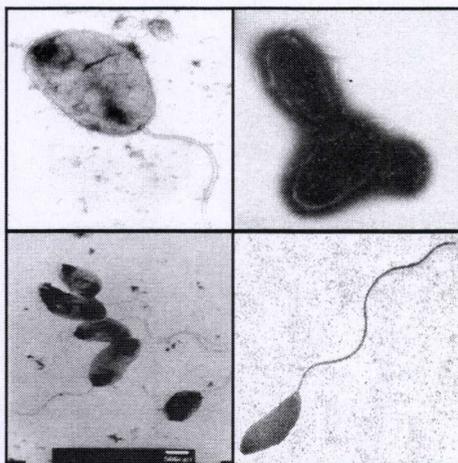


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54529

Improved taxonomy of the family *Vibrionaceae*



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Promoter: Professor Dr. Ir. Jean Swings
Academic Year: 2002-2003
Degree: Doctor in Sciences (Biochemistry)

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Acknowledgements

I am thankful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) for a 4-year scholarship (no. 2008361/98-6). I thank the staff of CNPq for excellent work. I thank the help of J. Vandenberghe, Dr. R. Robles, Prof. R. Cavalli, Prof. P. Sorgeloos and Prof. J. Swings during scholarship request.

I thank all the co-workers for their collaboration in different parts of this project. I am particularly indebted to Bart and Katrien for their commitment to this project. I thank Dr. B. Gomez-Gil, Dr. Y. Li, Prof. T. Sawabe and C. C. Thompson for their continued collaboration to this thesis. I thank Dr. Y. Ben-Haim and Prof. E. Rosenberg for the collaboration in the study of the coral pathogenic strains.

I thank all the staff of the Laboratory for Microbiology and of the LMG Bacteria Collection for all collaboration to this project. Since I arrived in Ghent in 1999, I have had the chance to meet and get along with all these very special people of the Lab. for Microbiology and of the LMG Bacteria collection. I am thankful to M. & M. Cnockaert, P. Dawindt, M. Demba Diallo, F. Depoorter, J. De Jaeger, D. Gevers, G. Goris, J. Heyrman, B. Hoste, G. Kindt, A. Struyvelt, P. Segers, R. Temmerman, K. Vandemeulebroecke, J. Vandenberghe, S. Van Eigen, S. Van Trappen. These are some of the names that come to my mind at this moment but the list is far more extensive.

I thank Margo and Jeroen for their friendship and all their aid throughout my thesis, including the writing of the "sammenvatting" of this work.

I thank K. Engelbeen, Dr. G. Huys, Prof. P. Vandamme, and C. Vereecke for their collaboration in different parts of this work.

I thank the members of the reading committee (Professors B. Austin, P. Vandamme, M. Vanechoutte, E. Stackebrandt and J. Swings) and the members of the examination committee (P. Grimont, M. Gyllenberg, J. Van Beeumen, T. Sawabe, P. Sorgeloos) for their evaluation and comments on this thesis.

I acknowledge my friend Prof. P. Abreu for teaching me the first steps towards microbiology.

I thank all the staff of the student home OBSG for their help and friendship.

I thank the help and friendship of Mrs. C. Vanlaar.

I thank my wife and collaborator Cristiane, my parents Antonio and Mayda, my mother-in-law Marly, my relatives and my son Mateus for their continued support and inspiration throughout this work.

I am deeply indebted to my mentor Prof. J. Swings, not only for his tremendous guidance, confidence and incentive during this project but also for his friendship and thoughtful insights into (micro)biology.

Ghent, May 10th 2003

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List of non-standard abbreviations

AFLP	Amplified fragment length polymorphism
ATCC	American type culture collection
BCCM TM /LMG	Belgian co-ordinated collections of microorganisms/laboratory for microbiology
BHI	Brain heart infusion
BLASTN	Basic local alignment search tool
CAIM	Collection of aquacultural important microorganisms
CDC	Center for disease control
CECT	Spanish collection of type cultures
CFU	Colony forming unity
DDBJ	DNA databank of Japan
DSMZ	German collection of microorganisms and cells
EDTA	Ethylene-diamine-tetra-acetic acid
EMBL	European molecular biology laboratory
FAFLP	Fluorescent amplified fragment length polymorphism
FAME	Fatty acid methyl ester
FISH	Fluorescent in situ hybridisation
HPLC	High-performance liquid chromatography
LSM	Laser scanning microscope
MA	Marine agar 2216
MEGA	Molecular evolutionary genetics analysis
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
ORF	Open reading frame
r	Pearson correlation coefficient
RAPD	Random amplified polymorphism DNA
RDP	Ribosomal data project
Rep	Repetitive extragenic palindrome
RFLP	Restriction fragment length polymorphism
SD	Similarity by Dice coefficient
TCBS	Thiosulphate citrate bile salts sucrose agar
TSA	Tryptone/trypticase soy agar
UPGMA	Unweighted pair group method with arithmetic averages
VBNC	Viable but nonculturable
WHO	World health organisation

CHAPTER 1. Overview of literature data

In 1854, the Italian physician Filippo Pacini (1812-1883) 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 (460-377 BC) (Blake, 1994). Pacini examined the intestinal mucosa of fatal victims of cholera 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 bad or pestilential airs). In the same period John Snow (1813-1858) studied the epidemiology of cholera in several cities of England including Birmingham, London, and Manchester (Snow, 1855; available online at <http://www.ph.ucla.edu/epi/snow/snowbook.html>). Cholera had killed tens of thousands of people in England between 1830s to 1850s (Brock, 1999; Mintz et al., 1994). 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 sewers and house-drains, as an effective means to contain the dissemination of the disease.

Nearly 30 years after Pacini identified for the first time the etiological agent of cholera, Robert Koch (1843-1910) obtained **pure cultures** of the deadly *V. cholerae* on gelatine plates. In August of 1883 Koch and his team went to Egypt where cholera had broken out resulting in about hundred thousand deaths in that country. In Alexandria 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 on 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 (Brock, 1999). In 1893, an outbreak of cholera occurred in Hamburg (Germany), with about eight thousand fatal victims. Koch was requested to study means towards an 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**

(Brock, 1999). The first non-pathogenic *Vibrio* species i.e. *V. fischeri* and *V. splendidus* and *Photobacterium phosphoreum* isolated from the aquatic environment were discovered by the Dutch microbiologist Martinus Beijerinck (1851-1931) in the late 1880s.

1.1. Occurrence and importance of the *Vibrionaceae*

In a recent study on the **bacterioplankton** of the Chesapeake Bay using fluorescent in situ hybridisation (FISH), Heidelberg et al. (2002a) found that *Gammaproteobacteria* comprises up to 10 % (3.1×10^8 cells/liter) of the total *Bacteria*, while *Vibrio* and *Photobacterium* comprise up to 4 % (2.1×10^8 cells/liter) of the total *Bacteria*. At the Italian coast (Ancona, Adriatic Sea) *Vibrio* counts of sea water have been reported to be as high as 10^5 colony forming units (CFU)/ml in summer months (Barbieri et al., 1999). In the North Sea, vibrios accounted only for 10^3 cells/ml (mainly particle-associated) when genus specific probes were used in FISH detection (Eilers et al., 2000a). 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 (Eiler et al., 2000b). Vibrios could not only rapidly respond to nutrient-enrichment experiments but also maintained viability for up to 50 days in starved conditions. These authors concluded the high rRNA content of vibrios provide the potential for such rapid responses which allow them to grow rapidly, out-competing other members of the bacterial community.

Vibrionaceae species appear particularly in high densities **in and/or on marine organisms**. *Photobacterium leiognathi* and *P. phosphoreum* are found in symbiotic association with fish and *V. logei* and *V. fischeri* with squids. These bacteria colonise the light organs of the host and play a role (via emission of light) in communication, prey attraction and predator avoidance (Fidopiastis et al., 1998; Ruby, 1996). In the light organs of the squid *Sepiolla* the abundance of vibrios can be as high as 10^{11} cells/organ (Fidopiastis et al., 1998; Nishiguchi, 2000). Newly hatched squids excrete a mucus matrix from the pores of the light organs whereby *Vibrio fischeri* cells present in sea water are caught. Subsequently, *V. fischeri* migrates into the organ and colonizes the crypt epithelium (Figure 1.1). Obviously, the flagella of *V. fischeri* play a crucial role in the colonisation of the light organs,

but hyperflagellated *V. fischeri* cells containing up to 16 flagella are defective in normal colonisation (Millikan and Ruby, 2002). *V. fischeri* cells entrapped in the light organs of squids can sense the density of conspecific cells by signalling molecules or pheromones (e.g. N-acyl homoserine lactones)

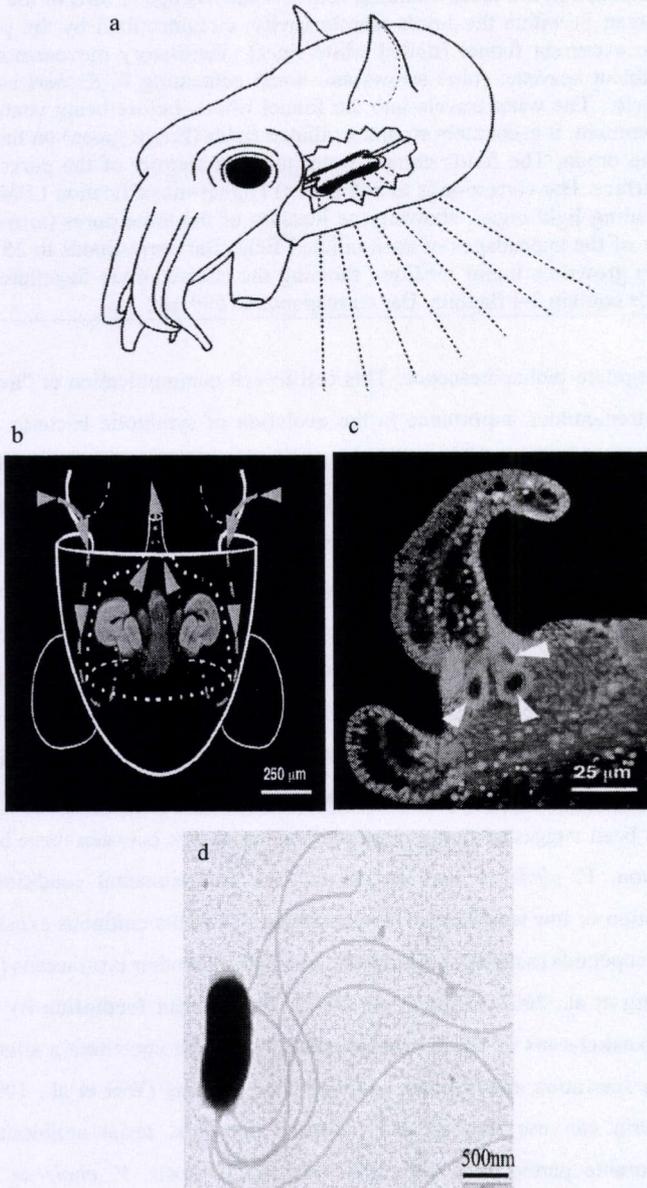


Figure 1.1. The symbiotic relationship between *Vibrio fischeri* and the squid *Euprymna scolopes*. Source: adapted from Millikan and Ruby (2002), Nyholm et al. (2000), and Ruby (1996). a) Schematic illustration of an adult squid *Euprymna scolopes* (about 4 cm in length), its light organ and the pattern of ventral light emission. b) Diagram illustrating the path of *V. fischeri* cells to the site of colonisation of the light organ. The diagram illustrates the outline of the host's body (solid white lines), superimposed over a laser-scanning confocal micrograph (LSM) of the nascent organ. The organ is within the host's mantle cavity, circumscribed by the posterior portion of the excurrent funnel (dotted white lines). Ventilatory movements of the host draw ambient seawater (blue arrows and lines) containing *V. fischeri* cells into the mantle cavity. The water travels into the funnel where, before being vented back into the environment, it encounters complex ciliated fields (bright green) on the lateral surfaces of the organ. The fields entrain water into the vicinity of the pores on the light organ surface. Bar corresponds to 250 μm . c) Higher-magnification LSM of one side of a hatchling light organ, showing the location of the three pores (arrows) that lie at the base of the appendages of each ciliated field. Bar corresponds to 25 μm . d) *Vibrio fischeri* grown on liquid medium, showing the characteristic flagellated cells. *V. fischeri* cells contain 2-4 flagella. Bar corresponds to 500 nm.

and thereby regulate bioluminescence. This cell to cell communication or "**quorum sensing**" has tremendous importance in the evolution of symbiotic bacteria. It has also been documented in the pathogens *V. anguillarum* (Milton et al., 1997), *V. cholerae* (Camara et al., 2000), *V. harveyi* (Lilley and Bassler, 2000; Manefield et al., 2000) and *V. vulnificus* (McDougald et al., 2000). 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 shrimps could be controlled by signalling antagonists produced by the algae *Delisea pulchra* (Manefield et al., 2000).

High *Vibrio* and *Photobacterium* numbers ($4.3 \times 10^6/\text{mm}^2$) attached to the external surface of zooplankton have also been reported (Heidelberg et al., 2002a, 2002b). It has been suggested that a close partnership occurs between these bacteria and zooplankton. *V. cholerae* may endure adverse environmental conditions e.g. nutrient starvation or low temperature by associating within the chitinous exoskeleton of its host i.e. copepods (nauplii), polychaetes, barnacle, and other crustaceans (Figure 1.2) (Heidelberg et al., 2002a; Lipp et al., 2002). **The biofilm formation by *Vibrio* spp. on the exoskeletons of these crustaceans** may in fact constitute a strategy to survive during starvation and/or other environmental stresses (Wai et al., 1999). In biofilms bacteria can use trapped and absorbed nutrients, resist antibiotics and establish favourable partnerships with other bacteria or hosts. *V. cholerae* moves

along and attaches to surfaces with the aid of the flagellum and pili which may act as adhesins. In as few as 15 minutes *V. cholerae* forms microcolonies on surfaces, and subsequently it produces exopolysaccharides which stabilize the pillars of the biofilm (Watnick and Kolter, 1999, 2000). A 15- μm thick biofilm, with pillars of cells and water channels, is formed within 72 hours. According to these authors the high ability of *V. cholerae* El Tor in forming densely packed biofilms in the environment gives a survival advantage to this organism which is the predominant cause of cholera world wide currently. **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 thus such outbreaks may be predicted by monitoring these parameters (Lipp et al., 2002). The wide ecological relationships and ability to cope with global climate changes may be a reflection of the high genome plasticity of vibrios (Figure 1.2) (Lipp et al., 2002). Recently a number of reports have highlighted the pathogenic potential of vibrios towards humans and marine animals (e.g. corals, gorgonians, shrimps) which may be coupled with rising of sea water temperature due to global warming (Kushmaro et al., 2001; Rosenberg and Ben-Haim, 2002; Martin et al., 2001; Sechi et al., 2000).

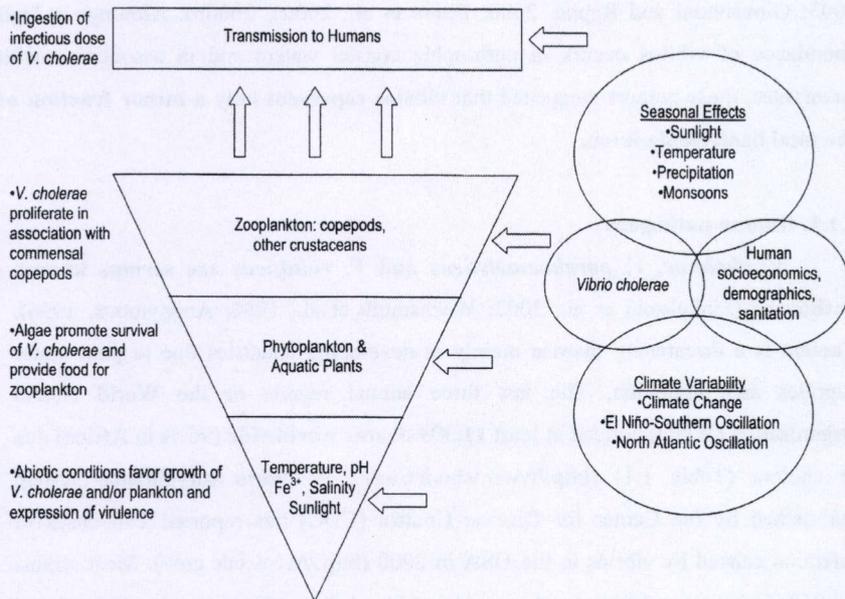


Figure 1.2. Hierarchical model for the environmental cholera transmission. Source: Lipp et al. (2002).

The application of **cultivation-independent techniques** such as direct extraction of nucleic acids from the environmental samples (e.g. water, gut tissue, sediment), subsequent 16S rDNA amplification and sequencing or alternatively FISH of filter-fixed cells with subsequent visualisation with epifluorescence microscope have provided efficient means of detecting, identifying and quantifying marine bacteria, including vibrios (Giovannoni and Rappe, 2000; Eilers et al., 2000a, 2000b). These approaches have shed light on the distribution and ecology of vibrios in the marine environment and overcame **the problem of the great plate anomaly**, i.e. the difference in the order of 10^2 - 10^3 found between direct cell counts with e.g. an epifluorescence microscope and the counts of CFU on e.g. marine agar (MA) plates (Azam, 2001). Vibrios may be in a **dormant state i.e. viable but non culturable (VBNC)** or alternatively they may not be able to grow on the selective media employed (Colwell and Grimes, 2000). Several authors have recently highlighted that the most abundant prokaryotic groups e.g. *Archaea*, *Cytophaga-Flavobacterium*, *Roseobacter*, SAR11, SAR86, SAR116, SAR 202 (*Alpha*- and/or *Betaproteobacteria*) found in the marine environment are not readily culturable (Cottrell and Kirchman, 2003; Giovannoni and Rappé, 2000; Eilers et al., 2000a, 2000b). Although a high abundance of vibrios occurs in eutrophic coastal waters and in association with eukaryotes, these authors suggested that **vibrios represent only a minor fraction of the total bacterioplankton.**

1.1.1. Human pathogens

V. cholerae, *V. parahaemolyticus* and *V. vulnificus* are serious human pathogens (Finkelstein et al., 2002; Wachsmuth et al., 1994; Anonymous, 1996). Cholera is a threatening disease mainly in developing countries due to poor water supplies and sanitation. The last three annual reports of the World Health Organization (WHO) noticed at least **11,399 deaths worldwide** (96 % in Africa) due to cholera (Table 1.1) (<http://www.who.int/wer>). A *Vibrio* surveillance system maintained by the Center for Disease Control (CDC) has reported 296 cases of infection caused by vibrios in the USA in 2000 (<http://www.cdc.gov/>). Most strains (n=268) were isolated from stool, wound and blood. From this collection 137, 64 and 27 isolates were identified as *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*,

respectively. Most patients (n=22 or 88 %) who died were infected by *V. vulnificus*. The CDC report also stated that most cases occur during the summer months and that seafood (e.g. oysters, shrimp, fish) had been consumed by the patients. *V. cholerae* enters the human host via contaminated food and/or water (Wachsmuth et al., 1994). In the intestine this bacterium adheres to the epithelium and there it produces an enterotoxin, **cholera toxin (CT)** (Reidl and Klose, 2002). This toxin causes an intense watery diarrhea which may lead to death, but appears to have no role when *V. cholerae* is in the environment (Reidl and Klose, 2002). According to Cotter and DiRita (2000) few virulence genes are involved in cholera disease. In addition to the essential role of CT in cholera, the **toxin-coregulated pilus (TCP)** encoded by *tcpA-tcpF* genes are pivotal for the colonisation 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 (Faruque et al., 1998; Reidl and Klose, 2002). Motility and chemotaxis also play a role in virulence (Watnick and Kolter, 2000).

Table 1.1. World statistics of cholera cases and deaths in the last three years.

	2000		2001		2002*	
	<u>Cases</u>	<u>Deaths</u>	<u>Cases</u>	<u>Deaths</u>	<u>Cases</u>	<u>Deaths</u>
Africa	118,932	4,610	173,359	2,590	121,568	3,753
America	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

*Data of the year of 2002 is incomplete. Number of cases and deaths in Brazil are indicated between brackets. Source: World Health Organisation (<http://www.who.int/en/>).

Studies of the genome of vibrios, particularly *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, have greatly improved current knowledge of their pathogenicity and epidemiology. For instance, the paradigm of cholera

epidemics held until recently was that cholera originated in a particular region of the globe and then spread to other places via human contact and/or contaminated material (Wachsmuth et al., 1994). It has now been pointed out that the genetic background of environmental and clinical *V. cholerae* strains is quite similar and that pathogenic strains may arise from nontoxigenic strains within the aquatic environment (Faruque et al., 1998). *Vibrio cholerae*, "once a harmless environmental organism, has become pathogenic via multiple horizontal gene transfers" (Heidelberg et al., 2000). The cholera toxin (CT) is encoded by genes which belonged originally to the genome of a lysogenic filamentous bacteriophage CTX Φ (Faruque et al., 1998), while the toxin co-regulated pilus gene (TCP) (a crucial intestinal colonisation factor) is part of a recently acquired pathogenicity island (Karaolis et al., 1998). It is clear that within the aquatic environment **transduction** is a major process in the generation of new pandemic *Vibrio* strains (Boyd et al., 2000; Nasu et al., 2000). Moreover, the role of **integrons** in the evolution of antibiotic resistance and in the acquisition of novel genetic capabilities to colonize new ecological niches has been well documented in several *Vibrio* species, including *V. cholerae* (Rowe-Magnus et al., 1998; Rowe-Magnus et al., 2001), *V. parahaemolyticus* (Tagomori et al., 2002) and *V. vulnificus* (Smith and Siebeling, 2003).

V. vulnificus is an important etiologic agent of wound infections and septicemia in humans (Anonymous, 1996; Finkelstein et al., 2002). Septicemia occurs mainly in immunosuppressed people and in subjects 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 the bacterium. A **capsular polysaccharide (CPS)** is the primary virulence factor in *V. vulnificus* pathogenesis (Wright et al., 2001). The presence of this factor correlates with the opaque colony phenotype and is thought to have a inflammatory role within the human body. Smith and Siebeling (2003) described four essential genes (i.e. wcvA, wcvF, wcvI and orf4) responsible for the synthesis of CPS. They showed further that mutation in any of these genes results in loss of capsule which is typical of an avirulent translucent colony phenotype (Wright et al., 2001). 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 (Cervený et al., 2002). A dose of 10^8 phages/mice significantly

reduced *V. vulnificus* from wounds and liver of mice. Estrogen seems to provide protection against *V. vulnificus* lipopolysaccharide-induced endotoxic shock in rats, reducing to half the mortality rate of infected animals (Merkel et al., 2001).

V. parahaemolyticus causes gastroenteritis in which hemolysins (TDH) encoded by the **thermostable direct hemolysin (tdh)** and/or thermostable related hemolysin (*trh*) genes play a crucial role (Nishibuchi and Kaper, 1995). It has been suggested that *V. parahaemolyticus* has acquired these genes via horizontal gene transfer (Nishibuchi and Kaper, 1995). Raimondi et al. (2000) has 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+} . High concentration of TDH increases the number of porin channels which result in ionic influx, culminating in cell swelling and death due to osmotic imbalance (Raimondi et al., 2000). Other toxins, proteases, cytolysins and pili may also play a role as virulence factors in both *V. parahaemolyticus* and *V. vulnificus*.

Other *Vibrio* species e.g. *V. alginolyticus*, *V. cincinnatiensis*, *V. fluvialis*, *V. furnisii*, *V. harveyi*, *V. hollisae*, *V. metschnikovii*, *V. mimicus* and *Photobacterium damsela* have been found in human infections sporadically (Farmer III, 1992; Farmer III and Hickman-Brenner, 1992). Apparently, they have a lesser importance as human pathogens (Farmer III and Hickman-Brenner, 1992).

1.1.2. Nutrient cycling

There are indications that vibrios play a role in the nutrient cycling in the aquatic environments by taking up dissolved organic matter (Sherr and Sherr, 2000). Vibrios may provide essential polyunsaturated fatty acids to the aquatic food web, which many aquatic organisms cannot produce *de novo* (Cottrell and Kirchman, 2003; Nichols, 2003). **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 (Cottrell and Kirchman, 2000a, 2000b; Cottrell et al., 2000; Riemann and Azam, 2002). *Vibrio harveyi*, for instance, excretes at least ten different chitin-degrading enzymes (Svitil et al., 1997). Accordingly, it was suggested that this ability may explain the ubiquitous occurrence of vibrios in aquatic settings (Riemann and Azam, 2002). Some *Vibrio* species are able to degrade toxic polycyclic aromatic hydrocarbons (PAHs) within marine polluted sediments (Hedlund and Staley, 2001). **Vibrios are important producers of antibiotics** among marine bacteria (Long and

Azam, 2001). Inhibitory compounds produced by certain *Vibrio* isolates reduced the group of other community members e.g. *Alfaproteobacteria* and *Alteromonas*. Long and Azam (2001) supposed that this strategy accounts for the microscale variations in competing bacterial populations.

1.1.3. Coral pathogens

Coral reefs are highly productive and very diverse ecosystems within coastal tropical environments, mainly in oligotrophic regions (Hoegh-Guldberg, 1999). These ecosystems are important sources of income for several countries via tourism, fishing, but also represent protection to coastal areas. Tourism in the Caribbean sea generates nearly 90 billion dollars annually (Hoegh-Guldberg, 1999). Coral bleaching i.e. the paling or the loss of color due to the disruption of symbiosis between coral host and symbiotic zooxanthellae is the most serious disease affecting corals.

Coral bleaching is believed to be linked to the increased sea water temperature due to recent global climate changes caused by greenhouse gas emissions, although other factors such as sea water eutrophication, sedimentation, light (ultraviolet radiation and photosynthetically active radiation), pollution by heavy metals, and reduction of salinity may also play a role. The strongest bleaching episodes have occurred during El Niño periods where surface sea water 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 recognized recently** (Ben-Haim and Rosenberg, 2002; Rosenberg and Ben-Haim, 2002). Coral bleaching is linked to the climate changes as two *Vibrio* species i.e. *V. shilonii* and *V. coralliilyticus* have been proven to bleach corals and their pathogenicity was shown to be temperature dependent (Figure 1.3).

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/cm³ in five days) within the coral tissues have been described in detail (Rosenberg and Ben-Haim, 2002). Within the coral tissues most *V. shilonii* cells become VBNC, but continue to be virulent.

V. coralliilyticus, another temperature dependent *V.* pathogen was shown to cause patchy necrosis of tissues of *Pocillopora damicornis* when the coral was

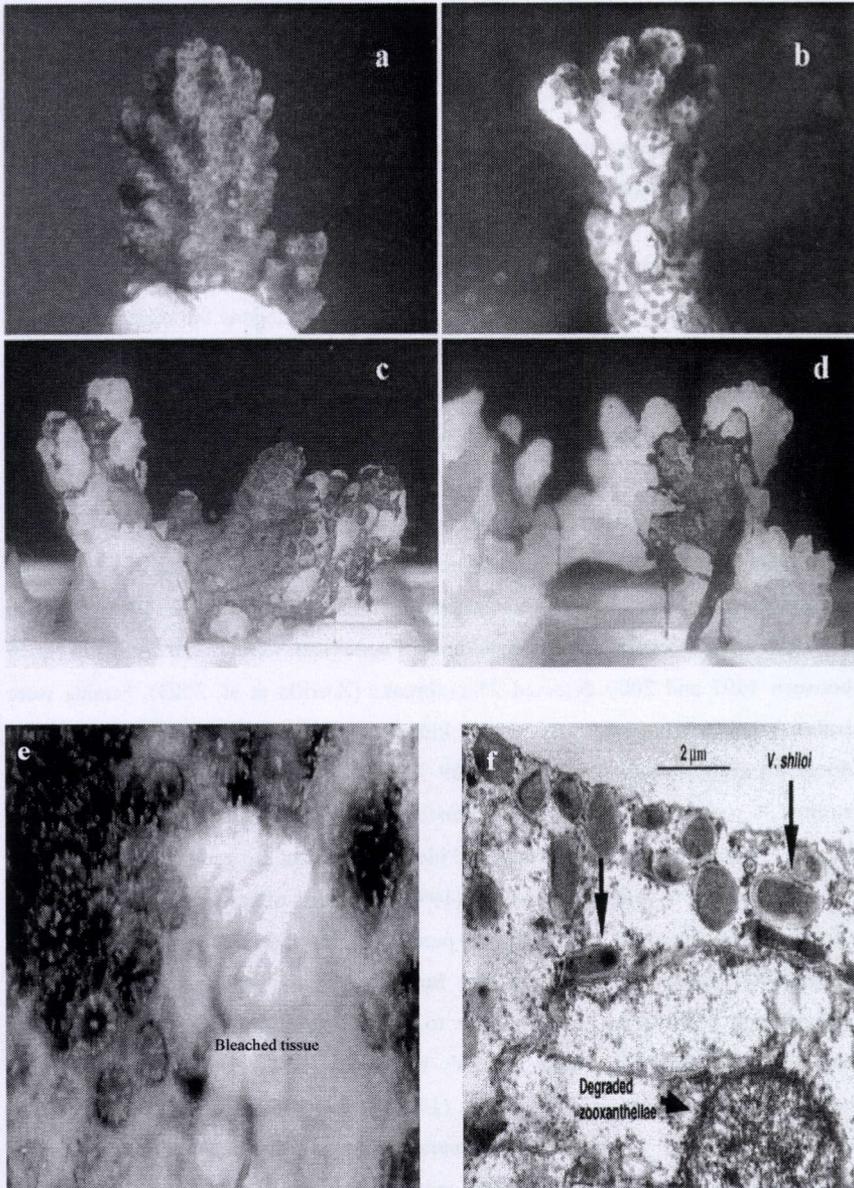


Figure 1.3. The two coral pathogens *V. coralliilyticus* and *V. shilonii*. Source: Rosenberg and Ben-Haim (2002). a-d) Series of pictures showing the infection process caused by *V. coralliilyticus* in the coral *Pocillopora damicornis*. a) healthy uninoculated coral, b) ~20 % lysis, c) 40 % tissue lysis and d) 80 % lysis after 14 days of inoculation. e) A colony of *Oculina patagonica* showing bleached (white) and healthy (green) tissues; f) *V. shilonii* cells infecting *O. patagonica*.

incubated at temperatures of 27 °C or higher (Ben-Haim and Rosenberg, 2002). Because tropical sea water temperatures have undergone warming in the past 100 years, and increases of 1-2 °C have been predicted by 2100 as a result of increased emission of greenhouse gases, it has been suggested that bleaching episodes will occur even more frequently (Hoegh-Guldberg, 1999).

1.1.4. Role of the *Vibrionaceae* in aquaculture

Vibrionaceae species are important bacterial pathogens for animals reared in aquaculture (Austin and Austin, 1999; Bergh et al., 2001; Lightner and Redman, 1998; Zorrilla et al., 2003). ***V. anguillarum*, *V. salmonicida* and *V. vulnificus* are among the main bacterial pathogens for several fish species, and *V. harveyi* for shrimps e.g. *Litopenaeus vannamei* and *Penaeus monodon*** (Austin and Austin, 1999; Lavilla-Pitogo et al., 1998; Leano et al., 1998). Mortalities caused by vibrios in reared fish and shellfish are very common during early larval stages and can occur suddenly, leading sometimes to total mortality (Hansen and Olafsen, 1999; Olafsen, 2001). A bacteriological survey in cultures of sea bream (*Sparus aurata* L.) in Spain between 1997 and 2000 detected 25 outbreaks (Zorrilla et al, 2003). Strains were isolated mainly from 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 several *Vibrio* isolates had phenotypes different from known *Vibrio* species and thus remained identified only at the genus level.

As stated by Austin and Austin (1999) **the mode of infection in fish** consists of three basic steps: (1) the bacterium penetrates into the host tissues by means of chemotactic motility; (2) within the host tissues the bacterium deploys iron sequestering systems e.g. siderophores to "steal" iron from the host, and (3) the bacterium eventually damages the fish by means of extracellular products e.g. haemolysins and proteases. Grisez et al. (1996) showed that the infection of turbot *S. maximus* larvae by *V. anguillarum* occurs in the intestinal epithelium, where the pathogen invades the blood stream and spreads towards different organs culminating in the fish death. More recently, Ringo et al. (2001) detected bacterial endocytosis by the pyloric caeca and midgut of arctic charr (*Salvelinus alpinus* L.) adults and suggested that the whole gastrointestinal tract of fish may be subjected to infection.

Internal symptoms of disease in fish caused by *Vibrionaceae* strains are intestinal necrosis, anaemia, ascitic fluid, petechial haemorrhages on muscle wall, liquid in the air bladder, haemorrhaging and/or bloody exudate in the peritoneum, swollen intestine, haemorrhaging in/on internal organs and pale mottled liver (Austin and Austin, 1999). **External symptoms** include sluggish behaviour, twirling, spiral or erratic movement, lethargy, darkened pigment eye damage/exophthalmia, haemorrhaging in the mouth, gill damage, white and/or dark nodules on the gills and/or skin, fin rot, haemorrhaging at the base of the fins, distended abdomen, haemorrhaging on the surfaces and muscles, ulcers, haemorrhaging 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. (2000a) established **the infection route of *Vibrio proteolyticus* CW8T2**. They first infected *Artemia* nauplii by inoculating the pathogen in the rearing water. One day later they detected the penetration of the bacterium in the gut epithelium with subsequent tissue damage, qualified by the authors as "devastating", and spread towards the *Artemia*'s body cavity. This study illustrates well the infectious capability of certain *Vibrio* strains and suggests that vibriosis in penaeid shrimp larvae rearing systems would be even more devastating taking into account the fragility of these larvae. Lavilla-Pitogo et al. (1998) 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 shrimps between 1992 and 1994 associated with concomitant prevalence of luminous vibrios in rearing water. Lavilla-Pitogo et al. (1998) 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 of e.g. Fidopiastis et al. (1998, 2002), McFall-Ngai (1999, 2002), Oxley et al. (2002) and Ruby (1996) 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. (2002) examined the bacterial flora of healthy wild and reared shrimps *Penaeus merguensis* and found high abundance of vibrios (including *V. logei* ca. 10^4 to 10^5 CFU/g of gut). The authors also highlighted that the bacterial flora of wild and reared penaeid shrimps is similar and suggested that shrimps may influence and/or select the composition of their gut microflora. In the light of the current knowledge about the bacterial population structure of certain human

pathogens e.g. *Neisseria* spp. (Maynard Smith et al., 2000), it is more likely that under favourable conditions (e.g. high nutrient loads and high animal density) within rearing systems a certain hyper-virulent strain (or clone) dominates the bacterial community and causes disease in shrimps and fish rather than the whole bacterial species. This view implies that only a minority of *Vibrio* strains are true pathogens and further underscores the idea that vibrios are opportunistic pathogens.

The pathogenic effects of vibrios are highly critical in aquaculture settings where organisms e.g. penaeid shrimps and salmonids are reared at high densities using very artificial and unstable conditions (Austin and Austin, 1999; Bergh et al., 2001; Lightner and Redman, 1998; Olafsen, 2001). To maintain the productivity of such an **intensive aquaculture**, high inputs of fish protein originated 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 favour the proliferation of vibrios and enhance their virulence and disease prevalence. Obviously, this highly intensive aquaculture has disastrous effects for the environment (Williams et al., 2000; Nailor et al., 1998, 2000). According to Nailor et al. (2000) some of the most serious negative environmental impacts are: (1) loss of wild fish (5 kg of wild fish has to be caught to feed each kg of carnivorous fish reared); (2) loss of natural habitats (e.g. mangroves); (3) use of wild seed to stock ponds; (4) impact of foreign fish and shellfish introduced in the wild and (5) effluent discharge (Naylor et al. 2000). **The spread of antibiotic resistance** from aquaculture settings towards natural environments has recently been shown (Huys et al., 1999). According to Roque et al. (2001) and Molina et al. (2002) about 70 % (n=100) of the vibrios isolated from aquaculture settings in Mexico are multiple-drug resistant. These authors further showed that several *Vibrio* isolates have acquired resistance towards the most employed antibiotics (e.g. enrofloxacin, florfenicol, trimethoprim and oxytetracycline) in shrimp rearing and suggested that the application of these antimicrobials since quite recently has led to the generation of resistant *Vibrio* strains. Ben-Haim et al. (2002) have recently advanced the hypothesis that aquaculture settings serve as foci or reservoir for pathogenic *Vibrio* strains: in certain periods of the year pathogenic vibrios would endure adverse environmental conditions within aquaculture settings and when favourable environmental conditions are re-established, vibrios would be able to cause disease in wild animals.

Alternatives towards a more environmentally sound aquaculture have been proposed (Bergh et al., 2001; Olafsen, 2001). Because certain *Vibrio* strains may be **potential probiotics and/or symbionts of commercially important organisms** such as penaeid shrimps, salmonids, flat fishes and abalones, recent studies have suggested such strains could act as biocontrol agents in aquaculture, diminishing the need for antibiotics and reducing effluent discharges (Verschuere et al., 2000b). The normal bacterial community associated with *L. vannamei* has recently been examined in order to find potential probiotic organisms (Gomez-Gil et al., 1998, 2000, 2002; Moss et al., 2000; Vandenberghe et al., 1999). Planktonic and particle-associated bacteria (arguable vibrios) seem to enhance survival and growth of reared *L. vannamei*. Moss et al. (2000) also reported *Vibrio* and *Aeromonas* compose up to 85 % (about 10^9 CFU/gram of gut tissue) of the bacterial flora in the gut of this shrimp, whereas Gomez-Gil et al. (1998) found a wealth of vibrios i.e. 10^5 CFU/g and 10^4 CFU/ml, respectively, in the hepatopancreas and the hemolymph of healthy *L. vannamei*.

Pujalte et al. (1999) 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 FISH the same authors determined vibrios accounted for up to 40 % (156 cells/ml) of the heterotrophic culturable flora grown on MA. In a successful recirculating rearing system for rotifers the *Vibrio* spp. abundance was up to 1.7×10^5 CFU/ml, suggesting that these bacteria were playing a positive role in the health of the rotifers (Suantika et al. 2001). Sawabe et al. (2003) estimated the abundance of *V. halioticoli* strains in the gut of several abalone (*Haliotis* spp.) species. The authors report *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 gut. *V. halioticoli* strains were found to produce high amounts of acetic and formic acid (up to 68.1 mM) which may in turn be used as energy source or precursor for protein synthesis by the abalones.

Because the use of probiotics in domestic animals e.g. pigs and chickens has had a certain success, several researchers advocate that the use of probiotic bacterial strains or selected mixtures will have a positive impact in health management of marine organisms (Irianto and Austin, 2002; Olafsen, 2001; Verschuere et al., 2000b). A considerable difference between the culture of domestic and aquatic animals is that the latter is in constant and intimate contact with a wealth of microorganisms e.g.

viruses, protozoa, fungi (Sherr and Sherr, 2000, 2002). Unfortunately, studies on the use of "probiotic" bacteria have not looked at the interactions with the aquatic microbial food web (Azam et al., 1983; Sherr and Sherr, 2000). The so called probiotic bacterial strains could well be fuelling the food web, giving rise to 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 (Thompson et al., 2002).

1.2. Classification of the *Vibrionaceae*

The beginning of bacterial taxonomy or systematics can be traced back to the times of the Swedish botanist C. von Linné (1707- 1778) who assigned the "animalcules" of A. van Leeuwenhoek (1632-1723) into a "specia dubia" group (Kandler, 1985, Drews, 2000). Taxonomy has undergone a tremendous change in the last few decades mainly due to technological developments (Schleifer and Stackebrandt, 1983). Today its scope includes the phylogeny, classification, nomenclature and identification of bacterial isolates (Goodfellow, 2000; Vandamme et al., 1996). One of the main aims of taxonomy is to provide useful classification schemes to be used at a variety of scientific and practical purposes. Ideally, such classifications should be stable, predictive, objective and highly informative (Goodfellow, 2000).

The genera *Vibrio* and *Photobacterium*, the two most important taxa within the family *Vibrionaceae*, are among the oldest bacterial genera (Kandler, 1985, Drews, 2000). The beginning of the taxonomy of vibrios can be traced back to the work of Pacini who provided the first description of *Vibrio cholerae*, the current type species of the family *Vibrionaceae*, while studying outbreaks of cholera in Florence in 1854. 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 7th edition of **Bergey's Manual of Determinative Bacteriology (Breed et al., 1957)**, the genus *Vibrio* belonged to the family *Spirillaceae* and consisted of 34 species which, with the exception of *V. cholerae* (= *V. comma*) and *V. metschnikovii*, were later reclassified into other genera e.g. *Campylobacter* (*C. fetus*, *C. jejuni*, *C. sputorum*), *Comamonas* (*C. terrigena*), *Pseudomonas* (*P. fluorescens*) or no longer accepted as

validly described species according to the **Approved List of Bacterial Names (Skerman et al., 1980)**. The genus *Photobacterium*, on the other side, harboured one species i.e. *P. phosphoreum* and was allocated into the genus *Bacterium* of the family *Bacteriaceae*.

The heterogeneity within the genus *Vibrio* was highlighted by Davis and Park (1962). By examining morphological and biochemical features of most species of the genus *Vibrio*, they showed that the genus *Vibrio* was quite artificial and they concluded that at least three genera would exist among the species examined. The foundation of the modern *Vibrio* taxonomy was laid by a number of numerical (phenetic) and/or polyphasic taxonomic studies by P. Baumann, R. Colwell, R. Sakasaki and J. Lee (Baumann et al., 1971; Baumann and Baumann, 1977; Baumann et al., 1980; Baumann et al., 1983; Colwell, 1970; Citarella and Colwell, 1970; Fujino et al. 1974; Lee et al., 1981; Reichelt et al., 1976; Tubiash et al., 1970; West et al., 1983). Most of these studies clustered large collections of strains on the basis of their ability to utilise different (ca. 50-150) compounds as sources of carbon and/or energy, enzyme activity (e.g. gelatinase, chitinase, DNase), salt tolerance, luminescence, growth at different temperatures, antibiograms, DNA base composition, morphological features, and other biochemical tests (e.g. oxidase, catalase, Voges-Proskauer, indole, nitrate reduction, arginine dihydrolase, lysine and ornithine decarboxylases). The clusters defined by phenotypic features were further refined and validated by DNA-DNA hybridisation experiments; phenotypic clusters having about 80 % similarity were found to correspond to DNA-DNA homology clusters having more than 80 % similarity (Baumann et al., 1977). This suggests that for the *Vibrionaceae* taxonomy one should use 80 % DNA-DNA similarity as the limit for species definition instead of the canonical 70 % proposed by Wayne et al. (1987).

In the 8th edition of the **Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974)** the family *Vibrionaceae* which was proposed by Véron (1965), comprised *Vibrio* and *Photobacterium* along with *Beneckea*, *Aeromonas*, *Plesiomonas* and *Lucibacterium*. The combination of *Vibrio* (*V. anguillarum*, *V. cholerae*, *V. costicola*, *V. fischeri*, *V. parahaemolyticus*) and *Photobacterium* (*P. mandapamensis*=*P. leiognathi* and *P. phosphoreum*) in a single family was an improvement of the taxonomy of these two related genera which were thought for a long time to be distantly related. Baumann et al. (1971) proposed the

genus *Beneckeia* 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. (1977, 1980, 1983) suggested that *Beneckeia* species and *Lucibacterium harveyi* should be reallocated to the genus *Vibrio*. *Aeromonas* and *Plesiomonas* should be placed into other families and *V. costicola* in another genus (Baumann et al., 1977; Baumann et al., 1980; Baumann et al., 1983). These authors also suspected that the evolution of *Vibrio* and *Photobacterium* species to be 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 (Baumann et al., 1984a, 1984b; Reichelt et al., 1976). These studies disclosed a **core group of related vibrios i.e. the *Vibrio harveyi* group consisting of *V. harveyi*, *V. campbellii*, *V. natriegens*, *V. alginolyticus* and *V. parahaemolyticus***. *V. harveyi* and *V. campbellii* were found to have 61-74 % DNA-DNA similarity, while *V. parahaemolyticus* and *V. alginolyticus* had 61-67 %. Reichelt et al. (1976) also proposed biotypes I and II for each *V. splendidus* and *V. pelagius*, but they suggested these biotypes could be in fact different species. The biotypes I and II of *V. splendidus* and *V. pelagius* showed at maximum 61 % and 58 % DNA-DNA similarity, respectively. Additionally, the biotypes of both species were clearly distinguishable by phenotypic features. Nevertheless, researchers have been using the biotype designation still today. Arias et al. (1997) have suggested that the two biotypes of *V. vulnificus* should be abolished. These biotypes should in fact be considered as different species according to the current species definition (Stackebrandt et al., 2002).

It is important to highlight that Baumann et al. (1983) had already realised the limited value of DNA-DNA hybridisation studies for bacterial taxonomy stating: "Regrettably in vitro DNA-DNA hybridisation is becoming the ultimate standard method despite its inherent limitations". To better index bacterial evolutionary relatedness they suggested the comparison of amino acid sequence differences of certain proteins e.g. glutamine synthetase (GS), superoxide dismutase (SOD) and alkaline phosphatase (AP) which were supposed to have different evolutionary rates and could be used in combination in order to distinguish closely and distantly related *Vibrio* species. Because the determination of amino acid sequence 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 i.e. GS and SOD (Baumann et al., 1983). On the basis of this analysis they concluded that *Beneckea* species, *Photobacterium fischeri* and *P. logei* should be transferred to the genus *Vibrio* (Baumann et al., 1980) (see also the 1th edition of **Bergey's Manual of Systematic Bacteriology; Krieg and Holt, 1984**). They also mentioned that they needed to apply a certain "subjective judgement" about the limits of the genus *Vibrio* because they found this genus was highly diverse. Several species e.g. *V. cholerae*, *V. fischeri*, *V. logei*, *V. costicola* (now *Salinivibrio costicola*) were distantly related to each other and to the *Beneckea* species.

In the 2th edition of the **Bergey's Manual of Systematic Bacteriology, 2002** (the outline is available at <http://dx.doi.org/10.1007/bergeysoutline200210>) the family *Vibrionaceae* encompasses six genera: (1) *Vibrio* (43 species); (2) *Photobacterium* (6 species); (3) *Listonella* (2 species); (4) *Salinivibrio* (1 species); (5) *Enhydrobacter* (1 species) and (6) *Allomonas* (1 species). *Allomonas* (Kalina et al., 1984) and *Enhydrobacter* (Staley et al., 1987) harbour a single species *A. enterica* and *E. aerosaccus*, respectively. These genera were tentatively allocated to the family *Vibrionaceae* based on phenotypic features and DNA hybridisation experiments, but so far there has not been a confirmation of their phylogenetic position based on 16S rDNA sequence data.

More recently Stackebrandt et al. (2002) have listed the main shortcomings of DNA-DNA hybridisation techniques to the study of bacterial taxonomy: (i) few labs can execute this technique, (ii) the methodology is the slowest and most problematic step in the species description, (iii) DNA-DNA data are not cumulative, and each new experiment requires inclusion of reference strains. Finally, Stackebrandt et al. (2002) concluded that the methodology cannot be improved and thus researchers should seek for new alternatives and new species proposals based on new approaches. Lan and Reeves (2000, 2001) have criticised the use DNA-DNA hybridisation and 16S rDNA sequences as standards. Alternatively they propose that bacterial classification should be based on phylogenetic and population genetic analyses of housekeeping genes.

1.3. Phylogeny of the *Vibrionaceae*

In the last two decades bacterial taxonomy was enriched with chronometers e.g. rRNAs (5S, 16S, 23S) to reconstruct bacterial phylogenies but also as taxonomic markers for identification. In many cases the phylogenies obtained by 16S rRNA sequencing pointed out to the inadequacy of grouping bacteria by the classical criteria e.g. morphology and biochemical features. The fairly close relationship of *Vibrio* and *Photobacterium* was promptly confirmed by this approach and both genera were placed within the Purple Bacteria, a diverse group of Gram negative, phototrophic and heterotrophic bacteria (Woese et al., 1985), later renamed *Proteobacteria* (Stackebrandt et al., 1988). More recently the *Proteobacteria* has been elevated to the rank of phylum (Figure 1.4) (Kerstens et al., 2003; 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 (Dorsch et al., 1992; Kita-Tsukamoto et al., 1993; Macdonell and Colwell, 1985; Pujalte et al., 1992; Ruimy et al., 1994). Alternatively other genes coding for e.g. 23S rDNA (Macián et al., 2001a, 2002b), RNA polymerase subunitB (*rpoB*) (Mollet et al., 1997), recombination-repair (*recA*), malate dehydrogenase (*mdh*), hemolysin (*hlyA*) and replication proteins (e.g. *dnaE* and *gyraseB=gyrB*) (Byun et al., 1999, Vuddhakul et al., 2000) have been used to study the evolutionary relationships among vibrios and related organisms.

1.3.1. 5S rRNA-based phylogeny

Macdonell and Colwell (1985) analysing the 5S rRNA of the superfamily I (*Vibrionaceae* + *Enterobacteriaceae*) correctly concluded that *V. marinus* (now *Moritella marina* in the family *Alteromonadaceae*), *V. psychroerythrus* (now *Colwellia psychroerythrus* in the family *Alteromonadaceae*) and *Aeromonas* spp. (now in the family *Aeromonadaceae*) were not authentic *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 found currently within the family *Alteromonadaceae*, but the genus *Listonella* which would encompass the species *V. anguillarum*, *V. pelagius*, *P. damsela* has not been recognised by many expert taxonomists in the field (Austin et al., 1995b). Further phenotypic and phylogenetic studies clearly evidenced that these three species should be retained in their original genera (Smith et al., 1991; Dorsch et al., 1992; Kita-Tsukamoto et al., 1993). The use of 5S rRNA sequences to reconstruct the phylogeny is clearly of limited use (Pujalte et al., 1992), probably due to its small size ca. 500 bp (Woese, 1987).

1.7.2. 16S rRNA-based phylogeny

The 16S rDNA molecule (about 1500 bp in length) consists of highly conserved regions which may reveal deep-branching (e.g. classes, phylum) relationships, but also variable regions which may discriminate species within the same genus. This feature has prompted researchers to use 16S rDNA both as a identification tool (Gaurger et al., 2002; Wiik et al., 1995) and as a phylogenetic marker. The Ribosomal Database Project II (<http://rdp.cme.msu.edu/html/>) consists of nearly 65,000 thousand 16S rDNA sequences. These entries can be easily queried using publicly available softwares e.g. BLAST (Altschul et al., 1990) and FASTA (Pearson and Lipman, 1988).

