptococcus pneumoniae* is about 10 to 15 (see the MLST website at http://www.mlst.net/new/index.htm). If we assume that each species of vibrios comprises 10 clonal complexes, we would, expect a multiplication of species of vibrios from the current 74 to 740 species.

Vibrios have been exploited in various ways. These organisms may be used for environmental monitoring (286, 288, 401), as well as to produce *N*-acyl homoserine lactone autoinducer molecules for controlling infections and biofilm formation (373). Vibrios are also used in vaccine (207) and probiotic (411) production. Bioremediation of polyaromatic hydrocarbons may be carried out by vibrios (161). Vibrios may also be

a source of novel polysaccharides and proteases (93, 316). Further exploration and description of the biodiversity among vibrios in the environment is an important topic for future research. Although there are now already 74 validly described species within this group, the biodiversity of the vibrios has not been fully covered yet. The ecological role, distribution, and abundance of the newly described species of vibrios is another important issue. Several new species were dominant on plates at the time of their isolation and appear to be ubiquitous in aquatic environments. For instance, *V. brasiliensis* was very abundant in cultures of *Nodipecten nodosus* larvae, while *V. neptunius* and *V. rotiferianus* were dominant in *Brachionus plicatilis* rearing systems. The pathogenicity of newly described species, including *E. norvegicus*, *V. brasiliensis*, *V. coralliilyticus*, *V. fortis*, *V. kanaloaei*, *V. pomeroyi*, *V. rotiferianus*, and *V. xuii*, for fish and crustacean models is now under investigation (B. Austin et al., unpublished data). Newly isolated strains associated with coral bleaching were allocated into the species *V. coralliilyticus*, *V. fortis*, and *V. rotiferianus* (Thompson, Gevers, et al., submitted). Do these culturable microorganisms really represent the dominant microbiota of those sites, or are they just overestimated on marine agar plates? Are there other dominant VBNC vibrios in the same habitats where the new species were isolated? If so, what is the taxonomic allocation and abundance of these VBNC *Vibrionaceae* strains? Are they related to the new species recently described? Apparently, eutrophic environments, e.g., certain estuarine ecosystems and aquaculture settings, favor the growth of culturable vibrios, and one would expect that these organisms are really dominant over other groups, including VBNC bacteria, but this remains to be proven (300).

Whole-genome sequencing of other vibrios is needed (116). The reduction in the costs of sequencing and the development of new sequencing technologies, e.g., resequencing, pyrosequencing, and micro- or nanochannel capillary electhrophoresis, will facilitate the sequencing of other whole vibrio genomes. The whole-genome sequencing of nonclinical vibrios abundantly found in the marine environment will help us to understand their ecology and better define their taxonomy and species boundaries. Comparative genomics of vibrios through the application of microarrays should facilitate the investigation of the gene repertoire at the species level (101) and may be an important tool for studying emerging pathogens of marine animals (333). Based on such new genomic information, the taxonomy of and species definition for vibrios will be reviewed in the next few years.

ACKNOWLEDGMENTS

F.L.T. acknowledges a Ph.D. scholarship (no. 2008361/98-6) from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) and a postdoctoral fellowship from BCCM/LMG Bacteria Collection. J.S. acknowledges grants from of the Fund for Scientific Research (FWO), Belgium.

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