Dorsch et al. (1992) determined almost complete 16S rRNA sequences of 10 *Vibrio* species and obtained results on the *Vibrio* core group which were in agreement with previous DNA-DNA homology data of Baumann et al. (1977). Dorsch et al. (1992) also indicated that *V. hollisae* should be allocated into a new genus. In 1993, Kita-Tsukamoto et al. presented a comprehensive phylogenetic study of the *Vibrionaceae*. Although this study was based on partial 16S rRNA sequences, Kita-Tsukamoto and co-workers selected a broad collection of 50 species including the type species of the family *Vibrionaceae*, *Vibrio cholerae*, most *Vibrio* species, and species of *Aeromonas*, *Deleya*, *Escherichia*, *Marinomonas*, *Pseudomonas* and *Shewanella*. The main outcomes of this study were (1) the circumscription of species (at least 99.3 % 16S rRNA similarity), genera (95-96 %) and family (90-91 %) borders within the *Vibrionaceae* and (2) 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. (1993) were

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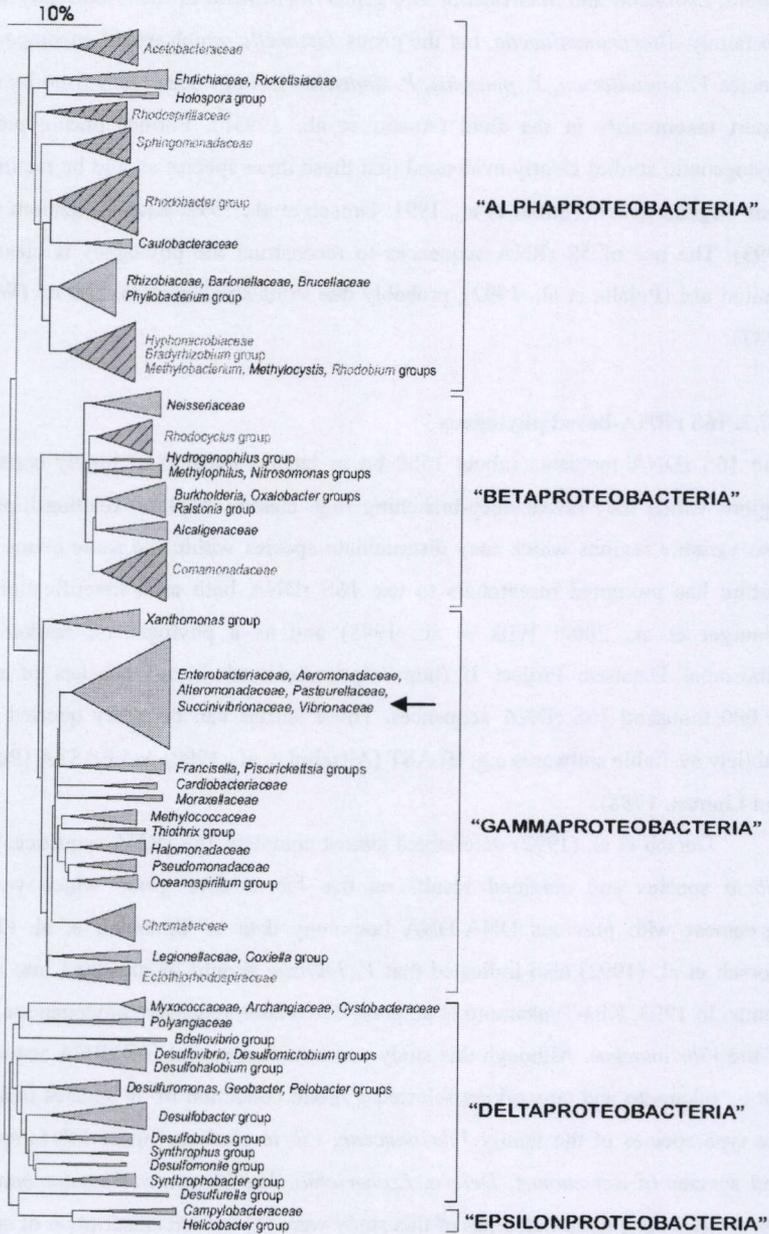


Figure 1.4. Phylogenetic tree of the *Proteobacteria* based on the 16S rDNA sequences. Source: Kersters et al. (2003).

further addressed by Mellado et al. (1996) who transferred *V. costicola* into *Salinivibrio costicola* and Urakawa et al. (1998b, 1999b) who transferred *V. marinus* into *Moritella marina* and *V. iliopiscarius* into *Photobacterium iliopiscarius*. According to Kita-Tsukamoto et al. (1993) *V. cholerae* and *V. mimicus* would correspond to a genus on their own. The psychrophilic vibrios *V. fischeri*, *V. logei*, *V. salmonicida* and relatives should be elevated to the genus rank. In both cases the status of these *Vibrio* species has not yet been fully determined. If *V. cholerae* and *V. mimicus* and the psychrophilic vibrios are to be elevated to the genus level, then one might argue the revival of *Beneckeia* to encompass all other remaining vibrios, an idea which was originally supported by Baumann et al. (1971).

1.4. Genomics of the *Vibrionaceae*

The study of the genomes of vibrios has led to important breakthroughs regarding all the aspects of their biology. Certain *Vibrio* species i.e. *V. anguillarum*, *V. cholerae*, *V. fluvialis*, *V. parahaemolyticus*, *V. vulnificus* have been shown to have two chromosomes (Yamaichi et al., 1999). These authors concluded that the split of the genome in two replicons would be advantageous for the rapid DNA replication normally observed in *V. parahaemolyticus*, a species with a short doubling time of 8 to 9 minutes. A detailed analysis of the genome of *V. cholerae* El Tor N16961 confirmed the presence of two chromosomes, a larger one 2,961,151 bp containing 2,770 open reading frames (ORFs) and a smaller one 1,072,914 bp containing 1,115 ORFs (Heidelberg et al., 2000). Most ORFs (>86 %) are coding. The majority of the genes required for growth and viability are localised on the larger chromosome. Significantly more genes encoding for DNA replication and repair, transcription, translation, cell-wall biosynthesis, a variety of central catabolic and biosynthetic pathways and pathogenicity genes are also localised on the larger chromosome. More hypothetical genes are found on chromosome two. Duplication of 105 genes in both chromosomes involved in chemotaxis and solute transport points out to the importance of these gene products in the biology of *V. cholerae*, particularly its ability to live in diverse environments. Because the smaller chromosome lacks rRNA operons and contains relatively less essential and/or functional genes and harbours an integron region, Heidelberg et al. (2000) hypothesised that this chromosome was a megaplasmid acquired by an ancestral

vibrio. These authors also suggest that under certain environmental conditions *V. cholerae* could generate **single-chromosome cells** which would not replicate (potentially VBNC) but would help the persistence of the species by producing enzymes (e.g. proteases and chitinases) that enhance the survival of *V. cholerae* cells in biofilms during adverse environmental conditions. Other researchers argue that the small chromosome may have arisen by excision from a single large ancestral genome (Waldor and Raychaudhuri, 2000; Tagomori et al., 2002). Comparative genomic analysis on *V. cholerae* and *V. parahaemolyticus* has reinforced this view. In *V. parahaemolyticus* also two chromosomes (3.3 and 1.9 Mb) occur, but the integron region is found on the larger chromosome. Most ORFs in the two chromosomes of *V. parahaemolyticus* have homologous in the genome of *V. cholerae*. Differences in the physical order of homologous genes through the chromosomes of *V. cholerae* and *V. parahaemolyticus* are due to intrachromosomal recombination and reshuffling. The toxin gene thermostable direct hemolysin (tdh) of *V. parahaemolyticus* occurs on the small chromosome, while the toxin genes CT and TCP of *V. cholerae* occur on the large chromosome.

1.5. Identification and typing of the *Vibrionaceae*

Vibrionaceae strains are Gram negative, usually motile rods, chemoorganotrophic, have a facultative fermentative metabolism and are found in aquatic habitats. They are generally able to grow on thiosulphate-citrate-bile salt-sucrose agar (TCBS) and are often oxidase-positive. An array of phenotypic and genomic techniques have become available for the identification of vibrios in the last three decades (Figure 1.5) (van Belkum et al., 2001; Olive and Bean, 1999; Salvelkoul et al., 1999; Rademaker et al., 1996; Vandamme et al., 1996). Ribotyping and PCR based techniques e.g. amplified fragment length polymorphism (AFLP), amplified ribosomal DNA restriction analysis (ARDRA), random amplified polymorphism DNA (RAPD), repetitive extragenic palindromes (rep), restriction fragment length polymorphism (RFLP), but also multilocus enzyme electrophoresis (MLEE) and latter on multilocus sequence typing (MLST) have yielded the most valuable information and new insights into the population structure of some species of the *Vibrionaceae* and also provided means of identifying these organisms. Below

we present some of the methodologies which have been mostly used for the identification and typing of vibrios.

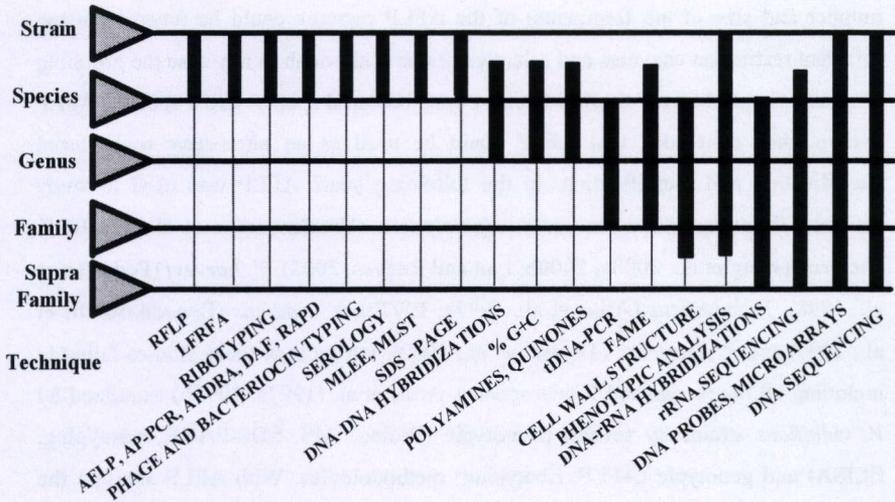


Figure 1.5. Overview of techniques and their respective taxonomic discriminatory power. Adapted from Vandamme et al. (1996).

1.5.1. Amplified Fragment Length Polymorphism (AFLP)

The AFLP technique consists of three main steps: (1) digestion of total genomic DNA with two restriction enzymes and subsequent ligation of the restriction halfsite-specific adaptors to all restriction fragments; (2) selective amplification of these fragments with two PCR primers that have corresponding adaptor- and restriction site sequences as their target sites and (3) 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 (Vos et al., 1995; Huys and Swings, 1999). Originally, Vos et al. (1995) used radioactive labelled primers, but now AFLP is mainly performed with fluorescently labelled primers. FAFLP indexes variation in the whole genome and thus is considered to give useful information on the short- and long-term evolution of bacterial strains (Lan and Reeves, 2002). Janssen et al. (1996) 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* and one *V. tubiashii*

strain. The grouping obtained by AFLP corresponded well to that obtained by DNA-DNA similarity data. Janssen et al. (1996) also reported that the complexity (i.e. number and size of the fragments) of the AFLP patterns could be tuned by using different restriction enzymes and selective primers, although in any case the grouping of strains should be very similar. Because each bacterial species had a specific AFLP pattern, they concluded that AFLP could be used as an alternative to bacterial classification and identification. In the following years AFLP was used to study several *Vibrionaceae* species i.e. *V. alginolyticus* (Vandenberghé et al., 1999), *V. cholerae* (Jiang et al., 2000a, 2000b; Lan and Reeves, 2002), *V. harveyi* (Pedersen et al., 1998), *V. vulnificus* (Arias et al., 1997a, 1997b), *V. wodanis* (Benediktsdóttir et al., 2000) and *P. damsela* (Thyssen et al., 2000), but most of these studies failed in including all the recognised *Vibrio* species. Arias et al. (1997a, 1997b) examined 80 *V. vulnificus* strains by several phenotypic (Biolog, API, SDS-PAGE, serotyping, ELISA) and genotypic (AFLP, ribotyping) methodologies. With AFLP analysis the authors were able to discriminate strains with identical ribotypes and thus they concluded AFLP is the most suitable and discriminatory tool for epidemiological studies on *V. vulnificus*. Other AFLP analysis 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 (Pedersen et al., 1998). Vandenberghé et al. (1999) discriminated pathogenic and probiotic *V. alginolyticus* strains using AFLP and concluded that this technique should be used to authenticate probiotic cultures prior to their use. Thyssen et al. (2000) used AFLP to differentiate the two subspecies of *Photobacterium damsela* i. e. *P. d.* subsp. *damsela* and *P. d.* subsp. *piscicida*.

Jiang et al. (2000a, 2000b) discriminated *V. cholerae* serogroups O1 and O139 using AFLP with *ApaI*-*TaqI* restriction enzymes. They found that the genetic background of environmental and clinical *V. cholerae* strains is quite similar and concluded that pathogenic strains may in fact arise from nontoxicogenic strains within the aquatic environment. Jiang et al. (2000a) demonstrated by AFLP analysis that the population structure of *V. cholerae* undergoes seasonal shifts. Certain clones are abundant during winter and others during summer. More recently Lan and Reeves (2002) examined 45 *V. cholerae* isolates of the seventh pandemic and partitioned these isolates in 38 FAFLP profiles. They concluded that FAFLP is the best tool for discriminating clones of the seventh pandemic and suggested the design of PCR

primers which target particular AFLP bands that could be used for epidemiological analysis.

1.5.2. Multilocus Enzyme Electrophoresis (MLEE) and Multilocus Sequence Typing (MLST)

MLEE was first applied in bacterial systematics in the 1980s and has become the standard technique for studies of population genetics (Caugant, 2000; Selander and Levin, 1980). MLST was developed recently as an improved adaptation of MLEE (Maiden et al., 1998). Both techniques index variation in housekeeping genes. MLST assigns alleles directly from the nucleotide sequences, while MLEE compares the electrophoretic mobility of the enzymes that the genes encode (Caugant, 2000, Feil and Spratt, 2001). Obviously, MLST has several advantages over MLEE e.g. higher discriminatory power because it detects synonymous and non-synonymous changes, accuracy and portability of the data, ease to perform and reproducibility (Maiden et al., 1998). 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** (Beltrán et al., 1999). A high genetic diversity as assessed by MLEE of 15 enzyme loci was observed among 107 diverse *V. cholerae* isolates (Farfán et al., 2000). These isolates displayed 99 different electrophoretic patterns, a high number of alleles (i.e. 2 to 7) per locus, but no significant clustering between serogroups, biotype and country of isolation was observed (Farfán et al., 2000). These authors applied MLST of six housekeeping enzyme loci on a subset of 31 *V. cholerae* serogroup O139 strains and found four distinct groups of strains (Farfán et al., 2002). They concluded that recombination, if any, has not occurred among these vibrios.

1.5.3. Random Amplified Polymorphism DNA (RAPD) and Repetive Extragenic Palindrome (rep-PCR)

Random Amplified Polymorphism DNA involves PCR amplification of random fragments of genomic DNA using arbitrary primers, while rep-PCR amplifies intervening sequences located between highly repetitive DNA motifs (Towner and Grundman, 2000). This technique have been used mainly with the aim of typing a few *Vibrio* species i.e. *V. alginolyticus* (Sudheesh et al., 2002) *V.*

cholerae (Rivera et al, 1995), *V. parahaemolyticus* (Wong et al., 2001) and *V. vulnificus* (Warner and Oliver, 1999) and it is thus difficult to determine its taxonomic resolution and value for the whole family *Vibrionaceae*. According to Sudheesh et al. (2002) *V. alginolyticus* and *V. parahaemolyticus* have different RAPD profiles and thus they can be reliably separated by this fast screening methodology. Wong and Lin (2001) compared RAPD, rep-PCR, PFGE and ribotyping and concluded that Rep-PCR is the most discriminatory of the techniques. Rivera et al. (1995) analysed 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.

1.5.4. Restriction Fragment Length Polymorphism (PCR-RFLP)

PCR-RFLP technique consists of the PCR amplification of certain genes e.g. 16S rDNA, *gyrB*, *rpoD* and subsequent restriction of the PCR products with endonucleases to obtain band patterns (Le Roux et al., 2002; Urakawa et al., 1997, 1998a, 1999a). According to Urakawa et al. (1997), who analysed the restriction patterns of the 16S rDNA of 35 *Vibrionaceae* species, this technique is useful for the classification and identification of *Vibrionaceae* strains. Although a closer examination of the data presented by these authors reveals e.g. that all *Vibrio* core group (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. *V. tubiashii* and *V. pelagius* showed identical genotypes and also *Photobacterium iliopiscarius*, *P. leignathi* and *P. phophoreum*. This is quite striking since the 16S rDNA sequence similarity between these *Vibrio* and *Photobacterium* species is only about 97.8 % and < 96.5%, respectively, clearly proving the low discriminatory power of PCR-RFLP in this particular study.

1.5.5. Ribotyping

Ribotyping consists of four main steps: (1) restriction of the bacterial chromosome with an endonuclease, (2) gel electrophoresis of the resulting fragments, (3) transfer of the fragments to a membrane and (4) hybridisation of the gel with a labelled probe complementary to the 16S and 23S rDNA (Grimont and Grimont, 1986). Ribotyping was one of the first fingerprinting techniques to be successfully

used in the taxonomy of vibrios, and has been particularly useful in the study of *V. cholerae* (Grimont and Grimont, 1986, 2001; Lan and Reeves, 1998; Pourshafie et al., 2000, 2002). A standardised ribotyping scheme was proposed as a reliable tool for epidemiological studies on *V. cholerae* (Popovic et al., 1993). In this scheme 214 *V. cholerae* O1 strains isolated from 35 countries were partitioned in 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 highlighted that certain clones were particular to certain regions e.g. the ribotype 8 was restricted to central Africa and the ribotype 5 was predominant in the Latin America epidemics. Using ribotyping to analyse 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 (Faruque et al., 1998). 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 (Coelho et al., 1995; Faruque et al., 1999). More recently, ribotyping has been used to assess the genomic diversity of environmental *Vibrio* strains associated with fish and oysters (Austin et al., 1997; Macián et al., 2000a; Pujalte et al., 1999). According to Austin et al. (1995b) closely related *Vibrio* species e.g. *V. anguillarum* and *V. ordalii* can be differentiated on the basis of ribotyping. Macián et al. (2000a) analysed 82 *V. splendidus*, 25 *V. harveyi* and 10 *V. tubiashii* strains isolated in one-year period and found 64, 17 and 9 different ribotypes, respectively; certain *V. splendidus* ribotypes were typical for summer, while others were for winter.

1.5.6. Phenotypic identification

Classical phenotypic identification, including tests for arginine dihydrolase, lysine and ornithine decarboxylases were among the most extensively used techniques to screen the *Vibrio* diversity associated with marine animals and their habitat and these tests have been proposed as reliable species identification schemes (Alsina and Blanch, 1994a, 1994b; Austin et al., 1997; Blanch et al., 1997; Macián et al., 1996; Ortigosa et al., 1989, 1994). However, variable results e.g. for arginine dihydrolase of some species have been reported, making their identification on this

basis difficult (Pujalte et al., 1992). Biolog was also one of the most used phenotypic techniques for the identification of *Vibrionaceae* strains in the last decade (Austin et al., 1995a, 1995b 1997; Vandenberghe et al., 1999). It consists of the inoculation of bacterial strains in 95 different compounds which may serve as carbon sources, and thus the grouping of the strains is based on the utilisation of these 95 carbon sources. One of the most important diagnostic phenotypic features for the identification of *Vibrio* species was the presence of flagella and thereby motility, but Sawabe et al. (1998) have described a non-motile species i.e. *V. haliotocoli*, suggesting that the presence of flagellum is not an essential diagnostic feature. Likewise, oxidase-negative *V. metschnikovii* and *V. gazogenes* strains have been documented and so *Vibrio* strains that fail to grow on TCBS (Alsina and Blanch, 1994). Colony variation is also a common feature among *Vibrio* species (Hickman et al., 1982; Watnick et al., 2001).

Fatty acids methyl esters profiling was evaluated for the differentiation of *Vibrionaceae* species (Lambert et al., 1983; Bertone et al., 1996). Apparently, differentiation at the genus level was possible, but similar FAME profiles among the different species examined was very striking and the authors thus concluded that this technique could only be used as an additional phenotypic feature. The ample phenotypic variability within *Vibrionaceae* species points out that classification and identification schemes should be based on genomic data.

1.5.7. Selective media and species specific probes

Detection of vibrios on selective media and subsequent colony hybridisation with species-specific probes based on variable regions e.g. V3 of the 16S rDNA has also been evaluated as a fast screening alternative tool for *V. harveyi* (Harris et al., 1996), *V. proteolyticus* (Muniesa-Pérez et al., 1996), *V. vulnificus* (Cerdeña-Cuellar et al., 2000), *V. anguillarum* (Martínez-Picado et al., 1996) and *V. scophthalmi* (Cerdeña-Cuellar, 2002). It was demonstrated that on the different selective media a number of *Vibrio* species, sometimes even not allocated to the same phylogenetic branches, were also able to grow, proving that those media were not quite selective. Species-specific media are thus yet to be formulated. Likewise, the specificity of certain probes e.g. for detection of *V. anguillarum* has not been proven as it hybridises also with *V. ordalii*, *V. diazotrophicus* and *V. navarrensis* (Martínez-Picado et al., 1996).

Similarly, the probe for *V. scophthalmi* detection was not evaluated against *V. ichthyoenteri*. Both species share nearly 100 % 16S rDNA similarity and there is thus a great chance of crosshybridisation. Interesting enough, *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. This suggests that misleading conclusions could arise from ecological studies using this probe (Cerdeña-Cuéllar, 2002). On the other hand the probe for *V. vulnificus* detection seemed to be very reliable (Cerdeña-Cuéllar et al., 2000).

1.6. Current bacterial species definitions

Because in this thesis we sought to improve the taxonomy of the *Vibrionaceae*, it is important to examine the species definition for bacteria. In the past, several definitions have been proposed. Several researchers think of bacterial species as "**condensed nodes in a cloudy and confluent taxonomic space**" (Vandamme et al., 1996). 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" (Vandamme et al., 1996; van Belkum et al., 2001). Throughout this work we have followed the species definition most recently recommended by the ad hoc committee for the re-evaluation of the species definition in bacteriology (Stackebrandt et al., 2002). According to this definition 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 will share at least 70 % DNA-DNA similarity at stringent conditions. Other authors suggest a more relaxed limit i.e. 50-70 % similarity (Rosselló-Mora and Amann, 2001). Young (2001) has pointed out that the phenotypic circumscription of the bacterial species boundaries is the most important criterium for species definition. Other researchers have developed a species definition based only on the 16S rDNA sequences to delineate marine bacterioplankton species (Hagström et al., 2002). These authors argued species would be entities having at least 97 % 16S rDNA similarity. However, a recent ecological and evolutionary theory has provided for the first time a species concept - **the ecotype** - based on the population genetics principles applied to MLST data (Cohan, 2002). According to this concept an ecotype is a clonal complex i.e. a cluster of strains that share 5 or 6

identical 500-bp gene fragments out of 7 housekeeping genes. In the case of non-clonal populations e.g. *Neisseria* spp., an ecotype has 4 identical loci out of 7 (Maiden et al., 1998). Cohan (2000) also argues that most of the currently recognised species thus harbour several different ecotypes each of which occupying a different ecological niche. This author concluded that recognised species could be in fact regarded as genus. This view has been supported by other population geneticists who propose a species genome definition based on phylogenetic and genetic analyses of housekeeping genes (Lan and Reeves, 2000; 2001). The idea that a bacterial species concept should be shaped by both evolutionary and ecological grounds has also been argued by other microbiologists (Ward, 1998). According to Ward each ecologically unique population is in fact a unique species.

Maynard Smith et al. (2000) see the *Neisseria* species as clusters, partially characteristic, but sharing some common identity with 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. (2000) 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. (2000) about *Neisseria* species holds for other or perhaps all currently circumscribed bacterial species it is yet to be proven. Accordingly, in the next years bacterial taxonomists will likely be faced with the goal of identifying clones and clonal complexes if they intend verifying the hypothesis of Maynard Smith and colleagues.

1.7. The conceptual framework and aim of this thesis

Between 1988 and 2001 a large collection of vibrios was constituted in the framework of several EU projects aimed at the characterisation of vibrios in aquaculture facilities in Africa, America, Asia and Europe. A collection of about

2,000 *Vibrio* isolates, stocked in the Laboratory for Microbiology, Ghent University, was analysed mainly by phenotypic methodologies (Austin et al., 1995b; Austin et al., 1997; Grisez et al., 1997; Huys et al., 2001; Pedersen et al., 1998; Vandenberghe et al., 1998; Vandenberghe et al., 1999; Vandenberghe et al., 2003; Verdonck et al., 1997). These projects had shown that (1) vibrios are abundant in the aquaculture environment and frequently associated with disease outbreaks (2) many vibrios did not belong to any known species, as revealed mainly by phenotypic techniques. In this context, we hypothesised that unknown *Vibrio* phenotypes are in fact novel *Vibrio* species that should be classified.

The study of Vandenberghe et al. (2003) analysed 1,473 *Vibrio* isolates by Biolog. The results of this analysis are shown in chapter 2 (section 2.1). The most striking result of this study was that 56 groups did not cluster with any of the type strains and thus remained unidentified. Vandenberghe et al. (2003) also concluded that a high phenotypic diversity exists among vibrios. In order to extend and refine the observations of Vandenberghe et al. (2003), my PhD work started with the hypothesis that unidentified Biolog phenotypes belong to species not yet described.

To test the hypothesis that unknown *Vibrio* phenotypes may belong to novel *Vibrio* species, We first selected a collection of 506 strains (see Figure 1.7; Table 1, Annex) comprising 155 unidentified *Vibrio* spp. distributed in 22 Biolog groups (Table 2, Annex) disclosed in the study of Vandenberghe et al. (2003), 242 *Vibrio* reference strains of different species (Vandenberghe et al., 2003), 53 *Vibrio* spp. isolates from abalones (Sawabe et al., 2002) and 56 type and reference strains of most species of the *Vibrionaceae* which are deposited in the LMGTM/BCCM Bacteria collection groups (Table 1, Annex). Most LMG reference strains were originally identified by Biolog. The *Vibrio* reference strains selected from Vandenberghe's study were assumed to be references because they clustered with type strains.

We then selected a highly discriminatory genomic tool i.e. FAFLP in order to screen the 506 strains and further group strains with similar genomes. Additional 16S rDNA sequences of representative strains was performed in order to obtain the phylogenetic allocation of the FAFLP groups. FAFLP was chosen amongst all currently available techniques because FAFLP has been proven to be in agreement with DNA-DNA hybridisation data for several model groups e.g. *Aeromonas* (Huys et al., 1996; Huys and Swings, 1999), *Agrobacterium* (Mougel et al., 2002), *Burkholderia* (Coenye et al., 1999a), *Xanthomonas* (Rademaker et al., 2000).

FAFLP pattern similarities around 70 % correlate with DNA-DNA similarities of 70 % and thus FAFLP may be a reliable alternative to the laborious DNA hybridisation experiments.

The results of the FAFLP screening of the 506 strains and the phylogenetic allocation of the FAFLP groups is presented in chapter 2 (section 2.2). Chapter 2 also comprises the results of the first screening of the 1,473 isolates by Biolog and other three studies (sections 2.3, 2.4, and 2.5). The study presented in section 2.3 reiterates part of the FAFLP data, but the studies presented in sections 2.4 and 2.5 focused on new sets of isolates (see Tables 3 and 4, Annex) which had not been included in the main study (section 2.2). Section 2.4 presents the study on *V. cholerae* which focused on the diversity of 96 pathogenic and environmental strains isolated in Brazil between 1991 and 2001 (Table 3, Annex), while section 2.5 presents the analyses of 30 presumptive *V. harveyi* by means of different fingerprinting techniques i.e. FAFLP and Rep-PCR (Table 4, Annex).

Most subsequent studies presented in Chapter 3 are sequels of the FAFLP study in section 2.2. Except for the FAFLP and 16S rDNA data presented in sections 3.4, 3.6, and 3.10, the new species descriptions presented in Chapter 3 reiterated and discussed in more detail parts of the FAFLP and 16S rDNA sequence data generated in section 2.2 (Chapter 2). In addition, for each new species description at least one extra 16S rDNA sequence of a second representative strain from the same FAFLP group was performed in order to confirm the monophyletic nature of the new group.

The main goal of this work was to improve the taxonomy of the family *Vibrionaceae* through the description of new taxa. The taxonomic position of known *Vibrionaceae* species was also analysed in order to better circumscribe this family. Additionally, the intraspecific polymorphism of four *Vibrio* species i.e. *V. campbellii*, *V. cholerae*, *V. haliotocoli*, and *V. harveyi* was evaluated in more detail by examining additional sets of isolates.

This PhD work comprises the following publications:

Chapter 2

Vandenbergh, J., **Thompson, F.L.**, Gomez-Gil, B. & Swings, J. 2003. Phenotypic diversity amongst *Vibrio* strains from marine aquaculture. *Aquaculture* 219, 9-20. (section 2.1).

Thompson, F.L., Hoste, B. Vandemeulebroecke, K. & Swings, J. 2001. J. Genomic diversity amongst *Vibrio* isolates from different sources determined by Amplified Fragment Length Polymorphism. *Systematic and Applied Microbiology* 24, 520-538. (section 2.2)

Sawabe, T., **Thompson, F.L.**, Heyrman, J., Cnockaert, M., Hayashi, K., Tanaka, R., Yoshimizu, M., Hoste, B., Swings, J. & Ezura, Y. 2002. Fluorescent amplified fragment length polymorphism and repetitive extragenic palindrome-PCR fingerprinting reveal host-specific genetic diversity of *Vibrio haliotocoli*-Like strains isolated from the gut of Japanese Abalone. *Applied and Environmental Microbiology* 68, 4140-4144. (section 2.3)

Thompson, F.L., Thompson, C.C., Vicente, A.C.P., Theophilo, G.N.D., Hofer, E. & Swings, J. 2003. Genomic diversity of clinical and environmental *Vibrio cholerae* strains isolated in Brazil between 1991 and 2001 as revealed by FAFLP analysis. *Journal of Clinical Microbiology*, May. (section 2.4)

Gomez-Gil, B., **Thompson, F.L.** & Swings, J. Identification of *V. campbellii* and *V. harveyi* using molecular fingerprinting techniques. In preparation. (section 2.5)

Chapter 3

Thompson, F.L., B. Hoste, C.C. Thompson, J. Goris, B. Gomez-Gil, L. Huys, De Vos, P. & J. Swings. 2002. *Enterovibrio norvegicus* gen. nov., sp. nov., isolated from the gut of turbot (*Scophthalmus maximus*) larvae: A new member of the family *Vibrionaceae*. *International Journal of Systematic and Evolutionary Microbiology* 52, 2015-2022. (section 3.1)

Thompson, F.L., Li, Y., Gomez-Gil, B., Thompson, C.C., Hoste, B., Vandemeulebroecke, K., Rupp, G.S., Pereira, A., De Bem, M. M., Sorgeloos, P. & Swings, J. 2003. *Vibrio neptunius* sp. nov., *V. brasiliensis* sp. nov. and *V. xuii* sp. nov., isolated from the marine aquaculture environment (bivalves, fish, rotifers and shrimps). *International Journal of Systematic and Evolutionary Microbiology* 53, 245-252. (section 3.2)

Thompson, F.L., Thompson, C.C., Li, Y., Gomez-Gil, B., Vandenbergh, J. & Swings, J. 2002. Description of *Vibrio kanaloae* sp. nov., *Vibrio pomeroyi* sp. nov. and *Vibrio chagasii* sp. nov., from sea water and marine animals. *International Journal of Systematic and Evolutionary Microbiology*, published online 12 July 2002. DOI 10.1099/ijs.0.02447-0. (section 3.3)

Ben-Haim, Y., **Thompson, F.L.**, Thompson, C.C., Cnockaert, M.C., Hoste, B., Swings, J., & Rosenberg, E. 2003. *Vibrio corallilyticus* sp. nov., a temperature-dependent pathogen of the coral *Pocillopora damicornis*. *International Journal of Systematic and Evolutionary Microbiology* 53, 309-315. (section 3.4)

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Chapter 1

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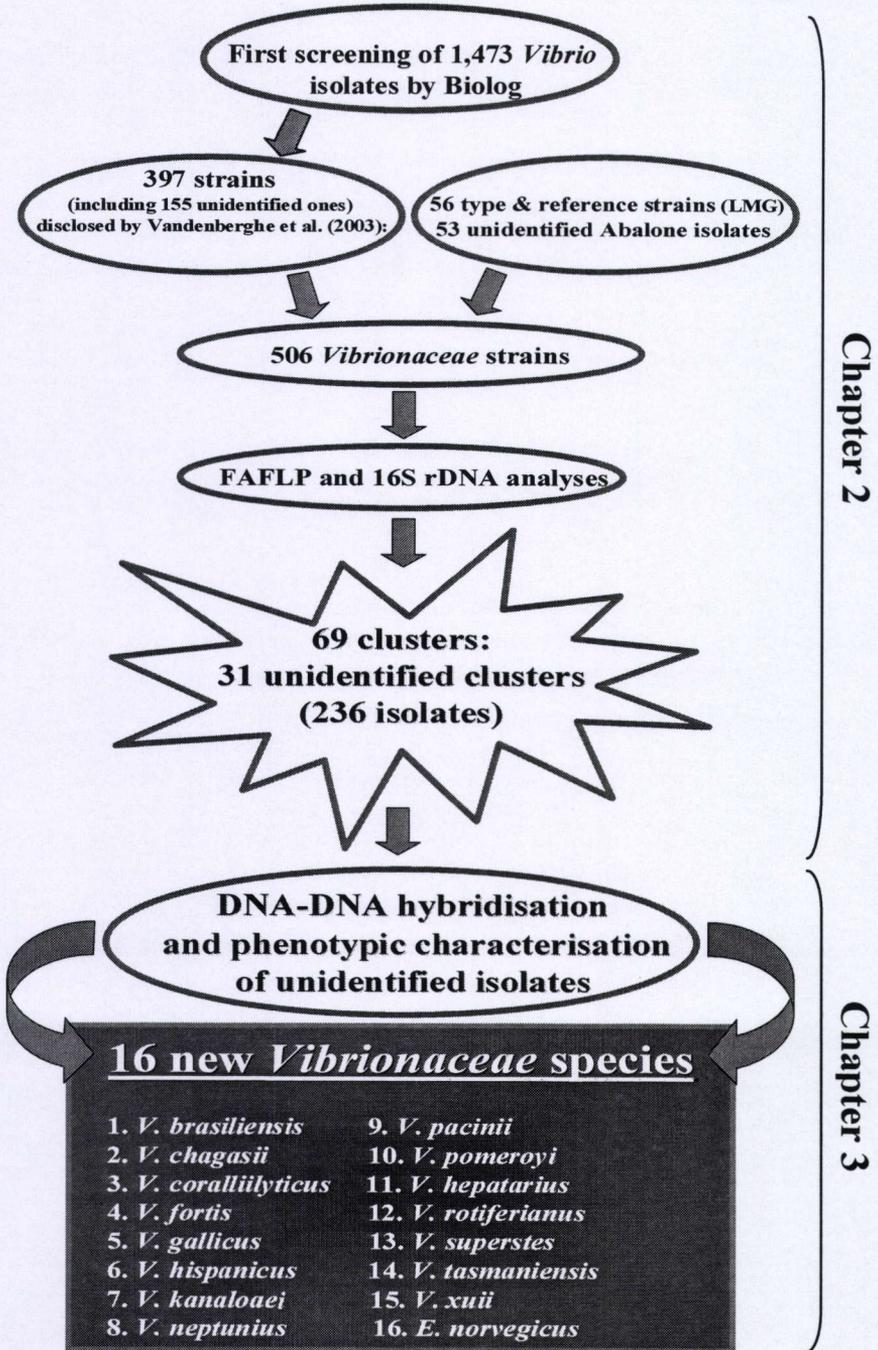


Figure 1.6. Overview of the present thesis.

Chapter 2

Chapter 1

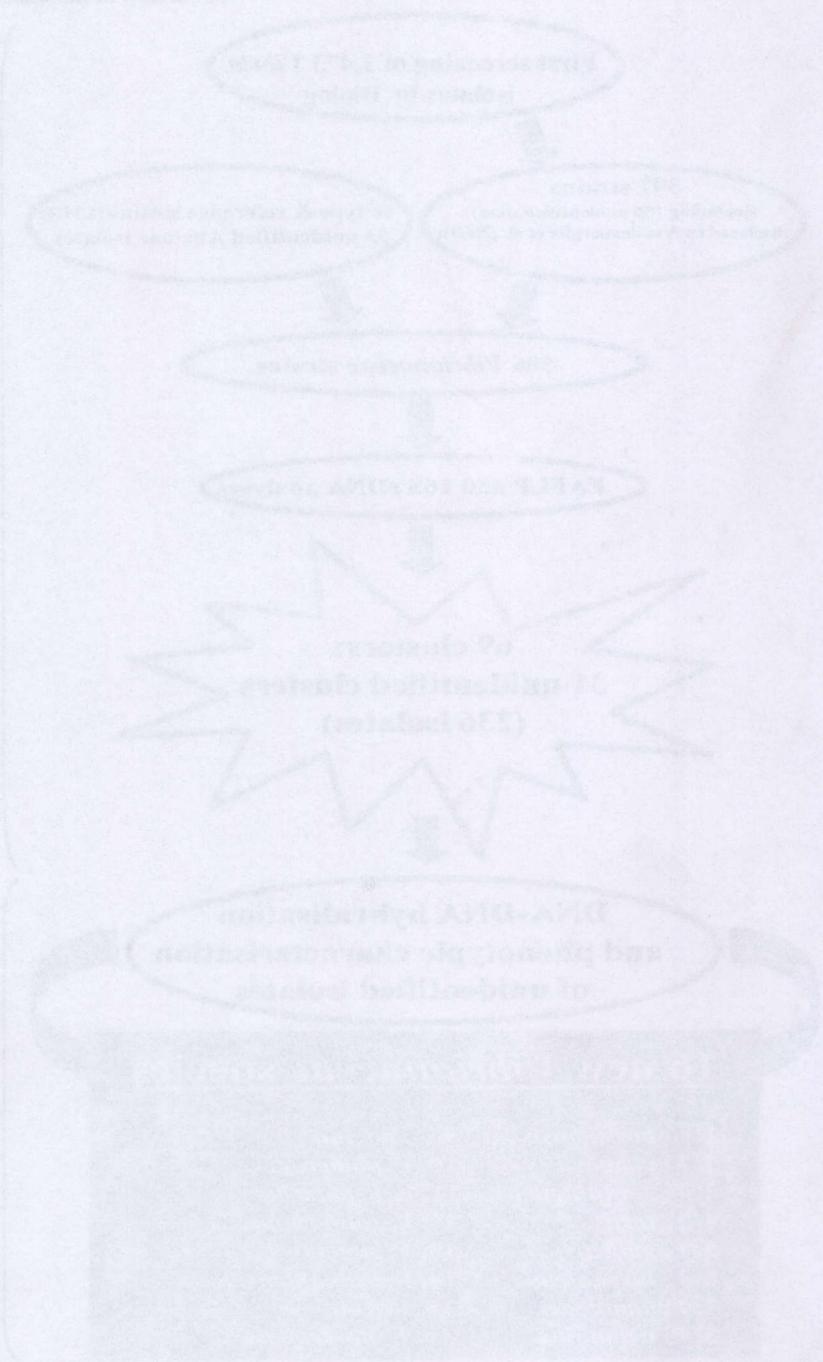


Figure 1. A flowchart of the project plan.

CHAPTER 2. Own work: establishing the diversity within the *Vibrionaceae*

2.1. Phenotypic diversity amongst *Vibrio* isolates from marine aquaculture systems

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Aquaculture 219 (2003), 9-20

Abstract

A total number of 1,473 *Vibrio* isolates were collected from different aquaculture systems in many countries. Isolates were obtained from bivalves (mussels, scallops, oysters), shrimp and fish, sea urchins, live feed (algae, *Artemia*, rotifers), seaweed, aquaculture market products and from the aquaculture environment (tank water, seawater, sediments). Eggs, healthy and diseased or dead larvae, and adult organisms were sampled from cold-water species and moderate- to warm-water species. All isolates were phenotypically characterised using the Biolog GN technique. Eighty-nine different clusters were obtained, of these clusters, only 33 were identified comprehending 992 isolates. The remaining 56 groups did not cluster with any of the included type strains and remained unidentified. Seventy-eight isolates did not cluster with any other strain. It was shown that the *Vibrio* genus is a phenotypically diverse group making the identification with the Biolog system difficult and unreliable.

Introduction

Vibrio is one of the most important bacterial genera in aquaculture, mostly because of their capacity to infect a wide range of aquatic organisms such as penaeid shrimp (Lightner, 1993), several fish species (Austin and Austin, 1999), and molluscs (Rheinheimer, 1992). Numerous *Vibrio* species, such as *Vibrio alginolyticus*, have been characterized as probionts (Gomez-Gil et al., 2000) as well as pathogens (Lee et al., 1996). Many attempts have been made to characterize and identify *Vibrios* from aquacultural environments based on phenotypic characters. Most attempts focus on

some cultured species or aquaculture system, and almost none deal with strains isolated from many sources. Recent studies have employed the Biolog system as a means to test a large number of phenotypic characters, but have been restricted to certain species such as *Penaeus chinensis* (Vandenberghe et al., 1998), striped bass (Nedoluha and Westhoff, 1997) and scallop (Nicolas et al., 1996). Also, Biolog GN has been employed to characterize isolates from a broader source, such as penaeid shrimp culture systems (Vandenberghe et al., 1999) and rotifer production systems (Verdonck et al., 1997). Phenotypic characterization and identification of the genus *Vibrio* has presented several difficulties due to its high biochemical diversity, and description of several new species has led to a constantly changing taxonomy of the *Vibrionaceae* (Alsina and Blanch, 1994a, 1994b). Due to intensive genomic characterization and the optimisation of the screening for phenotypic characteristics, 43 *Vibrio* species are now described (Euzéby, 1997). It has been shown that a polyphasic approach, based on phenotypic, chemotaxonomic and genomic data, improves bacterial taxonomy (Vandamme et al., 1996). Applying the principles of polyphasic taxonomy to the genus *Vibrio* will most probably increase the number of species in the future, as the genus has many new species still undescribed (Pedersen et al., 1998; Urakawa et al., 1999a; Thompson et al., 2001b). The present work aims to further unravel the taxonomic structure of the genus *Vibrio*, especially those species found in aquacultural systems, based on their phenotypic diversity and with the aid of the Biolog GN system.

Material and methods

Strain acquisition

From 1991 until 2001, 1,473 *Vibrio* isolates from the marine aquacultural environment were collected from all continents. Strains were isolated from various species of healthy, diseased, and dead bivalves (mussels, scallops, oysters), cultured and wild shrimp and fish, and sea urchins. Isolates were also collected from live (microalgae, *Artemia*, and rotifers) and artificial feeds, from seaweeds, and from aquaculture systems (tank water, sediments, and incoming water). Isolates also originated from eggs, larval stages, and adult organisms from cold-water and moderate- to warm-water species.

Storage of isolates

Suspensions of pure cultures were stored at -80 °C or in liquid nitrogen at -140 °C after addition of marine broth or trypticase soy broth (Becton Dickinson, Cockeysville, MD) supplemented with 2.0% (w/v) NaCl and 15% (v/v) glycerol. All strains were stored at the BCCM™/LMG Culture Collection (University of Ghent, Ghent, Belgium).

Isolate characterization

Gram stains were carried out on all isolates (Murray et al., 1994), and further phenotypic characterization was performed using the Biolog® GN technique (Biolog, Hayward, CA) as described by Austin et al. (1995). Isolates were grown on brain heart infusion (BHI; Difco, Detroit, MI) supplemented with 1.5% (w/v) bacteriological agar no. 1 (Oxoid, Basingstoke, England, UK) and with 1.5% (w/v) NaCl or on marine agar (MA; Difco) for 24 h at 25 °C. Inocula were prepared in 1.5% (w/v) NaCl solution and cell densities were photometrically standardized between 0.261 and 0.300 OD at 590 nm. The wells of the Biolog® GN microtiter plates were inoculated with the cell suspension and the microtiter plates were incubated for 24 h at 25 °C. Changes in colour were measured using a Multiscan Multisoft filter photometer (Labsystems, Helsinki, Finland) at 550 nm. For identification, the metabolic fingerprints of the isolates grown on BHI agar were compared to the metabolic fingerprints of 33 *Vibrio* type strains, *Listonella anguillarum*, *Listonella pelagia*, *Photobacterium damsela* subsp. *damsela* and *SaliniVibrio costicola*.

Comparison of the isolates was performed by numerical analysis using the Pearson product moment correlation coefficient (PPMCC) and hierarchical clustering with unweighted pair group method with arithmetic averages (UPGMA; Sneath and Sokal, 1973). The clustering results were validated using cophenetic correlation (Russek-Cohen and Colwell, 1996). Clusters were delineated at 80% and isolates clustering together at this level with type strains and reference strains were considered to belong to the same species (Priest and Austin, 1993). Clusters that harbour no type or reference strains were named according to one central strain in the cluster and thus remained unidentified.

Results and discussion

Eighty-nine different clusters (> 80% similarity) were obtained from a total of 1473 isolates analysed; from these clusters, only 33 (a total of 992 isolates) could be assigned to a species (Figure 2.1). Fifty-six remaining groups (481 isolates) did not cluster with any of the included type and reference strains and thus remained unidentified. Seventy-eight isolates formed single-member clusters.

Ten *Vibrio* species were not included in this analysis because they were not available at the time; these were *V. aerogenes*, *V. cyclitrophicus*, *V. diabolicus*, *V. lentus*, *V. natriegens*, *V. navarrensis*, *V. rumoiensis*, *V. tapetis*, and *V. wodanis*. The results showed that the majority of the included type and reference strains clustered in unique groups although some exceptions were observed. During previous studies on different subsets of strains included in this study, many correlations between the origin of isolates and their identity have been suggested (Austin et al., 1995b, 1996, 1997; Arias et al., 1997a; Dierckens et al., 1998; Grisez et al., 1997; Vandenberghe et al., 1998, 1999; Verdonck et al., 1994, 1997). Some additional correlations have been found in the present analysis. Results are discussed per cluster found, based on the position of the cluster in the dendrogram presented as Fig. 2.1.

V. harveyi (364 isolates)/*V. aestuarianus* (5 isolates) group

It was not possible to distinguish between *V. harveyi* and *V. aestuarianus* with this system since both type and reference strains clustered within the same group. Moreover, it was not possible to separate both type strains with the patterns obtained. High levels of heterogeneity were observed in the Biolog GN profiles of the different *V. harveyi* and *V. aestuarianus* isolates and the delineation of this group was done at 75%. *V. carchariae* was not considered as a separate species since Pedersen et al. (1998) have shown that *V. carchariae* is to be considered as junior synonym of *V. harveyi*. The phylogenetic study of Kita-Tsukamoto et al. (1993) of the *Vibrionaceae* based on 16S rRNA sequences shows that *V. harveyi* and *V. aestuarianus* belong to the core group of the genus *Vibrio*, but each species is more related with other *Vibrio* species than with one another. It was also shown by AFLP fingerprinting that some isolates identified as *V. harveyi*, using Biolog GN, were more closely related to *V. campbellii* (Vandenberghe et al., 1999). Therefore, it is concluded that the Biolog system was not useful to identify isolates related to either *V. harveyi* or *V. aestuarianus*.

70 75 80 85 90 95 100

Representative LMG strain number	IDENTIFICATION	Number of strains
LMG 4041 / LMG 7901	<i>V. Harveyi</i> / <i>V. aestuarianus</i>	369
LMG 13545T	<i>V. vulnificus</i>	25
LMG 19988	Vibrio STD3-932	4
LMG 19989	Vibrio STD3-1052	65
LMG 20036	Vibrio TAR L8	3
LMG 10936T	<i>V. tubiashii</i>	6
LMG 20008	Vibrio FKFO 99	44
LMG 19990	Vibrio STD3-996	3
LMG 19991	Vibrio STD3-874	2
LMG 11258T	<i>V. mediterranei</i>	46
LMG 11216T	<i>V. campbellii</i>	21
LMG 19840	Vibrio R3690	6
LMG 20032	Vibrio R3716	2
LMG 20010	Vibrio INCO 320	25
LMG 19272	Vibrio INCO 254	2
LMG 20012	Vibrio VIB 836	33
LMG 44081 / LMG 17719T	<i>V. alginolyticus</i> / <i>V. hollaise</i>	339
LMG 3772T	<i>V. parahaemolyticus</i>	7
LMG 19992	Vibrio STD3-541	2
LMG 2850T	<i>V. parahaemolyticus</i>	35
LMG 19993	Vibrio STD3-331	7
LMG 19994	Vibrio STD3-348	79
LMG 19995	Vibrio STD3-1008	12
LMG 19270	Vibrio INCO 222	10
LMG 4042T	<i>V. splendidus</i>	21
LMG 20011	Vibrio INCO 167	6
LMG 4437T	<i>L. anguillarum</i>	154
LMG 20013	Vibrio VIB 433	4
LMG 20014	Vibrio VIB 478	3
LMG 1165T	Vibrio VIB 386	2
LMG 19996	Vibrio STD3-988	12
LMG 4411	<i>L. anguillarum</i>	60
LMG 7981T	<i>V. cincinnatiensis</i>	11
LMG 10944	Vibrio VIB 377	2
LMG 7892T	<i>P. damselae</i>	40
LMG 20009	Vibrio FKFO 140	2
LMG 7910T	<i>V. furnissii</i>	5
LMG 7894T	<i>V. fluvialis</i>	9
LMG 19997	Vibrio STD3-1267	2
LMG 11664T	<i>V. metacrinikovii</i>	6
LMG 19998	Vibrio STD3-338	3
LMG 14011	<i>V. kugerii</i>	2
LMG 20033	Vibrio R3686	3
LMG 19999	Vibrio STD3-1057	3
LMG 19642	<i>V. pectenicida</i>	4
LMG 20000	Vibrio STD3-943	2
LMG 13950	Vibrio LMG 13950	7
LMG 14010T	<i>V. scopthalmi</i>	6
LMG 6460T	<i>S. costicola</i>	3
LMG 3895T	<i>V. nereis</i>	2
LMG 20015	Vibrio VIB 534	4
LMG 20016	Vibrio VIB 437	2
LMG 20001	Vibrio STD3-1018	17
LMG 20002	Vibrio STD3-1088	17
LMG 7896T	<i>V. miniculus</i>	15
LMG 20017	Vibrio VIB 791	2
LMG 3896T	<i>V. nigripulchritudo</i>	5
LMG 7897T	<i>V. orientalis</i>	8
LMG 19540T	<i>V. gazogenes</i>	4
LMG 7893T	<i>V. allosidrophilus</i>	12
LMG 20003	Vibrio STD3-341	12
LMG 20025	Vibrio TR38	4
LMG 20107	<i>V. mytili</i>	3
LMG 20026	Vibrio CG	2
LMG 10941	<i>L. pelagica</i>	3
LMG 20027	Vibrio IM25	2
LMG 20007	Vibrio TAR FT1	3
LMG 18542T	<i>V. haleticolli</i>	2
LMG 20030	Vibrio MJPXOM26	2
LMG 20031	Vibrio MJP3OM18	2
LMG 4414T	<i>V. fischeri</i>	5
LMG 20004	Vibrio STD3-865	3
LMG 20018	Vibrio VIB 610	2
LMG 20019	Vibrio VIB 717	4
LMG 20020	Vibrio VIB 734	3
LMG 10951	<i>V. ordalii</i>	11
LMG 20021	Vibrio VIB 739	3
LMG 4406	<i>V. cholerae</i>	3
LMG 20029	Vibrio MS1	2
LMG 20022	Vibrio VIB 583	2
LMG 20023	Vibrio VIB 782	8
LMG 20005	Vibrio STD3-536	2
LMG 10942	Vibrio LMG 10942	2
LMG 20028	Vibrio IM10	2
LMG 20109 / LMG 20110	<i>V. ichthyocenteri</i> / <i>V. parvaecida</i>	26
LMG 20112	Vibrio VIB 449	2
LMG 20108	<i>V. aestuariae</i>	5
LMG 19839	Vibrio R3710	24
LMG 20034	Vibrio R3733	2
LMG 20035	Vibrio R3810	6
LMG 20024	Vibrio VIB 637	2

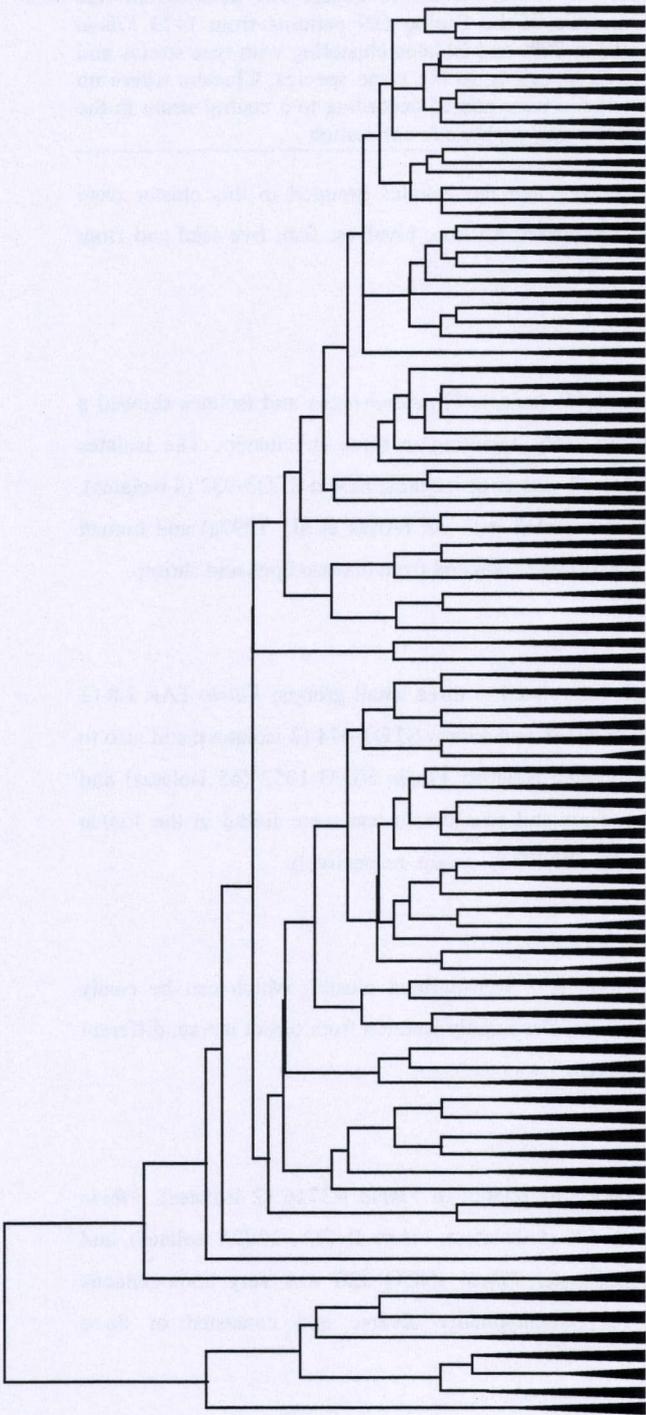


Figure 2.1. Biolog GN phenotypes within the *Vibrio* genus. The dendrogram was derived by UPGMA cluster analysis of the Biolog GN patterns from 1473 *Vibrio* isolates. Clusters were delineated at 80% and isolates clustering with type strains and reference strains were considered to belong to the same species. Clusters where no type or reference strains are included were named according to a central strain in the cluster. The results were validated using cophenetic correlation.

The analysis presented here showed that the isolates grouped in this cluster were frequently isolated from diseased penaeid shrimp, bivalves, fish, live feed and from seawater.

***V. vulnificus* (25 isolates)**

V. vulnificus was related to *V. harveyi*/*V. aestuarianus* and isolates showed a relative degree of phenotypic diversity, resulting in three subclusters. The isolates were also highly related with a small cluster of isolates, *Vibrio* STD3-932 (4 isolates). *V. vulnificus* was found to be associated with eel (Arias et al., 1997a) and human infections. *Vibrio* STD3-932 isolates were isolated from diseased penaeid shrimp.

***V. tubiashii* (6 isolates)**

The *V. tubiashii* cluster was related to three small groups: *Vibrio* TAR L8 (3 isolates), *Vibrio* STD3-996 (3 isolates), and *Vibrio* STD3-674 (2 isolates); and also to two groups of phenotypically diverse isolates: *Vibrio* STD3-1052 (65 isolates) and *Vibrio* FKFO 99 (44 isolates). Four and two subclusters were found in the *Vibrio* STD3-1052 group and in the *Vibrio* FKFO 99 group, respectively.

***V. mediterranei* (46 isolates)**

The *V. mediterranei* group is a well-defined cluster, which can be easily identified by Biolog. *V. mediterranei* was mainly isolated from turbot larvae, different species of cultured fish and bivalves.

***V. campbellii* (21 isolates)**

The *V. campbellii* cluster was related to *Vibrio* R3716 (2 isolates), *Vibrio* INCO 254 (2 isolates), *Vibrio* R3690 (6 isolates), *Vibrio* INCO 320 (25 isolates), and *Vibrio* VIB 836 (33 isolates). Cluster *Vibrio* INCO 320 was very homogeneous whereas *Vibrio* VIB 836 was phenotypically diverse and consisted of three

subclusters. Isolates from this cluster were mostly obtained from fish and live feed (*Artemia* and rotifers).

***V. alginolyticus* (332 isolates)/*V. hollisae* (7 isolates) group**

Isolates of *V. alginolyticus* and *V. hollisae* could not be separated using Biolog, although isolates of *V. hollisae* subclustered together. A high phenotypic diversity was observed in the cluster. Nevertheless, Vandenberghe et al. (1999) could identify isolates as *V. alginolyticus* with the Biolog GN system. Kita-Tsukamoto et al. (1993) found on the basis of phylogenetic relationships that *V. hollisae* has a low 16S rRNA similarity with *V. alginolyticus*. *V. alginolyticus* belongs to the core group of *Vibrio*, while *V. hollisae* belongs to a distant cluster together with *S. costicola* and *P. damsela*, some of this might even form a new family (Kita-Tsukamoto et al., 1993). *V. alginolyticus* isolates were isolated from practically all aquaculture sites from all over the world, making it perhaps the most ubiquitous *Vibrio* phenotype found.

***V. proteolyticus* (7 isolates)**

Isolates of *V. proteolyticus* were phenotypically related to *V. alginolyticus*/*V. hollisae*, and isolates of the *Vibrio* STD3-541 cluster (2 isolates). They generated a distinct Biolog profile. *V. proteolyticus* is closely related to *V. alginolyticus* by their 16S rRNA sequence (99% similarity) (Kita-Tsukamoto et al., 1993).

***V. parahaemolyticus* (35 isolates)**

V. parahaemolyticus generated a distinct Biolog profile with little phenotypic variation. Isolates originated from diseased penaeid shrimp.

***V. splendidus* (21 isolates)**

The *V. splendidus* cluster was phenotypically related to *Vibrio* STD3-1008 (12 isolates) and *Vibrio* INCO 222 (10 isolates), and more distantly to clusters *Vibrio* STD3-331 (7 isolates) and *Vibrio* STD3-348 (79 isolates). The *Vibrio* STD3-348 group was phenotypically heterogeneous resulting in four subclusters. Isolates of these clusters were found in fish, shrimp, and bivalves.

***L. anguillarum* (214 isolates)**

The isolates from *L. anguillarum* divided into two separate groups, with a similarity of only 70%. The level of heterogeneity of the Biolog profiles was so high that delineation of the two groups should be done at 75%. Also, Austin et al. (1997) found two *L. anguillarum* groups with no correlation with their serotypes and Biolog GN. Kühn et al. (1996) concluded that even with a high phenotypic heterogeneity Biolog GN is a reliable tool for the identification of *L. anguillarum* isolates. Four groups, *Vibrio* VIB 433 (4 isolates), *Vibrio* VIB 478 (3 isolates), *Vibrio* VIB 386 (2 isolates), and *Vibrio* STD3-988 (12 isolates), were found in between the two *L. anguillarum* clusters, therefore, these groups might also be *L. anguillarum* isolates, although their profiles showed clear differences with the profiles obtained from the *L. anguillarum* isolates. *L. anguillarum* was mostly isolated from different species of fish, but it was also recovered from sediments, rotifers, and seawater. Isolates of group *Vibrio* STD3-988 were mainly isolated from diseased penaeid shrimp.

V. cincinnatiensis

No isolates clustered together with the type strain of *V. cincinnatiensis* and this strain was phenotypically close to the *L. anguillarum* cluster.

***P. damsela* (40 isolates)**

P. damsela isolates showed a distinct Biolog pattern, making the Biolog system a good tool for *P. damsela* identification. This group was related to the *Vibrio* VIB 377 cluster (2 isolates). *P. damsela* was isolated from different species of fish and from diseased penaeid shrimp.

***V. furnissii* (5 isolates) and *V. fluvialis* (9 isolates)**

V. furnissii and *V. fluvialis* were phenotypically closely related and both showed a typical Biolog pattern. Phylogenetic analysis confirmed that these two species are highly related (Ruimy et al., 1994).

***V. metschnikovii* (6 isolates)**

V. metschnikovii isolates showed a certain degree of phenotypical heterogeneity within their cluster and were related to *Vibrio* STD3-1267 (2 isolates) and to *Vibrio* STD3-338 (3 isolates).

***V. logei* (2 isolates)**

Only two isolates were identified as *V. logei*. This cluster was phenotypically related to *Vibrio* R3666 (3 isolates) and to *Vibrio* STD3-1057 (3 isolates).

***V. pectenica* (4 isolates)**

V. pectenica isolates generated a highly typical Biolog profile; group *Vibrio* STD3-943 (2 isolates) was found to be related to it. The isolates were isolated only from diseased scallop (*Pecten maximus*) in France (Lambert et al., 1998), but this species was never recovered from diseased scallop (*N. nodosus*) larvae in Brazil despite a large group of isolates being analysed. It was never recovered from any other aquaculture source.

***V. scopthalmi* (6 isolates), *S. costicola* (3 isolates) and *V. nereis* (2 isolates)**

V. scopthalmi generated a similar Biolog profile to group *Vibrio* LMG 13950, both groups were phenotypically diverse. These isolates were related to *S. costicola*, *V. nereis*, *Vibrio* VIB 534 (4 isolates) and *Vibrio* VIB 437 (2 isolates). *Vibrio* LMG 13950 was isolated from different fish species.

***V. mimicus* (15 isolates), *V. nigripulchritudo* (5 isolates), and *V. orientalis* (8 isolates)**

The cluster of *V. mimicus* was a phenotypically diverse group and was related to *V. nigripulchritudo*, *V. orientalis*, *Vibrio* STD3-1018 (17 isolates), *Vibrio* STD3-1088 (17 isolates), and *Vibrio* VIB 791 (2 isolates). The clusters *Vibrio* STD3-1018 and *Vibrio* STD3-1088 were phenotypically highly diverse resulting in three subclusters. *V. mimicus* was isolated from different fish species and from diseased penaeid shrimp.

***V. gazogenes* (4 isolates), *V. diazotrophicus* (12 isolates), and *V. mytili* (3 isolates)**

V. gazogenes, *V. diazotrophicus*, and *V. mytili* were found to be closely related groups and showed a high degree of phenotypic diversity. These groups were also related to three phenotypic diverse *Vibrio* groups, *Vibrio* STD3-341 (12 isolates), *Vibrio* TR36 (4 isolates), and *Vibrio* C6 (2 isolates). *V. diazotrophicus* was isolated from live feed (rotifers and *Artemia*).

***L. pelagia* (3 isolates)**

The isolates from the *L. pelagia* group showed a typical Biolog profile and this group was related to the *Vibrio* IM25 (2 isolates) cluster.

***V. halioticoli* (2 isolates)**

A typical Biolog cluster was found for *V. halioticoli* group; *Vibrio* TAR FT1 group (3 isolates) was found to be closely related. Two other groups, *Vibrio* MJPXOM26 (2 isolates) and *Vibrio* MJP3OM16 (2 isolates) were phenotypically related on a low level with *V. halioticoli*.

***V. fisheri* (5 isolates)**

A distinct profile was observed for isolates from the *V. fisheri* group and were related at a low level with clusters *Vibrio* STD3-565 (2 isolates) and *Vibrio* VIB 510 (2 isolates).

***V. ordalii* (11 isolates)**

V. ordalii, a phenotypically diverse group, was highly related to *Vibrio* VIB 739 (3 isolates), *Vibrio* VIB 717 (4 isolates), and to *Vibrio* VIB 734 group (3 isolates). *V. ordalii* was only found in association with different fish species.

***V. cholerae* group**

Distinct Biolog profiles were observed for isolates from *V. cholerae* (3 isolates) and for the related *Vibrio* MS1 (2 isolates) group.

V. ichthyoenteri/V. penaeicida* group and *V. salmonicida

The Biolog GN patterns of these species were very different to those obtained from other *Vibrio* species, making them atypical *Vibrio* groups. The type and reference strains of *V. ichthyoenteri* and *V. penaeicida* could not be separated by the Biolog GN system and the cluster (26 isolates) was phenotypically diverse. Atypical *Vibrio* profiles were also obtained from cluster *Vibrio* VIB 449 (2 isolates), the diverse group of *V. salmonicida* isolates (5 isolates) and the four related groups, *Vibrio* R3710 (24 isolates), *Vibrio* R3733 (2 isolates), *Vibrio* R3810 (6 isolates), and *Vibrio* VIB 637 (2 isolates).

Other groups and unclustered *Vibrio* isolates

Characteristic profiles were obtained for isolates belonging to the *Vibrio* INCO 167 group (6 isolates), *Vibrio* FKFO 140 (2 isolates), *Vibrio* VIB 583 (2 isolates) and related group *Vibrio* VIB 782 (8 isolates), *Vibrio* STD3-536 (2 isolates), *Vibrio* LMG 10942 (2 isolates), and *Vibrio* IM10 (2 isolates). No clear phenotypic relations were found between these isolates and any of the included type or reference strains; therefore, they remained unidentified. The 78 unclustered *Vibrio* isolates were not included in the dendrogram (Fig. 2.1), all showed a low similarity index (<65%) with the clusters described above. Some unidentified *Vibrio* groups showed clear association with a particular host, i.e., *Vibrio* R3710, *Vibrio* R3733, *Vibrio* R3810, *Vibrio* R3716, *Vibrio* R3690, *Vibrio* FKFO 140, *Vibrio* R3666, *Vibrio* TAR FT1, and *Vibrio* VIB 782 were virtually all isolated from turbot larvae or from the tank water where they were cultured. *Vibrio* INCO 222 was isolated only from scallop larvae (*N. nodosus*) from Brazil. Some of the strains isolated from healthy turbot *Scophthalmus maximus* larvae in Norway (clusters LMG 19839 and LMG 19840) were identified as *Enterovibrio norvegicus*, a new genus related to *Vibrio* (Thompson et al., 2002). The results presented here showed that isolates from the aquaculture environment belonging to the genus *Vibrio* have highly variable phenotypes, especially regarding carbon utilization sources. The Biolog GN system did not permit the clear definition of certain species because in many cases more than one type strain was clustered together. It has been shown that Biolog GN identified correctly to species level only 57.1% of a varied selection of clinical relevant bacteria (Miller and Rhoden, 1991). In another study with type strains (ATCC), Biolog could positively identify 59% of the isolates (Klingler et al., 1992). Comparing identification of *Bradyrhizobium* strains with Biolog and with AFLP, it was demonstrated that Biolog groups poorly reflected the genotypic groups (Willems et al., 2000).

Many isolates did not cluster with any type species, but this can be due to a number of reasons, the single carbon source approach of Biolog GN does not have enough resolution capacity to differentiate between *Vibrio* species, many isolates from aquatic environments are new species yet undescribed, and because some type strains were not included in the analysis. To overcome these problems, it is recommended that a different approach should be taken, mainly employing the molecular techniques now available. Different molecular methods have been employed to differentiate

closely related species. Those that have been especially useful interrogate the whole genome, such as the repetitive extragenic palindromic elements (REP-PCR) (Versalovic et al., 1991) and amplified fragment length polymorphism (AFLP) (Rademaker et al., 2000). AFLP has been proven more robust to elucidate the taxonomic structure of *Vibrio* isolates from aquatic environments (Thompson et al., 2001b).

2.2. Genomic diversity amongst *Vibrio* isolates from different sources determined by Fluorescent Amplified Fragment Length Polymorphism

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Syst. Appl. Microbiol. (2001), 24, 520-538

Abstract

The genomic diversity among 506 strains of the family *Vibrionaceae* was analysed using Fluorescent Amplified Fragments Length Polymorphism (FAFLP). Isolates were from different sources (e.g. fish, mollusc, shrimp, rotifers, artemia, and their culture water) in different countries, mainly from the aquacultural environment. Clustering of the FAFLP band patterns resulted in 69 clusters. A majority of the actually known species of the family *Vibrionaceae* formed separate clusters. Certain species e.g. *V. alginolyticus*, *V. cholerae*, *V. cincinnatiensis*, *V. diabolicus*, *V. diazotrophicus*, *V. harveyi*, *V. logei*, *V. natrigens*, *V. nereis*, *V. splendidus* and *V. tubiashii* were found to be ubiquitous, whereas *V. haliotocoli*, *V. ichthyenteri*, *V. pectenocida* and *V. wodanis* appear to be exclusively associated with a particular host or geographical region. Three main categories of isolates could be distinguished: (i) isolates with genomes related (i.e. with $\geq 45\%$ FAFLP pattern similarity) to one of the known type strains; (ii) isolates clustering ($\geq 45\%$ pattern similarity) with more than one type strain; (iii) isolates with genomes unrelated ($< 45\%$ pattern similarity) to any of the type strains. The latter group consisted of 236 isolates distributed in 31 clusters indicating that many culturable taxa of the *Vibrionaceae* remain as yet to be described.

Introduction

The genus *Vibrio* along with *Listonella*, *Photobacterium*, *Salinivibrio* forms the family *Vibrionaceae* which belongs to the γ subdivision of the *Proteobacteria* (Farmer III, 1992; Stackebrandt et al., 1988). Members of this family are Gram negative, facultative anaerobes and inhabitants of brackish, estuarine and pelagic

waters and sediments. *Vibrios* are in high abundance in the marine environment and participate significantly in the nutrient cycling (Eilers et al., 2000a, 2000b; Hedlund and Staley, 2001; Ramaiah et al., 2000). Moreover, they form the dominant culturable microflora in the gut of fish, molluscs, and shrimps probably playing an important role in digestion and nutrition (Moss et al., 2000; Ringo and Birkbeck, 1999; Sawabe et al., 1998). Several *Vibrio* species are human and animal pathogens, others form a serious threat to fish, shellfish, and corals (Austin and Austin, 1999; Rosenberg et al., 1999). The use of *Vibrio* strains as probiotics has been reported (Austin et al., 1995a; Vandenberghe et al., 1999), although the role of certain *Vibrio* species (e.g. *V. alginolyticus*), in this respect, is still controversial.

The family *Vibrionaceae* which comprises 51 validated species has been under extensive investigation in the last decades, making it the best documented marine taxon (Kita-Tsukamoto et al., 1993). Major taxonomic modifications have been proposed recently, leading (i) to the creation of the new genera *Moritella* and *Salinivibrio*, harbouring the species *M. marina* (Urakawa et al., 1998b) and *S. costicola* (Mellado et al., 1996), and (ii) to the transfer of *Listonella damsela* (Smith et al., 1991) and *Vibrio iliopiscarius* (Urakawa et al., 1999b) to the genus *Photobacterium*. Until now, studies on the genetic diversity in the family *Vibrionaceae* have focused mainly on pathogenic species, i.e. *L. anguillarum* (Austin et al., 1995b), *P. damsela* subspecies *piscicida* (Thyssen et al., 2000), *V. cholerae* (Chun et al., 1999; Jiang et al., 2000a, 2000b), *V. harveyi* (Pedersen et al., 1998), *V. parahaemolyticus* (Maeda et al., 2000), *V. vulnificus* (Arias et al., 1997a), and *V. wodanis* (Benediktsdóttir et al., 2000). The family *Vibrionaceae* harbours many taxa not yet described (Austin et al. 1997; Pedersen et al., 1998; Urakawa et al., 1999a). As pointed out by Urakawa et al. (1999a) in a recent study on the diversity of psychrotrophic vibrios from Japanese coastal water, most isolates had different 16S rDNA RFLP patterns from those of the current *Vibrio* species and were thus supposed to represent new species.

In the present study we analysed 506 strains of the family *Vibrionaceae*, including 386 isolates originating from the marine aquacultural environment (between 1985 and 2001), by means of a high resolution genomic fingerprinting technique, i.e. fluorescent amplified fragment length polymorphism or FAFLP (Janssen et al., 1996). The application of AFLP has recently been shown to have a tremendous impact in the study of the diversity, taxonomy and phylogeny of several bacterial genera (e.g. see

Desai et al., 1999; Doignon-Bourcier et al., 2000; Gancheva et al., 1999; Willems et al., 2000). It also became clear that AFLP clustering reflects well DNA-DNA similarity. Due to its high discriminatory power, AFLP can be used as an identification tool (Coenye et al., 1999a; Hauben et al., 1999; Huys et al., 1996; Janssen et al., 1997; Rademaker et al., 2000), or for typing purposes (Duim et al., 2000; Jiang et al., 2000a, 2000b; Thyssen et al., 2000).

Material and Methods

Bacterial strains, growth condition and DNA extraction

The 506 strains used in this study are listed in Table 1 (Annex). Strains were grown on Marine Agar 2216E (Difco Co., USA) at 27 °C (*V. fischeri*, *V. logei*, *V. tapetis*, *V. salmonicida*, *V. wodanis* were grown at 18-19°C) for 24 hours except for *V. cholerae* which was grown on Brain Heart Infusion Agar (Difco Co., USA). All strains included in this study are deposited in the Research collection and/or in BCCMTM/LMG Bacteria Collection at Ghent University. Approximately 0.01 g of bacterial cells were harvested for DNA extraction following the technique of Pitcher et al. (1989). Concentration and purity of the DNAs were estimated measuring optical densities at 260, 234 and 280 nm in an Uvicom 941+ spectrophotometer (Kontron Instruments, Italy). DNA integrity was verified on a 1% Agarose gel in 1X TAE buffer (40 mM Tris/Acetate, 1 mM EDTA, pH 8.0).

FAFLP template preparation and PCR reactions

Template preparation was carried out essentially as described previously (Janssen et al., 1996). One µg of high-molecular-mass DNA was digested with *TaqI* (5'TCGA3') and *HindIII* (5'AAGCTT3') (Amersham Pharmacia Biotech, Sweden), followed by ligation of restriction half-site specific adapters to all restriction fragments with T4 ligase (Amersham Pharmacia Biotech, Sweden). Templates were precipitated in a solution containing 50% Isopropanol and 1.25 M NH₄OAc and dissolved in 100 µl T0.1E buffer (10 mM Tris-HCL, 0.1 mM EDTA, pH 8.0). Two subsequent PCR amplifications were applied. For the pre-selective PCR-amplification, 5 µl of template was mixed with 0.6 µl H00-ABI primer (5'GACTGCGTACCAGCTT3'; 1 µM), 0.6 µl T00-ABI primer (5'CGATGAGTCCTGACCGA3'; 5µM) and 18.7 µl of Amplification Core Mix

(Applied Biosystems, USA). The amplification reactions were performed in a GeneAmp PCR System 9600 thermocycler (Applied Biosystems, USA) using the following temperature program: 2 min at 72 °C and 20 cycles of 20 s at 94 °C, 30 s at 56 °C and 2 min at 72 °C. Pre-selective products were diluted in 130 µl T0.1E buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). In the selective PCR-amplification 2.0 µl of the diluted solution was mixed with 0.7 µl H01-6FAM primer (5'GACTGCGTACCAGCTTA3'; 1 µM), 0.7 µl T03-ABI (5'CGATGAGTCCTGACCGAG3'; 5µM), and 10 µl of Amplification Core Mix. The H01-6FAM primer is fluorescently labelled, and the selective bases at the 3'-end are underlined. The temperature profile of the selective amplification was as follows: (i) denaturation for 2 min at 94 °C, (ii) 10 cycles of : denaturation for 20 sec at 94 °C, annealing at decreasing stringency at 67-n °C for 30 sec (with n=the cycle number) and extension at 72 °C for 2 min, (iii) 20 cycles of: denaturation for 20 sec for 94 °C, annealing at 56 °C for 30 sec, and extension at 72 °C for 2 min, and (iv) final extension at 60°C for 30 min.

Polyacrylamide gel electrophoresis and numerical analysis

Separation of the selective PCR products was generated on 36 cm denaturing polyacrylamide gels (4.25% Acrylamide, 6 M Urea in 1 X TBE/89 mM Tris + 89 mM Boric acid + 2 mM EDTA, pH 8.3) on a ABI Prism 377 DNA sequencer (Applied Biosystems, USA). Before loading the samples (1 µl) on the gel, 1.5 µl of the selective product was mixed with a loading buffer (0.75 µl deionised Formamide, 0.25 µl Blue Dextran 50 mM EDTA solution, 0.5 µl GeneScan-500 TAMRA size standard, and 0.5 µl GeneScan-2500 TAMRA size standard) and heated at 95 °C for 3 min. The mix was kept on a thermobloc (-20 °C) while the gel was being loaded. The data were registered during electrophoresis run at 51 °C by the ABI Prism™ Data Collection Software (Applied Biosystems, USA) for 3.5 hours. Tracking and normalization of the lanes were performed by the GeneScan 3.1 software (Applied Biosystems, USA). Normalized tables of peaks, containing fragments of 50 to 536 base pairs, were transferred into BioNumerics 2.0 software (Applied Maths, Belgium) for numerical analysis. Clustering of the patterns was done using the Dice coefficient and the Ward algorithm (Sneath and Sokal, 1973). For fragment comparison, a band

position tolerance value of 0.5% was allowed to compensate for misalignment of homologous bands due to technical imperfections.

16S rDNA sequencing

The amplification and sequencing of the 16S rDNA gene was carried out as follows: First, a fragment of the 16S rDNA gene (corresponding to positions 8-1541 in the *Escherichia coli* numbering system) was amplified by PCR using the conserved primers pA (5'AGAGTTTGATCCTGGCTCAG3') and pH (5'AAGGAGGTGATCCAGCCGCA3') or MH1 (5'AGTTTGATCCTGGCTCAG3') and MH2 (5'TACCTTGTACGACTTCACCCCA3'). Each PCR reaction was composed of 16.75 µl sterile HPLC water, 2.5 µl PCR buffer (10 X), 2.5 µl dNTP's (2 mM), 0.25 µl pA primer (50 ng/µl), 0.25 µl pH primer (50 ng/µl), 0.25 µl AmpliTaq DNA Polymerase (1 U/µl), and 2.5 µl template DNA (0.01 µg/µl) and was performed using a GeneAmp PCR System 9600 thermocycler (Applied Biosystems, USA). The thermal program consisted of 5 min at 95 °C, 30 cycles of 1.5 min at 95 °C + 1 min at 55 °C + 2 min at 72 °C, and 10 min at 72 °C. The primers used gave a PCR product with approximately 1.5 kb long which was then purified using QIAquick PCR purification kit (Qiagen, German). Subsequently, three µl of purified product was mixed with 4 µl ABI Prism™ Big Dye Terminator™ Ready Reaction Mix and 3 µl primer (20 ng/µl). The eight primers used were 16F358 (5'CTCCTACGGGAGGCAGT3', positions 339 to 358 of the *E.coli* 16S rDNA sequence), 16F536 (5'CAGCAGCCGCGTAATAC3', positions 519 to 536), 16F926 (5'AACTCAAAGGAATTGACGG3', positions 908 to 926), 16F1112 (5'AGTCCCACAACGAGCGCAAC3', positions 1093 to 1112), 16F1241 (5'GCTACACACGTGCTACAATG3', positions 1222 to 1241), 16R339 (5'ACTGCTGCCTCCCGTAGGAG3', positions 358 to 339), 16R519 (5'GTATTACCGCGGCTGCTG3', positions 536 to 519), and 16R1093 (5'GTTGCGCTCGTTGCGGGACT3', positions 1112 to 1093), where F means forward primer and R reverse primer. The thermal program consisted of 30 cycles of 15 sec at 96 °C + 1 sec at 35 °C + 4 min at 60 °C. Sequencing products were run on 48 cm denaturing polyacrylamide gels [4.25% Acrylamide, 6 M Urea in 1X TBE (89 mM Tris + 89 mM Boric acid + 2 mM EDTA, pH 8.3)] on an ABI Prism 377 DNA Sequencer (Applied Biosystems, USA) at 51 °C for 10 hours, while the data were

registered by the ABI Prism™ Data Collection Software (Applied Biosystems, USA). Sequences were assembled with the AutoAssembler software (Applied Biosystems, USA). The phylogenetic allocation of the consensus sequences was obtained via EMBL server using the FASTA program (Pearson and Lipman, 1988).

Results and Discussion

Reproducibility

For reproducibility control, the band patterns of 70 strains were generated twice, starting from a new DNA isolation. The 70 pairs of band patterns were numerically analysed and the mean reproducibility value found for each of these patterns was $91\% \pm 3$. This value is in accordance with previous studies using AFLP (Janssen et al., 1997; Willems et al., 2000). Strains clustering at the reproducibility level or higher were indistinguishable by FAFLP.

Numerical analysis of the FAFLP patterns

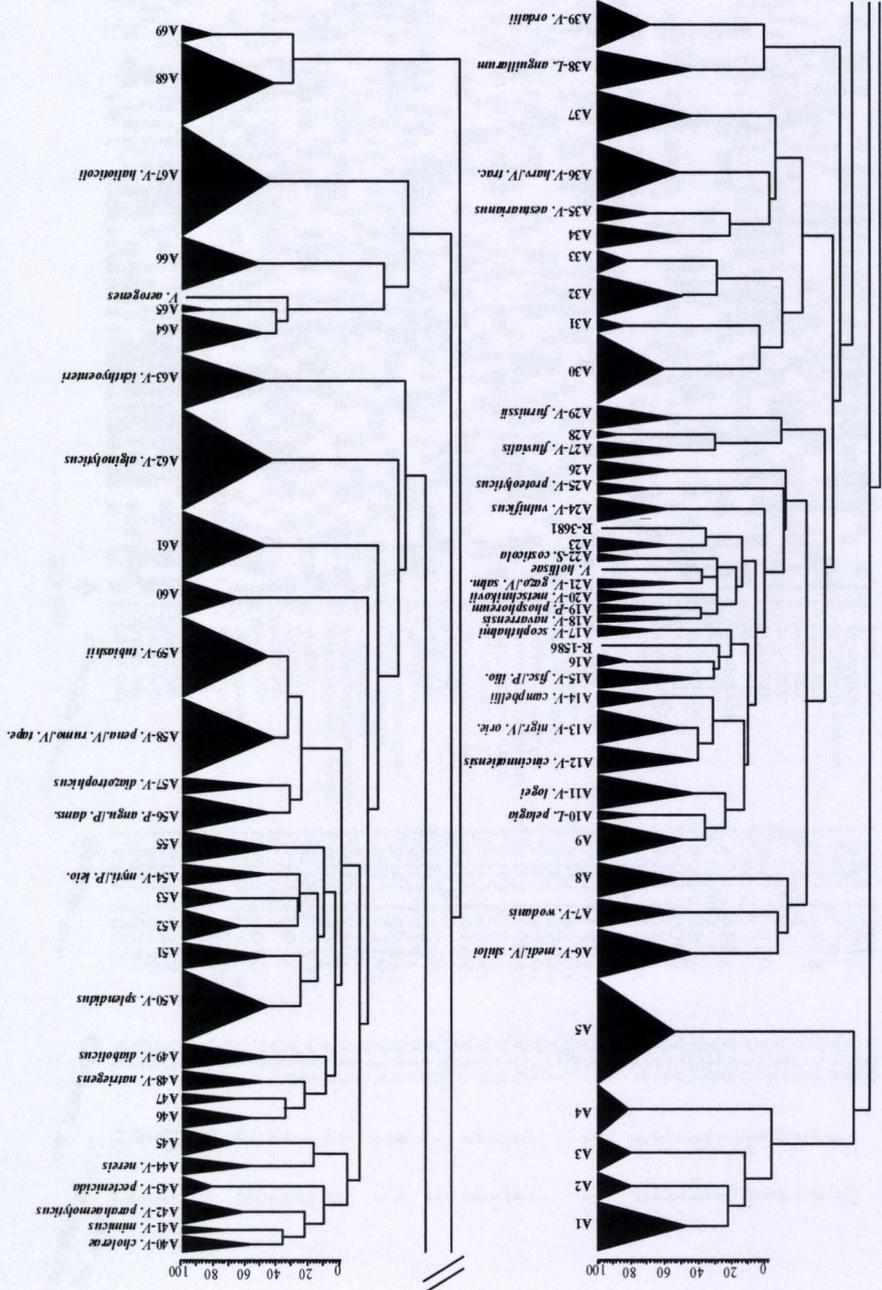
The clustering of the FAFLP band patterns of the 506 strains studied resulted in 69 clusters (A1 to A69) at a cut-off level of 45% similarity (Figure 2.2). This level of cluster delineation was selected based on previous studies on *Acinetobacter* (Janssen et al., 1996), *Aeromonas* (Huys and Swings, 1999), *Bradyrhizobium* (Willems et al., 2000), and *Vibrio* (Pedersen et al., 1998; Austin et al., 1995b; Austin et al., 1997). Figure 2.3 shows 83 FAFLP patterns of representative strains from each cluster. These patterns consisted of 102 ± 24 bands (50 to 536 base pairs in size). With a few exceptions, each actually recognised species showed a characteristic genome pattern and fell in separate clusters. Visual inspection revealed that some clusters e.g. A1, harboured more diverse isolates than other clusters e.g. A2, which were composed of highly related patterns, almost identical (Figure 2.4). Strains with indistinguishable genomes were isolated from the same source at the same date and place, suggesting the occurrence of one particular clone. Overall, three categories of isolates could be distinguished: (i) isolates with genomes related (i.e. with $\geq 45\%$ pattern similarity) to one of the known type strains. In these cases we identify to the known species (Table 1); (ii) isolates clustering ($\geq 45\%$ pattern similarity) with more than one type strain; (iii) isolates with genomes unrelated ($< 45\%$ pattern similarity) to any of the type strains.

Clusters

Cluster A1 consisted of ten diverse isolates (Fig. 2.4) originating from bivalve larvae in Brazil and UK, and from rotifers and turbot larvae in Belgium, whereas *clusters A2, A3 and A4* comprised six, seven and ten isolates, respectively, from bivalve larvae in UK and in Brazil. *Cluster A5* consisted 21 diverse isolates from rotifers and turbot larvae in Belgium and from bivalve larvae in Brazil. *Cluster A6* consisted of ten *V. mediterranei* strains. *V. mediterranei* LMG 11258^T and *V. shiloi* LMG 19703^T shared 56% band pattern similarity suggesting a high resemblance of their genomes. The coral bleaching *V. shiloi* (Banin et al., 2000) should be considered as a junior synonym of *V. mediterranei* (Thompson et al., submitted-a). *Cluster A7* comprised six *V. wodanis* strains isolated from diseased Atlantic salmon suffering of winter ulcer. *Cluster A8* consisted of seven highly related isolates from bivalve larvae in Brazil and one isolate from diseased shrimp larvae in China. *Clusters A9, A10, A11 and A12* harboured eight isolates, two *Listonella pelagia* strains, seven *V. logei* strains and six *V. cincinnatiensis* strains, respectively. These four clusters harboured strains from diverse sources and geographic origins. *Cluster A13* consisted of 7 strains from different sources, comprising the type strains *V. nigripulchritudo* LMG 3896^T, isolated from sea water in Hawaii (USA) and *V. orientalis* LMG 7897^T isolated from the Yellow Sea in China. Both species have mol% G+C of 45-47 (Farmer III and Hickman-Brenner, 1992), and about 96% 16S rDNA homology. Nevertheless, DNA-DNA hybridisation experiments proved that *V. nigripulchritudo* LMG 3896^T and *V. orientalis* LMG 7897^T are different species (unpublished data). The species allocation of the other five isolates of the *cluster A13* is unclear and further genomic and phenotypic analysis are needed. *Cluster A15* consisted of three *V. fischeri* strains and *Photobacterium iliopiscarius* 19543^T which showed 67% pattern similarity, suggesting a high resemblance of their genomes. The mol% G+C of these species is in the same range (i.e. 38 to 41%) which corroborates this observation. *V. fischeri* (former *Photobacterium fischeri*) has been assigned to the genus *Vibrio* based on studies of glutamine synthetase and superoxide dismutase (Baumann et al., 1980), but there have been suggestions on the transfer of *V. fischeri* into *Photobacterium* (Macdonell and Colwell, 1985). 16S rDNA analyses clearly shows that *V. fischeri* and *P. iliopiscarius* form distinct phylogenetic sub-branches in the Family *Vibrionaceae*. *P. iliopiscarius* appears along with all other *Photobacterium* species, whereas *V. fischeri* forms another sub-branch along with the psychrophilic species *V. wodanis*, *V.*

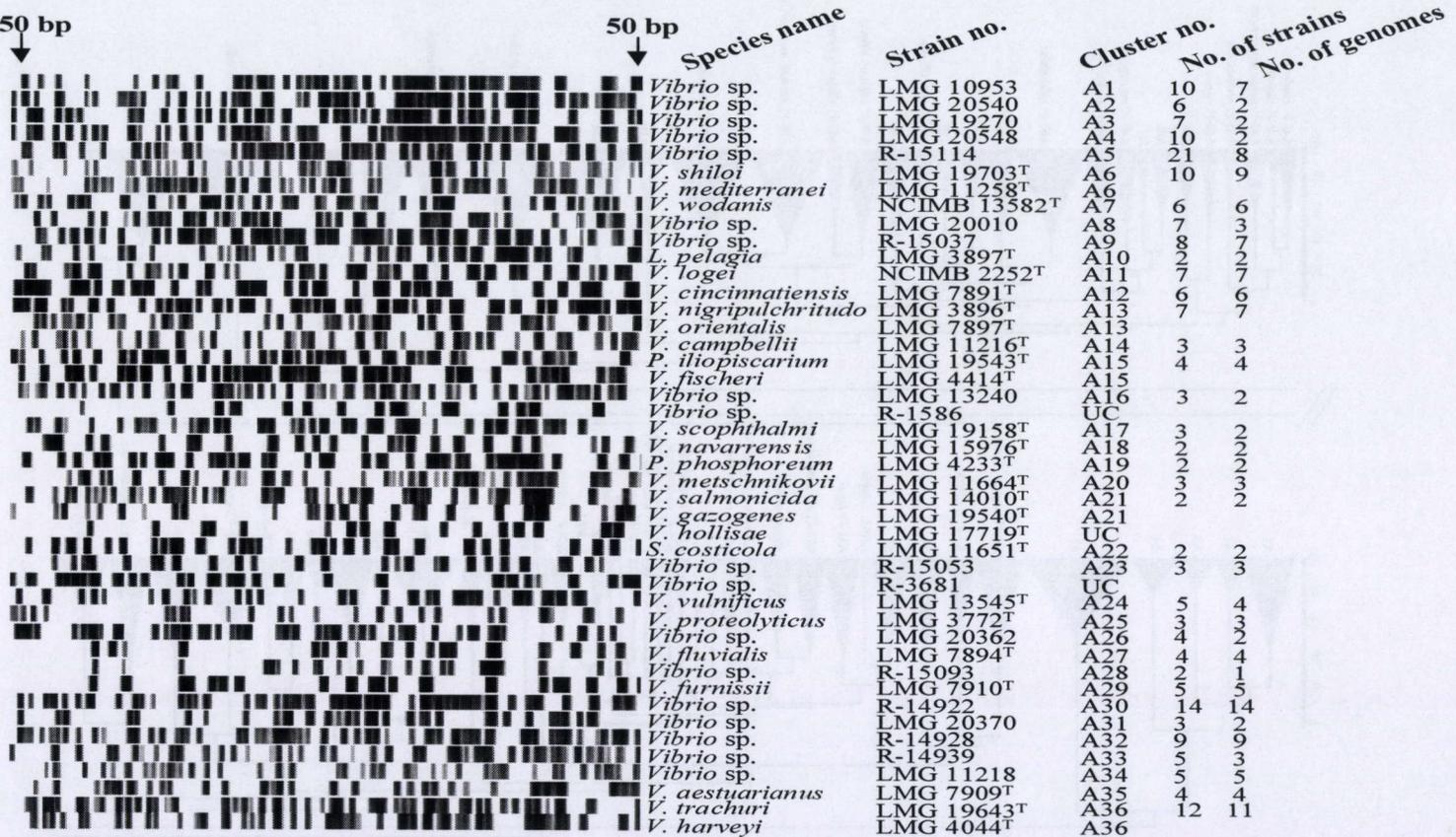
salmonicida and *V. logei* (unpublished data). Clusters A18, A19 and A20 harboured two *V. navarrensis* strains, two *P. phosphoreum* strains and three *V. metschnikovii* strains, respectively. Cluster A21 consisted of the type strains *V. gazogenes* LMG 19540^T (from sea water) and *V. salmonicida* 14010^T (from diseased fish). Both species shared 54% FAFLP pattern similarity, but the patterns also showed clear differences (Fig. 2.3). *V. gazogenes* and *V. salmonicida* show about 5% difference in their mol% G+C content (Farmer III and Hickman-Brenner, 1992), confirming their status as separate species. Cluster A22 comprised two *Salinivibrio costicola* strains isolated from such diverse sources as bacon curing brine and turbot and which had highly related genomes. Clusters A24 and A25 consisted of five *V. vulnificus* strains isolated from Eel in Sweden and from humans in United States, and three *V. proteolyticus* strains isolated from shrimps and fish, respectively. Clusters A30 and A32 represent ubiquitously occurring strains, whereas clusters A31 and A33 consisted of isolates from white shrimp in Ecuador and rotifers in Belgium, respectively. Cluster A36 harboured twelve *V. harveyi* strains from different sources. *V. harveyi* LMG 4044^T and LMG 7890 (former *V. carchariae* type strain) clustered at 79% similarity. It has been confirmed by means of DNA-DNA hybridisation experiments and phenotypical studies that the fish pathogen *Vibrio trachuri* (Iwamoto et al., 1995) is a junior synonym of *V. harveyi* (Thompson et al., submitted-a). Cluster A38 harboured eight diverse *L. anguillarum* strains from fish in Norway and Denmark. Previous analyses of these strains have shown that they are heterogeneous, with different serotypes and API20E profiles (Austin et al., 1995b). Cluster A39 harboured ten highly related strains of *V. ordalii* isolated from different species of fish in Japan and United States. These results confirm their very homogeneous genotypic and phenotypic features found previously (Austin et al., 1997). Cluster A40 consisted of four *V. cholerae* strains, from shrimp and fish in China, Germany and Mexico. The high genomic diversity of *V. cholerae* has recently been shown (Jiang et al., 2000a,

Figure 2.2. Dendrogram of the FAFLP patterns of 506 *Vibrionaceae* strains. A band based (Dice) cluster analysis (Ward) was used. The threshold for cluster delineation was 45%. The position of the type strains is indicated after the cluster number.



550 bp

50 bp



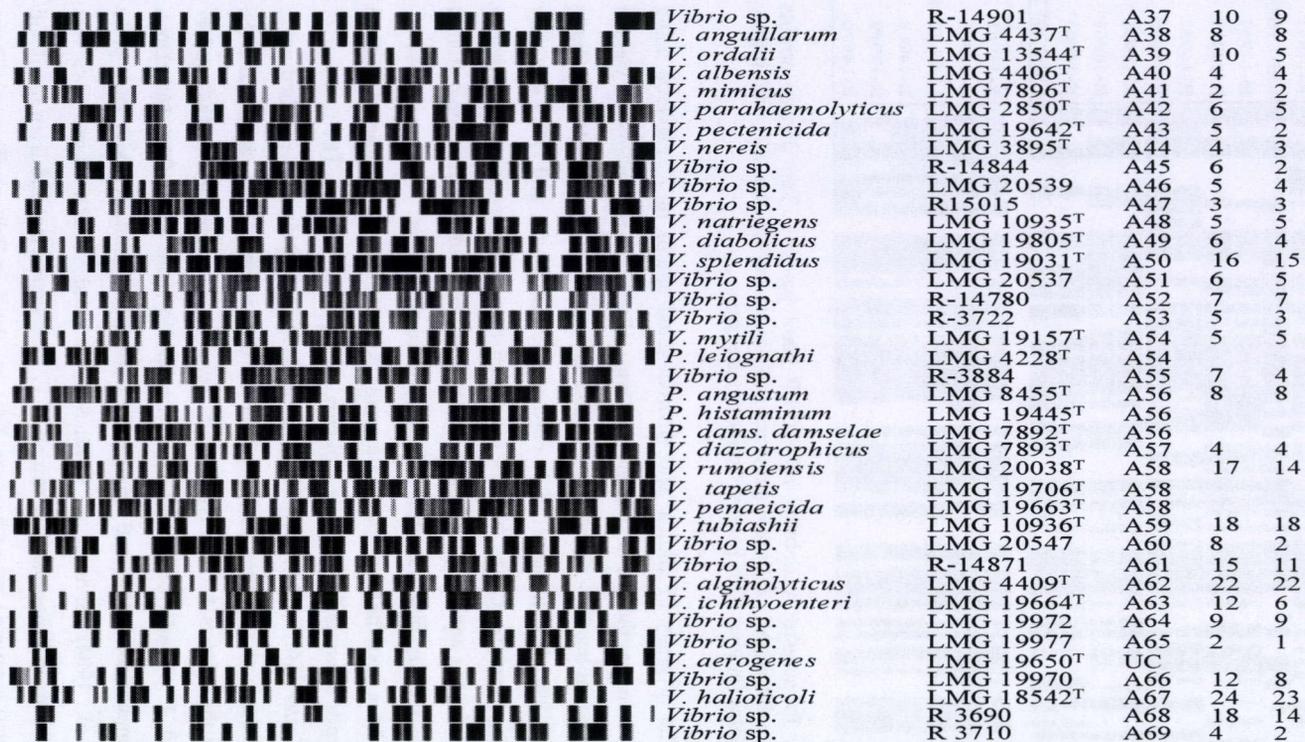


Figure 2.3. Representative FAFLP band patterns. 83 patterns representing the different clusters/type strains/unclustered strains. The average number of fragments (50 to 536 base pairs) of the FAFLP patterns shown was 102 ± 24 . The number of strains and distinguishable genomes per cluster is indicated in the two final columns.

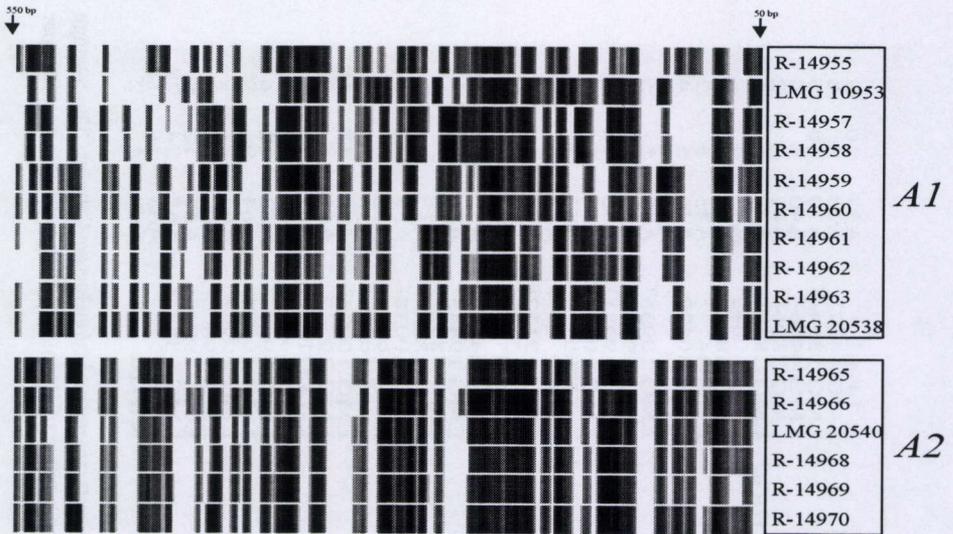


Figure 2.4. Intracluster variability of FAFLP band patterns. Ten diverse strains (*cluster A1*) with slightly different genomes and six strains (*cluster A2*) with highly related genomes (at least 82% pattern similarity) are shown.

, 2000b). The diversity of clinical and environmental strains of *V. cholerae* using AFLP has shown that the use of restriction enzyme *HindIII-TaqI* combination yielded a higher number of bands (> 50) than the *ApaI-TaqI* combination (20-30 bands) (Jiang et al., 2000b). The discrimination of *V. cholerae* serogroups O1 and O139 was only possible using *ApaI-TaqI*. The authors concluded that this enzyme combination is better suited for the analyses of clinical isolates, while *HindIII-TaqI* combination is more helpful for the discrimination of environmental isolates. Analyses of 67 *V. cholerae* isolates from the Chesapeake Bay using AFLP revealed the presence of three main genotypes separated by date of isolation (Jiang et al., 2000a). The fact that *V. cholerae* (*cluster A40*) and *V. mimicus* (*cluster A41*) are clearly distinguished using FAFLP illustrates the discriminatory power of this technique. It should also be stressed that the species *V. cholerae* and *V. mimicus*, *L. anguillarum* (*A38*) and *V. ordalli* (*A39*), *V. fluvialis* (*A27*) and *V. furnissii* (*A29*) share above 99% 16S rDNA similarity (unpublished data) and about 65% DNA-DNA similarity with each other (Brenner et al., 1984; Davis et al., 1982; Schiewe et al., 1982). *Cluster A43* was composed of four indistinguishable strains of *V. pectenida* isolated from moribund scallop (*Pecten maximus*) larvae between 1991 and 1995 in Brittany (France). These

results indicate that one persistent clone was responsible for the reported outbreaks in the hatcheries of Brittany during the period 1991 to 1995 (Lambert et al., 1998). *Cluster A49* consisted of six *V. diabolicus* strains from diverse origins. *V. diabolicus* LMG 19805^T and R-14784 originated from a hydrothermal vent polychaete, whereas R-14786 and R-14787 were isolated from mass cultures of probiont in a shrimp hatchery in Ecuador. Initially, these isolates were misidentified as *V. alginolyticus* using Biolog (Vandenberghe et al., 1999). *Cluster 50* comprised sixteen *V. splendidus* strains from fish and oyster in different countries. *V. splendidus* has been reported to be a very heterogeneous species and it has been found previously to form distinct ribotyping clusters (Mácian et al., 2000a). Analysis of the ribotypes of 84 *V. splendidus* strains isolated from the *Ostrea edulis* at the Mediterranean coast of Spain revealed the presence of 3 main clusters and a seasonal incidence of these groups. In the present study, representative *V. splendidus* isolates from oyster, found in the so-called ribotype cluster C (Mácian et al., 2000a), formed a sub-cluster (at 57% similarity) with other isolates from Spain and Greece. *Cluster A53* and *A55* consisted of five and seven highly related isolates from the gut of fish larvae in Norway. *Cluster A54* split in two sub-clusters; one sub-cluster consisted of three *Photobacterium leiognathi* strains from fish and the other harboured two highly related strains of *V. mytili* isolated from molluscs. Although belonging to different genera, those species showed similar genomes, sharing 49% pattern similarity. *Cluster A56* split in two sub-clusters; the first sub-cluster consisted of five *P. angustum* strains, whereas the second sub-cluster consisted of three *P. damsela* strains. Recently it has been proved that *P. histaminum* LMG 19445 (*cluster A56*) is a later synonym of *P. damsela* LMG 7892^T (Kimura et al., 2000). *Cluster A58* harboured 17 strains, distributed in four sub-clusters; the first sub-cluster consisted of two *V. penaeicida* strains from diseased prawn and oyster; the second sub-cluster consisted of two indistinguishable strains of *V. rumoiensis* from drain pool of fishery product processing plant in Japan which clustered at 68% with the first sub-cluster; the third sub-cluster harboured three indistinguishable *V. tapetis* strains from clam in France; and the fourth harboured 10 isolates from different sources. The relatedness of these isolates is unclear and further genomic and phenotypic analysis are needed. *V. penaeicida* and *V. rumoiensis* have 46-47 and 43 mol% G+C contents, respectively (Ishimaru et al., 1996; Yumoto et al., 1999), and share about 95% 16S rDNA homology with *V. tapetis*. The FAFLP patterns generated for *V. penaeicida*, *V. rumoiensis* and *V. tapetis* consisted of an

excessive number of bands (138 ± 12) which might have impeded their separation. *Cluster A60* consisted of eight isolates from bivalve larvae which were phylogenetic neighbours of *L. pelagia*, whereas *cluster A61* harboured fifteen diverse isolates closely related to *V. aestuarianus*. *Cluster A62* contained twenty two *V. alginolyticus* strains from different sources. Amongst *Vibrio* isolates from the gut of abalone quite some diversity occurred as they were found in *clusters A64, A65, A66* and *A67*. *Clusters A68* and *A69* contained eighteen and four isolates respectively, from the gut of turbot larvae in Norway.

Analysis of the 16S rDNA sequences of representative isolates from most FAFLP clusters clearly showed that they belong to the family *Vibrionaceae* (Table 2.1). Most unidentified clusters harboured isolates allocated in the so-called *Vibrio* core group (Dorsch et al., 1992), but clusters *A68* and *A69* were related to *V. hollisae*.

Taxonomic significance of FAFLP clusters

All species of the family *Vibrionaceae* (except *V. cyclotrophicus*, *V. lentus* and *P. profundum*) were included in this study. The clusters of *V. alginolyticus*, *L. anguillarum*, *V. cholerae*, *V. ordalii* and *V. splendidus* are well established and confirmed by previous studies on these species (Austin et al., 1995b; Austin et al., 1997; Vandenberghe et al., 1999). Moreover, the robustness of other FAFLP clusters i.e. *A6, A36, A68* and *A69* has been confirmed by DNA-DNA hybridisation experiments. *V. mediterranei* (*A6*) and *V. harveyi* strains (*A36*) exhibiting DNA homology higher than 70% showed FAFLP pattern similarity higher than 54% (Thompson et al., submitted-a; Thompson et al., submitted-b). Also, DNA-DNA hybridisation has been performed on representative isolates of the clusters *A68* and *A69* which share at least 32% FAFLP pattern similarity, and the DNA similarity among these clusters was higher than 86% (unpublished data), indicating that they represent a unique species. A total of 236 isolates distributed in 31 clusters had completely different genomes and thus remained unidentified (Fig. 2.2). This suggests that the genus *Vibrio* may harbour many taxa yet to be described. The usefulness of genomic fingerprinting techniques such as AFLP and rep-PCR was shown in determining the taxonomic relatedness of bacterial strains (Rademaker et al., 2000). AFLP has also been proposed as an alternative to DNA-DNA hybridisation (Coenye

et al., 1999a; Hauben et al., 1999; Huys et al., 1996; Janssen et al., 1997; Rademaker et al., 2000).

Ecological remarks

The *Vibrio* isolates studied are typical heterotrophs associated with a variety of marine aquacultural environments. Most isolates originated from cultured fish, molluscs and shrimps in Europe, America, Asia and Africa. Certain clusters e.g. *A9*, *A11*, *A12*, *A30*, *A32*, *A37*, *A40*, *A44*, *A46*, *A48*, *A49*, *A50*, *A57*, *A59*, *A61* and *A62* represent organisms with highly successful genomes since they occur in at least three continents and in different hosts. The ubiquitous occurrence of these groups may be due either to their easy spread via the aquatic environment or to their selective advantage acquired during evolution. Other clusters e.g. *A2*, *A3*, *A4*, *A7*, *A16*, *A17*, *A26*, *A28*, *A31*, *A33*, *A43*, *A45*, *A52*, *A53*, *A55*, *A60*, *A63* to *A69* appear to be exclusively associated with a particular host and geographical region. Clusters *A38*, *A39* and *A63* were found in association with different fish species, whereas cluster *A67* comprised mainly isolates from abalone.

This study was intended as a survey of the genomic diversity within the genus *Vibrio* and closest related genera. Although transduction via the aquatic environment may explain certain cases of genomic plasticity e.g. in the acquisition of pathogenicity islands (Hacker and Kaper, 2000), our results clearly show that the family *Vibrionaceae* harbours a great diversity of genomes of which several may belong to taxa as yet to be described. The robustness of FAFLP clusters and their phylogenetic position is under investigation with the aim of further improving the taxonomy of the Family *Vibrionaceae*.

Table 2.1. Phylogenetic allocation of representatives strains from most FAFLP clusters on basis of their 16S rDNA sequences. The closest phylogenetic neighbour of each strain was found using FASTA program.

Cluster	Strain number (accession no.)	Length (bp)	Closest neighbour	Similarity (%)
A1	LMG 10953 (AJ316167)	1468	<i>V. tubiashii</i> ATCC 19109 ^T (X74725)	97.9
A2	R-14968 (AJ316168)	1468	<i>V. tubiashii</i> ATCC 19109 ^T (X74725)	98.3
A3	LMG 19270 (AJ316169)	1468	<i>V. tubiashii</i> ATCC 19109 ^T (X74725)	98.2
A4	LMG 20548 (AJ316170)	1468	<i>V. tubiashii</i> ATCC 19109 ^T (X74725)	97.5
A5	LMG 20536 (AJ316171)	1468	<i>V. tubiashii</i> ATCC 19109 ^T (X74725)	97.2
A8	LMG 20546 (AJ316172)	1504	<i>V. tubiashii</i> ATCC 19109 ^T (X74725)	97.9
A9	R-15032 (AJ316173)	488	<i>V. orientalis</i> ATCC 33934 ^T (X74719)	96.7
A10	LMG 19995 (AJ316174)	469	<i>V. splendidus</i> ATCC 33789 (AB038030)	98.5
A11	R-15024 (AJ316175)	447	<i>V. vulnificus</i> ATCC 27562 ^T (X76333)	99.7
A12	R-15041 (AJ316176)	488	<i>V. orientalis</i> ATCC 74719 ^T (X74719)	96.7
A13	R-15048 (AJ316177)	488	<i>V. orientalis</i> ATCC 74719 ^T (X74719)	96.7
A16	LMG 13240 (AJ316178)	1468	<i>V. proteolyticus</i> ATCC 15338 ^T (X74723)	96.8
A17	LMG 20023 (AJ316179)	1463	<i>V. scophthalmi</i> CECT 4638 ^T (U46579)	98.5
A20	LMG 4426 (AJ316180)	479	<i>V. metschnikovii</i> CIP 69.14 ^T (X74711)	99.5
A23	R-15052 (AJ316181)	1435	<i>V. nereis</i> ATCC 25917 ^T (X74716)	96.1
A24	R-15063 (AJ316182)	443	<i>V. vulnificus</i> ATCC 27562 ^T (X76333)	100
A25	R-15065 (AJ316183)	457	<i>V. proteolyticus</i> ATCC 15338 ^T (X74723)	98.9
A26	LMG 20362 (AJ345063)	1468	<i>V. orientalis</i> ATCC 74719 ^T (X74719)	97.9
A27	R-15090 (AJ316184)	456	<i>V. fluvialis</i> ATCC 33809 ^T (X74703)	100
A30	R-14913 (AJ316185)	438	<i>V. campbellii</i> ATCC 25920 ^T (X74692)	99.7
A31	LMG 20370 (AJ345066)	1470	<i>V. campbellii</i> ATCC 25920 ^T (X74692)	98.7
A32	R-14928 (AJ316186)	441	<i>V. campbellii</i> ATCC 25920 ^T (X74692)	99.5
A33	R-14939 (AJ316187)	1468	<i>V. campbellii</i> ATCC 25920 ^T (X74692)	99.8
A37	LMG 20369 (AJ345065)	828	<i>V. campbellii</i> ATCC 25920 ^T (X74692)	99.7
A39	R-15101 (AJ316188)	479	<i>V. ordalii</i> ATCC 33509 ^T (X74718)	99.3
A40	LMG 19996 (AJ316189)	481	<i>V. cholerae</i> ATCC 14035 ^T (X74695)	99.7

Table 2.1. (continued).

A41	R-14850 (AJ316190)	474	<i>V. mimicus</i> ATCC 33653 ^T (X74713)	98.3
A42	R-14855 (AJ316191)	451	<i>V. parahaemolyticus</i> ATCC 17802 (X74720)	98.4
A45	LMG 20012 (AJ316192)	1507	<i>V. lentus</i> CECT 5110 ^T (AJ278881)	99.0
A46	LMG 20539 (AJ316193)	1494	<i>V. aestuarianus</i> KT0901 (AF172840)	98.1
A47	LMG 19999 (AJ316194)	1494	<i>V. furnissii</i> ATCC 35016 ^T (X76336)	96.4
A49	R-14784 (AJ316195)	911	<i>V. diabolicus</i> HE800 ^T (X99762)	98.6
A50	R-14789 (AJ316196)	444	<i>V. splendidus</i> ATCC 33125 ^T (X74724)	98.4
A51	R-14805 (AJ316197)	485	<i>V. splendidus</i> ATCC 33125 ^T (X74724)	98.5
A52	LMG 13219 (AJ316198)	471	<i>V. splendidus</i> ATCC 33789 (AB038030)	98.9
A53	R-3712 (AJ316199)	1435	<i>V. splendidus</i> ATCC 33789 (AB038030)	99.7
A55	R-3884 (AJ316200)	1507	<i>V. lentus</i> CECT 5110 ^T (AJ278881)	98.9
A59	R-14825 (AJ316201)	488	<i>V. tubiashii</i> ATCC 19109 ^T (X74725)	97.7
A60	LMG 20547 (AJ316202)	1505	<i>L. pelagia</i> CECT 4202 ^T (AJ293802)	98.4
A61	R-1556 (AJ316203)	1507	<i>V. aestuarianus</i> KT0901 (AF172840)	98.6
A62	R-14876 (AJ316204)	438	<i>V. alginolyticus</i> ATCC 17749 ^T (X74690)	99.0
A64	LMG 19972 (AJ316205)	1435	<i>V. haliotocoli</i> IAM 14596 ^T (AB000390)	98.7
A66	LMG 19970 (AJ316206)	1429	<i>V. haliotocoli</i> IAM 14596 ^T (AB000390)	98.7
A68	LMG 19840 (AJ316207)	1505	<i>V. hollisae</i> ATCC 33564 ^T (X74707)	94.1
A69	LMG 19839 (AJ316208)	1505	<i>V. hollisae</i> ATCC 33564 ^T (X74707)	94.1

2.3. Fluorescent amplified fragment length polymorphism and repetitive extragenic palindrome-PCR fingerprinting reveal host-specific genetic diversity of *Vibrio halioticoli*-Like strains isolated from the gut of Japanese Abalone

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Appl. Env. Microbiol. (2002), 68, 4140-4144

Abstract

When analyzed by fluorescent amplified fragment length polymorphism and repetitive extragenic palindrome-PCR fingerprinting, a total of 47 *Vibrio halioticoli* strains isolated from four Japanese abalone species and one turban shell species formed three clusters that roughly reflect the different species of host abalone from which they were isolated. The *V. halioticoli* isolates from turban shells were distributed evenly among the clusters. Representative isolates from two clusters were deemed separate species or subspecies by DNA-DNA hybridisation.

Introduction

Gut microbial ecology studies of human, mouse, and ruminant systems are quite advanced due to the development of tools for examining host-microbe interactions in an evolutionary context (time scale) and for identifying the interactions (Hooper and Gordon, 2001; Russel and Rychlik, 2001). Host characteristics such as innate immunity and nutrition suggest that gut microbes have coevolved with their hosts to develop various symbiotic, commensal, and pathogenic associations (Hooper and Gordon, 2001). With the exception of the *Vibrio halioticoli*-abalone relationship, these relationships in marine herbivores have not been well studied (Sawabe et al., 1995, 1998; Tanaka et al., 2002). Abalones have had conserved herbivorous feeding behavior (on algae) throughout their long evolutionary history. In one abalone species (*Haliotis discus hannai*), a unique alginolytic bacterium has been found in abundance

of 10^5 to 10^9 CFU/g of gut tissue, and both the abalone species and the bacterium have unique substrate specificities and alginate degradation activities, suggesting that the bacterium may contribute significantly to the host's digestion of alginate (Sawabe et al., 1995). This novel alginolytic and facultatively anaerobic bacterium was classified as *Vibrio halioticoli* (Sawabe et al., 1998). Recently, *V. halioticoli*-like strains have been found in the gut of three other species of *Haliotidae* abalone and one species of *Turbinidae* shell in Japan (T. Sawabe, N. Setoguchi, R. Tanaka, O. Setoguchi, M. Yoshimizu, and Y. Ezura, abstract from the Annual Scientific Meeting of the Australian Society for Microbiology 2000, Microbiol. Aust. 21:A119, 2000). *V. halioticoli* may be a key symbiotic microbe for digesting and converting alginate to available energy sources for the host like volatile short-chained fatty acids.

Genetic diversity among bacterial strains can be assessed by using Box, enterobacterial repetitive intergenic consensus, and repetitive extragenic palindrome (rep)-PCR genomic fingerprinting techniques (Rademaker et al., 1998, 2000; Versalovic et al., 1994). Another fingerprinting technique, amplified fragment length polymorphism (AFLP), has been used in bacterial taxonomy (Huys et al., 1996; Janssen et al., 1996; Thompson et al., 2001) and diversity studies of pathogenic bacteria for epidemiological purposes (Duim et al., 1999, 2000; Jiang et al., 2000a, 2000b). AFLP fingerprinting has a higher discriminating power than the Box, enterobacterial repetitive intergenic consensus, and rep-PCR fingerprinting techniques (Rademaker et al., 2000). However, results of both AFLP and rep-PCR fingerprinting techniques are in close agreement with those of DNA-DNA hybridization studies, and these techniques are regarded as the best tools available to date for determining the taxonomic and phylogenetic structures of bacterial populations (Duim et al., 2001; Nick et al., 1999). Recent studies comparing the similarity coefficients of genomic fingerprinting results and DNA-DNA hybridization values for *Stenotrophomonas* and *Xanthomonas* strains found that AFLP similarity values (Dice coefficients [SDs]) above 55 to 65% correlated with DNA homology values of 70 to 75% (Hauben et al., 1999; Rademaker et al., 2000).

Gut microbes, which play an important role in the host digestion system, may have coevolved with their hosts. To examine whether the host-gut microbe association of *V. halioticoli*-like strains is host specific, the genetic diversity of these *V. halioticoli*-like strains from abalone and turban shells collected from various locations

along coastal Japan were analyzed by both AFLP and rep-PCR fingerprinting techniques. Relatedness was further examined by DNA-DNA hybridization.

Material and Methods

The *V. halioticoli*-like strains used in this study were isolated from five host animals (Table 1, Annex): 17 strains were from the abalone species *H. discus hannai*, 14 were from *Haliotis discus discus*, 10 were from *Haliotis diversicolor supertexta*, 1 was from *Haliotis diversicolor diversicolor*, and 3 were from the turban shell species *Turbo cornutus*. Two strains were collected from seawater around abalone farms (Sawabe et al., Microbiol. Aust. 21:A119, 2000). All isolates except confirmed *V. halioticoli* strains IAM14569^T, IAM14597, IAM14598, and IAM14599 (Sawabe et al., 1998) were identified as *V. halioticoli*-like by using *V. halioticoli*-specific colony hybridization (Tanaka et al., 2002) and 16S ribosomal DNA PCR/restriction fragment length polymorphism analysis (Tanaka et al., 2001). Isolates used for fingerprint analysis were geographically distributed in Japan as follows: *H. discus hannai* isolates from the three Hokkaido sites and one Iwate site were 500 km apart, and *H. discus discus* isolates from Kanagawa and Izu Ohshima were 100 km apart (Table 1, Annex). All strains were maintained on ZoBell 2216E agar containing 0.5% sodium alginate (Sawabe et al., 1995).

Cells used for DNA extraction were cultured in ZoBell 2216E broth at 25°C for 24 h, harvested, and extracted with a Promega (Madison, Wis.) Wizard genomic DNA extraction kit according to the manufacturer's instructions.

Fluorescent AFLP patterns of the 47 strains were generated and analyzed as described previously (Thompson et al., 2001). Briefly, 1 µg of high-molecular-weight DNA was digested with *TaqI* and *HindIII*, followed by ligation of restriction half-site-specific adapters, and amplified by performing PCR twice with primers H00/T00 and H01-6FAM/T03 (Thompson et al., 2001). PCR products were separated on 36-cm denaturing polyacrylamide gels on an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, Calif.). GeneScan 3.1 software (Applied Biosystems) was used to track and normalize the lanes. Tables of data from normalized peaks containing fragments of 50 to 536 bp were transferred into BioNumerics 2.0 software (Applied Maths, Sint-Martens-Latem, Belgium) for numerical analysis. Clustering of the patterns was done by use of the Dice coefficient (SD) and the Ward algorithm (Sneath and Sokal, 1973).

rep-PCR fingerprinting was performed using the PCR conditions described previously by Rademaker et al. (1998) and reaction mixtures containing 1 μ l of DNA (50 ng μ l⁻¹), 5 μ l of 5x Gitschier buffer, 0.4 μ l of bovine serum albumin (10 mg ml⁻¹), 2.5 μ l of dimethyl sulfoxide (10 mg ml⁻¹), 1.25 μ l of a deoxynucleoside triphosphate mixture (100 mM each deoxynucleoside triphosphate), 1 μ l of GTG5 primer (0.3 μ g μ l⁻¹; Amersham Pharmacia Biotech, Uppsala, Sweden) (24), and 0.4 μ l of Taq DNA polymerase (5 U μ l⁻¹; Goldstar Red). PCR products were separated on a 1.5% (wt/vol) agarose gel with TAE buffer [1.21 g of Tris 2-amino-2 (hydroxymethyl)-1,3-propanediol liter⁻¹, 0.2 ml of 0.5 M EDTA liter⁻¹ (pH 8)] at a constant voltage of 55 V for 900 min at 4°C. Molecular markers (45.5% [vol/vol] 100-bp ruler; 36.5% [vol/vol] 500-bp ruler, and 18% [vol/vol] loading buffer) were loaded in the first and every sixth lane. After the gels were stained with ethidium bromide, the visualized patterns were digitalized. Normalization, recognition, and assignment of bands on the gel were performed using BioNumerics software (Applied Maths), and a dendrogram based on the Pearson similarity coefficient (r) was constructed (Hane et al., 1983).

Results and Discussion

AFLP analysis grouped the 47 *V. haliotocoli*-like strains into three main clusters (Figure 2.5). Cluster 1 (similarity Dice=SD, 48.6%) included the four *V. haliotocoli* reference strains, all *V. haliotocoli*-like strains isolated from *H. discus hannai*, two isolates from seawater from abalone farms, one isolate (TC4-2 [=LMG19963]) from *T. cornutus*, and four isolates (HDD4-1 [=LMG19974], HDD4-2, HDD5-1 [=LMG19975], and HDD5-2) from *H. discus discus*. Cluster 2 (SD, 54.6%) included 10 isolates from *H. discus discus* and 1 isolate (TC2-3) from *T. cornutus*. Cluster 3 (SD, 54.6%) included all isolates from *H. diversicolor aquatilis* and *H. diversicolor diversicolor* and isolate TC2-1 from *T. cornutus*. The low SDs (<20%) among these three groups indicate that they consist of isolates with divergent genomes (Fig. 2.5).

rep-PCR fingerprinting of these 47 strains resulted in a delineation of the three main clusters identical to the AFLP grouping. *V. haliotocoli*-like strains grouped in the same clusters according to their host abalone species, except for the rearrangement within cluster 1 of isolates HDD4-1 (=LMG19974), HDD4-2, HDD5-1 (=LMG19975), and HDD5-2. As with the AFLP clustering, isolates from seawater

and *T. cornutus* were found in the same clusters. Clusters 1, 2, and 3 had inner r values of 48.7, 60.6, and 59.0%, respectively, and the r value among the clusters was lower than 45%.

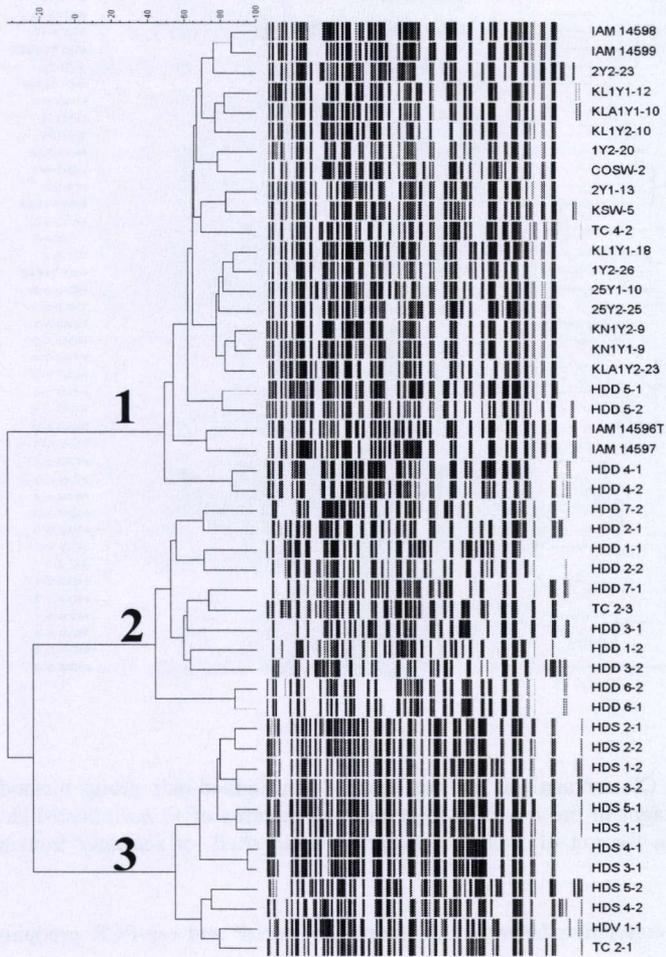


Figure 2.5. Dice coefficient-Ward algorithm cluster analysis of AFLP fingerprinting patterns of *V. halioticoli*-like strains isolated from the gut of Japanese abalone, turban shell, or seawater from abalone farms.

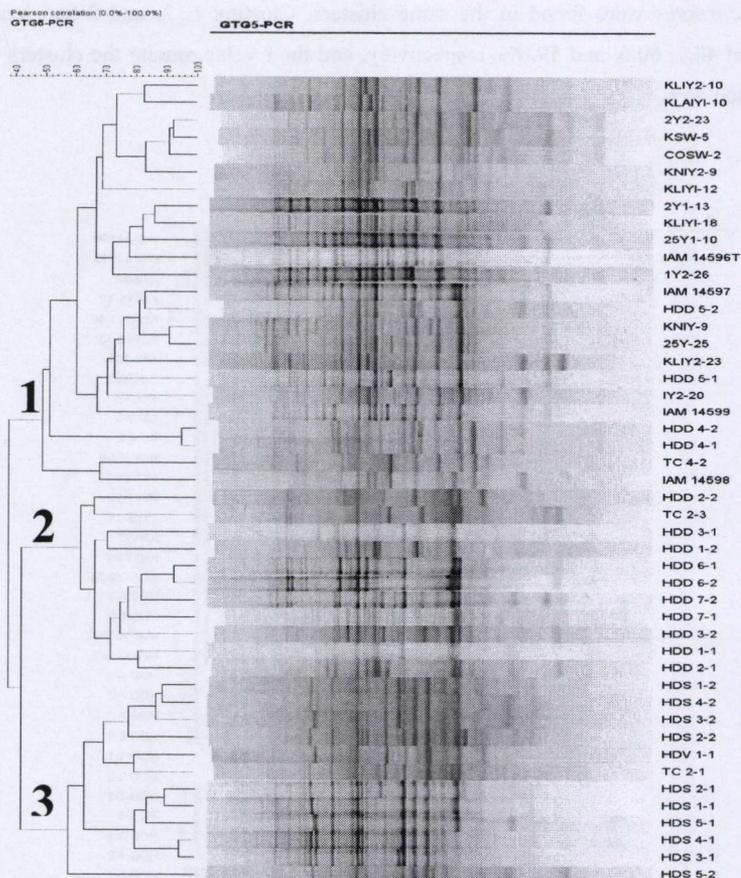


Figure 2.6. Cluster analysis by the Pearson unweighted-pair group method using average linkages of the rep-PCR fingerprinting patterns of *V. halioticoli*-like strains isolated from the gut of Japanese abalone, turban shell, or seawater from abalone farms.

The correlation between the fluorescent AFLP and rep-PCR groupings was found to be high. In addition, differences in geographical distribution, for example, of the strains from *H. discus hannai* from Hokkaido and Iwate and of the strains from *H. discus discus* from Kanagawa and Izu Ohshima, were not found to be distinct subgroups by either AFLP fingerprinting or rep-PCR fingerprinting analysis (Figs 2.5. and 2.6). Therefore, the only criteria for the grouping of the *V. halioticoli*-like strains by both fingerprinting techniques appears to be the abalone host species from which

the strains were isolated. *V. haliotocoli*-like isolates from abalone were divided into three clusters by both AFLP and rep-PCR fingerprinting analyses based on their abalone host species. However, *V. haliotocoli*-like strains from turban shells were not grouped in a single cluster (Figs. 2.5 and 2.6). In fact, the nearest neighbors of isolates TC2-1 and TC2-3 from wild turban shells were wild abalone isolates HDV1-1 (from *H. diversicolor diversicolor*) and HDD7-1 (from *H. discus discus*), with 79.4 and 73.4% SDs, respectively, by the AFLP analysis (Fig. 2.5). Alternatively, the host species of strain TC4-2 was cultured at Izu Oshima, and the nearest neighbours of TC4-2 were isolates from *H. discus hannai* (Fig. 2.5). These data lead to the hypothesis that *V. haliotocoli*-like isolates can be taken up into the gut of turban shells without strong specificity in the symbiotic associations between *V. haliotocoli* and their turban shell hosts. Rather low populations (below 20%) of *V. haliotocoli*-like strains in the gut of the turban shell (unpublished data) compared to the abundant populations (40 to 60%) in the gut of abalone suggests a transient or neutral relationship between turban shells and the bacterium. Results of DNA-DNA hybridization experiments with representative *V. haliotocoli*-like strains divide into the fingerprinting clusters.

In our study, AFLP fingerprinting similarities (measured as SDs) between cluster 1, which includes the *V. haliotocoli* type strains, and the other groups and those between cluster 2 and cluster 3 were below 20%. DNA-DNA hybridization experiments were performed with microdilution wells by a fluorometric direct binding methodology as previously described (Ezaki et al., 1988). DNAs of *V. haliotocoli* IAM14596^T (representative of AFLP cluster 1), HDD3-1 (representative of AFLP cluster 2), and HDS1-1 (representative of AFLP cluster 3) were labeled with photobiotin (Vector Laboratories, Burlingame, Calif.). Unlabeled single-stranded DNA from each of these strains was immobilized in microdilution wells (Immuron 200, FIA/LIA plate, black type; Greiner Labortechnik, Frickenhausen, Germany). Hybridization was performed at 45°C (Sawabe et al., 1998).

DNA-DNA relatedness levels between the biotinylated strain IAM14596^T and unlabeled strains HDD3-1 and HDS1-1 were 97 and 70%, respectively (Table 2.2). However, the DNA relatedness values between labeled HDD3-1 and HDS1-1 and the other unlabeled strains were all below 70% (Table 2). The results indicate that isolates belonging to AFLP clusters 2 and 3 (HDD3-1 and HDS1-1, respectively) can be defined as a species or subspecies that is distinct from authentic *V. haliotocoli* by using

a DNA relatedness of greater than 70 % as the criterion for defining a bacterial species (Wayne et al., 1987).

Table 2.2. DNA relatedness among *V. halioticoli* and related isolates

	AFLP cluster	DNA relatedness (%) with biotinylated DNA from:		
		IAM14596 ^T	HDD3-1	HDS1-1
<i>V. halioticoli</i> IAM14596 ^T	1	100	43	32
HDD3-1	2	97	100	67
HDS1-1	3	70	48	100

Conclusion

The genomic fingerprinting analysis reveals that host-driven (or host-dependent) DNA polymorphism rather than geographical or environmental factors accounts for the groupings observed in the *V. halioticoli*-like strains isolated from Japanese abalone (Fig. 2.5 and 2.6). Furthermore, cospeciation of *V. halioticoli*-related species and Japanese abalone may have occurred (Table 2.2). There are, however, about 70 abalone species in the world that populate coastal areas along France, South Africa, Australia, New Zealand, United States, and Taiwan. In the one regional example described in the present report, we have shown the strong role of host species specificity in the abalone-gut microbe relationship. One proposed ancestral abalone species (*Haliotis iris*, a New Zealand species) split into two main lineages according to molecular phylogenetic analysis using a sequence of abalone sperm lysine and morphological comparisons to the oldest abalone fossil records (Lee and Vacquire, 1995). A study of the distribution of the gut microbe *V. halioticoli* or related species in modern abalone species should be conducted to clarify the coevolution of abalone and *V. halioticoli*.

2.4. Genomic diversity of clinical and environmental *Vibrio cholerae* strains isolated in Brazil between 1991 and 2001 as revealed by FAFLP analysis

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J. Clin. Microbiol. (2003), 41, 1946-1950

Abstract

Vibrio cholerae is a ubiquitous and abundant organism in aquatic environments, particularly in coastal areas, estuaries and rivers. This organism was the cause of a considerable number of deaths in Brazil during the last decade. In this study we applied the genomic fingerprinting technique Fluorescent Amplified Fragment Length Polymorphism (FAFLP) to analyse 106 *V. cholerae* O1 and non-O1/non-O139 strains isolated from clinical specimens and environment between 1991 and 2001. Numerical analysis of the FAFLP patterns disclosed seven main groups of genomes, all of them originated from a variety of different places in different years, suggesting that *V. cholerae* is a very diverse species. Strains O1 and non-O1/non-O139 were distinguishable by FAFLP, although clinical and environmental strains clustered together in a few cases. The persistence of some strains of highly related genomes during several years and in completely different geographical regions suggests these strains are highly successful in adapting to changing environmental conditions.

Introduction

Between 1991 and 1998 at least 1296 deaths due to cholera occurred in Brazil, mostly in the North and Northeast regions of the country (Waldman et al., 2002). The lack of a water network supply and sanitation as well poverty were concluded to be the main reasons for this high rate of mortality. The study of the *V. cholerae* population structure as well as cholera molecular epidemiology may help us to understand the spread of this disease. Molecular typing techniques i.e. fluorescent amplified fragment length polymorphism (FAFLP), repetitive extragenic palindrome-

PCR (rep-PCR), multilocus enzyme electrophoresis (MLEE), multilocus sequence typing (MLST), randomly amplified polymorphic DNA (RAPD), and ribotyping have been extensively applied in the study of *V. cholerae* (Dijkshoorn et al., 2001). Although ribotyping has been perhaps the most used technique in the study of the diversity, epidemiology and evolution of *V. cholerae* (Wachsmuth et al., 1994), Lan and Reeves (1998) have recently highlighted that most of the variation in ribotyping is due to recombination events which take place at a very high rate (10^{-5} per cell per generation). In these cases ribotyping similarity between isolates does not reflect their origin from the same ancestor. It has been suggested that a robust and reproducible alternative is the use of FAFLP fingerprinting technique (Jiang et al., 2000a, 2000b; Lan and Reeves, 2002).

FAFLP analysis is performed basically in four steps (Janssen et al., 2001; Janssen et al., 1996): (1) digestion of total genomic DNA with two restriction enzymes, a "4-base cutter" (e.g. *TaqI*) which digests the DNA frequently and a "6-base cutter" (e.g. *ApaI*) which digests the DNA less frequently. Subsequent ligation of the restriction halfsite-specific adaptors to all restriction fragments is performed by using a DNA ligase; (2) selective amplification of these fragments with two PCR primers that have corresponding adaptor- and restriction site sequences as their target sites. Selective bases that flank the restriction site sequences are included in the primers to reduce the number of fragments amplified; (3) electrophoretic separation of the PCR products on polyacrilamide gels with selective detection of fragments which contain the fluorescently labelled primer and (4) computer assisted numerical analysis of the band patterns. FAFLP indexes variation in the whole genome and thus is considered to give useful information on the short- and long-term evolution of bacterial strains. In the present study we applied fluorescent AFLP analysis to uncover the genomic diversity of clinical and environmental *V. cholerae* O1 and non-O1/non-O139 isolates from Brazil during a 11-years sampling period, corresponding to the beginning of the seventh pandemic in Latin America.

Material and Methods

Bacterial strains, growth condition and DNA extraction

A total of 106 *V. cholerae* strains i.e. 10 reference strains and 96 isolates originated from different Brazilian states were examined in this study (Table 3, Annex; Figure 2.7). 87 strains originated from clinical specimens and 20 strains were

isolated from the environment. Strains were grown aerobically on Tryptone Soya agar (TSA; Oxoid) supplemented with 2 % (w/v) NaCl for 24 h at 28 °C. DNA was extracted following the methodology described by Pitcher et al. (1989). All strains included in this study are deposited in the BCCMTM/LMG Bacteria Collection at Ghent University (Ghent, Belgium) and in the Oswaldo Cruz Institute Collection (Rio de Janeiro, Brazil).

FAFLP template preparation and PCR reactions

Template preparation was carried out essentially as described previously (Jiang et al., 2000a; Thompson et al., 2001). Briefly, one µg of high-molecular-mass DNA was digested with *TaqI* and *ApaI* (Amersham Pharmacia Biotech, Sweden), followed by ligation of restriction half-site specific adapters to all restriction fragments with T4 ligase (Amersham Pharmacia Biotech, Sweden). An aliquot of 2.5 µl template was mixed with 0.8 µl A01-6FAM primer (5'GACTGCGTACAGGCCCA3'; 1 µM), 0.8 µl T01-ABI (5'CGATGAGTCCTGACCGAA3'; 5µM), and 12 µl of Amplification Core Mix (Applied Biosystems, USA). The A01-6FAM primer is fluorescently labelled, and the selective bases (adenosine) at the 3'-end are underlined. The amplification reactions were performed in a GeneAmp PCR System 9600 thermocycler (Applied Biosystems, USA) as described previously (Thompson et al., 2001).

Capillary electrophoresis and numerical analysis

Six µl of the selective PCR product was mixed with 24 µl deionised formamide, 1 µl GeneScan-500 TAMRA size standard and 1 µl GeneScan-2500 TAMRA size standard and heated at 95 °C for 3 min. Subsequently, the mix was chilled on ice for a few minutes. Separation of the selective PCR products was generated in a ABI 310 genetic analyser (Applied Biosystems, USA). Time and voltage of sample injection were 16 sec. and 15 kV, respectively. Each run was performed at 60 °C, for 28 minutes at 10mA and 15kV. Normalization of the patterns was performed by the GeneScan 3.1 software (Applied Biosystems, USA). Normalized tables of peaks, containing fragments of 50 to 536 base pairs, were transferred into BioNumerics 2.5 software (Applied Maths, Belgium) for numerical analyses. Pattern similarity was calculated using Pearson coefficient, and

dendrograms were obtained by Unweighted Pair Group Method of Arithmetic Averages (UPGMA) (Sneath and Sokal, 1973). Cophenetic correlation and a cluster cutoff algorithm based on the Point-Biserial Correlation (Jobson, 1996) were applied in order to evaluate the robustness of clusters. Additionally, Principal Component Analysis (PCA), a non-hierarchical technique, was performed on binarized FAFLP data in order to obtain the relatedness among strains in a three-dimensional space.

DNA hybridisation

DNA-DNA hybridisation was performed at stringent conditions using the microplate technique with photobiotin-labeled DNA at a temperature of 40 °C for 3 hours as described previously (Willems et al., 2001). DNA similarity values are mean of reciprocal and non-reciprocal reactions, each of which are performed in quadruplicates.

Results and Discussion

The results depicted in Figure 2.7 clearly denote that *V. cholerae* is a diverse species. The strains examined consist of seven main groups of genomes, not correlated to particular dates or places. This grouping was supported by cophenetic correlation and a cluster cutoff algorithm. *Cluster 1* (n = 70) was the largest group, including the type strain of *V. cholerae* LMG 21698^T and other established reference strains such as LMG 19996 (Thompson et al., 2001), LMG 21699, El Tor R-18308 and Amazon variant strains (Coelho et al., 1995). *Cluster 1* was composed of strains O1 and non-O1/non-O139 isolated from patients and environment. Overall isolates from the different serogroups formed sub-groups, whereas some clinical and environmental strains (e.g. R-18301 and R-18338; R-18251 and R18258) were highly related. Some clonal groups of strains (i.e. strains having about 95 % pattern similarity) were correlated by either the year or the region of isolation. For instance, strains R-18309, R-18316, R-18305 and R-18304 were all isolated in 1993, but at states at least 1500 Km far from each other. Strains R-18331, R-18332, R-18333, R-18355, R-18356, R-18359, R-18362, were all isolated in Amazonas state in 1991-1992 and were related to the majority of the cases of cholera in that region (Coelho et al., 1995). These strains have been referred to as a new variant of *V. cholerae* O1, the Amazon variant. According to many phenotypic and genotypic techniques, this variant is completely apart from other *V. cholerae* O1 strains (Coelho et al., 1995).

Genomic diversity of *V. cholerae*

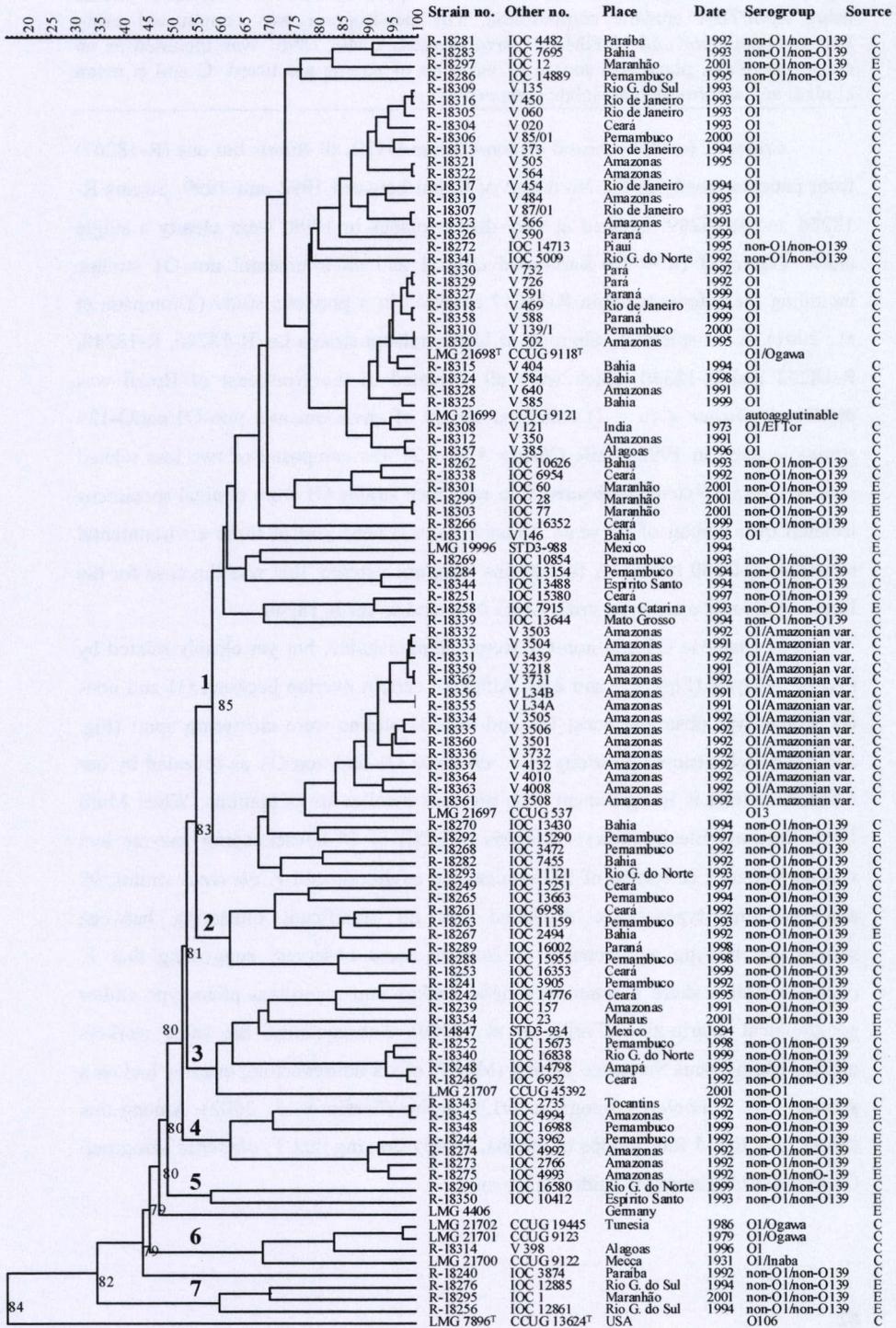


Figure 2.7. Dendrogram derived from FAFLP patterns of 106 *V. cholerae* strains using *Apal/TaqI* enzyme combination. The dendrogram was constructed using Pearson coefficient and UPGMA. *Vibrio mimicus* LMG 7896^T was included as an out-group. Year, place and source of isolation of strains are listed. C and E mean clinical and environmental isolates respectively.

Cluster 2 (n=8) consisted of non-O1/non-O139, all strains but one (R-18267) from patients mostly in the Northeast of Brazil between 1992 and 1999. Strains R-18288 and R-18289, isolated at very distant places in 1998, were clearly a single clone. *Cluster 3* (n = 10) harboured clinical and environmental non-O1 strains, including the reference strain R-14847 analysed in a previous study (Thompson et al., 2001). A conspicuous subgroup of highly related strains i.e. R-18246, R-18248, R-18252 and R-18340 which were all isolated in the Northeast of Brazil was disclosed. *Cluster 4* (n = 7) consisted mostly of environmental non-O1/nonO-139 strains isolated in 1992, while *Cluster 5* (n = 2) was composed of two less related strains. *Cluster 6* (n=4) harboured four reference strains O1 from clinical specimens isolated over a span of 65 years. *Cluster 7* (n=3) consisted of three environmental non-O1/non-O139 strains. A few strains were unclustered; this was the case for the former *Vibrio albensis* type strain LMG 4406 and so for R-18240.

V. cholerae O1 and non-O1 were distinguishable, but yet closely related by FAFLP analysis (Figs. 2.7 and 2.8). Although certain overlap between O1 and non-O1 strains was observed, most O1 and non-O1 strains were clustering apart (Fig. 2.8). The high genome plasticity of *V. cholerae* O1 and non-O1 as revealed by our FAFLP analysis is in agreement with previous detailed investigations. When Multi Locus Enzyme Electrophoresis analysis (MLEE) of 15 housekeeping enzyme loci was applied on a collection of 107 clinical and environmental *V. cholerae* strains, 99 electrophoretic types were disclosed and no significant clustering between serogroups, biotype and country of isolation were observed, suggesting that *V. cholerae* strains share a common genetic background regardless phenotype and/or geographical distribution (Farfán et al., 2000). Subsequently, the same workers applied Multi Locus Sequence Typing (MLST) of six housekeeping enzyme loci on a subset of 31 *V. cholerae* serogroup O139 strains (Farfán et al., 2002). Among this subset they found four groups of strains, clearly proving that *V. cholerae* serogroup O139 consists of multiple epidemic lineages.

Because the Amazon O1 population was so apart from the other *V. cholerae* strains as revealed by PCA analysis (Fig. 2.8), we decided to perform DNA-DNA hybridisation experiments to check if this population would not be in fact another species. *V. cholerae* strains, including the Amazon variant, were highly related i.e. ≥

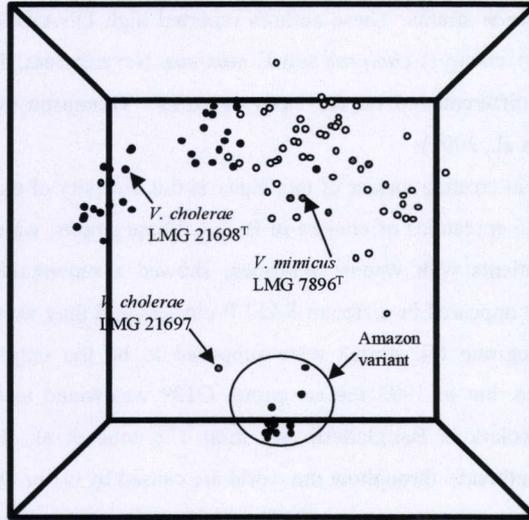


Figure 2.8. Three-dimensional representation of Principal Component Analysis derived from the FAFLP patterns (24 bands ± 4) of 106 *V. cholerae* strains. Black dots indicate O1 strains, while white and grey dots indicate strains non-O1 and other serogroups, respectively.

Table 2.3. DNA-DNA similarity values.

	1	2	3	4	5	6	7	8
<i>V. cholerae</i> Amazon v.								
1. R-18333	100							
2. R-18337	98	100						
3. R-18355	102	103	100					
<i>V. cholerae</i>								
4. R-18306	97	96	98	100				
5. R-18308	96	97	98	99	100			
6. R-18325	94	97	97	97	101	100		
7. LMG 21698 ^T	96	96	101	99	99	99	100	
<i>V. mimicus</i>								
8. LMG 7896 ^T	73	73	73	74	75	77	79	100

94 % similarity, despite their long span of isolation. (Table 2.3). This similarity level corresponded to FAFLP pair-wise pattern similarities of about 75 %, indicating the

higher discriminatory power of the latter technique. Our careful experiments not only proved that the Amazon variant belongs to the species *V. cholerae*, but also showed that *V. mimicus* LMG 7896^T is highly related to *V. cholerae* (Table 2.3). The species *V. mimicus* was proposed by Davis et al. in 1981 to encompass biochemically atypical *V. cholerae* strains. These authors reported high DNA-DNA hybridisation levels (i.e. 67 %) among *V. cholerae* and *V. mimicus*. Nevertheless, these two species can be clearly differentiated on the basis of AFLP (Thompson et al., 2001) and MLEE (Vieira et al., 2001).

Another interesting aspect of this study is the diversity of non-O1/non-O139 strains within the epidemics of cholera in Brazil. These strains, which were isolated mostly from patients with watery diarrhoea, showed a remarkably high genome diversity as they appeared in different FAFLP clusters and they were also related to O1 strains. Serogroup O1 strains were supposed to be the only responsible for epidemic cholera, but in 1993 the serogroup O139 was found to be the cause of epidemics of cholera in Bangladesh and India (Faruque et al., 1998). Although currently most outbreaks throughout the world are caused by O1 or O139 serogroups, in Brazil cholera cases due to non-O1/non-O139 strains have been observed. A typical example of such a successful lineage is the group represented by the strains R-18246, R-18248, R-18252, R-18340, which were isolated in the Northeast of Brazil during 1992-1999 (Fig. 2.7; cluster 3).

While the precise role of non-O1/non-O139 strains in the cholera outbreaks in Brazil is unknown at present, our results suggest that strains of different serogroups are highly related and may be important in the evolution of *V. cholerae* and cholera. Recent studies on the distribution of virulence genes in *V. cholerae* strains isolated in Brazil and worldwide, have demonstrated that both clinical and environmental strains of *Vibrio cholerae* of different serogroups may harbour virulence genes i.e. *ctxAB* and *tcpA* genes and are thus potentially pathogenic (Brazil et al., 2002; Chakraborty et al., 2000; Rivera et al., 2001). Lateral gene transfer of both virulence and antibiotic resistance genes is an important and ancient process that has been taking place in the evolution of Gram negative bacterial pathogens (Faruque et al., 1998; Karaolis et al., 1998; Rowe-Magnus et al., 2001; Rowe-Magnus et al., 2002). Bearing this process in mind and taking into consideration the high abundance of vibrios (i.e. $\sim 10^8$ cells.l⁻¹ or up to 4 % of *Bacteria*) in aquatic environments (Heidelberg et al., 2002a, 2002b), an important aspect to consider in the study of the evolution of cholera would be the

role of other *Vibrio* species (currently more than 55) as potential reservoirs of virulence genes (Li et al., 2002; Sechi et al., 2000). MLST of housekeeping and virulence genes is under way in order to better understand the population biology of vibrios including *V. cholerae*.

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2.5. Molecular identification of *V. campbellii* and *V. harveyi* isolates associated with disease in aquatic organisms

Bruno Gomez-Gil, Fabiano L. Thompson, and Jean Swings

Paper in preparation

Abstract

In this study we analysed forty-four *Vibrio* isolates previously identified phenotypically as *V. harveyi* and fifteen type and reference strains of *V. harveyi*, *V. campbellii*, *V. alginolyticus* and *V. parahaemolyticus*. Many of these *V. harveyi* isolates had been implicated in disease (e.g. luminous vibriosis) outbreaks in aquaculture settings. Numerical analyses of Fluorescent Amplified Fragment Polymorphism (FAFLP) and repetitive extragenic palindrome (rep-PCR) genomic fingerprinting band patterns revealed that (i) species of the *Vibrio* core group can be clearly differentiated using these tools and (ii) isolates phenotypically identified as *V. harveyi* belong in fact to the species *V. campbellii*. Further DNA-DNA experiments confirmed our findings with FAFLP and rep-PCR. We conclude that fingerprinting methodologies used are most valuable tools for the study of closely related *Vibrio* species.

Introduction

V. harveyi has been identified as one of the principal responsible for disease (i.e. vibriosis) in many cultured aquatic organisms e.g. penaeid shrimp (Lightner and Redman, 1998), several fish species (Austin and Austin, 1999), and mollusks (Rheinheimer 1993). Luminous vibrios have been implicated principally with disease outbreaks in shrimp larviculture facilities and in grow-out ponds (Leano et al., 1998).

Certain *V. harveyi* isolates have been proven to cause disease for shrimp larvae. *V. penaeicida* causes disease in juveniles and adults shrimps (Ishimura et al., 1995). For all other *Vibrio* species, no clear reproduction of the infectious process has been demonstrated, such as colonization, reproduction of the bacteria within the host, damage of tissues or cells. *V. campbellii* has also been found in disease outbreaks in

shrimp and proven pathogenic for shrimp larvae and juveniles. This species has been isolated from diseased turbot (*Colistium nudipinnis*) and brill (*C. guntheri*) larvae (Diggle et al. 2000). In many reports, no clear identification could be established and the isolates were called *V. campbellii*-like because they did not fully present the phenotypic characteristics of *V. campbellii* (Diggle et al., 2000). *V. campbellii* and *V. harveyi* are closely related species, having nearly 100 % 16S rDNA sequence similarity and up to 74 % DNA-DNA similarity (Baumann et al., 1984).

In the past, the identification of bacteria isolated from penaeid culture systems has been imprecise and represents hard work as it compromises performing many biochemical and/or physiological tests. Alternatively, molecular fingerprinting methodologies e.g. rep-PCR and AFLP and 16S rDNA sequencing became available in the last years. 16S rDNA sequences have been successfully used to identify presumptive isolates into the genus *Vibrio*, but these sequences are unable to resolve closely related species, such as the *Vibrio* core group (i.e. *V. alginolyticus*, *V. parahaemolyticus*, *V. harveyi*, *V. campbellii*, *V. natriegens*, and the newly described *V. rotiferianus* (Gomez-Gil et al. 2003). In contrast, rep-PCR and AFLP can discriminate strains beyond the species level (Rademaker et al., 2000). The aim of this study was to analyse isolates phenotypically identified as *V. harveyi* using FAFLP, rep-PCR and DNA-DNA hybridisations.

Material and Methods

The forty-four presumptive *V. harveyi* isolates and fifteen type and reference strains examined in this study are listed in Table 4 (Annex) and in Figure 2.9. *V. harveyi* strains were isolated from different aquacultural environments (mainly from the penaeid shrimp *Litopenaeus vannamei*, fish, molluscs, and seawater) in different countries in the 1990s. All strains are deposited in the LMG (University of Ghent, Belgium) and CAIM (CIAD, Mazatlan Unit, Mexico) collections. Phenotypic identification was performed according to the scheme of Alsina and Blanch (1994a) and Holt et al. (1994).

DNAs were extracted with Promega (Madison, USA) Wizard[®] Genomic DNA Purification kit (A1120) according to the manufacturer instructions. PCR reaction mix for rep-PCR contained 12.45 µl of water, 1.25 µl of dNTP mix (25 mM each), 2.5 µl DMSO, 5.0 µl of 5X Gitschier buffer, 0.4 µl BSA (10 mg ml⁻¹), 1.0 µl of each primer (0.3 µg µl⁻¹), 0.4 µl of Taq (5 U µl⁻¹, AmpliTaq; Applied Biosystems, Foster City,

California) and 1.0 μl of DNA (50 ng μl^{-1}) for final volume of 25 μl . Primers for rep-PCR were REP1R 5'- III ICG ICG ICA TCI GGC-3' and REP2 5'-ICG ICT TAT CIG GCC TAC-3'. Inosine (I) contains the purine base hypoxanthine capable to form Watson-Crick base pairs with A, G, C or T. BOX-PCR only employs the primer BOXA1R 5'- CTA CGG CAA GGC GAC GCT GAC G -3', and (GTG)₅ the primer 5'- GTG GTG GTG GTG GTG -3'. The amplification protocol for BOX was 95 °C for 2 min, followed by 35 cycles of 94 °C for 3 min, 92 °C for 30 s, 50 °C for 1 min and 65 °C for 8 min with a final extension of 65 °C for 8 min. The amplification protocol for REP and (GTG)₅ was 95 °C for 2 min, followed by 35 cycles of 94 °C for 3 min, 92 °C for 30 s, 40 °C for 1 min and 65 °C for 8 min with a final extension of 65 °C for 8 min.

PCR products were resolved on 1.5 % agarose gel in TAE buffer. 5 μl of a gel loading dye were mixed with the 25 μl of the PCR product and 10 μl of the mixture were loaded in the gel. In order to normalise the band patterns, 5 μl of the PCR molecular weight marker (Smartladder; Eurogenetec, Belgium) were added every five lanes. Gel electrophoreses was performed at 4-8 °C for 15 h at 55 V. The gel was stained in an ethidium bromide solution with TAE 1X buffer for 20 min, de-stained for 5 min and photographed with a digital system. The resulting images were processed with Bionumerics 2.5 software (Applied Maths, Belgium). Fluorescent amplified fragment length polymorphism (FAFLP) patterns were generated on an ABI Prism 377 DNA sequencer (Applied Biosystems, USA) and analysed as described previously (Thompson et al., 2001).

DNA-DNA hybridisation was performed at stringent conditions using the microplate technique with photobiotin-labeled DNA at a temperature of 40 °C for 3 hours as described previously (Willems et al., 2001). DNA similarity values are mean of reciprocal and non-reciprocal reactions, each of which are performed in quadruplicates.

Results and discussion

The fourty-four isolates examined here had the main phenotypic features of *V. harveyi* (Alsina and Blanch 1994a; Holt et al., 1994). These presumptive *V. harveyi* isolates grew on TCBS agar, were motile, fermented glucose, were oxidase-positive and sensitive to the vibriostatic agent 0/129 at 150 μg . They were arginine dihydrolase negative, and lysine and ornithine decarboxylase positive. Most, if not all, fourth-four

presumptive *V. harveyi* isolates were luminescent and utilised D-gluconate, L-glutamate, D-glucuronate, heptanoate, D-galactose, sucrose and grew at 40 °C. These isolates did not utilise L-histidine and L-arabinose.

The consensus analyses consisting of rep-PCR and FAFLP patterns of the 59 strains are shown in Figure 2.9. rep-PCR generated simpler band patterns (6 to 31 bands) than FAFLP (72 to 147 bands). Separated analyses of each fingerprint, suggests that FAFLP is the most discriminatory tool for the identification of closely related species such as those of the *Vibrio* core group, followed by GTG₅ (15 to 31 bands) REP (9 to 27 bands) and BOX (6 to 22 bands) (figures not shown).

Cluster one encompassed most of the presumptive *V. harveyi* isolates (n=31) and the type strain of *V. campbellii*. Whereas, cluster two (n=11) harboured the type and several reference strains of *V. harveyi*, including the coral pathogen LMG 20977 (former *V. shilonii*). Cluster three comprised the newly described *V. rotiferianus* (n=5), while clusters four (n=6) and five (n=4) harboured respectively the species *V. alginolyticus* and *V. parahaemolyticus*.

Because the isolates assigned to *V. campbellii* and to *V. harveyi* were very heterogeneous (Figure 2.9), we decided to perform DNA-DNA hybridisation with representative strains to check the robustness of the clusters based on the consensus genomic fingerprint. The results of our careful DNA-DNA hybridisation experiments are summarised in Table 2.4. Clearly, all representative isolates belong to the species *V. campbellii*, having at least 71 % DNA similarity.

Several isolates e.g. LMG 21363, R-16617, R-16619, R-16622, R-16614, R-16613, R-16616, R16630, R-16695 and LMG 21365 examined in this study had been involved in outbreaks of vibriosis in shrimp farms. Strains LMG 21363, R-16616 and R-16617 were originally identified as *V. harveyi* were reported as the aetiological agents of luminous vibriosis in Philippines (Leano et al., 1998). Our results clearly show that these strains belong to the species *V. campbellii*. This suggests that several outbreaks of luminous vibriosis may be caused in fact by *V. campbellii*.

Both *V. harveyi* and *V. campbellii* are very intriguing animal pathogens. Apparently they are able to cause disease in a wide range of aquatic organisms, including fish, molluscs, shrimps and corals. Although the present study did not reveal the mechanisms by which *V. harveyi* and *V. campbellii* cause disease, it showed the usefulness of FAFLP and rep-PCR for the molecular identification of these organisms.



R-16609	Nu-1, Nu-2, Na	<i>Litopenaeus stylosis nauplii</i>	Hatchery in Santa Cruz Gulf, Mexico
LMG 21362	Mi-a, R-16611	Seawater from <i>L. stylosis</i> broodstock tank	Hatchery in Santa Cruz Gulf, Mexico
R-16610	ML	<i>L. vannamei</i>	Mexico
R-16603	Z2	Seawater from <i>L. stylosis</i> broodstock tank	Hatchery in Santa Cruz Gulf, Mexico
LMG 21361	Z1 R-16602	Seawater from <i>L. stylosis</i> broodstock tank	Hatchery in Santa Cruz Gulf, Mexico
R-16604	Z3	Seawater from <i>L. stylosis</i> broodstock tank	Hatchery in Santa Cruz Gulf, Mexico
R-14899	VB629	Bkrabe	Venezuela
LMG 20369	8P	Hepatopancreas of <i>L. vannamei</i>	CENAIM, Ecuador
LMG 16835	VB404	Black Tiger prawn <i>P. monodon</i>	Thailand
LMG 16830	VB406	Black Tiger prawn <i>P. monodon</i>	Thailand
LMG 11216T	*	Seawater	Hawaii, USA
LMG 11256a	*	Seawater	Hawaii, USA
LMG 21363	PN801 R-16618	Diseased <i>P. monodon</i> juveniles	Philippines
2Me	2Me	<i>L. vannamei</i>	Mexico
R-16617	PL96-116	Diseased <i>P. monodon</i> postlarvae	Philippines
R-16606	1A-1	Near shore seawater	Santa Barbara Bay, Mexico
R-16619	HL34	Hemolymph from diseased juvenile <i>L. vannamei</i>	Guasave, Sinaloa, Mexico
R-16622	HL148	Hemolymph from diseased juvenile <i>L. vannamei</i>	Huatabampo, Sonora, Mexico
R-16631	SW-9702	Seawater	Bilo, Philippines
R-16614	STD3-1002	Diseased penaeid krabbe	Feng Cheng, China
R-16613	STD3-131	Diseased <i>L. vannamei</i> postlarvae	Ecuador
R-16616	IP18	<i>P. monodon</i> postlarvae with luminescent vibrios	Philippines
R-16630	HP130	Hepatopancreas from diseased juveniles	Guamuchil, Sinaloa, Mexico
R-16695	HL115	Hemolymph from diseased <i>L. vannamei</i> juvenile	Acacah, Ind. del Metatipe S. A. Nayarit
LMG 21365	HL150, R-16623	Hemolymph from diseased <i>L. vannamei</i> juvenile	La Brea, Mexico
R-16624	HL151	Hemolymph from diseased <i>L. vannamei</i> juvenile	La Brea, Mexico
R-16625	HL152	Hemolymph from diseased <i>L. vannamei</i> juvenile	La Brea, Mexico
R-16629	HL159	Hemolymph from diseased <i>L. vannamei</i> juvenile	La Brea, Mexico
R-16626	HL155	Hemolymph from diseased <i>L. vannamei</i> juvenile	La Brea, Mexico
R-16628	HL158	Hemolymph from diseased <i>L. vannamei</i> juvenile	La Brea, Mexico
R-16627	HL157	Hemolymph from diseased <i>L. vannamei</i> juvenile	La Brea, Mexico
LMG 21364	HL135, R-16620	Hemolymph from diseased <i>L. vannamei</i> juvenile	Sinaloa, Mexico
R-16621	HL136	Hemolymph from diseased <i>L. vannamei</i> juvenile	Sinaloa, Mexico
R-14951	VB22	Seabass <i>Dicentrarchus labrax</i>	Greece
R-14950	VB23	Sea bream <i>Sparus aurata</i>	Greece
R-14952	VB568	Turbot <i>Scophthalmus maximus</i>	Spain
LMG 20977	Former <i>V. shikoi</i> AK2	Diseased coral <i>Oculina patagonica</i>	Israel
LMG 7890	Former <i>V. carchariae</i>	Kiloby of brown shark <i>Cardathium plumbeus</i>	Baltimore, Maryland, USA
LMG 4044T	*	Dead amphipod <i>Talorchestia</i> sp.	Massachusetts, USA
LMG 11226	*	Seawater	Hawaii, USA
LMG 19643	Former <i>V. nashui</i>	Japanese homemaker <i>Triachurus japonicus</i>	Namazu, Japan
R-16608	11M	<i>L. stylosis</i> nauplii	Hatchery in Mazatlan, Sin., Mexico
R-16607	10M	<i>L. stylosis</i> nauplii	Hatchery in Mazatlan, Sin., Mexico
R-16605	1a	<i>L. stylosis</i> krabbe	Hatchery in Santa Cruz Gulf, Mexico
LMG 21460T	RFT 16, CAM 577T	Ronifer <i>Baccharium plankton</i> flow-through system	ARC, Gent, Belgium
LMG 21458	RFT 61, CAM 575	Ronifer <i>Baccharium plankton</i> flow-through system	ARC, Gent, Belgium
LMG 21459	RFT 13, CAM 576	Ronifer <i>Baccharium plankton</i> flow-through system	ARC, Gent, Belgium
LMG 21456	RFT 51, CAM 573	Ronifer <i>Baccharium plankton</i> flow-through system	ARC, Gent, Belgium
LMG 21457	RFT 41, CAM 574	Ronifer <i>Baccharium plankton</i> flow-through system	ARC, Gent, Belgium
R-14893	INC032	Healthy invasive brine <i>Cinnostoeus gigas</i>	Genivry sea farm, UK
LMG 4495T	ATCC 17749T	Spotted horse mackerel <i>Trachurus trachurus</i>	Japan
R-14896	VB 342	Crab	France
R-14891	STD3-1208	<i>L. vannamei</i> krabbe	CENAIM (Ecuador)
R-14892	INC 0 81	Healthy brine larvae <i>Nodipetern nodosus</i>	LCCM, Flomispolis, Brazil
LMG 2174	ATCC 14582	<i>Pinus palustris</i> submerged in seawater	Port Huemene, USA
R-14854	STD3-94X, HL54	Diseased <i>L. vannamei</i> juvenile	Sinaloa, Mexico
LMG 2850T	*	Disease of human	Japan
LMG 16842	VB 456	Shrimp	Thailand
LMG 16839	VB 459		

V. campbellii

V. harveyi

Ronifer

Alga

Para.

Figure 2.9. Consensus dendrogram calculated based on the concatenated band patterns of rep-PCR and FALP using Dice and Ward. A band position tolerance of 0.3 % was allowed to compensate technical errors. *, indicates a type and/or reference strain. The numbers of the strains examined by DNA-DNA hybridisation are underlined. *Rotife.*, *V. rotiferianus*. *Algin.*, *V. alginolyticus*. *Para.*, *V. parahaemolyticus*. *V. alginolyticus* and *V. parahaemolyticus* were included as out-groups. Strains LMG 11659, LMG 20370 and R-14928 were omitted from the dendrogram.

Table 2.4. DNA-DNA similarity among *Vibrio* strains

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	FAFLP* group
<i>V. campbellii</i>																
1. LMG 11216 ^T	100															A14
2. LMG 21363	79	100														-
3. LMG 21365	71	96	100													-
4. R-14899	78	94	92	100												A37
5. LMG 21362	74	95	90	90	100											-
6. LMG 21361	82	99	90	93	93	100										-
7. LMG 20369	76	88	79	89	81	90	100									A37
8. LMG 16835	78	87	80	80	81	91	86	100								A37
9. LMG 21364	82	90	86	86	83	88	90	83	100							-
<i>V. harveyi</i>																
10. LMG 4044 ^T	62	64	61	59	57	65	59	65	65	100						A36
11. LMG 11659	68									70	100					A30
12. LMG 20370	66									71	85	100				A31
13. R-14928	64									65	74	77	100			A32
<i>V. alginolyticus</i>																
14. LMG 4409 ^T	44	40	39	36	40	41	38	42	41	46				100		A62
<i>V. natriegens</i>																
15. LMG 10935 ^T	35	34	32	32	31	34	30	34	34	35				36	100	A48

*FAFLP groups delineated by Thompson et al. (2001).

CHAPTER 3. Own work: new taxa and reclassifications within the
Vibrionaceae

3.1. *Enterovibrio norvegicus* gen. nov., sp. nov., isolated from the gut
of turbot (*Scophthalmus maximus*) larvae: A new member
of the family *Vibrionaceae*

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Int. J. Syst. Evol. Microbiol. (2002), 52, 2015-2022

Abstract

Twenty-two isolates which originated from the gut of healthy cultured turbot larvae in Norway were investigated using a polyphasic approach. AFLP fingerprinting analysis showed that the isolates have typical patterns and form two main groups. Phylogenetic analysis revealed that the isolates belong to the γ -subclass of the *Proteobacteria*, with *Vibrio hollisae* as their closest neighbour. DNA-DNA hybridisation, chemotaxonomic and phenotypical analyses further proved that these isolates represent a tight new taxon different from the currently species in the family *Vibrionaceae*. We propose to accommodate these novel isolates in the new genus *Enterovibrio*, with *E. norvegicus* as the type species. Isolates are motile by polar flagellum, oxidase, catalase, arginine dihydrolase, β -galactosidase positive and Voges-Proskauer negative. They produce indole, do not reduce nitrate, and are resistant to vibriostatic agent 0/129. The mol % G+C of DNA of *E. norvegicus* is 47.1 to 47.9 %. The type strain is *E. norvegicus* LMG 19839^T (EMBL accession number AJ316208).

Introduction

Turbot *Scophthalmus maximus* is a commercially important species which has been under intensive fishing and rearing in different continents. Recently, there have been several reports showing that the intestinal microflora of seawater fish is dominated by members of the family *Vibrionaceae* and that the gut microflora plays

an important role in the health of early life stages of fish (Hansen and Olafsen, 1999; Ringo and Birkbeck, 1999). Mortalities in early larval stages of intensively cultured marine fish are often very high and related to bacterial infections in which opportunistic bacteria play an important role (Ishimura et al., 1996; Diggles et al., 2000). On the other hand, it has also been proved that certain *Vibrio* strains isolated from the gut of *S. maximus* larvae, when added in the culture water, improved larval survival and growth (Huys et al., 2001). The culturable microflora from the gut of fish larvae has been the subject of many reports, but mostly remained identified at the family and/or the genus level (Blanch et al., 1997; Ringo and Gatesoupe, 1998). It is likely that phenotype-based fingerprinting techniques applied in many of the previous studies have hampered the correct taxonomic identification of several taxa (Cerdà-Cuéllar et al., 1997; Hansen and Olafsen, 1999; Onarheim et al., 1994; Ringo and Birbeck, 1999). The usefulness of genomic fingerprinting techniques such as Amplified Fragment Length Polymorphism (AFLP), Pulsed-Field Gel Electrophoresis (PFGE) and Repetitive Elements Palindrome (rep-PCR) for studies on bacterial evolution, phylogeny and taxonomy have been recognised recently (Rademaker et al., 2000; Van Belkum et al., 2001). Several researchers have also suggested that AFLP could be an alternative to whole genome sequencing and DNA-DNA hybridisation experiments (Coenye et al., 1999a; Huys et al., 1996; Janssen, 2001).

In the present study we describe phenotypic and genomic features of 22 isolates originated from the gut of turbot larvae. We further show that phenotypically the isolates resemble the genus *Vibrio*, but at the genomic level they clearly represent a novel genus for which we propose the name *Enterovibrio* with *E. norvegicus* as the type species.

Material and Methods

We analysed 22 isolates [LMG 19839^T (=CAIM430), LMG 19840 (=CAIM427), LMG 19841 (=CAIM436), LMG 19842 (=CAIM451), LMG 20957 (=CAIM428), R-3719, R-3668, R-3678, R-3708, R-3717, R-3727, R-3729, R-3731, R-3749, R-3759, R-3764, R-3929, R-3773, R-3792, R-3814, R-3819, R-3847] which were isolated from the gut of healthy turbot *Scophthalmus maximus* larvae at the Aquaculture Research Station of Austevoll (Norway) during the summer of 1997 as described previously (Huys et al., 2001) (Table 1, Annex). All strains included in this study are deposited in the BCCMTM/LMG Bacteria Collection at Ghent University

and in the CAIM collection of the Centre of Research on Nutrition and Development (CIAD) in Mazatlan, Mexico. Strains were grown on Marine agar 2216E (MA; Difco) at 28 °C for 24 hours unless otherwise stated. Colony morphology was examined on cultures grown on Thiosulphate-citrate-bile-salts-sucrose agar (TCBS; Difco) and Tryptone soya agar (Oxoid) supplemented with 2 % (w/v) NaCl using a stereoscopic microscope. Cell morphology was examined on wet mounts using a phase-contrast microscope.

Bacterial DNA was extracted following the technique of Pitcher et al. (1989). Fluorescent Amplified Fragment Length Polymorphism (AFLP) patterns and 16S rDNA sequences were generated on an ABI Prism 377 DNA sequencer (Applied Biosystems) and analysed as described previously (Thompson et al., 2001). The consensus sequences were transferred into BioNumerics 2.0 software (Applied Maths), and phylogenetic trees were constructed based on the neighbour joining (Saitou and Nei, 1986) and on the maximum parsimony methods. The 16S rDNA sequences of the type strains of *Vibrio ichthyenteri* and *V. penaeicida* were accomplished in the course of the present study. The FAFLP patterns of all *Enterovibrio norvegicus* strains and the 16S rDNA sequences AJ316207 and AJ316208 were taken from section 2.2 (Chapter 2). The 16S rDNA sequence of the other type strains included in this study were retrieved from EMBL database. DNA-DNA hybridisation was performed at stringent conditions using the microplate technique with photobiotin-labeled DNA at a temperature of 38 °C for 3 hours as described previously (Willems et al., 2001). The mol % G+C of DNA was determined by HPLC (Tamaoka and Komagata, 1984).

Phenotypic characterisation of the isolates was performed using API 20E, API ZYM (bioMérieux) and Biolog GN metabolic fingerprinting (Biolog) following the instructions of the manufacturer, with slight modifications (Thompson et al., 2001). Classical phenotypic tests were performed as described previously (Baumann et al., 1984; Delafield et al., 1965; Farmer III and Whickman-Brenner, 1992; Thompson et al., 2001; Vandamme et al., 1998). Antibiograms were carried out using the disc diffusion methodology (Acar and Goldstein, 1996) using commercial discs (Oxoid). The inhibition zone of each antibiotic was measured on strains grown on Brain Heart Infusion Broth (BHI; Difco) supplemented with 1.5 % (w/v) bacteriological agar no.1 (Oxoid) and with 1.5 % (w/v) NaCl for 24 hours at 28 °C. Fatty acid methyl esters (FAME) analysis was carried out as described by Huys et al. (2001). Isolates were

grown on Trypticase Soy Broth (Becton Dickinson) supplemented with 1.5 % (w/v) Bacto agar (Becton Dickinson) and 1.5 % (w/v) NaCl and on MA at 28 °C for 24 hours. Approximately 50 mg of cells were harvested and the fatty acid were isolated, and analysed using the Microbial Identification System software package, version 3.9 (Microbial ID).

Results and Discussion

The fingerprinting analysis of the whole genome of *Enterovibrio* isolates clearly showed that they possess typical AFLP patterns consisting of 78 ± 9 bands (Figure 3.1). An intraspecific genomic diversity exists among the 22 *Enterovibrio* isolates,

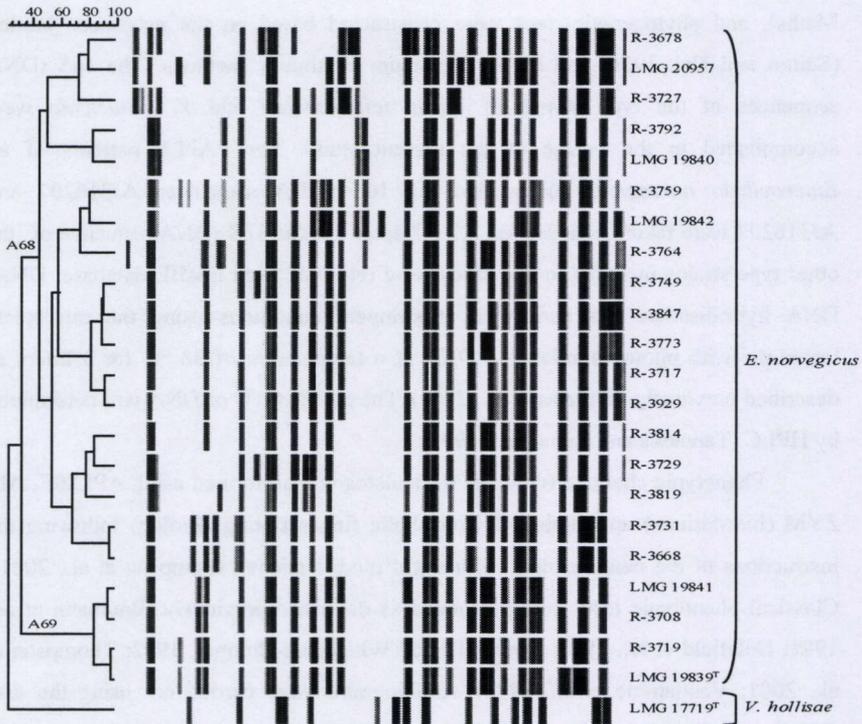


Figure 3.1. Dendrogram of the AFLP patterns of 22 *Enterovibrio norvegicus* isolates. *Vibrio hollisae* LMG 17719^T, the closest phylogenetic neighbour of *E. norvegicus*, was included as an out-group. A band based (Dice) cluster analysis (Ward) was used.

and at least two main groups of genomes exist, corresponding to the AFLP clusters A68 and A69 found in a previous study (Thompson et al., 2001). Some isolates (R-3749, R-3847 and R-3773; R-3717 and R-3929; R-3731 and R-3668; LMG 19841 and R-3708; R-3719 and LMG 19839^T) clustered at the reproducibility level (\geq i.e. 88 % pattern similarity), and were thus indistinguishable by AFLP. Visual examination and numerical analysis of the AFLP patterns of *Enterovibrio* isolates revealed that they form a cluster completely apart from the currently known species in the family *Vibrionaceae* (Thompson et al., 2001), supporting our conclusion that *Enterovibrio* possess a unique genome.

Figure 3.2 shows a neighbour joining tree with the estimated positions of most representatives of *Vibrionaceae* and the bootstrap values after 500 simulations, based on the almost complete 16S rDNA sequences. Six main branches could be distinguished within this family by both neighbour joining and maximum parsimony methods. The first branch harboured *Enterovibrio* isolates. *Vibrio hollisae* formed a second branch sharing only 94 % 16S rDNA similarity with *Enterovibrio*, its closest neighbour. The 16S rDNA sequences of these two taxa clearly indicate that they represent two new genera within the family *Vibrionaceae*. It has been previously concluded that 95-96 % 16S rDNA similarity would be the level for circumscribing different genera within the family *Vibrionaceae* (Kita-Tsukamoto et al., 1993). It has been suggested that *V. hollisae* should be elevated to the genus rank because of its great divergence with other *Vibrio* species (Dorsch et al., 1992; Kita-Tsukamoto, et al., 1993). The third branch harboured the *Photobacterium* species which had 16S rDNA sequence similarity values ranging from 93.9 to 97.8 %. The fourth branch consisted of psychrophilic species *V. fischeri*, *V. wodanis*, *V. salmonicida* and *V. logei* which had similarities ranging between 95.2 and 98.3 %. The fifth branch harboured all other *Vibrio* species (except *V. hollisae*), *Listonella pelagia* and *L. anguillarum* which had similarities of 92.9 to 99.2 %. *Enterovibrio* was distantly related to *V. cholerae*, the type species of the family *Vibrionaceae*, having both only 91.6 % 16S rDNA similarity. Some members of the fourth branch have been referred to as the *Vibrio* core group (Dorsch et al., 1992). It is noticeable that this branch is quite diverse. The genus *Listonella*, which was proposed based on the 5S rDNA (Macdonell and Colwell, 1985), cannot be distinguished from the other vibrios. The 16S rDNA sequences of *V. ichthyenteri* LMG 19664^T and *V. penaeicida* LMG

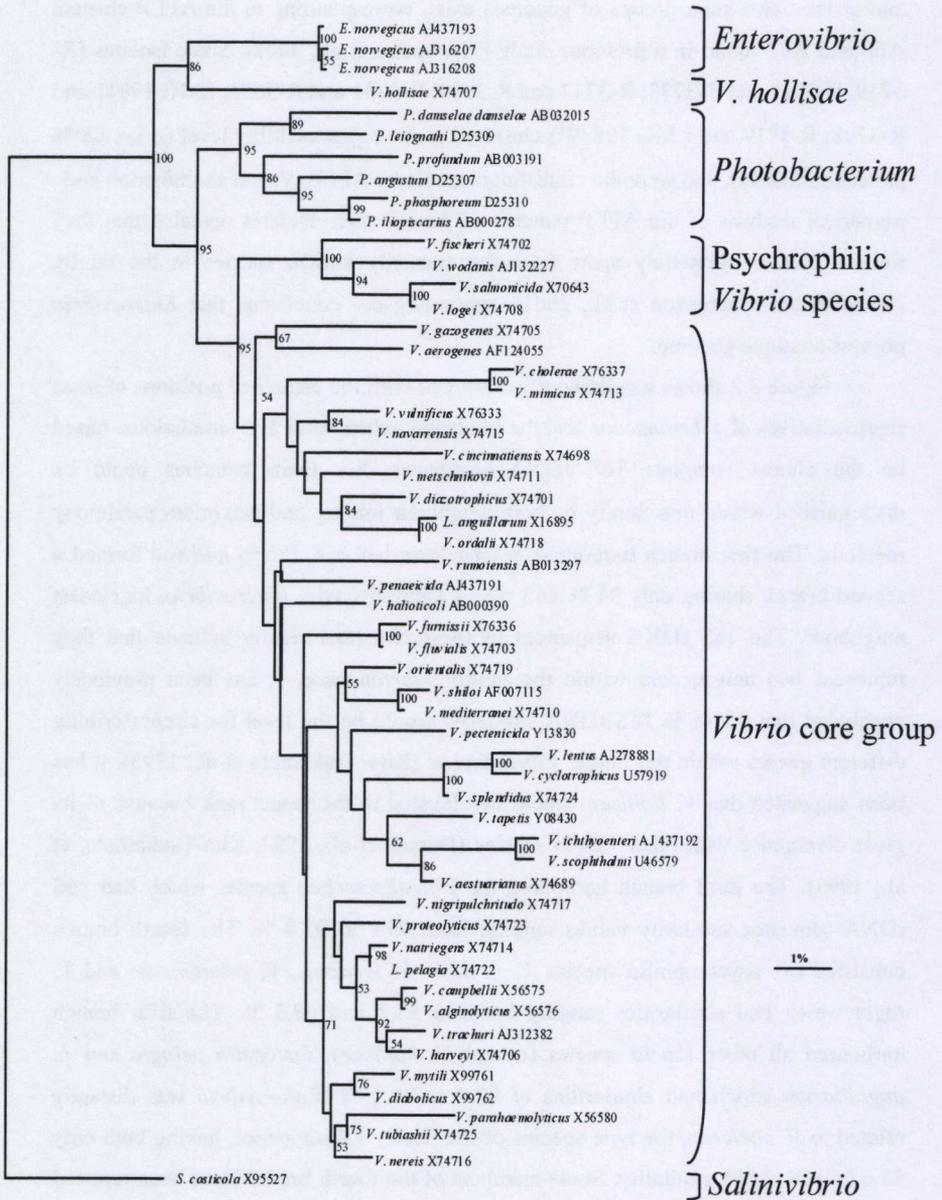


Figure 3.2. Phylogenetic tree with the estimated positions of most representatives of the family *Vibrionaceae* using the neighbour joining method based on almost complete 16S rDNA sequences. Bootstrap values after 500 simulations are shown. Bar, 1% estimated sequence divergence.

19663^T determined in this study were found to belong to the fifth branch and were closely related with *V. scopthalmi* (99.2 %) and *V. nigripulchritudo* (97.2 %), respectively. *Salinivibrio costicola* formed the sixth branch having 92.6 % similarity toward *Enterovibrio* isolates. The 16S rDNA similarity of *Enterovibrio* isolates towards representative species of related genera i.e. *Shewanella benthica* (X82131), *Moritella marina* (X74711), and *Pseudoalteromonas haloplanktis* (X67024) was 90.7 %, 89.5 %, and 89.1 %, respectively. Based on the 16S rDNA similarity values among *Enterovibrio* isolates and other members of the family *Vibrionaceae*, it can be concluded that *Enterovibrio* isolates represent a new taxon in this family.

The DNA-DNA hybridisation experiments revealed that *Enterovibrio* isolates form a single tightly related group which exhibits at least 87 % DNA similarity (Table 3.1). *Enterovibrio* showed only 22 % DNA similarity towards its closest phylogenetic neighbour, *V. hollisae*. These results confirmed our findings with AFLP and 16S sequence analyses and proved that *E. norvegicus* should be regarded as a novel taxon. *Enterovibrio* isolates possessed a mol % G+C of DNA content ranging from 47.1 to 47.9 %.

Table 3.1. DNA similarity among *E. norvegicus* isolates and *V. hollisae* LMG 17719^T and their mol % G+C.

	DNA similarity values with:						Mol % G+C
	1	2	3	4	5	6	
<i>E. norvegicus</i>							47.9
1. LMG 19840	100						
2. LMG 20957	94	100					47.1
3. LMG 19839 ^T	87	103	100				47.5
4. LMG 19841	92	104	99	100			47.4
5. LMG 19842	91	99	91	92	100		47.6
<i>V. hollisae</i>							
6. LMG 17719 ^T	20	21	22	22	21	100	48.5

Enterovibrio isolates shared the main phenotypic features of the genus *Vibrio*. Although, some useful characteristics to differentiate *Enterovibrio* from other genera of the family *Vibrionaceae* were found and are listed in Table 3.2. *Enterovibrio norvegicus* is easily differentiated from *Photobacterium* and *Salinivibrio* species since it produces indole but not acetoin. It is also distinguished from *Salinivibrio* strains by its β -galactosidase activity and absence of gelatinase activity. Most vibrios reduce nitrate (except *V. cyclotrophicus*, *V. gazogenes*, *V. metschnikovii*, *V. salmonicida*) and

utilise pyruvate (except *V. haliotocoli*, *V. tapetis*), whereas *Enterovibrio* isolates do not.

Table 3.2. Useful features for differentiating of genera in the family *Vibrionaceae*.

	<i>Enterovibrio</i>	<i>Photobacterium</i>	<i>Salinivibrio</i>	<i>Vibrio</i>
ONPG	+	+*	-	+*
Gelatinase activity	-	-**	+	+*
Acetoin production	-	+*	+	-**
Indole production	+	-	-	+*
Arginine dihydrolase	+	+	+	-*
Nitrate reduction	-	+*	-	+**
Susceptibility to 0/129 (150 µg)	-	+	+	+**
Utilization of:				
Citrate	-	-	-	+*
Pyruvate	-	+**	+	+**
Propionate	-	-	+	+*
L-Proline	-	-**	+	+**
D-Alanine	-	-	+	+**
Aconitate	-	-	-	+**
Mol% G+C of DNA	47.1-47.9	40-44	49-50.5	38.8- 50.6 [†]

*over 65 % of the species show this feature (exceptions are listed below for each feature). ** over 85 % of the species show this feature. [†]over 65 % of the species have not a G+C content within the range 47-48. Data were obtained from Alsina and Blanch (1994a), Benediktsdóttir et al. (2000), Borrego et al. (1996), Hedlund and Staley (2001), Ishimaru et al. (1995), Ishimaru et al. (1996), Lambert et al. (1998), Macián et al. (2001), Onarheim et al. (1994), Pujalte et al. (1993), Ragueneis et al. (1997), Sawabe et al. (1998), Urdaci et al. (1991) and Yumoto et al. (1999).

Less common reactions :

Beta-galactosidase activity (ONPG) negative: *P. damsela*, *P. iliopiscarius*, *V. alginolyticus*, *V. campbellii*, *V. scopthalmi*, *V. diabolicus*, *V. fischeri*, *V. harveyi*, *V. hollisiae*, *V. ichthyoenteri*, *V. metschnikovii*, *V. nereis*, *V. ordalii*, *V. parahaemolyticus*, *V. proteolyticus*, *V. furnissii*, *V. salmonicida*, and *V. wodanis*.

Gelatinase positive: *P. angustum*.

Gelatinase negative: *V. agarivorans*, *V. cincinnatiensis*, *V. diazotrophicus*, *V. haliotocoli*, *V. hollisiae*, *V. ichthyoenteri*, *V. mediterranei*, *V. mytili*, *V. natriegens*, *V. pelagius*, *V. rumoiensis*, *V. salmonicida*, and *V. scopthalmi*.

Acetoin production negative: *P. angustum*.

Acetoin production positive: *V. alginolyticus*, *V. anguillarum*, *V. cholerae*, *V. diabolicus*, *V. gazogenes*, and *V. metschnikovii*.

Indole production positive: *V. aerogenes*, *V. agarivorans*, *V. cincinnatiensis*, *V. cyclotrophicus*, *V. gazogenes*, *V. ichthyoenteri*, *V. metschnikovii*, *V. mytili*, *V. natriegens*, *V. pectenocida*, *V. rumoiensis*, and *V. scopthalmi*.

Arginine dihydrolase positive or variable (v): *V. aestuariamus*, *V. anguillarum*, *V. cyclotrophicus*, *V. fluvialis*, *V. furnissii*, *V. nereis*, *V. proteolyticus*, *V. scopthalmi*, *V.*

diazotrophicus (v), *V. splendidus* (v), *V. tubiashii* (v), *V. mediterranei* (v), *V. metschnikovii* (v), *V. mimicus* (v), *V. orientalis* (v), *V. splendidus* (v) and *V. tubiashii* (v).

Nitrate reduction negative (v): *V. cyclotrophicus*, *V. gazogenes*, *V. metschnikovii*, *V. salmonicida* and *P. angustum* (v).

Susceptibility to O/129 Vibriostatic agent (150µg): *V. aerogenes*, and *V. lentus* are not susceptible.

Utilization of:

Citrate negative or variable (v): *V. agarivorans*, *V. campbellii* (v), *V. cholerae* (v), *V. fischeri* (v), *V. harveyi* (v), *V. haliotocoli*, *V. hollisae*, *V. ichthyenteri*, *V. logei*, *V. mediterranei*, *V. metschnikovii* (v), *V. salmonicida*, *V. splendidus* (v), *V. scophthalmi*, *V. pectenica*, and *V. tapetis*.

Pyruvate negative: *P. phosphoreum*, *V. haliotocoli*, and *V. tapetis*.

Propionate negative: *P. damsela*, *V. cyclotrophicus*, *V. fischeri*, *V. haliotocoli*, *V. lentus*, *V. logei*, *V. metschnikovii*, *V. ordalii*, *V. orientalis*, *V. proteolyticus*, and *V. tubiashii*.

L-Proline negative: *P. damsela*, *V. fischeri*, *V. logei*, *V. navarrensis*, *V. pectenica*, *V. tapetis* are negative.

D-alanine negative: *V. agarivorans*.

Aconitate negative: *V. haliotocoli* and *V. tapetis*.

Mol % G+C of DNA: *V. alginolyticus* (45-47 %), *V. campbellii* (46-48 %), *V. cholerae* (47-49 %), *V. diazotrophicus* (45.9-47.5 %), *V. gazogenes* (47 %), *V. harveyi* (46-48 %), *V. navarrensis* (45-47 %), *V. parahaemolyticus* (46-48 %), *V. penaeicida* (46.2-47 %), *V. natrigens* (46-47 %), *V. nereis* (46-47 %), *V. nigripulchritudo* (46-47 %), *V. pelagius* (45-47 %), and *V. vulnificus* (46-48 %).

Table 3.3. Useful features for differentiating arginine dihydrolase and indole positive species of the family *Vibrionaceae*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
FAME*:															
14:0	2.7±0.8	5.5±0.7	5.6±0.7	4±1	3.6±0.3	4±0.3	8.3±1.8	5.1±0.5	4.1	5.4± 0.5	10±5.6	3.5±0.3	9.4	4.9±1	5.7±1.5
16:0	20.9±1	23.1±1	27.2±3	21.7±3	14.3±2	14.8±2	18.1±3	19.2±1	21	14.3±3	21.2±6	14.6±1	27.7	17.9±1	18.5±3
16:1 ω9c	3.5±0.3	ND	1.2±0.4	ND	ND	ND	ND	ND	1.2	0.4	ND	0.4±0	ND	0.6	ND
18:1 ω9c	2.7±0.2	ND	ND	0.1	0.2	0.3	ND	0.3±0.1	0.7	0.2	ND	0.2	5.3	0.4	ND
18:1 ω7c	15±2	16.1±1	12.3±0	17.5±1	17.8±1	22.2±0	20.8±2	22.8±1	16.2	23.1±1	22.4±11	15.5±1	12	23.7±3	16.4±6
ONPG	+	v*	+	+	v	+	v	v	+	-	.*	-	v	+	+
Gelatinase activity	-	+	+	-	+	+	+	+	+	v	+	v	+	+	-
Acetoin Production	-	-	+	-	-	-	v	v	v	-	-	v	-	-	+
NO ₃ ⁻ reduction	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+
Susceptibility to O/129 (150 µg)	-	+	+	+	+	+	-	+	-	+	ND	v	-	+	+
Utilization of D-mannitol	v [†]	+	+	+	+	+	v	v	v	v	+	v	+	+	-
Acid from sucrose	-	+	+	+	+	+	+	+	-	+	+	-	v	+	v
Growth on/at:															
8 % NaCl	-	v	-	+	v	+	v	v	-	+	+	+	v	v	v
35 °C	-	+	+	+	+	+	v	+	+	+	+	+	v	v	

Taxa are indicated as follow: 1, *E. norvegicus* (n=22); 2, *V. aestuarianus* (n=3); 3, *V. anguillarum* (n=3); 4, *V. diazotrophicus* (n=3); 5, *V. fluvialis* (n=2); 6, *V. furnissii* (n=2); 7, *V. mediterranei* (n=5); 8, *V. metschnikovii* (n=3); 9, *V. mimicus* (n=1); 10, *V. nereis* (n=3); 11, *V. orientalis* (n=2); 12, *V. proteolyticus* (n=2); 13, *V. splendidus* (n=1); 14, *V. tubiashii* (n=3); 15, *P. damsela* subsp. *damsela* (n=2). *Our own data. †=95 % of the *Enterovibrio* isolates do not utilise D-mannitol. ND=not detected. v=variable. Phenotypic data were obtained from Alsina and Blanch (1994). *Vibrio diazotrophicus*, *V. mediterranei*, *V. metschnikovii*, *V. mimicus*, *V. orientalis*, *V. splendidus* and *V. tubiashii* show variable arginine dihydrolase reaction.

Moreover, *Enterovibrio* is not susceptible to the vibriostatic agent 0/129 as most vibrios (except *V. lentus* and *V. aerogenes*). The mol % G+C of the DNA of *Enterovibrio* partially overlap the mol %G+C span of vibrios. *Enterovibrio norvegicus* possess typical fatty acid patterns and phenotypical features which differentiate it from other arginine dihydrolase and indole positive species of the family *Vibrionaceae* (Table 3.3).

Genomic and phenotypic features of *Enterovibrio* isolates presented in this study clearly prove that they represent a new taxon within the family *Vibrionaceae*. Therefore, we propose to include these isolates into the new genus named *Enterovibrio* with *E. norvegicus* as the type species.

Description of *Enterovibrio* gen. nov.

Enterovibrio (En.te.ro.vib'ri.o Gr. n. *enteron* intestine; L. n. *vibrio* that which vibrates; N.L. n. *Enterovibrio* enteric vibrio). Gram negative, motile, oxidase and catalase positive. *Enterovibrio* strains have a mol % G+C of DNA content ranging from 47.1 to 47.9 %. The most abundant fatty acids are 16:1 ω 7c and/or 15 iso 2-OH, 16:0 and 18:1 ω 7c. Chemoheterotrophic, mesophilic and moderately halophilic. *Enterovibrio* strains utilise dextrin, N-acetyl-D-glucosamine and α -D-glucose as sole carbon source. Arginine dihydrolase, indole and β -galactosidase are positive. Voges-Prokauer, Lysine and Ornithine decarboxylase are negative. Nitrate is not reduced. Resistant to vibriostatic agent 0/129 (10 μ g and 150 μ g). Member of the γ -subclass of the *Proteobacteria*. The type species is *Enterovibrio norvegicus*.

Description of *Enterovibrio norvegicus* sp. nov.

Enterovibrio norvegicus (nor.ve'gi.cus M.L. adj. *norvegicus* of Norway, where the organism was isolated). Description is as for the genus with the following additional features. Cells are 0.8 μ m in width and 1.0-1.2 μ m in length, motile by means of a polar flagellum when grown in liquid medium. They form smooth-rounded colonies with raised margins, beige in colour and about 1 mm in diameter after two days of incubation on MA at 27-28 °C. These facultative anaerobic isolates also grew well on TSA and BHI agar supplemented with 1.5 % NaCl. The isolates grew slowly on TCBS, forming green colonies after three days at 28 °C. No growth occurs on 0 and \geq 8.0 % NaCl. No growth occurs at 4 and \geq 35 °C. Prolific growth

occurs in media containing 2 % NaCl at 20-28 °C. *Enterovibrio norvegicus* has both an oxidative and a fermentative metabolism. Aerobic utilisation of different carbon sources is summarised in Table 3.4. All strains ferment α -D-glucose. None of the strains ferment arabinose, amygdalin, melibiose, sucrose, L-rhamnose, D-sorbitol, M-inositol and D-mannitol. *Enterovibrio norvegicus* produces β -galactosidase, phosphatase alkaline, esterase, esterase lipase, lipase, leucine arylamidase, phosphatase acid, naphthol-AS-BI-phosphohydrolase, but not tryptophane deaminase, urease, gelatinase, DNase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, α -galactosidase, β -glucuronidase, H₂S, and acetoin. *Enterovibrio* isolates are not luminescent, are methyl red-negative, and do not degrade PHB. *Enterovibrio* isolates are resistant to the vibriostatic agent O/129 at 10 and 150 μ g, streptomycin (10 μ g), trimethoprim (1.2 μ g) and fusidic acid (10 μ g). *E. norvegicus* isolates show intermediate susceptibility to penicillin G (10 U), novobiocin, (5 μ g), chloramphenicol (30 μ g), polymixin B (300 U), ampicillin (10 μ g), oxytetracycline (30 μ g) and nalidixic acid (30 μ g). The major fatty acids of *Enterovibrio* isolates grown on TSA are summed feature 3 (35.8 % \pm 0.5; comprising 16:1 ω 7c and/or 15 iso 2-OH), 16:0 (20.9 % \pm 1.5), 18:1 ω 7c (15.0 % \pm 2.0), 12:0 (4.2 % \pm 0.6), 16:1 ω 9c (3.5 % \pm 0.3), 16:0 iso (2.9 % \pm 1.8), 14:0 (2.7 % \pm 0.8), 18:1 ω 9c (2.7 % \pm 0.2), summed feature 2 (2.5 % \pm 0.4; comprising 14:0 3-OH and/or 16:1 iso I and/or unidentified fatty acid with equivalent chain length value of 10.928 and/or 12:0 ALDE), 12:0 3-OH (1.9 % \pm 0.4), 18:0 (1.8 % \pm 0.5), unidentified fatty acid with equivalent chain length value of 12.484 (1.1 % \pm 0.2), summed feature 7 (0.9 % \pm 0.3; 19:1 ω 6c and/or unidentified fatty acid with equivalent chain length value of 18.846), and 14:0 iso (0.6 % \pm 0.5). The major fatty acids of *Enterovibrio* isolates grown on MA are summed feature 3 (36.2 % \pm 0.9; comprising 16:1 ω 7c and/or 15 iso 2-OH), 16:0 (19.3 % \pm 0.7), 18:1 ω 7c (14.8 % \pm 1.1), 12:0 (3.4 % \pm 0.2), 18:1 ω 9c (3.1 % \pm 0.2), 16:1 ω 9c (3.1 % \pm 0.2), 12:0 3-OH (2.9 % \pm 0.2), 16:0 iso (2.6 % \pm 0.5), summed feature 2 (2.4 % \pm 0.2; comprising 14:0 3-OH and/or 16:1 iso I and/or unidentified fatty acid with equivalent chain length value of 10.928 and/or 12:0 ALDE), 18:0 (2.4 % \pm 0.2), 17:1 ω 8c (2.1 % \pm 0.2), 17:0 (1.4 % \pm 0.2), 14:0 (1.3 % \pm 0.2) and 18:0 iso (1.0 % \pm 0.1). Isolated from the gut of the turbot *Scophthalmus maximus* larvae. The 16S rDNA sequences of strains LMG 19840 LMG 19842 are deposited in the EMBL under the accession numbers AJ316207 and AJ 437193,

respectively. The type strain of this species is LMG 19839^T (EMBL accession number AJ316208). The mol % G+C of the type strain is 47.7.

Table 3.4. Variable phenotypical features of *Enterovibrio norvegicus* isolates.

	<i>E. norvegicus</i> (n=22)	LMG 19839 ^T
Substrates:		
D-Mannose	16*	+
Maltose, Acetic acid	15	+
Inosine	14	-
D-Trehalose	12	+
D-Fructose	12	+
Uridine	9	-
L-Alanine	8	-
L-Glutamic acid	6	-
β -Hydroxy butyric acid	5	-
D,L-Lactic acid	4	-
Psicose	4	+
Malonic acid, succinic acid, gentiobiose	2	-
Glycogen, D-mannitol, D-sorbitol, D-galacturonic acid, L-alanyl-glycine, L-asparagine, thymidine	1	-

*Numbers indicate the number of positive isolates.

All isolates utilised dextrin, N-acetyl-D-glucosamine and α -D-glucose as sole carbon source. None of the isolates utilised α -cyclodextrin, N-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, cellobiose, i-erythritol, L-fructose, D-galactose, α -D-lactose lactulose, D-melibiose, β -methyl-D-glucoside, D-raffinose, L-rhamnose, methyl pyruvate, mono-methyl succinate, cis-aconitic acid, citric acid, formic acid, D-galactonic acid, D-galacturonic acid, γ -hydroxybutyric acid, p-hydroxy phenylacetic acid, itaconic acid, α -ketobutyric acid, α -keto valeric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, bromosuccinic acid, succinamic acid, glucuronamide, alaninamide, D-alanine, glycyl-L-glutamic acid, L-histidine, hydroxy L-proline, L-leucine, L-ornithine, L-phenyl alanine, L-pyro glutamic acid, D-serine, γ -amino butyric acid, urocanic acid, phenyl ethylamine, putrescine, 2-amino ethanol, 2,3-butanediol, glycerol and glycerol phosphate.

3.2. *Vibrio neptunius* sp. nov., *V. brasiliensis* sp. nov. and *V. xuii* sp. nov., isolated from the marine aquaculture environment (bivalves, fish, rotifers and shrimps)

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Int. J. Syst. Evol. Microbiol. (2003), 53, 245-252

Abstract

The FAFLP groups A5 (21 isolates), A8 (6 isolates) and A23 (3 isolates) distinguished in a former paper (Thompson et al., 2001) were examined in more depth. These three groups were phylogenetically related to *V. tubiashii*, but DNA-DNA hybridisation experiments proved that the three AFLP groups are in fact new species. Chemotaxonomic and phenotypical analyses further revealed several differences among the thirty isolates and known *Vibrio* species. We propose to accommodate these isolates into three novel species, namely *V. neptunius* (type strain is LMG 20536^T; EMBL accession no. is AJ316171; G+C content of the type strain is 46.0 mol %), *V. brasiliensis* (type strain is LMG 20546^T; EMBL accession no. is AJ316172; G+C content of the type strain is 45.9 mol %) and *V. xuii* (type strain is LMG 21346^T; EMBL accession no. is AJ316181; G+C content of the type strain is 46.6 mol %). These species can be differentiated on the basis of phenotypical features, including fatty acid composition (particularly 14:0 iso, 14:0 iso 3-OH, 16:0 iso, 16:0, 17:0 and 17:1 ω8c), enzyme activity, and utilisation and fermentation of various carbon sources.

Introduction

It is well recognised that bacteria play a pivotal role in the cycling of dissolved and particulate organic matter in aquatic ecosystems (Sherr and Sherr, 2000). There has been increasingly evidence that bacteria also fuel food webs in marine aquaculture systems and influence the health of cultured marine organisms (Hansen and Olafsen,

1999; Thompson et al., 2002a). Vibrios are highly abundant in aquatic ecosystems particularly in eutrophic environments, accounting for up to 14-45 % (i.e. 10^4 - 10^5 cell/ml) of the culturable microbiota (Eilers et al., 2000a, 2002b; Suantika et al., 2001). Moreover, vibrios are present in high numbers in a successful recirculating system for rotifers (Suantika et al., 2001), and are also part of the normal flora of penaeid shrimps (Gomez-Gil et al., 1998). Certain *Vibrio* strains stimulate reproduction and ameliorate growth rates of molluscs and rotifers, and protect *Artemia* against bacterial infections, whereas other *Vibrio* strains constitute serious pathogens or potential pathogens for the same organisms (Riquelme et al., 2001; Verschuere et al., 2000a).

Recently we surveyed the genomic diversity of 506 *Vibrionaceae* strains by means of the fluorescent amplified fragment length polymorphism technique (FAFLP; Thompson et al., 2001). Many isolates from the aquacultural environment possess genomes different from the currently known *Vibrio* species and are thus potentially new species. In the present study we describe additional genomic and phenotypic characteristics of a subset of thirty isolates distributed in the FAFLP groups A5, A8 and A23. FAFLP cluster A5 represented mainly the dominant culturable bacterial microflora of a recirculating system for rotifers (Suantika et al., 2000). Group A8 was abundant in cultures of bivalve *Nodipecten nodosus* larvae at Florianópolis, south of Brazil, whereas group A23 was found to be ubiquitous and in association with cultured shrimps in China and Ecuador and in cultures of *N. nodosus* larvae in Brazil.

Material and Methods

Bacterial strains, growth conditions and DNA isolation

Strains used in this study are described in Table 1 (Annex). Strains were grown aerobically on Tryptone Soya agar TSA (Oxoid) supplemented with 2 % (w/v) NaCl for 24 h at 28 °C. DNA was extracted following the methodology described by Pitcher et al. (1989). All strains included in this study are deposited in the BCCMTM/LMG Bacteria Collection at Ghent University and in the CAIM collection of the Centre for Research on Nutrition and Development (CIAD) in Mazatlán, México.

Genotypic analyses

Selective amplification of restriction fragments (FAFLP) and sequencing of the almost complete 16S rDNA sequences were accomplished essentially as described previously (Thompson et al., 2001). Alignment of the 16S rDNA sequences, distance estimations (Jukes and Cantor, 1969), clustering by neighbour joining (Saitou and Nei, 1987), maximum likelihood and maximum parsimony methods and stability of the clusters (bootstrap analysis with 1000 replicates) were performed with the software BioNumerics 2.5 (Applied Maths). DNA-DNA hybridisation experiments using photobiotin-labelled DNAs were run at stringent conditions (39 °C) following the methodology described by Willems et al. (2001). The mol % G+C of DNA was determined by HPLC (Mesbah et al., 1989).

Phenotypic characterisation

Biochemical characterisation of the isolates was performed using API20E and APIZYM (bioMérieux) test strips and metabolic fingerprinting was carried out by means of Biolog GN2 microtiterplates (Biolog). Preparations were done according to the manufacturer instructions, with slight modifications (Thompson et al., 2002b). Classical bacteriological tests were performed as described previously (Baumann et al., 1984; Farmer III and Whickman-Brenner, 1992; Thompson et al., 2002b; Vandamme et al., 1998). Antibiograms were carried out using the disc diffusion methodology (Acar and Goldstein, 1996) using commercial discs (Oxoid). The inhibition zone of each antibiotic was measured on strains grown on Iso-sensitest agar (Oxoid) supplemented with 1.5 % (w/v) NaCl for 24 hours at 28 °C. Fatty acid methyl esters (FAME) analysis was carried out as described by Huys et al. (1994). Isolates were grown on Trypticase Soy Broth (Becton Dickinson) supplemented with 1.5 % (w/v) Bacto agar (Becton Dickinson) and 1.5 % (w/v) NaCl at 28 °C for 24 hours. Approximately 50 mg of cells were harvested and the fatty acids were isolated following the recommendations of the manufacturer using the Microbial Identification System manual and software package, version 3.9 (Microbial ID).

Results and Discussion

The 30 *Vibrio* isolates formed three groups by FAFLP fingerprinting analysis. FAFLP groups A5, A8 and A23 had complex band patterns consisting of 126 ± 14 bands, 115 ± 7 and 83 ± 14 (50-536 bp in size), respectively (Figure 3.3). The three

FAFLP groups were clearly different from known *Vibrio* species (Thompson et al., 2001), suggesting that they represent new species. Isolates of the group A5 had at least 75 % pair-wise pattern similarity, and less than 71 % pair-wise pattern similarity towards other *Vibrio* species. Surprisingly these strains which were isolated over a four-year period and in different places showed a remarkable resemblance of genomes. For instance, LMG 20536^T isolated in 1998 at Florianópolis island (Brazil) and LMG 20612 isolated in 1996 at the ARC (Belgium), had 87.5 % pattern similarity. Some strains e.g. LMG 20614 and R-15108 and R-15111 and R-15112 clustered at the reproducibility level of FAFLP (i.e. ≥ 88 % pattern similarity), and were thus indistinguishable by FAFLP. Isolates of the FAFLP groups A8 and A23 showed mutual similarities of at least 82 % and 62 % and similarity levels below 73 % and 54 % towards other *Vibrio* species, respectively.

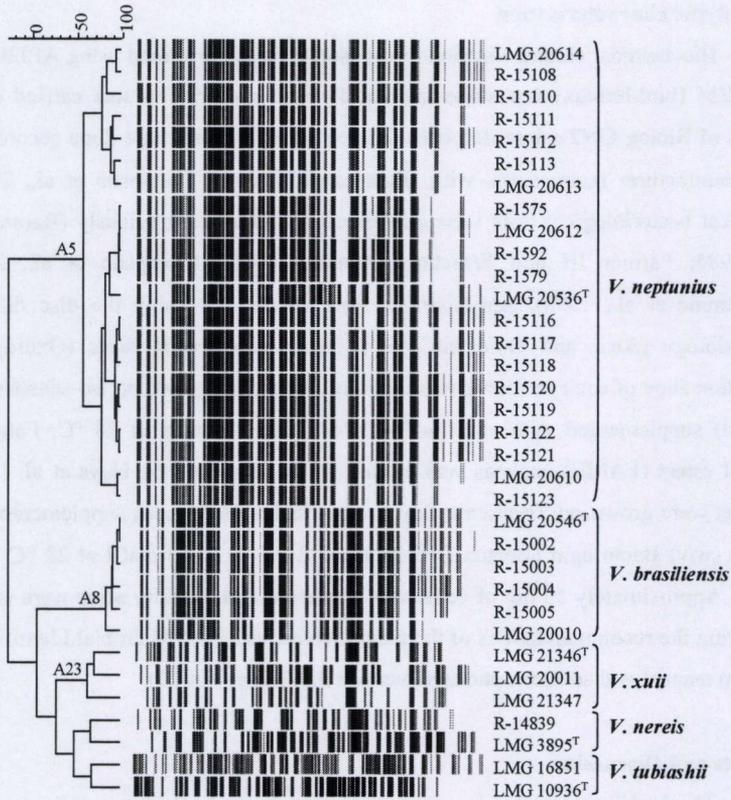


Figure 3.3. Dendrogram of the FAFLP patterns of the 30 marine aquaculture *Vibrio* isolates. *V. tubiashii* and *V. nereis* were included as out-groups. A band based (Dice) cluster analysis (Ward) was used.

likelihood and maximum parsimony were in agreement and the closest phylogenetic neighbours of the three novel *Vibrio* species were *V. tubiashii* (98 to 98.8 %), *V. nereis* (97.6 to 98.8 %), *V. coralliilyticus* (96.8 to 98.5 %), *V. mytili* (96.8 to 98.2 %) and *V. diabolicus* (97.1 to 98.1 %) (Figure 3.4). *V. coralliilyticus* and LMG 20536^T were closely related to each other having 98.2 % 16S rDNA similarity and so were LMG 20546^T and LMG 21346^T (98.4 %). *V. neptunius* had 97.2 % 16S rDNA similarity towards *V. brasiliensis* and *V. xuii*. Similarity levels of the three proposed new species towards other genera of the family *Vibrionaceae* were below 95 %.

Two representative isolates of each FAFLP group were chosen for DNA hybridisation experiments. The DNA similarity levels within each FAFLP group were ≥ 93 %, but lower than 67 % towards other phylogenetic related *Vibrio* species (Table 2). DNA hybridisations confirmed the FAFLP grouping and further revealed other interesting relationships. For instance, FAFLP group A5 was found to be highly related (64-66 % DNA similarity) to a novel coral pathogenic species, *V. coralliilyticus* (Ben-Haim et al., in press); *V. coralliilyticus* belongs to the FAFLP clusters A1 to A3 (Thompson et al., 2001).

Table 3.5. DNA-DNA similarity and DNA G+C content of marine aquaculture *Vibrio* isolates and related *Vibrio* species

	1	2	3	4	5	6	7	8	9	10	11	12	Mol % G+C
<i>V. mediterranei</i>													
1. LMG 11258 ^T	100												43.8
<i>V. mytili</i>													
2. LMG 19157 ^T	21	100											44.6
<i>V. diabolicus</i>													
3. LMG 19805 ^T	22	37	100										45.6
<i>V. coralliilyticus</i>													
4. LMG 20984 ^T	19	22	23	100									46.2
<i>V. nereis</i>													
5. LMG 3895 ^T	25	30	34	32	100								45.9
<i>V. tubiashii</i>													
6. LMG 10936 ^T	22	35	25	30	34	100							44.8
<i>V. neptunius</i>													
7. LMG 20536 ^T	20	25	26	64	34	34	100						46.0
8. LMG 20613	24	27	27	66	37	38	93	100					45.3
<i>V. brasiliensis</i>													
9. LMG 20546 ^T	17	20	22	25	32	34	32	29	100				45.9
10. LMG 20010	16	21	22	26	32	34	32	28	100	100			45.9
<i>V. xuii</i>													
11. LMG 21346 ^T					50	31					100		46.6
12. LMG 21347	16	28	26	24	41	27	29	26	25	26	94	100	47.1

The thirty *Vibrio* isolates examined in this study had the main phenotypical and chemotaxonomic features of the genus *Vibrio* (Bertone et al., 1996; Farmer III and Hickman-Brenner, 1992; Lambert et al., 1983). They were slightly curved rods, gram negative, oxidase- and catalase-positive and motile by means of at least one polar flagellum. The major fatty acids were summed feature 3 (comprising 16:1 ω 7c and/or 15 iso 2-OH), 16:0, 18:1 ω 7c, 14:0, accounting for ≥ 68 % of the total fatty acid composition. These facultative anaerobic isolates grew on Thiosulphate-citrate-bilesalt-sucrose agar (TCBS) forming yellow colonies, but they did not grow without NaCl or in presence of the vibriostatic agent 0/129 at 10 and 150 μ g per disc (except LMG 21346^T). Prolific growth occurred in media containing 2.5 % (w/v) NaCl at 28 °C. None of the isolates fermented inositol, sorbitol, rhamnose and melibiose. All isolates utilised dextrin, N-acetyl-D-glucosamine, D-fructose, α -D-glucose, maltose, D-mannose, psicose, D-trehalose, D,L-lactic acid, succinic acid, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, L-serine, inosine, uridine and thymidine as sole carbon source. None of the isolates utilised adonitol, D-arabitol, i-erythritol, L-fucose, m-inositol, α -lactose, α -D-lactose lactulose, D-melibiose, D-raffinose, L-rhamnose, xylitol, cys-aconitic acid (except LMG 21346^T), citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, α -hydroxy butyric acid, itaconic acid, α -keto valeric acid, malonic acid, quinic acid, D-saccharic acid, sebacic acid, succinamic acid, glucuronamide, L-histidine, L-leucine, L-pyroglutamic acid, D,L-carnitine, urocanic acid and phenyl ethylamine. None of the isolates were luminescent, but they reduced nitrate, and were Voges-Proskauer and methyl red positive. The thirty isolates produced indole, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase and valine arylamidase (except LMG 20613), but they did not produce urease, H₂S, lysine and ornithine decarboxylases, α -chymotrypsin, α -galactosidase, β -glucuronidase, β -glucosidase and α -fucosidase. The thirty isolates were sensitive to chloramphenicol (30 μ g per disc) (except LMG 21346^T), tetracycline (30 μ g per disc), polymixin B (300 U), and resistant to and kanamycin (30 μ g per disc).

We propose to accommodate the 30 *Vibrio* isolates examined in the present study in three novel species i.e. *V. neptunius*, *V. brasiliensis* and *V. xiii*. The three novel *Vibrio* species can be differentiated from each other and from other *Vibrio*

species by a number of phenotypical features (Table 3.6). Quantitative and qualitative differences in the fatty acid composition of these novel species were detected. Of special interest where the fatty acids 14:0 iso, 14:0 iso 3-OH and 16:0 iso which appeared at a higher concentration in the group A8, and the fatty acids 16:0, 17:0 and 17:1 ω8c which were at a higher concentration in the group A5.

Table 3.6. Useful features for differentiating of *V. neptunius*, *V. brasiliensis* and *V. xuii* from closely related *Vibrio* species

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Utilization of:																
Cellobiose	-	+	+	+	+	+	-	-	+	v	+	+	+	-	v	+
D-Galactose	-	+	-	+	v	+	+	+	+	+	+	+	+	-	v	+
Gentiobiose	-	+	v	+	+	ND	-	-	+	+	ND	v	+	-	-	-
β-hydroxy butiric acid	-	+	+	-	-	-	-	ND	-	+	ND	-	-	+	v	v
Growth on																
8 % (w/v) NaCl	-	-	+	v	-	+	-	ND	+	v	ND	v	+	+	v	v
Fermentation of:																
Mannitol	-	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+
Amygdaline	-	+	+	+	+	ND	-	-	+	+	ND	+	+	-	+	-
Melibiose	-	-	-	-	-	ND	-	-	-	-	+	v	-	-	+	v
Enzyme activity:																
Gelatinase	+	+	-	+	+	+	+	+	-	+	+	v	-	v	+	-
β-galactosidase	-	+	-	+	+	-	+	-	+	v	ND	ND	+	-	v	+
N-acetyl-β-glucosaminidase	+	-	-	+	+	ND	-	-	-	+	ND	+	+	ND	-	+
FAME composition:																
14:0 iso	0.2 ± 0.1	3.3 ± 0.4	1.2 ± 0.1	0.3**	0.0	0.0	0.5	0.2	0.3	1.4	0.0	1.8	0.0	0.2	0.0	0.0
14:0 iso 3-OH	0.1 ± 0.1	1.3 ± 0.2	0.9 ± 0.1	0.2	0.0	0.0	0.3	0.3	0.3	0.8	0.0	0.5	0.3	0.3	0.0	0.0
16:0	18.0 ± 0.8	11.3 ± 0.3	12.5 ± 0.6	23.2	28.6	30.5	15	14.4	24.5	16.1	24.7	10.9	18.8	12.9	20.8	17.3
16:0 iso	0.5 ± 0.1	10.5 ± 0.6	5.5 ± 0.4	2.2	0.4	0.0	0.8	1.7	2.2	4.9	0.0	4.3	1.5	1.1	0.0	0.0
17:0	2.3 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	0.3	0.0	0.1	2.5	1.6	0.3	0.7	0.0	0.0	0.0	1.9	0.0	0.1
17:1 ω8c	2.1 ± 0.1	0.7 ± 0.1	0.5	0.5	0.0	0.0	1.8	2.5	0.3	0.9	0.0	0.2	0.0	4.6	0.0	0.1
17:1 ω6c	1.2 ± 0.1	0.3 ± 0.1	0.2	0.0	0.0	0.0	0.6	0.7	0.0	0.2	0.0	0.0	0.0	1.3	0.0	0.0
18:1 ω7c	17.8 ± 1.6	17.3 ± 0.3	21.0 ± 2.4	15.9	12.5	7.5	18.2	17.4	16.5	16.8	8.7	17.2	19.9	22.6	12.0	25.4

*Phenotypic data were obtained from Bem-Haim et al. (1984); Baumann et al. (1984); Farmer III and Hickman-Brenner (1992); Hedlund and Staley (2001); Macián et al. (2001a; 2001b); Pujalte et al. (1993) and Raguénès et al. (1997). Fatty acids are mean ± SD. ND, no data available. v, variable feature. **Fatty acid profiles of know *Vibrio* species (type strains) are from our own database. 1, *V. neptunius* (n=21); 2, *V. brasiliensis* (n=6); 3, *V. xuii* (n=3); 4, *V. aestuarianus*; 5, *V. anguillarum*; 6, *V. cicytrophicus*; 7, *V. corallilyticus*; 8, *V. diabolicus*; 9, *V. diazotrophicus*; 10, *V. fluvialis*; 11, *V. lentus*; 12, *V. mediterranei*; 13, *V. mytili*; 14, *V. nereis*; 15, *V. splendidi*; 16, *V. tubiashii*.

Description of *V. neptunius* sp. nov.

V. neptunius (nep.tu'nius. L. masc. adj., *neptunius* of Neptune, the Roman god of the sea). Cells are 1 μm in width and 2.3-3 μm in length. They form translucent, convex, no-swarming, smooth-rounded colonies with entire margin, beige in colour and about 3 mm on TSA after 48 h incubation at 28 °C; colonies are yellow, umbonate, round, entire, smooth, shiny and transparent and of 2-3 mm in size on TCBS after 24 h at 28 °C. No growth occurs on 0 and ≥ 8.0 % (w/v) NaCl. No growth occurs at 4 and ≥ 40 °C. Strains are facultative anaerobic and ferment D-glucose and sucrose. None of the strains ferment mannitol and amygdalin. All strains utilise citrate, glycogen, D-mannose, β -methyl-D-glucoside, sucrose, D-serine, L-threonine, glucose-1-phosphate, and glucose-6-phosphate as sole carbon source. None of the strains utilise tween 80, N-acetyl-D-galactosamine, L-arabinose, cellobiose, D-galactose, gentiobiose, D-mannitol, D-sorbitol, turanose, mono methyl succinate, D-gluconic acid, β -hydroxy butyric acid, γ -hydroxy butyric acid, *p*-hydroxy phenylacetic acid, hydroxy L-proline, L-phenyl alanine, D,L-carnitine, γ -amino-butyric acid, putrescine, and 2,3-butanediol as sole carbon source. □ Strains produce gelatinase, tryptophane deaminase, trypsin, N-acetyl- β -glucosaminidase, but they do not produce cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -galactosidase and α -glucosidase. Arginine dihydrolase is variable, but positive for the type strain. The major fatty acids of *V. neptunius* are summed feature 3 (35.7 % \pm 0.9; comprising 16:1 ω 7c and/or 15 iso 2-OH), 16:0 (18 % \pm 0.8), 18:1 ω 7c (17.8 % \pm 1.6), 14:0 (5.5 % \pm 0.6), summed feature 2 (2.4 % \pm 0.3; comprising 14:0 3-OH and/or 16:1 iso I and/or unidentified fatty acid with equivalent chain length value of 10.928 and/or 12:0 ALDE), 17:0 (2.3 % \pm 0.2), 17:1 ω 8c (2.1 % \pm 0.1), 12:0 3-OH (2.1 % \pm 0.6), 12:0 (1.9 % \pm 0.3), 15:0 (1.7 % \pm 0.3), 17:0 iso (1.5 % \pm 0.1), 15:0 iso (1.2 % \pm 0.3), 17:1 ω 6c (1.2 % \pm 0.1), 13:0 iso (1.0 % \pm 0.0), 16:1 ω 7c alcohol (0.9 % \pm 0.3), 11 methyl 18:1 ω 7c (0.6 % \pm 0.3), 16:0 iso (0.5 % \pm 0.1), 14:0 iso (0.2 % \pm 0.1), 14: iso 3-OH (0.1 % \pm 0.1). Strains are resistant to ampicillin (25 μg per disc). Additional phenotypical features are listed in Table 3.7. The 16S rDNA sequences of strains LMG 20536^T and LMG 20613 are deposited in the EMBL under the accession numbers AJ316171 and AJ490150, respectively. The type strain of this species is LMG 20536^T (CAIM 532^T) isolated from bivalve (*Nodipecten nodosus*) larvae in the south of Brazil. The mol % G+C of the type strain is 46. 0.

Description of *V. brasiliensis* sp. nov.

V. brasiliensis (bra.sili.en'sis. N.L. masc. adj., *brasiliensis* from Brazil). Cells are 1 µm in width and 2.5-3 µm in length. They form translucent, convex, smooth-rounded colonies with entire margin, beige in colour and 2.5-3 mm in size on TSA after 48 h incubation at 28 °C. Colonies are yellow, umbonate, wavy, shiny, translucent, round with scalloped margin, and about 3 mm in size on TCBS after 24 h incubation at 28 °C. No growth occurs on 0 and ≥ 8.0 % NaCl. No growth occurs at 4 and ≥ 45 °C. Strains are facultative anaerobic and ferment D-glucose, sucrose, mannitol and amygdalin. None of the strains ferment arabinose. All strains utilise α-cyclodextrin, glycogen, cellobiose, gentiobiose, D-galactose, gentiobiose, α-D-glucose, D-mannitol, β-methyl D-glucoside, sucrose, methyl pyruvate, β-hydroxy butyric acid, bromo succinic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-ornithine, L-proline, D-serine, L-threonine, and glycerol as sole carbon source. None of the strains utilise N-acetyl-D-galactosamine, adonitol, γ-hydroxy butyric acid, P-hydroxy phenylacetic acid, α-keto glutaric acid, α-keto valeric acid, alaninamide, L-phenyl alanine, 2-amino ethanol, 2,3-butanediol, D,L-α-glycerol phosphate, glucose-1-phosphate and glucose-6-phosphate as sole carbon source. All strains produce arginine dihydrolase, β-galactosidase and gelatinase. None of the strains produce trypsin, acid phosphatase, α-glucosidase and N-acetyl-β-glucosaminidase. The most abundant fatty acids are summed feature 3 (34.7 % ± 1.0; comprising 16:1 ω7c and/or 15 iso 2-OH), 18:1ω7c (17.3 % ± 0.3), 16:0 (11.3 % ± 0.3), 16:0 iso (10.5 % ± 0.6), 14:0 (4.6 % ± 0.1), 14:0 iso (3.3 % ± 0.4), 15:0 iso (1.8 % ± 0.1), summed feature 2 (1.8 % ± 0.3; comprising 14:0 3-OH and/or 16:1 iso I and/or unidentified fatty acid with equivalent chain length value of 10.928 and/or 12:0 ALDE), 12:0 3-OH (1.5 % ± 0.3), 12:0 (1.4 % ± 0.2), 17:0 iso (1.4 % ± 0.1), 14:0 iso 3-OH (1.3 % ± 0.2), 18:0 iso (1.1 % ± 0.0), 15:0 (1.0 % ± 0.1), 13:0 iso (1.0 % ± 0.0), 17:1 ω8c (0.7 % ± 0.1), 17:0 (0.6 % ± 0.1), 17:1 ω6c (0.3 % ± 0.1) and 16:1 ω7c alcohol (0.3 % ± 0.0). Additional phenotypical features are listed in Table 3.7. Isolated from bivalve (*Nodipecten nodosus*) larvae in the south of Brazil. The 16S rDNA sequences of strains LMG 20546^T and LMG 20010 are deposited in the EMBL under the accession numbers AJ316172 and AJ490151, respectively. The type strain of this species is LMG 20546^T (CAIM 495^T). The mol % G+C of the type strain is 45.9.

Description of *V. xuii* sp. nov.

V. xuii (xu'i.i N.L. gen. n. xuii of Xu, in honour of the microbiologist Huai-Shu Xu). Cells are 1 μm in width and 2-3 μm in length. They form translucent, convex, smooth-rounded colonies with entire margin, beige in colour and 3-4 mm in size on TSA after 48 h incubation at 28 °C. Colonies are yellow, convex, round, entire, shiny, translucent, and about 2 mm in size on TCBS after 24 h incubation at 28 °C. No growth occurs on 0 and ≥ 10.0 % NaCl. No growth occurs at 4 and ≥ 45 °C. This facultative anaerobic organism ferments glucose, mannitol, sucrose, amygdalin, and arabinose. *V. xuii* utilises α -cyclodextrin, tween 40, tween 80, N-acetyl-D-galactosamine, L-arabinose, cellobiose, D-mannitol, D-mannose, D-sorbitol, sucrose, methyl pyruvate, mono methyl succinate, acetic acid, D-gluconic acid, β -hydroxy butyric acid, γ -hydroxy butyric acid, p-hydroxy phenylacetic acid, α -keto glutaric acid, D-alanine, glycyl-L-glutamic acid, L-proline, L-threonine, 2,3-butanediol, glycerol and D,L- α -glycerol phosphate as sole carbon source. *V. xuii* does not utilise D-galactose, gentiobise, β -methyl D-glucoside, D-raffinose, α -keto butyric acid, propionic acid, D-serine, quinic acid, sebacic acid, hydroxy L-proline, 2-amino ethanol, glucose-1-phosphate as sole carbon source. *V. xuii* produces arginine dihydrolase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and tryptophane deaminase. *V. xuii* does not produce cystine arylamidase, trypsin, β -galactosidase, α -glucosidase, N-acetyl- β -glucosaminidase and gelatinase. The major fatty acids are summed feature 3 (38.7 % \pm 1.5; comprising 16:1 ω 7c and/or 15 iso 2-OH), 18:1 ω 7c (21.0 % \pm 2.4), 16:0 (12.5 % \pm 0.6), 16:0 iso (5.5 % \pm 0.4), 14:0 (3.5 % \pm 0.3), 12:0 (3.2 % \pm 0), summed feature 2 (2.6 % \pm 0; comprising 14:0 3-OH and/or 16:1 iso I and/or unidentified fatty acid with equivalent chain length value of 10.928 and/or 12:0 ALDE), 12:0 3-OH (1.4 % \pm 0), 14:0 iso (1.2 % \pm 0.1), 15:0 iso (1.1 % 0.1), 15:0 (0.9 % \pm 0.1) and 14:0 iso 3-OH (0.9 % \pm 0.1), 17:0 (0.5 % \pm 0.1) and 17:1 ω 6c (0.2 % \pm 0). *V. xuii* is sensitive to ampicillin (25 μg per disc). Additional phenotypical features are listed in Table 3.7. The 16S rDNA sequences of strains LMG 21346^T and LMG 21347 are deposited in EMBL under the accession nos. AJ316181 and AJ490152. The type strain of this species (LMG 21346^T, CAIM 467^T) was isolated from shrimp culture water in China. The mol % G+C of the type strain is 46.6.

Table 3.7. Variable phenotypical features of *V. neptunius*, *V. brasiliensis* and *V. xiii* isolates

	<i>V. neptunius</i> (n=21)	LMG 20536 ^T	<i>V. brasiliensis</i> (n=6)	LMG 20546 ^T	<i>V. xiii</i> (n=3)	LMG 21346 ^T
Enzyme Activity:						
Arginine dihydrolase	18*	+				
Lipase (C14), valine arylamidase and trypsin	20	+				
Cystine arylamidase					2	+
Substrates:						
α -cyclodextrin, α -keto butiric acid, α -keto glutaric acid, alaninamide, 2-amino ethanol, glycerol	4	-				
Tween 40	4	+				
Tween 80					1	+
L-Arabinose			4	-		
Gentiobiose					2	+
D-sorbitol, γ -amino-butyric acid			5	+		
Turanose			5	+	2	+
Methyl Pyruvate, L-proline, L-threonine, glycyl-L-glutamic acid	16	+				
Mono methyl succinate						
Acetic acid	12	+				
cis-Aconitic acid					1	+
D-Gluconic acid						
Propionic acid	8	+				
Bromo succinic acid	12	+				
D-Alanine	12	+				
Hydroxy L-proline						
L-Ornithine	8	+				
D-Serine					2	+
Putrescine						
D-L- α -glycerol phosphate	12	+			2	+
Susceptibility to:						
0/129 (10 and 150 μ g), chloramphenicol (30 μ g) and tetracycline (30 μ g)					2	-

*Numbers indicate the number of positive isolates.

3.3. Description of *Vibrio kanaloaei* sp. nov., *Vibrio pomeroyi* sp. nov. and *Vibrio chagasii* sp. nov., from sea water and marine animals

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Int. J. Syst. Evol. Microbiol. (2003), 53, May

Abstract

The taxonomic position of the FAFLP fingerprinting groups A46 (five isolates), A51 (six isolates), A52 (five isolates) and A53 (seven isolates) obtained in a previous study were further analysed through a polyphasic approach. The twenty-three isolates were phylogenetically related to *V. splendidus*, but DNA-DNA hybridization experiments proved that they belong to three new species. Chemotaxonomic and phenotypic analyses further disclosed several differentiating features between the 23 isolates and known *Vibrio* species. We propose the names *V. kanaloaei* (type strain is LMG 20539^T; EMBL accession no. AJ316193; mol % G+C content of the type strain is 44.7), *V. pomeroyi* (type strain is LMG 20537^T; EMBL accession no. is AJ491290; mol % G+C content of the type strain is 44.1) and *V. chagasii* (type strain is LMG 21353^T; EMBL accession no. is AJ316199; mol % G+C content of the type strain is 44.6) to encompass the five isolates of A46, the six isolates of A51 and the 12 isolates of A52/A53, respectively. The three new species can be distinguished from known *Vibrio* species by several phenotypical features, including utilisation and fermentation of various carbon sources, β -galactosidase activity, and fatty acid content (particularly 12:0, 14:0, 14:0 iso and 16:0 iso).

Introduction

Several *Vibrio* species are ubiquitous in aquatic ecosystems and display an extraordinarily high growth rate, which makes them highly successful and dominant particularly in eutrophic environments (Aiyar et al., 2002, Macián et al., 2000a). In this study we report on the taxonomic analysis of four unidentified groups of vibrios i.e. A46 (5 isolates), A51 (6 isolates), A52 (7 isolates) and A53 (5 isolates) found

previously (Thompson et al., 2001). These isolates were phylogenetically related to *V. splendidus*, a ubiquitous luminous marine bacterium which was first described by the early microbial ecologist Beijerinck in 1900. *V. splendidus* strains have constantly been found in association with cultured oysters (*Ostrea edulis*) in the Mediterranean sea throughout the years, suggesting a close relationship between the bacterium and the host invertebrate (Macián et al., 2000a). *V. splendidus* has also been involved as an etiological agent of septicaemia in various species of fish (Austin and Austin, 1999) and as the causative agent of bacillary necrosis of oyster larvae (Sugumar et al., 1998). Our polyphasic taxonomic study, including genomic, phenotypic and chemotaxonomic analyses revealed that the 23 isolates belong to three new species, for which we propose the names *V. kanaloaei*, *V. pomeroyi* and *V. chagasii*. *V. kanaloaei* was found to be ubiquitous in the aquatic environment, whereas *V. pomeroyi* isolates were abundant in cultures of *Nodipecten nodosus* larvae in the south of Brazil. *V. chagasii* isolates were the regular inhabitants of rotifer cultures in Greece (Verdonck et al., 1997).

Material and Methods

Bacterial strains, growth conditions and DNA isolation

Strains used in this study are listed in Table 1 (Annex). Strains were grown aerobically on Tryptone Soy Agar (TSA; Oxoid) supplemented with 2 % (w/v) NaCl for 24 h at 28 °C. DNA was extracted following the methodology described by Pitcher et al. (1989). All strains included in this study are deposited in the BCCMTM/LMG Bacteria Collection at Ghent University and in the CAIM collection of the Centre for Research on Nutrition and Development (CIAD) in Mazatlán, México.

Genotypic analyses

Selective amplification of restriction fragments (FAFLP) and sequencing of the almost complete 16S rDNA sequences were accomplished essentially as described previously (Thompson et al., 2001). Alignment of the 16S rDNA sequences, distance estimations (Jukes and Cantor, 1969), clustering by neighbour joining (Saitou and Nei, 1987), maximum likelihood and maximum parsimony methods and stability of the clusters (Bootstrap analysis with 1000 replicates) were performed with the software BioNumerics 2.5 (Applied Maths). Rep-PCR fingerprinting was performed essentially

as described previously (Sawabe et al., 2002). DNA-DNA hybridisation experiments using photobiotin-labelled DNAs were run at stringent conditions (39 °C) following the methodology described by Willems et al. (2001). Hybridisations were performed in four replicates. DNA similarity values are the mean of reciprocal and non-reciprocal reactions. The mol % G+C of DNA was determined by HPLC (Mesbah et al., 1989).

Phenotypic analyses

Phenotypic characterisation of the isolates was performed using API20E and APIZYM (bioMérieux) and Biolog GN metabolic fingerprinting (Biolog) following the instructions of the manufacturer, with slight modifications (Thompson et al., 2002b). Classical phenotypic tests were performed as described previously (Baumann et al., 1984; Farmer III and Whickman-Brenner, 1992; Murray et al., 1994; Thompson et al., 2002b; Vandamme et al., 1998). Antibiograms were carried out using the disc diffusion methodology (Acar and Goldstein, 1996) using commercial discs (Oxoid). The inhibition zone of each antibiotic was measured on strains grown on Iso-sensitest agar (Oxoid) supplemented with 1.5 % (w/v) NaCl for 24 hours at 28 °C. Fatty acid methyl esters (FAME) analysis was carried out as described by Huys et al. (1994). Isolates were grown on Trypticase Soy Broth (Becton Dickinson) supplemented with 1.5 % (w/v) Bacto agar (Becton Dickinson) and 1.5 % (w/v) NaCl at 28 °C for 24 hours. Approximately 50 mg of cells were harvested and the fatty acid were isolated following the recommendations of the manufacturer using the Microbial Identification System manual and software package, version 3.9 (Microbial ID).

Results and Discussion

The FAFLP patterns of groups A46, A51 and A52/A53 consisted of 116 ± 19 , 126 ± 11 and 119 ± 14 bands respectively. The FAFLP patterns of these groups were clearly different from their closest phylogenetic neighbours (Figure 3.5) and from other known *Vibrio* species (Thompson et al., 2001). Isolates of the groups A46, A51 and A52/A53 had mutual pair-wise similarities of at least 57.2 %, 72.2 % and 60 % respectively. Representative strains of each FAFLP group were also distinguishable from other closely related *Vibrio* species on the basis of repetitive chromosomal elements analysis (data not shown).

The 16S rDNA sequences of at least three representative isolates of each FAFLP group were performed. FAFLP group A46 (LMG 20539^T, 1494 bp, EMBL accession

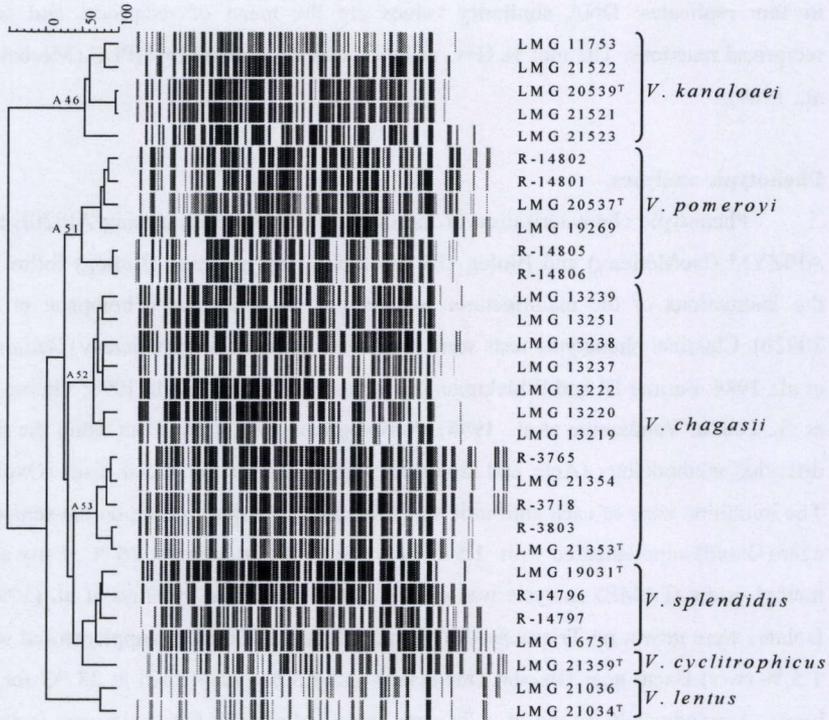


Figure 3.5. Dendrogram of the FAFLP patterns of *V. kanaloaei* (n=5), *V. pomeroyi* (n=6) and *V. chagasii* (n=12). *V. splendidus*, *V. cyclitrophicus* and *V. lentus* were included as out-groups. A band based (Dice) cluster analysis (Ward) was used.

no. AJ316193; LMG 21522, 488 bp, AJ490153; LMG 21523, 454 bp, AJ490154), FAFLP group A51 (LMG 20537^T, 1507 bp, EMBL accession no. AJ491290; LMG 21351, 485 bp, EMBL accession no. AJ316197; LMG 21352, 468 bp, AJ190156) and FAFLP groups A52 and A53 (LMG 21353^T, 1435 bp, AJ316199; LMG 13237, 1435 bp, EMBL accession no. AJ490157; LMG 21354, 887 bp, EMBL accession no. AJ490158; LMG 13219, 471 bp, AJ316198) were allocated to the genus *Vibrio* by FASTA program (Pearson & Lipman, 1986).

V. kanaloaei sp. nov., *V. pomeroyi* sp. nov. and *V. chagasii* sp. nov.

The 16S rDNA sequence similarity within each FAFLP group was $\geq 99\%$. Isolates LMG 20539^T, LMG 21522 and LMG 21523 had 99.5% 16S rDNA similarity, while LMG 20537^T, LMG 21351 and LMG 21352 had 99.4%. Isolates LMG 21353^T and LMG 13237 had 99.7%. The similarity between the representative isolates of each FAFLP group was at least 97.4%. The phylogenetic trees based on almost complete sequences and using neighbour joining, maximum likelihood and maximum parsimony methods were all in agreement and revealed that the three novel *Vibrio* species are closely related to *V. splendidus* (98.0, 99.1 and 98.5% similarity, respectively), *V. lentus* (97.8, 98.4 and 98.2%), *V. cyclitrophicus* (97.0, 98.3 and 97.7%), *V. mediterranei* (95.7, 97.2 and 97.8%) and *V. orientalis* (96.0, 97.1 and 97.6%) (Figure 3.6). The 16S rDNA similarity of the three novel species towards other *Vibrio* species and other genera of the family *Vibrionaceae* was below 97% and 93.5%, respectively.

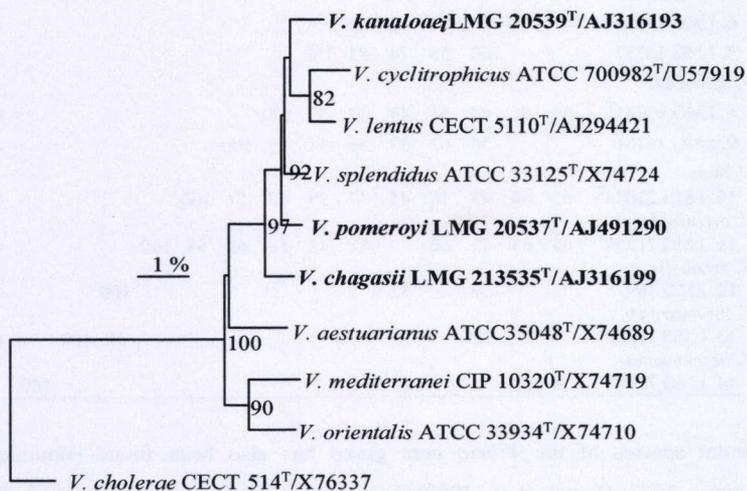


Figure 3.6. Phylogenetic tree with the estimated positions of *V. kanaloaei*, *V. pomeroyi* and *V. chagasii*, using the neighbour joining method based on the almost complete 16S rDNA sequences. Bootstrap values ($> 50\%$) after 1000 simulations are shown. Bar, 1% estimated sequence divergence.

At least two representative isolates of each FAFLP group were chosen for further DNA hybridisation experiments. The isolates of A46, A51 and A52/A53 had

89, 77 and 72 % mutual DNA-DNA similarity respectively, but ≤ 65 % towards other *Vibrio* species. These results confirm thus their status as new species (Table 3.8). DNA hybridisation experiments further confirmed that the three novel species and other *Vibrio* species in the same phylogenetic branch have intermediate DNA-DNA relatedness. Macián et al. (2001a) has already demonstrated that *V. lentus* and *V. splendidus* have 59 % DNA-DNA similarity. High DNA-DNA similarity between

Table 3.8. DNA-DNA binding values and G+C content of all *Vibrio* strains examined

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Mol % of G+C
<i>V. kanaloaei</i>															44.7
1. LMG 20539 ^T	100														44.6
2. LMG 21523	89	100													
<i>V. pomeroyi</i>															
3. LMG 20537 ^T	64	65	100												44.1
4. LMG 21352			77	100											44.0
<i>V. chagasii</i>															
5. LMG 21353 ^T	49	48	50	49	100										44.6
6. LMG 13219			53	54	72	100									44.5
7. LMG 13237			60	53	74	85	100								44.4
<i>V. splendidus</i>															
8. LMG 19031 ^T	64	61	44	54	44	47	39	100							45.0
9. LMG 16751			56	62	53	56	54	70	100						44.5
<i>V. lentus</i>															
10. LMG 21034 ^T	62	60	43	56	45	47	39	62	57	100					45.2
<i>V. cyclitrophicus</i>															
11. LMG 21359 ^T	65	63	45	60	49	51	42	59	61	58	100				44.2
<i>V. orientalis</i>															
12. LMG 7897 ^T			36		32							100			44.4
<i>V. mediterranei</i>															
13. LMG 11258 ^T			27		25							30	100		43.8
<i>V. aestuarianus</i>															
14. LMG 7909 ^T	24	24												100	

different species of the *Vibrio* core group has also been found (Baumann and Baumann, 1977; Dorsch et al., 1992). For instance, *V. harveyi* and *V. campbellii* have up to 74 % DNA-DNA similarity and very similar phenotypes, but they can be clearly distinguished by genomic fingerprinting techniques such as FAFLP and rep-PCR (Thompson et al., 2001; Gomez-Gil et al., unpublished). *V. cyclitrophicus* (Hedlund and Staley, 2001) was reported to have a mol % of G+C of 39, but our results clearly show that this bacterium has a mol % of G+C of 44.2. Measurements of mol % G+C of DNA by renaturation methods, as the one used by Hedlund and Staley (2001), are prompt to errors caused by low quality DNAs (i.e. fragmented DNA and/or

contaminated DNA with proteins and/or RNA) and thus may influence significantly the results (Mesbah et al., 1989).

The three novel *Vibrio* species examined in this study shared the main phenotypical and chemotaxonomic features of the genus *Vibrio* (Bertone et al., 1996; Farmer III and Hickman-Brenner, 1992; Lambert et al., 1983). The three novel *Vibrio* species had several phenotypical features in common; the 23 isolates were Gram-negative, facultative anaerobic, catalase and oxidase positive and showed prolific growth on TCBS agar, forming yellow colonies (except strains LMG 21357^T and LMG 13251 which formed green colonies). Isolates were motile by at least one polar flagellum, susceptible to 10 and 150 µg of O/129 (strain LMG 21523 was resistant towards both concentrations) and did not grow in the absence of NaCl. The predominant fatty acids were summed feature 3 (comprising 16:1 ω7c and/or 15 iso 2-OH), 16:0, 18:1 ω7c, 14:0 and 12:0 accounting for > 80 % of the total cellular fatty acid composition.

The three novel species fermented D-glucose and mannitol, but not inositol and rhamnose. Strains of the novel species utilised α-D-glucose, dextrin, glycogen, N-acetyl-D-glucosamine, D-fructose, maltose, D-trehalose, D,L-lactic acid, succinic acid, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, mono methyl succinate, glycyl-L-aspartic acid, L-threonine, inosine and glycerol as sole carbon source. None of the new species utilised N-acetyl-D-galactosamine, adonitol, D-arabitol, i-erythritol, L-fucose, M-inositol, α-lactose, D-melibiose, D-raffinose, L-rhamnose, turanose, xylitol, citric acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, γ-hydroxy butyric acid, P-hydroxy phenylacetic acid, itaconic acid, α-keto butyric acid, α-keto valeric acid, malonic acid, L-leucine, L-pyro glutamic acid, D,L-carnitine, γ-amino-butyric acid, urocanic acid, phenyl ethylamine, 2-amino ethanol and 2,3-butanediol. Strains of the three species produced indole, alkaline phosphatase, esterase (C4), esterase lipase (C8), naphthol-AS-BI-phosphohydrolase, but not lysine and ornithine decarboxylases, H₂S, urease, cystine arylamidase, α-galactosidase, β-glucuronidase, α- and β-glucosidase, α-mannosidase and α-fucosidase. The three novel species were non-luminescent, reduced nitrate, and were methyl red positive (A46 isolates were methyl red negative). FAFLP groups A51 and A52/A53 were susceptible to the vibriostatic agent (10 and 150 µg per disc).

Table 3.9. Variable features among *V. kanaloaei*, *V. pomeroyi* and *V. chagasii* strains.

	<i>V. kanaloaei</i> (n=5)	LMG 20539 ^T	<i>V. pomeroyi</i> (n=6)	LMG 20537 ^T	<i>V. chagasii</i> (n=12)	LMG 21353 ^T
Fermentation of:						
Melibiose			2	-		
Sorbitol			2	-		
Utilisation of:						
Tween 40			5	+		
Tween 80	4*	+	4	+		
D-galactose	4	+			5	-
Gentiobiose	2	+				
α -D-lactose lactulose	1	-				
L-arabinose	4	-				
D-mannose	3	+				
β -methyl D-glucoside	1	+				
Psicose	4	+	3	-		
Methyl piruvate	4	+				
D-sorbitol			3	-	3	-
Acetic acid	4	+	3	-	4	-
Cis-aconitic acid	2	+	4	+	2	-
Formic acid	3	+			1	-
D-gluconic acid			3	+	2	-
D-gluconic acid	1	-			5	-
α -hydroxy butiric acid	1	-			1	-
Propionic acid	3	+	3	-	2	-
Quinic acid	1	+	1	-	3	-
Bromo succinic acid	4	+	3	-	8	+
Succinamic acid					2	-
Glucuronamide	2	-			2	-
Alaninamide	2	+	3	-	2	-
Glycyl-L-glutamic acid	3	+				
L-histidine	3	+				
Hydroxy L-proline			2	-	1	-
Uridine	4	+				
L-serine					9	+
D, L α -glycerol phosphate					3	-
Putrescine	3	-				
Glucose-1-phosphate	1	+	3	-	4	-
Glucose-6-phosphate	3	+	4	+	5	-
Growth at/on:						
4 °C					7	+
8 % (w/v) NaCl	3	-			5	+
Production of:						
Arginine dihydrolase	2	+	3	+	7	+
Gelatinase			5	+		
Acetoin			5	+		
Lipase (14)					2	-
Valine arylamidase	3	+			1	-
Trypsin					10	+
Acid phosphatase					8	-
<i>N</i> -acetyl- β -glucosaminidase	3	+			5	-
Susceptibility to 0129 (10 and 150 μ g), chloramphenicol (30 μ g), and tetracyclin (30 μ g)	4	+				
Susceptibility to ampicillin (25 μ g)	1	-				

*Numbers indicate the number of positive isolates

Table 3.10. Useful features for differentiating of *V. kanaloaei*, *V. pomeroyi* and *V. chagasii* from closest phylogenetic related *Vibrio* species.

	<i>V. kanaloaei</i> (n=5)	<i>V. pomeroyi</i> (n=6)	<i>V. chagasii</i> (n=12)	<i>V. splendidus</i>	<i>V. lentus</i>	<i>V. cyclitrophicus</i>	<i>V. orientalis</i>	<i>V. mediterranei</i>
Growth at/on:								
4 °C	+	+	v	v	v	+	+	-
8 % (w/v) NaCl	v	+	v	v		+	+	v
10 % (w/v) NaCl	-	-	-	-	ND	+	-	-
Susceptibility to:								
0129 (150 µg)	v	+	+	+	-	ND	ND	+
Polymixin B (300 U)	v	+	+	ND	ND	-	ND	ND
Ampicilin (25 µg)	v	-	-	ND	ND	+	ND	ND
Nitrate reduction	+	+	+	+	+	-	+	+
Fermentation of:								
Sucrose	+	v	-	v	-	+	+	+
Melibiose	-	v	-	-	v	ND	-	v
Arabinose	+	-	-	-	-	-	-	ND
Utilisation of:								
Cellobiose	-	+	+	v	+	+	+	+
Gentiobiose	v	-	-	ND	ND	ND	ND	ND
L-alanine	+	+	+	v	+	ND	+	+
β-galactosidase activity	-	+	-	v	ND	-	ND	+
Indole production	+	+	+	+	v	-	+	+
Fatty acids:								
Summed feature 3	39.2 ± 0.2	32.9 ± 1.6	38.4 ± 3.5	35	41.7	35.3	35.4	33.4
16:0	25.6 ± 1	29.2 ± 1.7	22.4 ± 3.9	29.6	24.7	30.7	27.6	17.4
14:0	5.0 ± 0.3	10.5 ± 0.4	7.2 ± 3.5	9	8.8	7.96	20.6	8.4
12:0	4.2 ± 0.1	8.9 ± 1.2	3.8 ± 2.0	8.7	5.3	7.7	4.9	8.4
18:1 w7c	10.2 ± 1	7.6 ± 1.8	9.7 ± 1.6	8.2	8.7	7.5	4.3	18.9
16:0 iso	0.0	0.0	5.2 ± 2.6	0.0	0.0	0.0	0.0	1.9
Summed feature 2	2.1 ± 0.6	4.1 ± 0.6	3.3 ± 1.3	3.5	2.6	3.5	3.2	3.8
12:0 3-OH	3.4 ± 0.1	3.9 ± 0.6	2.7 ± 1.4	3.41	2.5	2.1	2.0	3.1
14:0 iso	0.0	0.0	1.1 ± 0.7	0.0	0.0	0.0	0.0	0.8

Phenotypic data were obtained from Baumann et al. (1984); Farmer III and Hickman-Brenner (1992); Hedlund and Staley (2001); Macián et al. (2001a, 2001b); Pujalte et al. (1993). Fatty acids are mean ± SD. ND, no data available. v, variable feature. **Fatty acid profiles of known *Vibrio* species (type strains) are from our own database.

polymixin B (300 U), tetracyclin (30 µg per disc), chloramphenicol (30 µg per disc), but resistant to ampicillin (25 µg per disc). None of the 23 isolates grew on 0 and \geq 10.0 % NaCl and at \geq 35 °C. Additional phenotypical features found to be variable among the three novel *Vibrio* species are listed in Table 3.9.

Genomic and phenotypic evidence presented in this study clearly indicate that the 23 isolates should be accommodate in three new *Vibrio* species, i.e. *V. kanaloaei*, *V. pomeroyi* and *V. chagasii*. Although the new species had the main phenotypical traits of the genus *Vibrio*, several useful differentiating features were disclosed which discriminate them from known *Vibrio* species (Table 3.10).

Description of *V. kanaloaei*

Vibrio kanaloaei (ka.na.lo'aei L. masc. adj. *kanaloaei* of Kanaloa, the Hawaiian god of the sea and of the seaman). Cells are slightly curved, 1 µm in width and 2-3 µm in length and motile by at least one polar flagellum. They form translucent, convex, non-swarming, smooth-rounded colonies with entire margin, beige in colour and of about 5 mm on TSA after 48 h incubation at 28 °C. Strains formed yellow, translucent, 5-10 mm colonies on TCBS agar. All strains fermented sucrose and arabinose but not sorbitol, melibiose and amygdalin. Growth at 4 °C. No growth in absence of NaCl. All strains utilise Tween 40, D-mannitol, sucrose, mono methyl succinate, α -keto glutaric acid, D-alanine, L-alanine, L-ornithine, L-proline, L-serine and L-threonine. None of the strains utilise cellobiose, D-sorbitol, D-saccharic acid, sebacic acid, succinamic acid, Hydroxy L-proline, L-phenyl alanine, D,L- α -glycerol phosphate. Strains produce leucine arylamidase, trypsin, tryptophane deaminase, acetoin, gelatinase, but not α -chymotrypsin, α - and β -galactosidase and lysine. Arginine dihydrolase is variable, but positive for the type strain. The major fatty acids of *V. kanaloaei* are summed feature 3 (39.2 % \pm 0.2; comprising 16:1 ω 7c and/or 15 iso 2-OH), 16:0 (25.6 % \pm 1.0), 14:0 (5.0 % \pm 0.3), 12:0 (4.2 % \pm 0.1), 18:1 ω 7c (10.2 % \pm 1.0), summed feature 2 (2.1 % \pm 0.6; comprising 14:0 3-OH and/or 16:1 iso I and/or unidentified fatty acid with equivalent chain length value of 10.928 and/or 12:0 ALDE), 12:0 3-OH (3.4 % \pm 0.1), 18:00 (1.0 % \pm 0.1) and 16:0 3-OH (0.3 % \pm 0.1). Additional phenotypical features are listed in Table 3. The 16S rDNA sequences of strains LMG 20539^T, LMG 21522, LMG 21523 are deposited in the EMBL under the accession numbers AJ316193, AJ490153 and AJ490154,

respectively. The type strain of this species is LMG 20539^T (CAIM 485^T), isolated from diseased oyster (*Ostrea edulis*) larvae in France. The mol % G+C of DNA of the type strain is 44.5.

Description of *V. pomeroyi* sp. nov.

Vibrio pomeroyi (po.me.roy'i N.L. gen. n. *pomeroyi* of Pomeroy, in honour of the north American microbial ecologist L. R. Pomeroy). Cells are slightly curved, 1 µm in width and 2-3 µm in length and motile by at least one polar flagellum. They form translucent, convex, no-swarming, smooth-rounded colonies with entire margin, beige in colour and of about 3 mm on TSA after 48 h incubation at 28 °C. Strains (except LMG 20537^T) formed yellow translucent colonies on TCBS agar. Growth at 4 °C. No growth in absence of NaCl. All strains utilise D-galactose, cellobiose, mono methyl succinate, sucrose, glycyl-L-glutamic acid, L-serine, L-threonine, inosine, uridine and thymidine. None of the strains utilise α-cyclodextrin, gentiobiose, α-D-lactose lactulose, putrescine, formic acid, D-glucuronic acid, α-hydroxy butyric acid, α -keto glutaric acid, quinic acid, D-saccharic acid, sebacic acid, succinamic acid, glucuronamide, L-histidine, hydroxy L-proline, L-leucine, L-phenyl alanine, L-pyro glutamic acid, D-serine, D,L-carnitine and D,L-α-glycerol phosphate. Strains produce β-galactosidase, acid phosphatase, but not lipase (C14), tryptophane deaminase and valine arylamidase. Arginine dihydrolase is variable, but positive for the type strain. The major fatty acids of *V. pomeroyi* are summed feature 3 (32.9 % ± 1.6; comprising 16:1 ω7c and/or 15 iso 2-OH), 16:0 (29.2 % ± 1.7), 14:0 (10.5 % ± 0.4), 12:0 (8.9 % ± 1.2), 18:1 ω7c (7.6 % ± 1.8), summed feature 2 (4.1 % ± 0.6; comprising 14:0 3-OH and/or 16:1 iso I and/or unidentified fatty acid with equivalent chain length value of 10.928 and/or 12:0 ALDE), 12:0 3-OH (3.9 % ± 0.6), 18:00 (1.6 % ± 0.2) and 16:0 3-OH (0.7 % ± 0.1). Additional phenotypical features are listed in Table 3. The 16S rDNA sequences of strains LMG 20537^T, LMG 21351 and LMG 21352 are deposited in the EMBL under the accession numbers AJ491290, AJ316197 and AJ490152 respectively. The type strain of this species is LMG 20537^T (CAIM 578^T), isolated of bivalve (*Nodipecten nodosus*) larvae in the south of Brazil. The mol % G+C of DNA of the type strain is 44.1.

Description of *V. chagasii* sp. nov.

Vibrio chagasii (cha.ga.si.i N.L. gen. n. *chagasii* of Chagas, in honour to the Brazilian physician and microbiologist C. Chagas). Cells are slightly curved, 1 μm in width and 2-3 μm in length and motile by means of at least one polar flagellum. They form opaque, convex, non-swarming, smooth-rounded colonies with entire margin, dark beige in colour and 3-4 mm on TSA after 48 h incubation at 28 °C. No growth in absence of NaCl. All strains (except LMG 13251) formed green transparent colonies on TCBS agar. All strains utilise tween 40, tween 80, cellobiose, L-alanine, D-mannitol, psicose, α -keto glutaric acid as sole carbon source. None of the strains utilise L-arabinose, β -methyl D-glucoside, α -cyclodextrin, gentiobiose, α -D-lactose lactulose, putrescine, β -hydroxy butyric acid, S-saccharic acid, sebacic acid, L-ornithine and L-phenyl alanine. Strains do not produce tryptophane deaminase. The most abundant fatty acids in *V. chagasii* are summed feature 3 (38.4 % \pm 3.5), 16:0 (22.4 % \pm 3.9), 18:1 ω 7c (9.7 % \pm 1.6), 14:0 (7.2 % \pm 3.5), 16:0 iso (5.2 % \pm 2.6), 12:0 (3.8 % \pm 2.0), summed feature 2 (3.3 % \pm 1.3), 12:0 3-OH (2.7 % \pm 1.4), 18:0 (1.1 % \pm 0.5), 14:0 iso (1.1 % \pm 0.7), 15:0 (0.6 % \pm 0.3), 17:0 (0.5 % \pm 0.3), 14:0 iso 3-OH (0.5 % \pm 0.3). Additional phenotypical features are listed in Table 3. The 16S rDNA sequences of strains LMG 21353^T, LMG 13237 and LMG 21354 are deposited in the EMBL under the accession numbers AJ316199, AJ490157 and AJ490158, respectively. The type strain of this species is LMG 21353^T (CAIM 431^T), isolated from the gut of turbot larvae (*Scophthalmus maximus*) in Norway. The mol % G+C of DNA of the type strain is 44.5.

3.4. *Vibrio coralliilyticus* sp. nov., a temperature-dependent pathogen of the coral *Pocillopora damicornis*

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Int. J. Syst. Evol. Microbiol. (2003), 53, 309-315

Abstract

Vibrio sp. YB1^T (= ATCC BAA-450^T = LMG 20984^T), the etiological agent of tissue lysis of the coral *Pocillopora damicornis* was characterized as a new *Vibrio* species on the basis of 16S rDNA sequence, DNA-DNA hybridization data (mol % G + C is 45.6), AFLP and GTG₅-PCR genomic fingerprinting patterns and phenotypic properties, including the cellular fatty acid profile. The predominant fatty acids were 16:0 and 18:1 ω 7c. The name *Vibrio coralliilyticus* sp. nov. is proposed for the new coral pathogenic species. In addition to strain YB1^T which was isolated from the Indian Ocean, five additional strains of *V. coralliilyticus* have been isolated, three from diseased *P. damicornis* in the Red Sea, one from diseased oyster larvae (Kent, UK) and one from bivalve larvae (Brazil). The six *V. coralliilyticus* strains showed high genotypic and phenotypic similarities, and all were pathogenic to *P. damicornis*. The closest phylogenetic neighbors to *V. coralliilyticus* are *V. tubiashii*, *V. nereis* and *V. shiloi*.

Introduction

During the last two decades there has been a large increase in the frequency and distribution of coral diseases (Hoegh-Guldberg, 1999; Peters, 1997; Richardson, 1998). These diseases have altered both total abundance and species diversity (Loya et al., 2001). The causative agent(s) of only a few of these diseases have been reported: bleaching of *Oculina patagonica* by *Vibrio shiloi* (Kushmaro et al., 1996, 1997), black band disease by a microbial consortium (Carlton and Richardson, 1995), sea fan disease by *Aspergillus sydowii* (Geiser et al., 1998; Smith et al., 1996) and coral white plague possibly by a *Sphingomonas* sp. (Richardson et al., 1998). The etiological

agents of most of coral diseases are presently unknown. What is known is that most, if not all, the diseases occur at higher-than-normal seawater temperatures (Hoegh-Guldberg 1999; Rosenberg and Ben-Haim 2002). Since temperatures are expected to rise considerably during this century, it is likely that coral disease will become even more prevalent. Thus, there is an increasing need to identify and characterise coral pathogens.

Recently, we isolated a novel temperature-dependent pathogen of the coral *Pocillopora damicornis*, tentatively named *Vibrio* sp. YB1. At water temperatures above 26°C, a pure culture of this pathogenic strain caused a rapid destruction of the coral tissue within two weeks (Ben-Haim and Rosenberg, 2002). The present study was carried out in order to characterize *Vibrio* sp. YB1 and related strains using a polyphasic approach.

Material and Methods

Micro-organisms, media and growth conditions

V. coralliilyticus YB1^T (ATCC BAA-450^T, LMG 20984^T) was isolated from a diseased coral, *Pocillopora damicornis*, in the Indian Ocean near Zanzibar 1999, as previously described (Ben-Haim and Rosenberg, 2002). Strains LMG 21348, LMG 21349 and LMG 21350 were isolated from three different diseased *P. damicornis* colonies on the Eilat coral reef, Red Sea 2001. The bacteria were isolated from the crushed tissues of the corals, as described previously (Ben-Haim and Rosenberg, 2002). *V. shiloi* ATCC BAA-91^T (LMG 19703^T, DSM 13774^T) was isolated from the bleached coral *Oculina patagonica* (Kushmaro et al., 1996, 2001). The other two *Vibrio* strains (LMG 10953=NCIMB 2165 and LMG 20538=INCO 83) analysed in this study were obtained from BCCMTM/LMG Bacteria Collection at Ghent University (Table 1, Annex). Strains were routinely grown at 30°C in MBT medium (1.8 % marine broth 2216, [Difco], 0.9 % NaCl, 0.5 % Tryptone, [Difco]), on MB 2216 agar (1.8 % marine broth, 0.9 % NaCl, 1.8 % Agar), TSA medium (Tryptone soya agar; Oxoid) supplemented with 2 % NaCl, or on TCBS (Thiosulphate-citrate-bilesalts-sucrose; Difco) agar adjusted to 3 % NaCl. Liquid cultures were prepared in 125 ml flasks containing 10 ml MBT, incubated with shaking (160 rpm) for 24-48 h. Cultures were stored either at -70°C in 15 % glycerol or as lyophilized cells.

DNA isolation and genomic fingerprinting (FAFLP, BOX-PCR, GTG₅-PCR)

Bacterial DNA was extracted following the technique of Pitcher et al. (1989). FAFLP patterns were generated and analysed as described previously (Thompson et al., 2001). Briefly, 1 µg of high-molecular-weight DNA was digested with *TaqI* and *HindIII*, followed by ligation of restriction half-site specific adapters to all restricted fragments. Subsequently, two PCR amplification reactions were applied using primers H00/T00 and H01-6FAM/T03. Separation of the PCR products was generated on 36 cm denaturing polyacrylamide gels on an ABI Prism 377 DNA Sequencer (Applied Biosystems). Tracking and normalization of the lanes were performed by the GeneScan 3.1 software (Applied Biosystems). Normalized tables of peaks, containing fragments of 50 to 536 bp, were analysed with BioNumerics 2.0 software (Applied Maths). Similarity among band patterns was calculated using Dice similarity coefficient, and dendrograms were built using the Ward algorithm (Sneath and Sokal, 1973).

Rep-PCR fingerprinting using GTG₅ and BOX primers was performed as described previously (Rademaker et al., 1998). Briefly, GTG₅ -PCR reactions consisted of 1 µl of template DNA (50 ng µl⁻¹), 5 µl 5 x Gitschier buffer, 0.4 µl bovine serum albumin (10 mg ml⁻¹), 2.5 µl dimethyl sulfoxide (10 mg ml⁻¹), 1.25 µl of a dNTP mixture (100 mM of each dNTP), 1 µl GTG₅ primer or 1 µl BOX primer (both 0.3 µg µl⁻¹; Amersham Pharmacia Biotech, Sweden), and 0.4 µl *Taq* DNA polymerase (5U µl⁻¹; Goldstar RedTM). The PCR products generated with GTG₅ primer were electrophoresed in a 1.5 % agarose gel (w/v) and 1 x TAE buffer (1.21 g l⁻¹ Tris 2-amino-2 (hydroxymethyl)- 1,3 propanediol, 0.2 ml l⁻¹ 0.5 M EDTA, pH 8) at a constant 55 V for 900 min at 4°C. The PCR products generated with BOX primer were electrophoresed in a 2 % agarose gel (w/v) and 1 x TBE buffer (89 mM Tris + 89 mM boric acid + 2 mM EDTA, pH 8.3) at a constant 130 V for 222 min at 4°C. After staining with ethidium bromide, the digitized patterns were normalized and numerically analyzed using the software BioNumerics 2.0 (Applied Maths). Similarity among patterns was calculated based on the Pearson similarity coefficient, and dendrograms were built using UPGMA (Sneath and Sokal, 1973).

16S rDNA sequencing

Almost complete 16S rDNA sequences were obtained following the methodology described previously (Thompson et al., 2001). Briefly, fragments of the

16S rDNA were amplified by PCR using the conserved primers pA (16F27) and pH (16R1522) or MH1 (16F27) and MH2 (16R1485). Subsequently, purified products were used as templates for sequencing amplification using the ABI Prism™ Big Dye Terminator™ Ready Reaction Mix and eight primers (16F358, 16F536, 16F926, 16F1112, 16F1241, 16R339, 16R519 and 16R1093). Purified sequencing products were run on 48 cm denaturing polyacrylamide gels on an ABI Prism 377 DNA Sequencer. Sequences were assembled with the AutoAssembler software (Applied Biosystems). The consensus sequences were transferred into BioNumerics 2.0 software, where a phylogenetic tree was constructed based on the neighbour joining method (Saitou and Nei, 1986). The phylogenetic position of the consensus sequences was obtained using FASTA program (Pearson and Lipman, 1988) and compared to known *Vibrio* 16S rDNA sequences (Doresh et al., 1992; Ruimy et al., 1994; Mellado et al., 1996; Denner et al., 2002).

DNA-DNA hybridization experiments and mol % G+C measurement

DNA-DNA hybridization was performed at stringent conditions using Ezaki's microplate technique with photobiotin-labeled DNA at a temperature of 39°C for 3 h as described previously (Willems et al., 2001). Hybridization values are means of the reciprocal and non-reciprocal values each of which was performed in four replicates. The mol % G+C of DNA was determined by HPLC (Tamaoka and Komagata, 1984).

Phenotypic characterisation

Colony morphology was examined using a stereoscopic microscope. Cell morphology was examined by scanning electron microscopy (JEOL 840A). Exponentially growing bacteria in MBT medium were adhered to a carbon-coated grid and negatively stained with 1 % uranyl acetate. Classical phenotypical tests were performed by standard methodologies (Farmer and Hickman-Brenner, 1992). Biochemical tests were performed by API-20 NE (micromethod tests for the identification of non-enteric Gram-negative rods, BioMérieux). The standard API-20 NE protocol was used except that media were adjusted to 3 % NaCl. NaCl tolerance was determined in MBT medium containing varying concentrations (1-15 %) of NaCl. Sensitivity to the vibriostatic compound 0129 (2,4-diamino-6,7-diisopropylpteridine; Sigma) was determined after incubation for 48 h at 30°C on MB

Agar containing 30 µg of the compound on a disc. Sensitivity to antibiotics was examined using either the minimal inhibition concentration (MIC) method for erythromycin (24 µg ml⁻¹), tetracycline (20 µg ml⁻¹), chloramphenicol (6 µg ml⁻¹) and kanamycin (50 µg ml⁻¹), or by the paper disc method for penicillin (10 µg disc⁻¹) and ampicillin (10 µg disc⁻¹). Growth was also tested on MB agar containing 200 µg ml⁻¹ gentamycin. Carbon utilization was carried out using Biolog GN2 MicroPlate™ (Biolog Inc. Hayward CA, USA). Pure cultures (12-18 h growth) were harvested from MB Agar plates and suspended in 20 ml GN/GP Inoculating Fluid (Biolog), adjusted to 3 % NaCl, to a density of 0.130 to 0.143 at A₆₀₀. The suspension was then distributed into Biolog GN2 microwell plates. Each plate contained 96 microwells with 95 different carbon sources in each and tetrazolium violet as an indicator of metabolic activity. The plates were incubated for 48 h at 30°C. Wells that changed to purple were marked positive for metabolic utilization. Fatty acid analysis was performed following the protocol of the Microbial Identification System (Microbial ID Inc., USA) as described previously (Huys et al., 1994). Strains for analysis were grown on TSA for 48 h at 28°C. The ability of each strain to infect the coral *P. damicornis* in controlled aquaria experiments was examined as described previously (Ben-Haim and Rosenberg 2002).

Results

V. coralliilyticus YB1^T is a Gram-negative, motile, rod-shaped bacterium (1.2-1.5 x 0.8 µm) that has a single polar, sheathed flagellum (Figure 3.7A). These properties, together with its ability to form yellow colonies on TCBS agar and its sensitivity to vibriostatic compound 0129, suggests that YB1^T is a species of the genus *Vibrio* (Farmer and Hickman-Brenner, 1992). The 16S rDNA sequences of YB1^T (1465 bp in length; EMBL accession no. AJ 440005), LMG 21349 (1468 bp in length; EMBL accession no. AJ440004) and LMG 10953 (1468 bp in length; EMBL accession no. AJ316167) were allocated to the γ-Proteobacteria using the FASTA program. The above three *Vibrio* strains formed a tight cluster with more than 99 % 16S rDNA similarity (Figure 3.7B). Their closest phylogenetic neighbours were *V. tubiashii* (97.2 %), *V. nereis* (96.8 %) and *V. shiloi* (96.6 %). AFLP, BOX-PCR and GTG₅-PCR analysis revealed that the strains possess typical genomes, consisting of 102 ± 7, 19 ± 3 and 23 ± 2 bands, respectively (Figure 3.8).

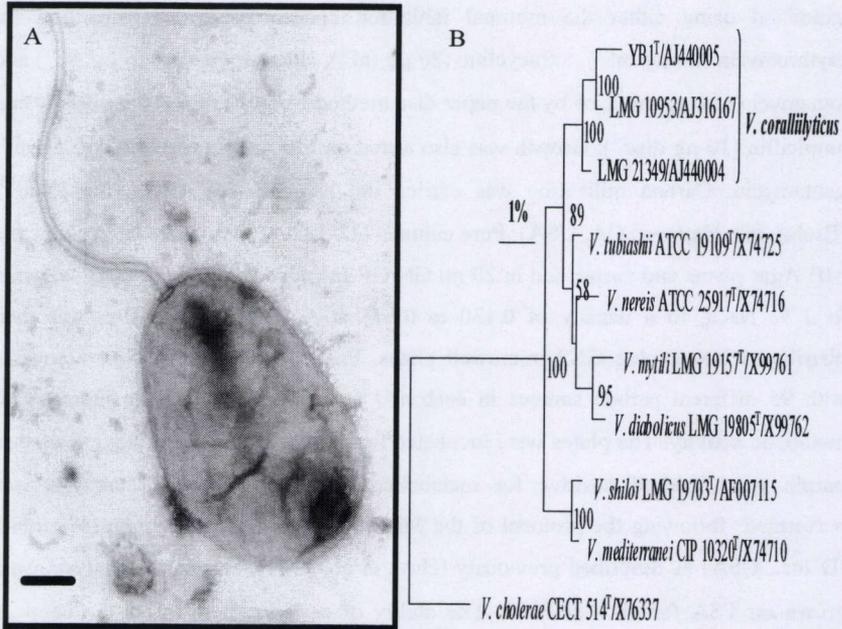


Figure 3.7. The bacterium *V. coralliilyticus*. A) Electron micrograph of negatively stained *V. coralliilyticus* YB1^T. Bar, 0.2 μm . B) Phylogenetic tree showing the relationships between *V. coralliilyticus* and other representative species of the family *Vibrionaceae*, based on the neighbour joining method based on the almost complete 16S rDNA sequences. Bootstrap values after 1000 simulations are shown. Bar, 1 % estimated sequence divergence.

The inner AFLP and GTG₅-PCR pattern similarities were higher than 64 %. It has recently been shown that AFLP and rep-PCR similarities around 65 % represent more than 70 % DNA-DNA similarity (Rademaker et al., 2000). It was clearly demonstrated by the three fingerprinting methodologies that the six *Vibrio* isolates form a tight genomic group, which is distinguishable from all other closely related *Vibrio* species. Surprisingly, strains YB1^T and LMG 21350 showed very similar patterns by AFLP and GTG₅-PCR, although YB1^T originated from the Indian Ocean and LMG 21350 from the Red Sea. Overall, the strains have less than 40 % pattern similarity towards their closest phylogenetic neighbours analysed by the three fingerprinting methodologies. DNA-DNA hybridization data (Table 3.11) confirmed that the six isolates form a single genomic group with DNA-DNA similarities higher than 85 %. DNA-DNA similarity to *V. nereis* and *V. tubiashii* was only 31 %

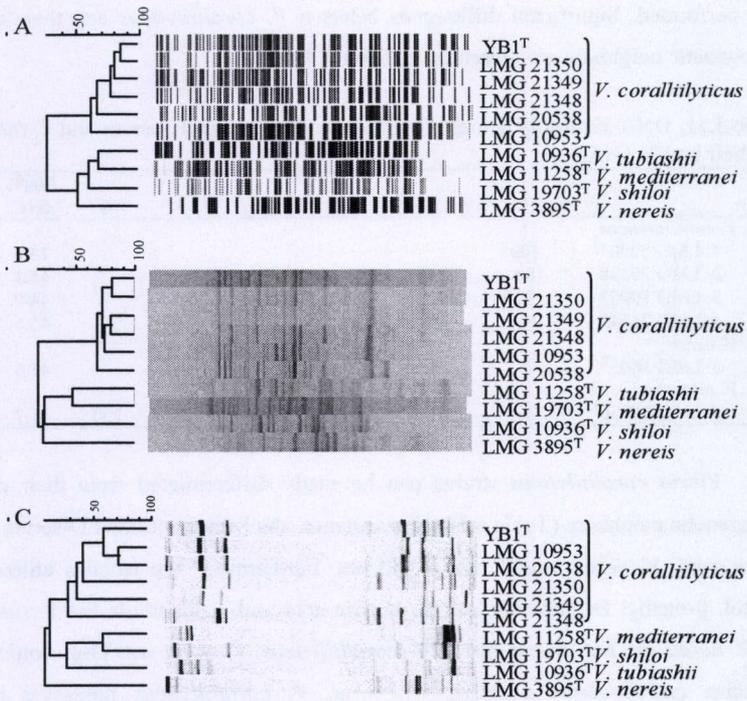


Figure 3.8. Genomic fingerprinting of *V. coralliilyticus*. A) AFLP, B) BOX and C) GTG₅ clustering analysis of *V. coralliilyticus* (n=6) and closest phylogenetic neighbours.

and 27 % respectively. DNA-DNA similarities to *V. shiloi*, *V. diabolicus* and *V. mytili* were lower than 24 %. Their mol % G + C of their DNA ranged from 44.9-45.6 %. *Vibrio coralliilyticus* conforms to the description of the family *Vibrionaceae* and of the genus *Vibrio* (Farmer and Hickman-Brenner, 1992). The cellular fatty acid compositions of six *V. coralliilyticus* strains are presented in Table 3.12. The *V. coralliilyticus* strains had similar overall fatty acid profiles, congruent with those known for the family *Vibrionaceae* (Bertone et al., 1996; Urdaci et al., 1990). Biochemical tests and carbon compound utilization differences between the six isolates are shown in Table 3.13. Of the 117 tests performed, the strains gave the same qualitative result in 110 cases. YB1^T and LMG 21350 gave identical results in all 117 phenotypical tests, although YB1^T was isolated from the Indian Ocean and LMG 21350 from the Red Sea. The other strains differed from YB1^T in only 1-5 of the 117

tests performed. Significant differences between *V. coralliilyticus* and their closest phylogenetic neighbors are presented in Table 3.14.

Table 3.11. DNA similarity among *V. coralliilyticus* strains, *V. nereis* and *V. tubiashii* and their mol % G+C.

	1	2	3	4	5	6	Mol% G+C
<i>V. coralliilyticus</i>							
1. LMG 20984 ^T	100						45.6
2. LMG 20538	86	100					45.1
3. LMG 10953	98	97	100				44.9
4. LMG 21349	87	88	89	100			45.6
<i>V. nereis</i>							
5. LMG 3895 ^T	27	31	30	30	100		45.6
<i>V. tubiashii</i>							
6. LMG 10936 ^T	26	27	26	26	25	100	45.7

Vibrio coralliilyticus strains can be easily differentiated from their closest phylogenetic neighbors (Table 3.14). For instance, the isolates utilized D-serine while *V. tubiashii*, *V. nereis* and *V. shiloi* did not. Furthermore, the isolates utilized inositol, β -methyl D-glucoside, α -keto butyric acid and alaninamide but *V. tubiashii* and *V. nereis* did not. In contrast to *V. coralliilyticus*, *V. shiloi* was D-gluconic acid, L-leucine and D-serine negative. Moreover, *V. coralliilyticus* possesses higher amounts of the fatty acids 17:0 and 17:0 iso than *V. tubiashii* and *V. nereis* and higher amounts of 17:0 and 18:1 ω 7c than *V. shiloi*.

Table 3.12. Fatty acid profiles of *V. coralliilyticus* strains.

	YB1 ^T	LMG 21348	LMG 21349	LMG 21350	LMG 10953	LMG 20538
Fatty acid:						
12:0	2.2	3.4	3.8	3.8	2.8	3.6
12:0 3-OH	2.7	3.1	3.9	3.7	2.0	2.8
13:0 iso	2.6	2.3	2.6	2.4	2.8	1.7
14:0	6.5	8.1	7.8	8.3	6.9	8.5
14:0 iso	0.5	0.2	0.3	0.2	0.7	0.8
15:0	1.3	1.8	2.0	1.9	1.8	2.8
15:0 iso	2.8	1.1	1.3	1.5	2.3	1.0
15:0 iso 3-OH	1.7	1.3	1.5	1.4	1.1	0.6
16:0	14.1	15.2	14.0	15.5	14.6	16.5
17:0	2.0	2.6	3.0	2.4	2.5	2.8
17:0 iso	3.2	1.7	1.8	2.0	2.4	0.9
17:1 ω 8c	1.3	1.7	2.1	1.7	1.6	2.1
18:1 ω 7c	18.2	20.6	18.0	18.6	19.1	14.5

Table 3.13. Biochemical test and carbon compound utilization differences between *Vibrio coralliilyticus* strains*

	YB1 [†]	LMG 10953	LMG 20538	LMG 21348	LMG 21349	LMG 21350
Arginine dehydrolase	+	-	-	-	-	+
Carbon compound utilization:						
Citrate	+	+	+	+	-	+
β-methyl D-glucoside	-	-	-	+	+	-
Propionic acid	+	+	+	+	-	+
Methyl pyruvate	+	+	+	-	+	+
Glucose-1-phosphate	+	+	+	+	-	+
D-mannitol	+	-	+	+	+	+

* Except for tests included here, all six *V. coralliilyticus* strains gave the same qualitative results with API-20 NE system and Biolog GN2 MicroPlate™ test (see species description).

Table 3.14. Differentiating phenotypical features of *V. coralliilyticus* and closest related species.

	<i>V. coralliilyticus</i>	<i>V. tubiashii</i>	<i>V. nereis</i>	<i>V. shiloi</i>
Utilization of:				
Cellobiose	-	+	-	+
D-galactose	+	+	-	+
m-inositol	+	-	-	V
B-methyl D-glucoside	+	-	-	+
Acetic acid	+	-	V	+
cis-aconitic acid	+	-	+	+
D-gluconic acid	+	+	+	-
β-hydroxy butyric acid	-	V	+	V
A-keto butyric acid	+	-	-	V
Alaninamide	+	-	-	+
L-histidine	+	-	V	+
Hydroxy L-proline	+	-	+	+
L-leucine	+	-	+	-
D-serine	+	-	-	-
Fermentation of:				
Amygdalin	-	+	+	+
Melibiose	-	V	-	V
Growth on 8 % NaCl	-	V	+	V
FAME composition:				
17:0	2.6 ± 0.3	0.5 ± 0.6	1.2 ± 1.1	0.2 ± 0.0
17:0 iso	2.0 ± 0.8	0.0	0.4 ± 0.5	4.2 ± 2.1
17:1 ω8c	1.8 ± 0.3	1.0 ± 1.4	2.7 ± 2.8	0.6 ± 0.6
18:1 ω7c	18.2 ± 2.0	21.6 ± 5.2	21.6 ± 1.5	12.1 ± 1.9

FAME analysis included *V. coralliilyticus* (n=6), *V. tubiashii* (n=4), *V. nereis* (n=2), *V. shiloi* (n=5). Phenotypic data for *V. nereis* and *V. tubiashii* were obtained from Holt et al. (1994). V, variable.

Because of the high genotypic and phenotypic similarities between the six *Vibrio* isolates, we examined the ability of each isolate to infect the coral *P. damicornis* in controlled aquaria experiments. All six strains were pathogenic, causing similar tissue damage within two weeks at 29 °C, whereas *P. damicornis* corals infected with *V. shiloi* under similar experimental conditions remained healthy.

Discussion

Vibrio sp. YB1^T has been reported to be the etiological agent of tissue damage to the coral *P. damicornis* (Ben-Haim and Rosenberg, 2002). From the data presented here, the strain is clearly a new *Vibrio* species. During the course of this investigation three additional pathogenic *Vibrio* strains (LMG 21348, LMG 21349, LMG 21350) were isolated from diseased *P. damicornis* in the Red Sea, and other two strains LMG 20538 and LMG 10953 present in the BCCMTM/LMG Bacteria Collection were also proven to be closely related to *Vibrio* sp. YB1^T. These six strains form a tight cluster, based on genotypic and phenotypic properties, that is significantly different from other *Vibrio* species. Based upon these results, we propose the name *V. coralliilyticus* for this novel pathogenic species.

One of the most interesting findings of this study was the demonstration of the power of bacterial taxonomy to uncover novel coral pathogenic strains. *Vibrio* strains LMG 10953 and LMG 20538, isolated from diseased *Crassostrea gigas* larvae and *Nodipecten nodosus* larvae, respectively, were shown to be pathogenic to the coral *P. damicornis*. Strain LMG 10953 was previously identified as *V. tubiashii* (Hada et al., 1984). Our results clearly demonstrate that this strain belongs to the new species *V. coralliilyticus*.

The fact that coral pathogenic strains of *V. coralliilyticus* are widely distributed is important in understanding the source of coral disease and attempts to prevent their spread. *V. coralliilyticus* infects and causes tissue damage to its host coral only at water temperatures above 25°C. It is likely that during the winter when temperatures are lower, the bacterium is present in different hosts, possibly bivalve larvae. Clearly, the taxonomic studies described here open new and unexpected avenues of investigation regarding the transmission of coral diseases.

Description of *Vibrio coralliilyticus* sp. nov.

Vibrio coralliilyticus (co.ral.lii.ly'ti.cus; L.n.. corallium, coral; Gr. adj. lytikos, dissolving; M.L. adj. *coralliilyticus*, coral dissolving). *Vibrio coralliilyticus* conforms to the description of the family *Vibrionaceae* and of the genus *Vibrio* (Farmer and Hickman-Brenner, 1992). Cells are Gram-negative, non-spore-forming rods (1.2-1.5 x 0.8 μm) that are motile by a single polar, sheathed flagellum when grown on solid or liquid medium. Colonies are cream colored on marine agar and yellow on TCBS agar after 48 h incubation at 30°C, have smooth edges and do not luminesce. The predominant cellular fatty acids are 16:0 and 18:1 ω 7c. *V. coralliilyticus* strains are oxidase- and catalase- positive. Produce acid from glucose. Reduce nitrate to nitrite and produce indole. *V. coralliilyticus* strains show positive reactions for β -glucosidase, β -galactosidase and gelatinase and are urease negative. Cells utilize D- and L- alanine, L-histidine, hydroxyproline, L-ornithine, L-proline, D-serine, thymidine, D-mannitol, L-aspartic acid, L-histidine, α -aminobutyric acid, D-galactose, methyl succinate, D-gluconic acid, succinic acid, bromosuccinic acid, alaninamide, L-serine, L-threonine, uridine, glycerol, glucose 6-phosphate, dextrin, glycogen, D,L- α -glycerol phosphate, Tween 40 and 80, N-acetyl glucosamine, D-fructose, maltose, D-mannose, D-psicose, sucrose, L-alanyl-glycine, L-asparagine, L-glutamic acid, glycy-L-aspartic acid and glycy-L-glutamic acid. Susceptible to 2,4-diamino-6,7-diisopropylpteridine (0129, vibriostatic agent), erythromycin, tetracycline, chloramphenicol and gentamycin and resistant to kanamycin, ampicillin and penicillin. Growth occurred over a wide range of salinity conditions (1-7 % NaCl); bacteria failed to grow in media containing 0% and 8 % or higher NaCl. Variable biochemical features are summarized in Table 3.13. The type strain YB1^T (LMG 20984^T, ATCC BAA-450^T) was isolated from a diseased coral, *Pocillopora damicornis*, in the Indian Ocean. Further strains of *Vibrio coralliilyticus* have been isolated from diseased *P. damicornis* in the Red Sea and from larvae of oyster in the Atlantic Ocean. All six strains described here are pathogenic to the coral *P. damicornis*. The mol % G+C of the type strain is 45.6. The EMBL accession number is AJ440005.

**3.5. Description of *Vibrio fortis* sp. nov. and *V. hepatarius* sp. nov.,
isolated
from aquatic animals and the marine environment**

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Int. J. Syst. Evol. Microbiol. (2003), in press

Abstract

In this study we evaluated the taxonomic position of nineteen *Vibrio* isolates disclosed in a previous study. Phylogenetic analysis based on the 16S rDNA sequences partitioned these isolates in two groups closely related (98.8 to 99.1 % similarity) to *V. pelagius* and *V. xuii*, respectively. DNA-DNA hybridisation experiments further showed that these groups had less than 70 % similarity towards other *Vibrio* species. We propose to accommodate the two groups in two new *Vibrio* species i.e. *V. fortis* sp. nov. (type strain is LMG 21557^T = CAIM 629^T, EMBL accession no. AJ514916; mol % of G+C is 45.6) and *V. hepatarius* sp. nov. (type strain is LMG 20362^T = CAIM 693^T; EMBL accession no. AJ345063; mol % of G+C is 45.6). Useful phenotypic features for discriminating *V. fortis* and *V. hepatarius* from other *Vibrio* species include production of indole and acetoin, utilisation of cellobiose, fermentation of amygdaline, melibiose and mannitol, β -galactosidase and tryptophane deaminase activity and fatty acid composition.

Introduction

Vibrios are among the most abundant culturable microbes in aquatic environments (Heideberg et al., 2002a). A recent study on the bacterioplankton of Chesapeake Bay has shown *Vibrio* and *Photobacterium* comprise up to 4 % (2×10^8 cells/liter) of the total *Bacteria* (Heidelberg et al., 2002b). High *Vibrio* and *Photobacterium* numbers ($4.3 \times 10^6/\text{mm}^2$) were also reported attached to the external surface of zooplankton. It was concluded a close partnership exists between these bacteria and the zooplankton (Heidelberg et al., 2002a; Lipp et al., 2002). Vibrios also

belong to the normal microflora of the shrimp *Litopenaeus vannamei* (Vandenberghé et al., 1999). Moss et al. (2000) reported *Vibrio* and *Aeromonas* compose up to 85 % (about 10^9 CFU/gram of gut tissue) of the bacterial flora in the gut of this shrimp, whereas Gomez-Gil et al. (1998) found high abundance of vibrios i.e. 10^5 CFU/g and 10^4 CFU/ml, respectively, in the hepatopancreas and the hemolymph of healthy *L. vannamei*. Certain *Vibrio* strains have been reported as potential probiotics for this shrimp (Gomez-Gil et al., 1998, 2000, 2002). The use of probiotics, i.e. live microorganisms which when administered in adequate amounts confer a health benefit on the host, has been claimed to reduce the need for medication (e.g. antibiotics and pesticides) and water exchange which are massively used in intensive shrimp rearing (Verschuere et al., 2000).

We have demonstrated that the genus *Vibrio* harbours a wealth of diverse genomes representing both cosmopolitan and endemic species as yet to be described (Thompson et al., 2001). The exact ecological role of several of these groups is unknown at present. In this study we report on the taxonomic characterisation of the FAFLP clusters A9, A26 and A60 disclosed in a former study (Thompson et al., 2001). Group A9 (n=8) was found to be ubiquitous in the marine environment, being associated with both diseased and healthy aquatic animals. Group A26 consisted of three isolates originated from the hepatopancreas of wild healthy adults of *L. vannamei* from Ecuador. Recent results may suggest that these isolates have probiotic properties for *L. vannamei* under culture conditions (Gullian and Rodriguez, *in press*). A representative strain LMG 20362^T showed high levels of colonization in the hepatopancreas of *L. vannamei*, out-competing and excluding the shrimp pathogen *V. harveyi*. Additionally, this strain seems to enhance shrimps health and weight (Gullian and Rodriguez, *in press*). Group 60 (n=8) was restricted to cultures of bivalve larvae (*Nodipecten nodosus*) in the south of Brazil. We propose to accommodate A9 and A60 isolates in a new *Vibrio* species i.e. *V. fortis* and A26 isolates in another i.e. *V. hepatarius*.

Material and Methods

Bacterial strains, growth conditions and DNA isolation

Strains characterised in this study are listed in Table 1 (Annex). Strains were grown aerobically on tryptone soy agar (TSA; Oxoid) supplemented with 2 % (w/v) NaCl for 24 h at 28 °C. DNA was extracted following the methodology described by

Pitcher et al. (1989). All strains included in this study are deposited in the BCCMTM/LMG Bacteria Collection at Ghent University and in the CAIM collection of the Centre for Research on Nutrition and Development (CIAD) in Mazatlán, México.

Genotypic analyses

Sequencing of the almost complete 16S rDNA sequences were accomplished essentially as described previously (Thompson et al., 2001). Alignment of the 16S rDNA sequences, distance estimations (Jukes and Cantor, 1969), clustering by neighbour joining (Saitou and Nei, 1987), maximum likelihood and maximum parsimony methods and stability of the clusters (Bootstrap analysis with 1000 replicates) were performed with the software BioNumerics 2.5 (Applied Maths). DNA-DNA hybridisation experiments using photobiotin-labelled DNAs were run at stringent conditions (39 °C) following the methodology described by Willems et al. (2001). Hybridisations were performed in four replicates. DNA binding values are the mean of reciprocal and non-reciprocal reactions. The mol % G+C of DNA was determined by HPLC (Mesbah et al., 1989).

Phenotypic analyses

Phenotypic characterisation of the isolates was performed using API 20E, API ZYM (bioMérieux) and Biolog GN metabolic fingerprinting (Biolog) following the instructions of the manufacturer, with slight modifications (Thompson et al., 2002). Classical phenotypic tests were performed as described previously (Baumann et al., 1984; Delafield et al., 1965; Farmer III and Whickman-Brenner, 1992; Thompson et al., 2002; Vandamme et al., 1998). Antibigrams were carried out using the disc diffusion methodology (Acar and Goldstein, 1996) using commercial discs (Oxoid). The inhibition zone of each antibiotic was measured on strains grown on Iso-sensitest agar (Oxoid) supplemented with 1.5 % (w/v) NaCl for 24 hours at 28 °C. Fatty acid methyl esters (FAME) analysis was carried out as described by Huys et al. (2001). Isolates were grown on Trypticase Soy Broth (Becton Dickinson) supplemented with 1.5 % (w/v) Bacto agar (Becton Dickinson) and 1.5 % (w/v) NaCl.

Results and Discussion

The phylogenetic position of five *V. fortis* representative strains LMG 21557^T (EMBL accession no. AJ514916), LMG 21558 (AJ514913), LMG 20547 (AJ316202), LMG 21566 (AJ514917), LMG 21562 (AJ514915), from both FAFLP groups A9 and A60, were analysed by means of 16S rDNA sequences. These isolates had nearly identical 16S rDNA sequences (Figure 3.9), and their closest neighbour was *V. pelagius* CECT 4202^T (EMBL accession no. AJ293802) with 98.8 % similarity. When we used the sequence of Ruimy et al. (1994), i.e. EMBL accession no. X74722, the similarity between the five novel isolates and *V. pelagius* dropped to 97.4 %. Macián et al. (2000) have already highlighted that most probably the latter EMBL entry is in fact a *V. natriegens* sequence rather than a *V. pelagius* one. The other phylogenetic neighbours of *V. fortis*, having at maximum 97.8 % similarity, are shown in Fig. 3.9. *V. cholerae*, *V. mimicus* and *V. metschnikovii* were the most distant phylogenetic relatives of *V. fortis* within the genus *Vibrio*, having 92.5-92.9 % similarity. *Vibrio hepatarius* LMG 20362^T was closest related to the newly described *V. xuii* (99.1 % similarity) (Thompson et al., 2003), and to *V. tubiashii* and *V. nereis* (98.6-99 %) (Figure 3.9). *Vibrio hepatarius* was related to *V. mytili*, *V. diabolicus* and *V. orientalis* (98-98.2 %), and its most distant relatives within the genus *Vibrio* were *V. mimicus* and *V. salmonicida* (92.9 %).

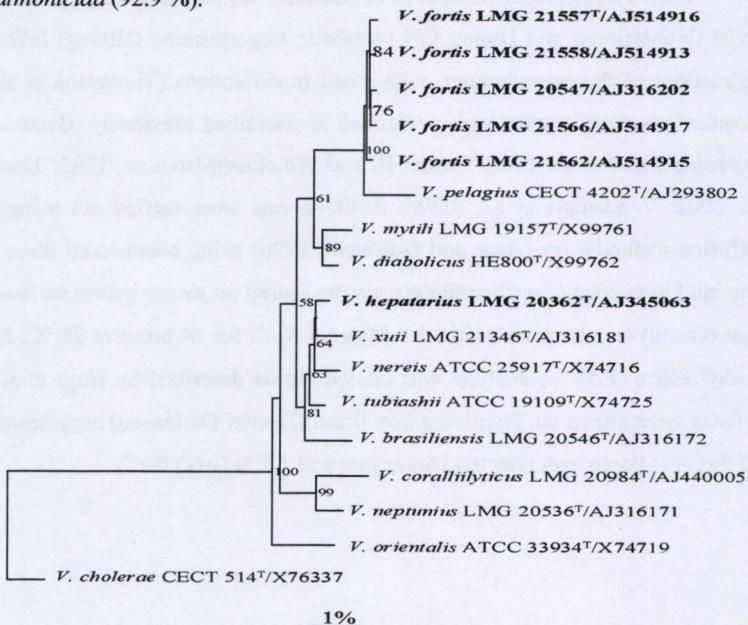


Figure 3.9. Phylogenetic tree with the estimated position of *V. fortis* and *V. hepatarius* using the neighbour joining method based on the almost complete 16S rDNA sequences. Bootstrap values (> 50 %) after 1000 simulations are shown. Bar, 1 % estimated sequence divergence.

The results of DNA-DNA hybridisation experiments are summarised in Table 3.15. We chose four representative isolates from cluster A9, because this group was found to be very heterogeneous by FAFLP analysis, and two representative isolates from A60 which was a very tight FAFLP cluster (Thompson et al., 2001). The six *V. fortis* isolates formed a single new species with at least 70 % DNA-DNA similarity, and at maximum 66 % similarity towards *V. pelagius*. Whereas *V. hepatarius* LMG 20362^T had at maximum 66 % similarity towards *V. orientalis*. The two new species had < 45 % DNA-DNA similarity towards the newly described *V. corallilyticus* (Ben-Haim et al., 2003) and *V. neptunius*, *V. brasiliensis* and *V. xuii* (Thompson et al., 2003). These results corroborate our previous findings with FAFLP fingerprinting which suggested that the groups A9, A26 and A60 were new species within the genus *Vibrio* (Thompson et al., 2001).

The two novel *Vibrio* species represented by the 19 isolates examined in this study shared the main phenotypic and chemotaxonomic features of the genus *Vibrio* (Bertone et al., 1996; Farmer III and Hickman-Brenner, 1992; Lambert et al., 1983). They were facultative anaerobic, oxidase positive and showed prolific growth on thiosulfate-citrate-bile salts-sucrose agar (TCBS). Isolates were slightly curved rods, motile, susceptible to the vibriostatic agent 0/129 (except LMG 21568 and LMG 21559), and had growth stimulated by NaCl. In spite of their similarity towards other *Vibrio* species, the two new species showed several differentiating phenotypic features (Table 3.16). We therefore propose to accommodate A9 and A60 isolates in a new species i.e. *V. fortis* and A26 isolates in another i.e. *V. hepatarius*. Because several phylogenetic neighbours (i.e. *V. neptunius*, *V. brasiliensis*, *V. xuii*, *V. corallilyticus*) of *V. fortis* and *V. hepatarius* were phenotypically analysed and described using the same methodologies used in this study, we assume that these results are largely comparable. Similar phenotypic methodologies have also been applied to the description of *V. diabolicus* (Ragu n s et al., 1997), *V. mytili* (Pujalte et al., 1993) and the two novel species in this study, suggesting that comparisons may be reliable.

Table 3.15. DNA-DNA binding values and mol % G+C of DNA of *Vibrio* strains examined.

	Mol % G+C	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
<i>V. fortis</i>																			
1. LMG 21557 ^T	45.6	100																	
2. LMG 21558	45.3	95	100																
3. LMG 21562	45.6	78	85	100															
4. LMG 21561	45.8	70		81	100														
5. LMG 20547	45.9	78	89	100	83	100													
6. LMG 21565	45.9	71		91	85	98	100												
<i>V. pelagius</i>																			
7. LMG 3897 ^T	45.7	58		65	66	66	65	100											
<i>V. coralliilyticus</i>																			
8. LMG 20984 ^T	45.6	34	40	33		30			100										
<i>V. neptunius</i>																			
9. LMG 20536 ^T	46.0	30	37	30		27				100									
<i>V. brasiliensis</i>																			
10. LMG 20546 ^T	45.9	35	44	34		34			39	40	100								
<i>V. xuii</i>																			
11. LMG 21346 ^T	46.6	30	38	29		27			34	32	39	100							
<i>V. hepatarius</i>																			
12. LMG 20362 ^T	45.5	23				25			40	34	42	30	100						
<i>V. diabolicus</i>																			
13. LMG 19805 ^T	45.6	24				25							28	100					
<i>V. tubiashii</i>																			
14. LMG 10936 ^T	44.8	20				23							29	24	100				
<i>V. nereis</i>																			
15. LMG 3895 ^T	45.9	23				26							30	32	26	100			
<i>V. orientalis</i>																			
16. LMG 7897 ^T	45.6	23				26							66	27	30	32	100		
<i>V. mytili</i>																			
17. LMG 19157 ^T	44.6	20				22							22	33	20	26	22	100	

Table 3.16. Differentiating features among *V. fortis*, *V. hepatarius* and closely related *Vibrio* species

	1	2	3	4	5	6	7	8	9	10	11	12
Production of:												
Indole	+	+	+	+	+	-	+	+	-	V	+	+
Acetoin	V+	+	+	+	+	-	-	-	-	-	-	-
Utilization of:												
Cellobiose	+	+	-	+	+	-	-	-	+	-	+	+
D-Galactose	+	V+	-	+	-	+	+	+	+	-	+	+
Gentiobiose	+	V-	-	+	V	V	-	-	+	-	ND	-
Growth on 10 % (w/v) NaCl	V-	+	-	-	+	-	-	ND	+	+	+	v
Nitrate reduction	V+	+	+	+	+	+	+	+	+	+	+	+
Fermentation of:												
Mannitol	+	+	-	+	+	+	-	+	+	-	+	+
Amygdaline	V+	+	-	+	+	-	-	-	+	-	-	-
Melibiose	V+	-	-	-	-	-	-	-	-	-	-	v
Enzyme activity:												
β -galactosidase	V+	-	-	+	-	+	+	-	+	-	-	+
Tryptophane deaminase	+	V+	+	-	+	-	-	+	+	-	-	-
FAME composition:												
14:0 iso	2.7 \pm 1.5	1.3 \pm 1.1	0.2 \pm 0.1	3.3 \pm 0.4	1.2 \pm 0.1	2.5	0.5	0.2	0.0	0.2	0.6	0.0
14:0 iso 3-OH	1.1 \pm 0.6	0.8 \pm 0.6	0.1 \pm 0.1	1.3 \pm 0.2	0.9 \pm 0.1	1.0	0.3	0.3	0.3	0.3	0.0	0.0
16:0	18 \pm 7	20.0 \pm 2.8	18.0 \pm 0.8	11.3 \pm 0.3	12.5 \pm 0.6	12	15	14.4	18.8	12.9	27.6	17.3
16:0 iso	8.4 \pm 4.3	4.8 \pm 3.9	0.5 \pm 0.1	10.5 \pm 0.6	5.5 \pm 0.4	7.5	0.8	1.7	1.5	1.1	0.0	0.0
17:0	0.5 \pm 0.9	0.3 \pm 0.1	2.3 \pm 0.2	0.6 \pm 0.1	0.5 \pm 0.1	0.5	2.5	1.6	0.0	1.9	0.0	0.1
17:1 ω 8c	0.7 \pm 1.9	0.2 \pm 0.2	2.1 \pm 0.1	0.7 \pm 0.1	0.5	0.5	1.8	2.5	0.0	4.6	0.0	0.1
17:1 ω 6c	0.1 \pm 0.1	0.0	1.2 \pm 0.1	0.3 \pm 0.1	0.2	0.0	0.6	0.7	0.0	1.3	0.0	0.0
18:1 ω 7c	11.9 \pm 2.9	15.5 \pm 0.5	17.8 \pm 1.6	17.3 \pm 0.3	21.0 \pm 2.4	23	18.2	17.4	19.9	22.6	4.3	25.4

1, *V. fortis* (n= 16); 2, *V. hepatarius* (n=3); 3, *V. neptunius* (Thompson et al., 2003); 4, *V. brasiliensis* (Thompson et al., 2003); 5, *V. xiii* (Thompson et al., 2003); 6, *V. pelagius* (Alsina and Blanch, 1994); 7, *V. coralliilyticus* (Bem-Haim et al., 2003); 8, *V. diabolicus* (Raguénès et al., 1997); 9, *V. mytili* (Pujalte et al., 1993); 10, *V. nereis* (Alsina and Blanch, 1994); 11, *V. orientalis* (Alsina and Blanch, 1994); 12, *V. tubiashii* (Alsina and Blanch, 1994). Fatty acids are mean \pm SD. ND, no data available. V, variable feature. V is followed by the type strain feature. Utilisation of gentiobiose, fermentation of amygdaline and melibiose, enzyme activities, fatty acid profiles of know *Vibrio* species are from our own database.

Description of *V. fortis* sp. nov.

Vibrio fortis (for.tis. M. L. adj. *fortis* of strong, a strong bacterium). Cells are slightly curved, 1 μm in width and 3 μm in length. They form translucent to opaque, low-convex, non-swarming, smooth-rounded colonies with entire margin, beige in colour and of about 4 mm in diameter on TSA after 48 h incubation at 28 °C. Strains formed yellow and/or green, translucent, smooth-rounded colonies of 4-5 mm on TCBS. All strains have a facultative anaerobic metabolism and ferment glucose and mannitol, but not inositol, sorbitol and rhamnose. Growth occurs at 4 to 35 °C and in media containing 1-8 % (w/v) NaCl. Prolific growth occurs at 28 °C in media containing 2.5 % (w/v) NaCl. The following features are positive for all strains: oxidase, catalase, indole, β -galactosidase and tryptophane deaminase. All strains utilise dextrin, glycogen, Tween 40, Tween 80, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, cellobiose, D-fructose, D-galactose, gentiobiose, α -D-glucose, maltose, D-mannitol, D-mannose, psicose, D-sorbitol, sucrose, D-trehalose, methyl pyruvate, mono methyl succinate, D-gluconic acid, D,L-lactic acid, succinic acid, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-proline, L-serine, L-threonine, inosine, thymidine, glycerol as sole carbon source. None of the strains utilise adonitol, D-arabitol, i-erythritol, L-fucose, M-inositol, L-rhamnose, xylitol, citric acid, formic acid, D-galactonic acid lactone, D-glucuronic acid, α -hydroxy butyric acid, P-hydroxy phenylacetic acid, itaconic acid, α -keto butyric acid, malonic acid, D-saccharic acid, sebacic acid, succinamic acid, glucuronamide, alaninamide, hydroxy L-proline, L-phenyl alanine, L-pyro glutamic acid, D,L-carnitine, urocanic acid, phenyl ethylamine, 2-amino ethanol, 2,3-butanediol, glucose-1-phosphate as sole carbon source. The following tests are negative for all strains: arginine dihydrolase, lysine and ornithine decarboxylases, H₂S production and urease. The most abundant fatty acids are summed feature 3 (37.5 % \pm 2.9; comprising 16:1 ω 7c and/or 15:0 iso 2-OH), 16:0 (18.0 % \pm 7.0), 18:1 ω 7c (11.9 % \pm 2.9), 16:0 iso (8.4 % \pm 4.3), 14:0 (5.3 % \pm 1.4), 12:0 (3.6 % \pm 1.2), 14:0 iso (2.7 % \pm 1.5), summed feature 2 (2.4 % \pm 0.4; comprising 14:0 3-OH and/or 16:1 iso I and/or unidentified fatty acid with equivalent chain length value of 10.928 and/or 12:0 ALDE), 12:0 3-OH (1.5 % \pm 0.4), 18:0 (1.2 % \pm 0.6), 14:0 iso 3-OH (1.1 % \pm 0.6), 17:1 ω 8c (0.7 % \pm 1.9), unknown 12,484 (0.6 % \pm 0.2), 18:0 iso (0.6 % \pm 0.3), 15:0 (0.6 % \pm 1.2), 17:0 (0.5 % \pm 0.9), 12:0 iso (0.4 %

± 0.2), summed feature 7 (0.4 % ± 0.2; comprising 19:1 ω6c and/or unidentified fatty acid with equivalent chain length value of 18.846), 12:0 iso 3-OH (0.3 % ± 0.2), 15:1 ω8c (0.3 % ± 0.9), 20:1 ω7c (0.2 % ± 0.1), 16:1 ω5c (0.2 % ± 0.1), 16:1 ω7c alcohol (0.2 % ± 0.1), unknown 11,799 (0.2 % ± 0.1), 18:1 ω5c (0.1 % ± 0.1), 11:0 3-OH (0.1 % ± 0.5), 10:0 3-OH (0.1 % ± 0.4), 12:0 2-OH (0.1 % ± 0.0), 16:0 3-OH (0.1 % ± 0.3), 11 methyl 18:1 ω7c (0.1 % ± 0.1), 17:0 anteiso (0.1 % ± 0.3), 13:0 (0.1 % ± 0.2) and 17:1 ω6c (0.1 % ± 0.1). All strains are susceptible towards tetracycline (30 µg) and chloramphenicol (30 µg), but intermediate resistant to polymyxin (300 U). Additional phenotypic features are listed in Table 3.17. The 16S rDNA sequences of strains LMG 21557^T, LMG 21558, LMG 20547, LMG 21566, LMG 21562 are deposited in the EMBL under the accession number AJ514916, AJ514913, AJ316202, AJ514917 and AJ514915. The type strain of this species is LMG 21557^T (CAIM 629^T), isolated from white shrimp *Litopenaeus vannamei* in Ecuador. The mol % G+C of the type strain is 45.6.

Description of *V. hepatarius* sp. nov.

Vibrio hepatarius (he.pa.ta.ri.us. L. masc. adj. a bacterium of or belonging to the liver). Cells are slightly curved, 1 µm in width and 2-3 µm in length. They form translucent, convex, non-swarming, smooth-rounded colonies with entire margin, beige in colour and of about 6 mm in diameter on TSA after 48 h incubation at 28 °C. Strains formed yellow, translucent, smooth-rounded colonies of 6 mm on TCBS. All strains have a facultative anaerobic metabolism and ferment glucose, mannitol, sucrose, and amygdalin, but not inositol, rhamnose, melibiose and arabinose. Growth occurs at 4 to 35 °C and in media containing 0-8 % (w/v) NaCl. Prolific growth occurs at 28 °C in media containing 2.5 % (w/v) NaCl. The following tests are positive for all strains: oxidase, catalase, indole, NO₃ reduction and acetoin. All strains utilise dextrin, N-acetyl-D-glucosamine, cellobiose, D-fructose, α-D-glucose, maltose, psicose, sucrose, D-trehalose, methyl pyruvate, inosine and glycerol as sole carbon source. None of the strains utilise glycogen, Tween 40, N-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, i-erythritol, L-fucose, D-galactose, gentiobiose, M-inositol, α-lactose, α-D-lactose lactulose, D-melibiose D-raffinose, L-rhamnose, turanose, xylitol, mono methyl succinate, acetic acid, cis-aconitic acid, citric acid, formic acid, D-galactonic acido lactone, D-galacturonic acid, D-gluconic

acid, D-glucosaminic acid, D-glucuronic acid, α -hydroxy butyric acid, γ -hydroxy butyric acid, P-hydroxy phenylacetic acid, Itaconic acid, α -keto butyric acid, α -keto valeric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, bromo succinic acid, succinamic acid, glucuronamide, alaninamide, D-alanine, L-aspartic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy L-proline, L-leucine, L-ornithine, L-phenyl alanine, L-pyro glutamic acid, D-serine, L-threonine, D,L-carnitine, γ -amino-butyrac acid, urocanic acid, uridine, thymidine, phenyl ethylamine, putrescine, 2-amino ethanol, 2,3-butanediol, D,L- α -glycerol phosphate, glucose-1-phosphate and glucose-6-phosphate as sole carbon source. The following tests are negative for all strains: β -galactosidase, lysine and ornithine decarboxylases, H₂S production and urease. The most abundant fatty acids are summed feature 3 (28.4 % \pm 15.2; comprising 16:1 ω 7c and/or 15:0 iso 2-OH), 16:0 (14.6 \pm 9.6), 15:0 iso (13.2 % \pm 22.1), 18:1 ω 7c (10.3 \pm 9.9), 14:0 (5.4 % \pm 1.4), 16:0 iso (4.7 \pm 2.7), 13:0 iso (3.7 % \pm 5.4), 17:0 iso (2.5 % \pm 3.7), 12:0 (2.2 % \pm 1.9), summed feature 2 (2.2 % \pm 0.3; comprising 14:0 3-OH and/or 16:1 iso I and/or unidentified fatty acid with equivalent chain length value of 10-928 and/or 12:0 ALDE), 14:0 iso (2.0 \pm 1.4), 15:0 anteiso (1.9 % \pm 2.9), 12:0 3-OH (1.1 % \pm 1.0), 17:1 ω 5c iso (1.1 % \pm 1.9), 17:1 ω 10c iso (0.8 % \pm 1.4), unknown 12.484 (0.5 % \pm 0.5), 14:0 3-OH iso (0.5 \pm 0.6), 17:0 anteiso (0.5 % \pm 0.6), 13:0 anteiso (0.5 % \pm 0.8), 16:1 ω 7c alcohol (0.4 % \pm 0.6) and 15:0 (0.4 % \pm 0.4), 17:0 (0.2 \pm 0.2), 17:1 ω 8c (0.1 \pm 0.2). All strains are susceptible towards the vibriostatic agent 0/129 (10 and 150 μ g), polymyxin (300 U), tetracycline (30 μ g) and chloramphenicol (30 μ g), but intermediate resistant to ampicillin (25 μ g). Additional phenotypic features are listed in Table 3.18. The 16S rDNA sequences of strain LMG 20362^T is deposited in the EMBL under the accession number AJ345063. The type strain of this species is LMG 20362^T (CAIM 693^T), isolated from white shrimp *Litopenaeus vannamei* in Ecuador. The mol % G+C of the type strain is 45.5.

Table 3.17. Variable features among *V. fortis* strains.

	<i>V. fortis</i> (n = 16)	LMG 21557 ¹
Enzyme activity:		
β-galactosidase	15	+
Gelatinase	3	-
Production of:		
Acetoin	13	+
Nitrate reduction	15	+
Growth on 10 % (w/v) NaCl	1	-
Growth at 4 °C	5	-
Utilisation of :		
Uridine	15	+
Acetic acid	12	+
D-alanine	11	+
Putrescine	8	+
L-ornithine, propionic acid	7	+
Bromo succinic acid	6	+
Cis-aconitic acid	4	+
Quinic acid	3	+
D-serine	11	-
β-hydroxy butyric acid	5	-
D,L-α-glycerol phosphate	4	-
α-lactose	3	-
α-D-lactose lactulose, D-melibiose, β-methyl D-glucoside, D-raffinose, turanose, L-histidine, γ-amino-butyric acid	2	-
N-acetyl-D-galactosamine, L-arabinose, D-galacturonic acid, D-glucosaminic acid, β-hydroxy butyric acid, α-keto glutaric acid, α-keto glutaric acid, α-keto valeric acid, L- leucine	1	-
Fermentation of:		
Sucrose	6	+
Melibiose	10	+
Amygdaline	9	+
Arabinose	1	-
Susceptibility to:		
0/129 (10 µg per disc)	14	+
0/129 (150 µg per disc)	15	+
Ampicilin (25 µg per disc)	2	+

*Numbers indicate the number of positive isolates.

Table 3.18. Variable features among *V. hepatarius* strains.

	<i>V. hepatarius</i> (n = 3)	LMG 20362 ¹
Arginine dihydrolase	2*	-
Tryptophane deaminase	2	+
Gelatinase	1	-
Fermentation of sorbitol	2	-
Susceptibility to ampicillin	1	I
Growth in 8 % NaCl	1	-
Utilisation of:		
α-cyclodextrin, Tween 80, D-galactose, β-methyl D-glucoside, D-sorbitol, β-hydroxy butyric acid, alaninamide, glycyl-L-aspartic acid, L-ornithine, L-serine, thymidine, L- asparagine	1	+
Gentiobiose, D-mannose, D-gluconic acid, quinic acid, L-alanyl-glycine, hydroxy L-proline	1	-
D-galactonic acido lactone, α-keto glutaric acid, D,L-lactic acid, succinic acid, L-alanine, L-proline	2	+
L-glutamic acid, glycyl-L-glutamic acid	2	-

*Numbers indicate the number of positive isolates. I, intermediate.

**3.6. *Vibrio gallicus* sp. nov., isolated from the gut of
French abalone *Haliotis tuberculata***

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Int. J. Syst. Evol. Microbiol., submitted

Abstract

Five alginolytic, facultative anaerobic, non-motile bacteria were isolated from the gut of abalone *Haliotis tuberculata*. Phylogenetic analysis based on almost complete 16S rDNA sequences indicated that these strains are related to *V. halioticoli* and *V. superstes* (97.5 % 16S rDNA similarity). DNA-DNA hybridization and AFLP fingerprinting demonstrated that the five strains constitute a single species different from all currently known vibrios. The name *Vibrio gallicus* sp. nov. (LMG 21330^T =CIP 107863^T=HT2-1^T; mol% G+C of DNA is 43.6-44.3) is proposed to encompass this new taxon. Several phenotypic features were disclosed which discriminate *V. gallicus* from other *Vibrio* species. *V. gallicus*, *V. halioticoli* and *V. superstes* can be differentiated on the basis of several features including arginine dihydrolase, β -galactosidase, indole and utilisation of different carbon compounds.

Introduction

Vibrio halioticoli and genetically related species are alginolytic, non-motile, fermentative and abundant in the gut of *Haliotis* abalones in Japan and South Africa (Sawabe et al., 1995, 2002). Our hypothesis is that *V. halioticoli* contributes in the digestion of alginate which is a abundant polysaccharide in kelps ingested by abalones (Sawabe et al., 2003). *V. halioticoli* may convert alginate into volatile short chained fatty acids via fermentation (Sawabe et al., 2003). Nearly 80 species of abalones are known and they appear in offshore areas worldwide, but little is known about the presence of *V. halioticoli*-like bacteria in the gut of these molluscs. Recently we isolated five dominant strains from the gut of French abalones (*Haliotis tuberculata*). Phenotypically these isolates resembled *V. halioticoli*. Here we present a detailed polyphasic analysis of these five isolates. Collectively, genomic phenotypic and

phylogenetic data demonstrate that the five *Vibrio* isolates represent a new *Vibrio* species i.e. *V. gallicus*.

Material and Methods

Five strains of *V. gallicus* i.e. LMG 21330^T=CIP 107863^T=HT2-1, LMG 21329=CIP 107864=HT1-3, CIP 107865=HT1-12, CIP 107866=HT2-6, and CIP 107867=HT3-3 were isolated from the gut of French abalones *H. tuberculata*. Abalones collected at the coastal area of Brest (Britany) by Scuba diving in February, 2001. Strains were cultured on ZoBell 2216E agar (Oppenheimer and ZoBell, 1952) and stored at -80 °C in 10 % glycerol. A total of 78 phenotypic characteristics, including alginase activity were determined as described previously (Baumann and Schubert, 1984; Hidaka and Sakai, 1968; Holt et al., 1994; Leifson, 1963; Ostel and Holt, 1982; West et al., 1977). The phenotypic characterization was done at 20 °C.

Bacterial DNAs were prepared by the procedure of Marmur (1961). Mol % G+C content of DNAs were determined by HPLC (Tamaoka and Komagata, 1984). DNA-DNA hybridization experiments were performed in microdilution wells using a fluorometric direct binding method previously described by Ezaki et al. (1988, 1989). FAFLP and 16S rDNA sequence analyses were performed as described previously (Thompson et al., 2001).

Results and Discussion

The five *V. gallicus* isolates had high levels of 16S rDNA similarity i.e. 99.7 to 99.9 %. Similarity levels below 97.5 % were found with other *Vibrio* species. The closest phylogenetic neighbor of LMG 21330^T was *V. halioticoli* (Figure 3.10a). LMG 21330^T and LMG 21329 had FAFLP pattern similarity of 79 % (Figure 3.10b). The FAFLP pattern similarity of *V. gallicus* towards *V. halioticoli*, *V. rumoiensis* and *V. harveyi* was 48, 47 and 44 %, respectively. Whereas the FAFLP pattern similarity of LMG 21330^T and LMG 21329 towards other *Vibrio* species was below 44 %, clearly pointing out that this novel species is different from other vibrios (Thompson et al., 2001).

The five *V. gallicus* isolates had at least 86 % DNA-DNA similarity, but only 16 to 24 % similarity towards *V. agarivorans*, *V. fischeri*, *V. halioticoli*, *V. logei* and *V.*

superstes. These results corroborate our findings with FAFLP analysis and clearly show that *V. gallicus* isolates are apart from *V. haliotocoli*.

The five *V. gallicus* isolates have the main phenotypic features of the genus *Vibrio* (except for the absence of flagella). The strains are Gram-negative and non-motile. No flagellated cells were observed by TEM observations, but short tubular projections were observed in *V. gallicus* cells similar to those of *V. campbellii* (Allen and Baumann, 1971). Proper function of the tubular projections has never been clarified (Allen and Baumann, 1971). No peritrichous flagella were observed when the strains were

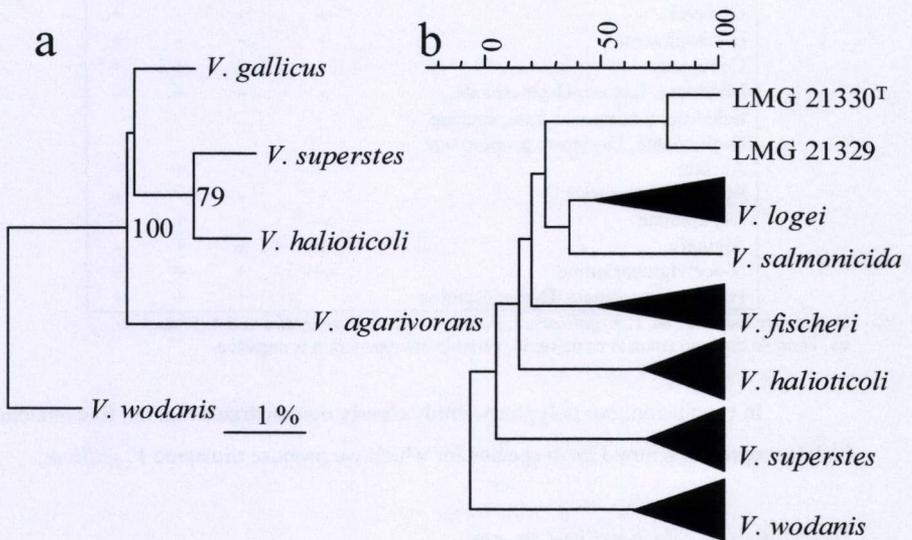


Figure 3.10. Neighbour-Joining tree (a) and FAFLP dendrogram (b) showing the relationship between *V. gallicus* and its closest neighbours. Dendrogram based on the FAFLP band patterns of *V. gallicus* (LMG 21329 and LMG 21330^T) and its closest phylogenetic neighbours using Dice and Ward and a band position tolerance of 0.2%.

cultivated on solid media. Strains required salt for growth, did not accumulate poly- β -hydroxybutyrate and were oxidase positive (Table 3.19). *V. gallicus* isolates were phenotypically related to *V. haliotocoli*, although the new species differed from *V. haliotocoli* by at least four traits i.e. arginine dihydrolase, β -galactosidase, utilisation of fumarate and succinate (Table 3.19).

Table 3.19. Phenotypic characteristics for distinguishing *V. gallicus* from other phylogenetically related *Vibrio* species.

Test	1 (n=5)	2	3	4
Motility	-	-	-	+
Luminescence	-	-	-	+
Production of :				
Amylase	v+	-	-	-
Alginase	+	+	+	-
Lysine decarboxylase, Lipase	-	-	-	+
Arginine dihydrolase	+	-	-	-
Indole production	+	+	-	-
β -galactosidase	-	+	-	-
Utilization of:				
D-mannose, D-galactose	-	-	+	+
Glycerol	v-	+	-	+
α -ketoglutarate	-	-	-	+
Cellobiose	-	-	+	+
Melibiose, lactose, D-glucronate, trehalose, γ -aminobutyrate, sucrose, D-gluconate, D-xylose, propinonate	-	-	+	-
Acetate	-	-	+	+
Pyruvate, L-tyrosine	-	-	-	+
L-glutamate	v-	-	+	+
Alginate	+	+	+	-
N-acetylglucosamine	v+	+	+	+
Fumarate, succinate, D-glucosamine	-	+	+	+

Species are indicated as: 1. *V. gallicus*, 2. *V. halioticoli*, 3. *V. superstes* and 4. *V. fischeri*. v+, variable but type strain is positive; v-, variable but type strain is negative.

In conclusion, our polyphasic study clearly demonstrates that the five abalone isolates represent a new *Vibrio* species for which we propose the name *V. gallicus*.

Description of *Vibrio gallicus* sp. nov.

Vibrio gallicus (*ga'lli.cus*. N.L. masc adj. *gallicus* from France).

The bacterium is Gram-negative, facultatively anaerobic, non-motile, non-flagellated. Cells are rod shaped, with rounded ends (0.6-0.8 x 1.2-1.3 μ m) when grown on ZoBell 2216E broth. No endospores or capsules are formed. Flagellation is not observed when the organism is cultivated on solidified media and/or in liquid media. Colonies on ZoBell 2216E agar are beige, circular, and smooth and convex with entire edge. Sodium ions are essential for growth. The bacterium is a mesophilic and neutrophilic chemo-organotroph which grows at temperatures between 15 and 30 °C. No growth occurs at 37 °C. The bacterium is positive for the following features: acid production from glucose, arginine dihydrolase, nitrate reduction, indole production, hydrolysis of alginate, oxidase, catalase, and assimilation of D-fructose, D-glucose, maltose,

D-mannitol and alginate. The following tests are negative: gas production from glucose, acetoin production, lysine and ornithine decarboxylase, β -galactosidase, luminescence, pigmentation, requirement for organic growth factors. The organism does not hydrolyse gelatin, chitin, Tween 80 and agar and does not accumulate poly- β -hydroxybutyrate; The bacterium does not assimilate D-mannose, sucrose, D-gluconate, D-sorbitol, α -ketoglutarate, D-galactose, cellobiose, melibiose, lactose, D-gluconate, trehalose, putrescine, γ -aminobutyrate, acetate, pyruvate, L-tyrosine, propionate, D-glucosamine, fumarate, succinate, meso-erythritol, D-xylose, L-arabinose, citrate, DL-malate, δ -aminovariate, and aconitate. The G+C content of DNA is 43.6-44.3 mol %. The type strain is LMG 21330^T=CIP 107863^T. The bacterium was isolated from the gut of French abalone *Haliotis tuberculata*.

3.7. *Vibrio hispanicus* sp. nov., isolated from *Artemia* sp. and seawater in Spain

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Int. J. Syst. Evol. Microbiol. (2003), submitted

Abstract

Three Gram-negative, motile rod-shaped bacteria were isolated from *Artemia* sp. and seawater in Barcelona (Spain) between 1990 and 1991. Isolates were fermentative, oxidase positive, sensitive to the vibriostatic agent O/129, arginine dihydrolase positive, lysine and ornithine decarboxylases negative, and can grow in the absence of NaCl. They differ from phenotypically related species by their ability to grow at 4 °C and utilize L-rhamnose. Cloning of the 16S rRNA of LMG 13240^T produced two different 16S rDNA genes, which differ in 15 bases (1 %). Comparison of these sequences with those deposited in GenBank showed close relationship with *Vibrio proteolyticus* (97.6 %), *V. diazotrophicus* (97.6 %), *V. campbellii* (96.8 %), and *V. alginolyticus* (96.8 %). The DNA-DNA similarity between LMG 13240^T and LMG 13211 was 103 %. The DNA-DNA similarity of these two strains towards closely phylogenetic neighbours was below 30 %. It is concluded that the three *Vibrio* isolates analysed here belong to a new species for which the name of *Vibrio hispanicus* is proposed. The type strain is LMG 13240^T (=CAIM 525^T; EMBL accession no. AJ316178).

Introduction

Artemia spp. nauplii and cysts have a diverse bacterial flora, and many of the bacterial genera and species found are those ubiquitous in the seawater. It has been suggested that the microflora of *Artemia* nauplii is significantly influenced by the surrounding water (Igarashi et al., 1989). *Artemia* spp. cysts have been found to harbour low abundance of vibrios (Dehasque et al., 1991; Igarashi, 1989). After

hatching, the bacterial density counted on TCBS agar (presumably vibrios), can increase dramatically to up to 10^3 CFU nauplius⁻¹ (Hameed 2000) or 10^6 to 10^8 CFU ml⁻¹ of *Artemia* nauplii homogenates (Lopez-Torres et al., 2001). In the surrounding water (Dehasque et al., 1991), the abundance of vibrios is about 10^6 CFU ml⁻¹, suggesting vibrios colonize *Artemia* when it hatches exposing itself for the first time to the surrounding environment. High numbers of *Vibrio* taxa associated with *Artemia* cultures have been reported (Verdonck et al., 1994). In three marine fish hatcheries, 58 % to 87 % of the isolates were identified as *Vibrio* by FAME (Verdonck et al., 1994).

In this study we present a detailed polyphasic characterisation of the three new *Vibrio* isolates (LMG 13240^T, LMG 13213, and LMG 13211) originated from *Artemia* and seawater. We further propose a new species *V. hispanicus* to encompass these isolates. The three new isolates were characterised phenotypically in former studies (Austin et al., 1995b; Vandenberghe et al., 2003) and by FAFLP genomic fingerprinting (Thompson et al., 2001). According to Vandenberghe et al. (2003), the three *Vibrio* isolates clustered with the type strain of *Vibrio gazogenes*. All the three *Vibrio* isolates have been proven to be pathogenic to the Atlantic salmon (*Salmo salar*) (Austin et al. 1995b). They had unique ribotyping patterns, and two of them (LMG 13213 and LMG 13240) carried a 4.4 Kbp plasmid (Austin et al., 1995b). FAFLP analysis of the three *Vibrio* isolates showed that they formed a separated cluster (named A16) with mutual similarity of > 80 %, not assigned to any type strain included in the analysis (Thompson et al., 2001). The FAFLP band pattern similarity of the three isolates towards their closest phylogenetic neighbours was below 45 % and was concluded that they belong to a new *Vibrio* species (Thompson et al., 2001).

Material and Methods

Strains LMG 13240^T (= CAIM 525^T, =VIB 213) and LMG 13213 (= CAIM 524, = VIB 186) were isolated from *Artemia* sp. in 1991, while strain LMG 13211 (= CAIM 523, = VIB 184) was isolated from seawater in 1990. All the strains were isolated in a fish hatchery in Barcelona, Spain as described elsewhere (Verdonck et al., 1994). All strains are deposited at the BCCMTM/LMG bacteria collection and at the CAIM (Collection of Aquacultural Important Microorganisms; CIAD, A.C. Mazatlán, México).

Phenotypic characterisation of the strains was performed as described previously (Gomez-Gil et al., 2003). Antibiotic sensitivity was estimated by the disk diffusion test (Bauer et al., 1966) on Iso-Sensitest agar + 1.5 % (w/v) NaCl. GLC analysis of methylated fatty acid was performed as described by Osterhout et al. (1991), but the cells were grown on TSA (Difco) + 1.5 % (w/v) NaCl and incubated at 28 °C. Growth of the three strains at different NaCl concentrations was tested in microtiter plates (five replicates) with TSB (Bioxon). Inoculated plates were incubated for 48 h at 30 °C, and the turbidity measured by optical density at 610 nm.

Determination of mol % G+C content of the DNA of strain LMG 13240^T was determined according to Mesbah et al. (1989) and modified by Logan et al. (2000). DNA-DNA hybridisation was done following the methodology described by Willems et al. (2001) at 39 °C. Almost complete 16S rDNA sequences was amplified with primers V16S-9F (5'AGAGTTTGATCATGGCTCAG 3') and V16S-1491R (5'AGCGCTACCTTGTTACGACTT 3'). PCR reaction mixes comprised 18.4 µl sterile water, 1.3 µl dNTP mix (2.5 mM each), 2.5 µl 10X PCR buffer with 25 mM MgCl₂, 0.26 µl of each primer (0.25 µg µl⁻¹), 0.2 µl Taq (5 U µl⁻¹, Promega) and 2.0 µl template DNA for a final volume of 25 µl. The temperature profile of the PCR was: 94 °C for 2 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C, and a final extension step of 5 min at 72 °C. Purified 16S rDNA PCR products were ligated into a pGEM-T cloning vector (Promega). Transformation was performed by heat shock at 37 °C for 20 seconds using DH5α competent cells (Gibco-Invitrogen). Cells were then incubated at 37 °C in SOC medium for one hour, centrifuged, plated in LB medium containing ampicillin (60 µg ml⁻¹), and incubated overnight at 37°C. Plasmid extraction was performed using a miniprep kit (Promega) following the instructions of the manufacturer. Positive clones were identified by *Eco*RI digestion and gel electrophoresis. Bidirectional sequencing was carried out using labeled T7 / SP6 universal primers and a Li-cor IR² DNA sequencer as described by the manufacturer.

The type 1 16S rDNA sequence (accession number AY254039) of LMG 13240^T was compared to the sequences deposited in GenBank (BLASTN; Altschul et al., 1990) and Ribosomal Database Project II (RDP, Sequence Match ver. 2.7; Cole et al. 2003). Sequences of the closest species and of isolate LMG 13240^T were aligned with Clustal X (ver. 1.8) program (Thompson et al., 1997). Tree topology (neighbor-

joining; Saitou and Nei, 1987), and stability of groupings (bootstrap analysis, 1000 replicates) were performed with the MEGA program (ver. 2.1, Kumar et al. 2001) with *Vibrio cholerae* El Tor strain as outlier.

Results and Discussion

Phenotypic characters including fatty acid composition of the three *V. hispanicus* isolates place them within the genus *Vibrio* (Bertone et al., 1996). *V. hispanicus* is lysine decarboxylase negative, grows at 0 % NaCl and at 4 °C. In addition, *V. hispanicus* utilizes L-rhamnose and ferments sucrose, indicating that it can be clearly differentiated from other vibrios, including *V. diazotrophicus* and *V. proteolyticus* (Table 3.20).

Table 3.20. Some phenotypic differences between *V. hispanicus* and related *Vibrio* species. Species are indicated as: 1, *V. hispanicus* sp. nov.; 2, *V. diazotrophicus*; 3, *V. fluvialis*; 4, *V. furnissii* 5, *V. metschnikovii*. +, positive; -, negative; (+); v, variable. Data from Alsina and Blanch 1994a and Holt et al. 1994.

Test	1	2	3	4	5	6
α -galactosidase	+	+	+	+	+	-
Lysine decarboxylase	-	-	-	-	v	+
Growth at:						
0 % NaCl	+	-	v	v	v	-
4 °C	+	v	-	-	v	-
Indole	+	+	v	v	v	+
Oxidase	+	+	+	+	-	+
Gas from glucose	-	-	-	+	-	-
Resistant to 0/129 (10 μ g)	-	v	+	+	v	v
Gelatinase	-	-	+	v	+	v
Utilization of L-rhamnose	+	-	-	-	-	-
Fermentation of sucrose	+	+	+	+	+	-

Members of the genus *Vibrio* require Na^+ for growth with few exceptions i.e. *V. cholerae* and *V. mimicus* (Alsina and Blanch, 1994a). Some strains of *V. fluvialis*, *V. furnissii*, and *V. metschnikovii* are also able to grow in absence of Na^+ (Alsina and Blanch, 1994a). The optimal Na^+ concentration for many marine bacteria is between 70 and 300 mM (Reichelt and Baumann, 1974), well below the concentration of the seawater (450-480 mM Na^+). Although, many vibrios have been isolated from hypersaline environments (4185 mM Na^+) where *Artemia* cysts are harvested (Straub and Dixon, 1993). The three new *Vibrio* isolates were able to grow at 1.0 mM of

NaCl, but best growth was obtained at 1024 mM and no growth was detected at 2048 mM (Figure 3.11).

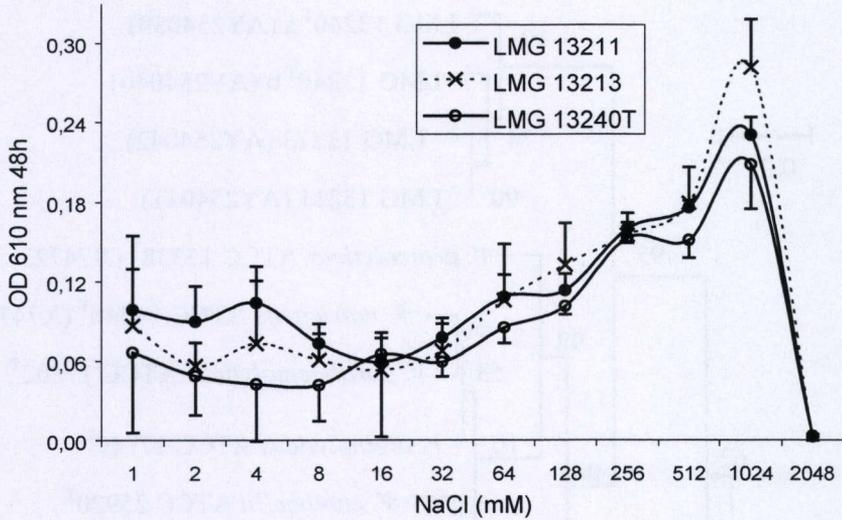


Figure 3.11. Growth of *V. hispanicus* sp. nov. strains at different NaCl concentrations after 48 h at 30 °C (Mean and standard deviation, n = 5).

The three new *V. hispanicus* isolates had very similar 16S rDNA sequences, with more than 99 % similarity (Figure 3.12). The closest phylogenetic neighbour of the three *V. hispanicus* isolates were *V. proteolyticus* (97.6 % BLASTN, 0.906 RDP), *V. diazotrophicus* (97.6 % BLASTN, 0.900 RDP), *V. campbellii* (96.8 % BLASTN, 0.857 RDP), *V. alginolyticus* (96.8 % BLASTN, 0.876 RDP), *V. natriegens* (96.7 % BLASTN, 0.867 RDP), *V. parahaemolyticus* (96.6 % BLASTN, 0.871 RDP), *V. nigripulchritudo* (96.6 % BLASTN, 0.882 RDP), *V. vulnificus* (97.1 % BLASTN, 0.867 RDP), and *V. harveyi* (96.4 % BLASTN, 0.875 RDP). Phylogenetic analysis with the neighbor-joining methodology (Figure 2) clustered *V. hispanicus* sp. nov. strains close to the *Vibrio* core group (Dorsch et al., 1992). The type strain LMG 13240^T produced two different 16S rDNA genes, which differ in 15 bases (1 %): five bases within the variable region 1 (VR-1), five bases within the variable region 7 (VR-7), and five bases scattered in conserved regions. Originally, the 16S rDNA sequence of LMG 13240^T (EMBL accession no. AJ316178) had 17 ambiguities

(Thompson et al., 2001). The position of these ambiguities is in the same places as the two different 16S rDNA sequences performed for the same strain in the present study.

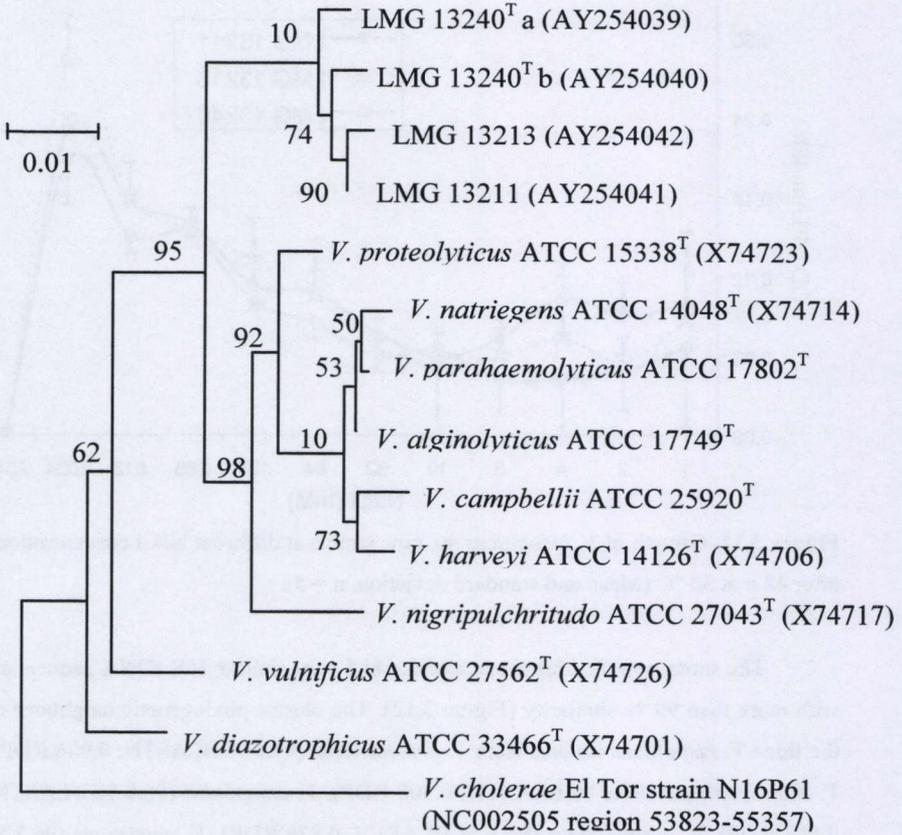


Figure 3.12. Phylogenetic tree of *Vibrio hispanicus* sp. nov. strains with the closest *Vibrio* species based on the almost complete sequences of the 16S rDNA using the Neighbor-joining methodology (pairwise deletion, Kimura 2-parameter, Gamma 0.4). Numbers at nodes indicate the level of bootstrap support (1000 replicates). Bar = 1 % divergence.

The DNA-DNA similarity between LMG 13240^T and LMG 13211 was 103 %. The DNA-DNA similarity of these two strains towards *V. proteolyticus*, *V. harveyi*, *V. nigripulchritudo*, *V. pelagius*, *V. fluvialis*, and *V. natriegens* was below 30 %.

Phenotypic and genotypic data presented here clearly support the creation of a new *Vibrio* species i.e. *Vibrio hispanicus* to encompass the strains LMG 13240^T, LMG 13211, and LMG 13213.

Description of *Vibrio hispanicus* sp. nov.

Vibrio hispanicus (his.pa' ni.cus. N.L. masc. adj. *hispanicus* from Spain).

Gram-negative motile rods with polar flagellation. Bright yellow, small (1–3 mm), circular colonies on TCBS agar. Non-luminescent, translucent, and non-swarming colonies on Marine agar. Growth occurs in media containing 0, 2.5, 6.0, 8.0, and 10 % NaCl (w/v), but not in 12.0 %. Growth occurs at 4, 30, 35, and 40 °C. Oxidase, indole, citrate, nitrite reduction, and α -galactosidase positive. Strains are fermentative. Arginine dihydrolase is positive, but lysine and ornithine decarboxylases and L-tyrosine are negative. Tryptophane deaminase, H₂S, gas from glucose production, Voges-Proskauer, gelatinase and urease are negative. *V. hispanicus* sp. nov. is susceptible to the vibriostatic agent O/129 at 10 and 150 μ g, to polymixin B (300 U), but resistant to streptomycin (25 μ g) and to gentamicin (10 μ g). It is positive for α -D-glucose, β -hydroxy butyric acid, β -methyl D-glucoside, D-glucuronic acid (LMG 13213 weak), cellobiose, D,L-lactic acid, dextrin, D-fructose, D-galactose, D-gluconic acid, D-mannitol, D-mannose, D-raffinose (LMG 13240 and LMG 13213 weak), D-saccharic acid (LMG 13213 weak), D-trehalose, gentiobiose, inosine, L-asparagine, L-aspartic acid (LMG 13240 weak), L-glutamic acid, L-rhamnose, L-serine, maltose, methyl pyruvate, N-acetyl-D-glucosamine, psicose, sucrose, thymidine, and uridine utilisation as sole carbon source. It is negative for 2,3-butanediol, 2-amino ethanol, acetic acid, adonitol, alaninamide, α -cyclodextrin, α -D-lactose lactulose, α -hydroxy butyric acid, α -keto butyric acid, α -keto glutaric acid, α -keto valeric acid, bromo succinic acid, cis-aconitic acid, citric acid, D,L- α -glycerol phosphate, D,L-carnitine, D-alanine, D-arabitol, D-galactonic acido lactone, D-galacturonic acid, D-glucosaminic acid, D-melibiose, D-serine, D-sorbitol, formic acid, γ -amino-butyric acid, γ -hydroxy butyric acid, glucose-1-phosphate, glucose-6-phosphate, glucuronamide, glycogen, glycyl-L-aspartic acid, glycyl-L-glutamic acid, hydroxy L-proline, i-erythritol, itaconic acid, L-alanine, L-alanyl-glycine, L-fucose, L-histidine, L-leucine, L-ornithine, L-phenyl alanine, L-proline, L-pyro glutamic acid, L-threonine, malonic acid, *myo*-inositol, mono methyl succinate, N-acetyl-D-galactosamine, phenyl ethylamine, P-hydroxy phenylacetic acid, propionic acid, putrescine, quinic acid, sebacic acid, succinamic acid, turanose, Tween 40, Tween 80, urocanic acid, and xylitol as sole carbon source. Acid and alkaline phosphatases, β -

Chapter 3

glucosidase, esterase, esterase lipase, leucine arylamidase, and naphthol-AS-BI-phosphohydrolase are positive. α -chymotrypsin, α -fucosidase, α -glucosidase, α -mannosidase, β -galactosidase, β -glucuronidase, cystine arylamidase, lipase, N-acetyl- β -glucosaminidase, trypsin, and valine arylamidase are negative. The most abundant fatty acids are, in descending order (mean of three strains, minimum and maximum): summed feature 3 (16:1 ω 7c and/or 15 iso 2-OH, 37.2; 35.8, 39.1), 16:0 (25.7; 22.8-27.9), 18:1 ω 7c (16.7; 15.5, 17.8), 14:0 (5.9; 5.3, 6.5), 12:0 (4.4; 4.2, 4.8), summed feature 2 (14:0 3-OH and/or 16:1 iso I, 3.2; 3.1, 3.4), 12:0 3-OH (2.6; 2.5, 2.8), and 18:0 (1.1; 0.9, 1.2). Additional phenotypic differences are listed in Table 2. The G+C content of the DNA is 42.8 mol %. The type strain is LMG 13240^T (= CAIM 525^T) and the reference strains are LMG 13211 (= CAIM 523) and LMG 13213 (= CAIM 524) all isolated from *Artemia* sp. and its culture water in Barcelona, Spain.

Table 3.21. Phenotypic differences among *V. hispanicus* isolates

Test	LMG 13240 ^T	LMG 13211	LMG 13213
Utilization of:			
α -lactose	-	+	+
Glycerol, amygdalin	+	-	+
succinic acid	+	+	-
Susceptibility to:			
ampicillin (30 μ g)	I	I	S
amikacin (30 μ g)	I	R	I
Kanamycin (30 μ g) oxytetracycline (30 μ g)	R	R	I
Tetracycline	I	R	R

3.8. *Vibrio pacinii* sp. nov., a new species isolated from cultured aquatic organisms

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Int. J. Syst. Evol. Microbiol. (2003), in press

Abstract

Three strains were isolated from cultured aquatic organisms, they were Gram-negative, oxidase positive, motile, fermentative, arginine dihydrolase positive, lysine and ornithine decarboxylase negative, and sensitive to the vibriostatic agent 0/129. These strains differ from other related *Vibrio* species by several phenotypic features including acetoin and indole production, utilisation of amygdalin and D-mannitol. Comparison of the 16S rDNA sequence showed close relationship to the recently described *Vibrio kanaloaei* (96.6 %), *Vibrio pomeroyi* (96.4 %), and to *Vibrio furnissii* (96.6 %), but DNA-DNA hybridisation experiments proved the three isolates form a tight new species with ≤ 30 % DNA-DNA similarity towards its closest phylogenetic neighbours. *Vibrio pacinii* sp. nov. is proposed, with LMG 19999^T (= CAIM 530^T; DNA G+C content of 44.9 mol %; The Genbank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of LMG 19999^T is AJ316194) as the type strain.

Introduction

Vibrio is an important genus in the culture of marine and estuarine animals in that many species have been responsible for causing disease in fish (Hjeltnes and Roberts, 1993), crustacea (Lightner, 1993), and molluscs (Austin, 1988). Some *Vibrio* species may also have a beneficial effect in the host organisms either as symbionts of squids (Mcfall-Ngai, 2002) or probionts (Gomez-Gil et al., 2000). Several studies have investigated the phenotypic diversity of vibrios in aquaculture environments (Vandenberghe et al., 1998, 2003). Recently, genotypic techniques have also been employed to better characterise the diversity of vibrios. Thompson et al. (2001)

characterised 506 strains with Fluorescent Amplified Fragment Length Polymorphism (FAFLP) and found many strains that did not cluster with any known type strains, and thus might be considered as potential new species of the genus *Vibrio*. This analysis disclosed a group (so called A47) of three tightly related strains (i.e. LMG 13245, LMG 19999^T and LMG 21514). These strains grouped apart from known type strains of *Vibrio* species, having FAFLP pattern similarity above 70 %, but only 52 % with their closest FAFLP neighbour, strain LMG 10953 (FAFLP group A1). A phenotypic analysis of 1476 *Vibrio* isolates with the Biolog GN (Biolog, Hayward, Ca.) system clustered strain LMG 19999^T and LMG 13245 in a separate group so called STD3-1057 related to *Vibrio logei*, and strain LMG 21514 in the *Vibrio ordalii* group (Vandenberghe et al., 2003). While Austin et al. (1995) reported that strain LMG 13245 had a unique ribotype and lipopolysaccharide profiles, had no plasmid content, belonged to an unknown serogroup and was pathogenic for the Atlantic salmon (*Salmo salar*). Phenotypic and genotypic methodologies clearly point out that the bacterioflora, particularly vibrios, found in aquaculture environments, represent a highly diverse group with many potential new species yet to be detected and described.

Material and Methods

Strain LMG 19999^T (=CAIM 530^T, =STD3-1057^T) was isolated from healthy shrimp larvae (*Penaeus chinensis*) in Dahua hatchery in Laizhou (Shandong Province, China) during the spring of 1996 (Vandenberghe et al., 1998). Strain LMG 13245 (=CAIM 526, =VIB 218) was isolated from Seabass (*Dicentrarchus labrax*) in Spain 1991, while strain LMG 21514 (=CAIM 466, =VIB 847) from Atlantic salmon (*Salmo salar*) in Tasmania during the 1990s. All strains are deposited at the BCCMTM/LMG bacteria collection and at the CAIM (Collection of Aquacultural Important Microorganisms; CIAD, A.C. Mazatlán, México).

Strains were grown aerobically on tryptone soy agar (TSA; Oxoid) supplemented with 2 % (w/v) NaCl for 24 h at 28 °C unless otherwise stated. Phenotypic characterization was done with the API 20E (BioMerieux) to determine the biochemical and nutritional properties of the strains tested, the API ZYM (BioMerieux) for the evaluation of enzymes produced by the strains, and the Biolog GN2 to test the ability to utilize different carbon sources. All the systems employed were used according to the manufactures instructions, but sterile saline solution (SSS,

1.5 % w/v NaCl) was used to prepare the inocula in every case. Additional phenotypic tests and confirmation of doubtful test obtained by the previously described systems, were performed following the methodologies of Lanyi (1987) and Austin and Lee (1992). Antibiotic sensitivity was estimated by the disk diffusion test (Bauer et al., 1966) on Iso-Sensitest agar + 1.5 % (w/v) NaCl. GLC analysis of methylated fatty acid was performed as described by Osterhout et al. (1991), but the cells were grown on TSA (Difco) + 1.5 % (w/v) NaCl and incubated at 28 °C. Determination of mol % G+C content of the DNA of strain LMG 19999^T was determined according to Mesbah et al. (1989) and modified by Logan et al. (2000).

Strain LMG 19999^T (EMBL accession no. AJ316194) was further analysed by sequencing the 16S rDNA as described by Thompson et al. (2001). Sequence similarities were obtained with the Jukes-Cantor model (Gamma 0.4, pairwise deletion; standard error (SE) estimated by bootstrap method, 1000 replications and random number seed = 67137) with the MEGA program (ver. 2.1, Kumar et al., 2001). LMG 19999^T sequence was also compared to the sequences deposited in GenBank (BLASTN; Altschul et al., 1990) and in the Ribosomal Database Project (RDP; Maidak et al., 1999). Sequences of the closest species and of isolate LMG 19999^T were aligned with Clustal X (ver. 1.8) program (Thompson et al., 1997). Tree topology (neighbour-joining; Saitou and Nei, 1987), and stability of groupings (bootstrap analysis, 1000 replicates) were performed with the Phylo_win software (Galtier et al., 1996) with *Vibrio cholerae* as outlier. DNA-DNA hybridisation was done following the methodology described by Willems et al. (2001) at 39 °C.

Results and Discussion

The three isolates grew as round yellow bright colonies on TCBS agar, no swarming or luminescence was observed, arginine dihydrolase positive, lysine and ornithine decarboxylase negative (A+, L-, O-). The A+, L-, O- vibrios represent a diverse group comprising many species (Alsina and Blanch, 1994a, 1994b). According to the identification scheme provided by Alsina and Blanch (1994b) strains LMG 19999^T, LMG 21514, and LMG 13245 would be identified as *Vibrio splendidus* II or *Vibrio furnissii*. All three isolates presented many characters that differentiate it from other A+, L-, O- vibrios e.g. positive Voges-Proskauer (except LMG 13245, and positive only in *Vibrio anguillarum*, *Vibrio metschnikovii* and *Vibrio tapetis*); negative indole (also negative for *Vibrio mytili* and *V. tapetis*, variable for *V.*

splendidus II); utilisation of amygdalin (also by *Vibrio diabolicus*) but not of D-mannitol (also not by *Vibrio nereis* and *V. tapetis*) (Table 3.22). The fatty acid content of *Vibrio pacinii* strains varied considerable, 28 were detected in strain LMG 13245, 23 in LMG 19999^T, and only 10 in LMG 21514 (see description and Table 3.23). The FAME profiles are in good agreement with those of representatives of the genus *Vibrio* (Bertone et al., 1996), although two fatty acids were present in higher concentrations. Fatty acids useful for differentiation are shown in Table 3.22.

16S rDNA sequence similarities from distance matrix calculation and from comparison of the LMG 19999^T strain to sequences deposited in GenBank (BLASTN) and the Ribosomal Database Project II (RDP, if available) indicated close relationship to several *Vibrio* species (Figure 3.13), including *V. metschnikovii* CIP 69.14^T (d = 0.028, SE = 0.005; 97.4 % BLASTN; 87.0 % RDP, 1360 uniquely occurring oligomers), *Vibrio kanaloaei* LMG 20539^T (d = 0.029, SE = 0.005; 96.6 % BLASTN), *Vibrio pomeroyi* LMG 20537^T (d = 0.029, SE = 0.005; 96.4 % BLASTN), *Vibrio aestuarianus* ATCC 35048^T (d = 0.031, SE = 0.005; 97.7 % BLASTN; 87.7 % RDP, 1345 uniquely occurring oligomers), *V. tapetis* CECT 4600^T (d = 0.033, SE = 0.005; 97.3 % BLASTN; 87.2 % RDP, 1387 uniquely occurring oligomers), and *V. furnissii* ATCC 35016^T (d = 0.035, SE = 0.005; 96.6 % BLASTN; 87.2 % RDP, 1453 uniquely occurring oligomers). The range of the 16S rDNA similarity within the *Vibrio* genus

for *V. pacinii* was between 0.091 and 0.028.

DNA-DNA hybridisation experiments revealed high similarity between the pairs LMG 19999^T and LMG 21514 (79 %), LMG 19999^T and LMG 13245 (92 %) and LMG 21514 and LMG 13245 (85 %). Whereas the similarity of strain LMG 19999^T with its closest phylogenetic neighbours was, in decreasing order, 30 % (28 % reciprocal value) with *V. aestuarianus* LMG 7909^T, 28 % (24 % reciprocal value) with *V. kanaloaei* LMG 20539^T, 28 % (17 % reciprocal value) with *V. pomeroyi* LMG 20537^T, 21 % with *V. furnissii* LMG 7910^T, 18 % (21.8 % reciprocal value) with *V. metschnikovii* LMG 11664^T, and 16 % (17 % reciprocal value) with *V. tapetis* LMG 19706^T.

Table 3.22. Phenotypic characteristics differentiating *Vibrio pacinii* sp. nov. from its closest neighbours and from other arginine positive, lysine and ornithine negative *Vibrio* species.

Test	1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Arginine dihydrolase	+	+	+	+	+	-	v	+	+	v	v	+	+	v	v	v	-	v
Lysine decarboxylase	-	-	-	v	-	+	-	-	-	v	v	-	-	+	-	-	-	-
Ornithine decarboxylase	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	nd	+
Resis. 0/129 10 µg	-	-	-	-	+	nd	v	+	+	-	-	nd	v	-	-	v	-	-
Indole	-	+	+	+	v	+	+	v	v	+	+	-	v	+	+	+	-	+
Voges-Proskauer	v	+	v	-	+	+	-	-	-	+	+	-	-	-	-	-	+	-
ONPG	v	-	+	+	+	-	+	v	+	+	+	+	-	+	v	v	+	+
Citrate	+	-	-	+	+	+	+	+	+	-	-	+	+	+	+	+	-	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Gelatinase	v	+	+	+	+	+	-	+	v	-	+	-	v	+	+	+	+	+
Urease	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	v
Swarming	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-
Luminescence	-	-	-	-	-	-	nd	-	-	-	-	-	-	+	-	-	nd	-
Growth in/at:																		
6% NaCl	+	+	+	+	+	+	+	+	+	+	v	+	+	+	+	v	-	+
8% NaCl	+	v	+	v	-	nd	+	v	+	v	v	+	+	+	v	v	-	v
10% NaCl	-	-	-	-	+	nd	v	v	+	-	v	+	v	-	-	-	-	-
35 °C	v	-	-	+	+	+	+	+	+	v	+	+	+	+	v	-	-	v
40 °C	-	-	-	-	-	+	v	+	+	-	+	-	v	-	-	-	nd	-
4 °C	+	+	+	+	-	+	v	-	-	-	-	-	v	+	v	-	+	-
Utilisation of:																		
D-mannitol	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+
D-Mannose	+	+	+	+	+	+	-	+	v	+	+	-	-	+	+	-	-	+
myo-Inositol	-	-	-	-	v	-	-	-	-	+	+	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	v	-	-	+
Amygdalin	+	-	-	nd	-	+	-	v	-	nd	-	nd	-	nd	-	-	-	-
L-Arabinose	-	+	-	+	+	-	+	+	+	-	-	+	v	-	-	-	-	-
FAME composition:																		
16:0	18.7	25.6	29.2	21.0	22.0	14.4	24.0	16.0	16.5	17.4	20.0	18.8	12.9	27.6	29.6	nd	1.5	17.3
16:0 iso	9.0	0.0	0.0	2.7	3.0	1.7	1.9	4.9	1.0	1.9	1.4	1.5	1.1	0.0	0.0	nd	0.0	0.0

*Taxa are indicated as: 1, *V. pacinii*; 2, *V. i*; 3, *V. pomeroyi*, 4, *V. aestuarianus*; 5, *V. anguillarum*; 6, *V. diabolicus*; 7, *V. diazotrophicus*; 8, *V. fluvialis*; 9, *V. furnissii*; 10, *V. mediterranei*; 11, *V. metschnikovii*; 12, *V. mytili*; 13, *V. nereis*; 14, *V. orientalis*, 15, *splendidus* I; 16, *V. splendidus* II; 17, *V. tapetis*; 18, *V. tubiashiii*. The data shown was obtained from Pujalte et al. (1993), Raguenees et al. (1997), Novoa et al. (1998), Alsina & Blanch (1994a, b), Farmer (1992), Thompson et al. (in press), and Baumann & Schubert (1983). +, positive; -, negative; v, variable; nd, no data available.

Collectively, phenotypic characterisation, 16S rDNA sequences, DNA-DNA similarity and FAFLP fingerprinting (Thompson et al., 2001), presented here provide solid evidence to propose strains LMG 19999^T, LMG 21514, and LMG 13245 as members of a new species of the genus *Vibrio*, for which the name *V. pacinii* is proposed.

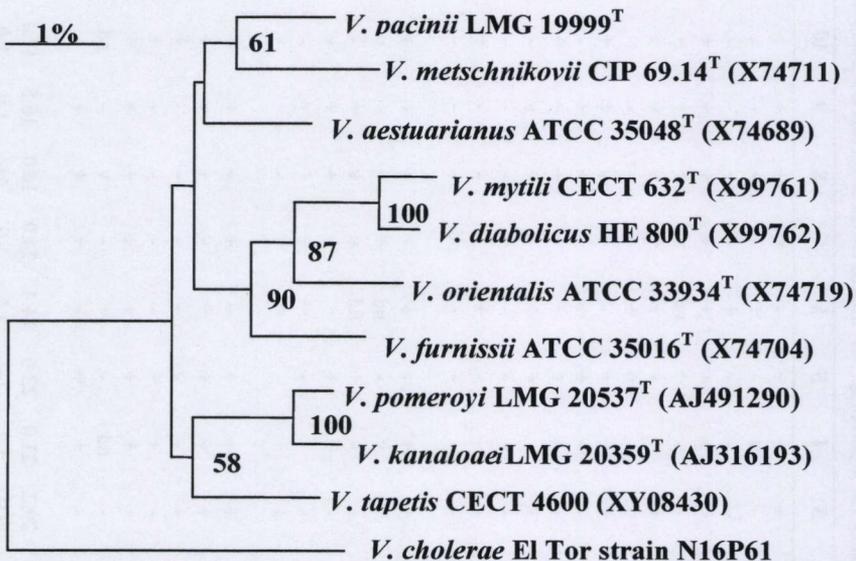


Figure 3.13. Consensus phylogenetic dendrogram of strain LMG 19999^T (= CAIM 530^T) and the closest *Vibrio* species derived from the almost complete 16S rDNA sequence data. Tree topology obtained by Neighbor Joining (0.4 Gamma correction, pairwise deletion, Jukes-Cantor correction). Numbers at nodes indicate the level of bootstrap support (1000 replicates). Bar 1 % divergence.

Description of *Vibrio pacinii* sp. nov.

Vibrio pacinii (pa.ci'nii. N.L. n. masc. pacini of Pacini, named after the Italian anatomist Filippo Pacini, who first discovered the causal agent of cholera). Gram-negative rods, motile, with polar flagella. Non-luminescent colonies, non-pigmented, translucent on marine agar with no swarming. Round, firm, yellow bright colonies on TCBS agar, 1.5-2.8 mm in diameter. Grows in the presence of 1.5, 2.5, 6.0, and 8.0 % NaCl but not at 0, 10.0 and 12.0 %; can grow at 4, 30, 35 °C but not at 40 °C in TSB. Oxidase and catalase positive, ferment glucose and lactose; arginine dihydrolase and L-tyrosine positive, lysine and ornithine decarboxylase negative. Positive for the Voges-Proskauer test (except strain LMG 13245), methyl red, tryptophane deaminase, nitrate reduction; negative for indol production, H₂S, and urease. Test for citrate utilisation is weakly positive, except strain LMG 13245 which is strongly positive. Positive activity of acid phosphatase (LMG 13245 weak), α -glucosidase (LMG 21514 weak), alkaline phosphatase, esterase (C4), esterase lipase (C8) (LMG 13245 weak), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, and valine arylamidase (LMG 13245 and LMG 21514 weak). Negative activity of α -chymotrypsin (LMG 19999^T is weakly positive), α -fucosidase, α -galactosidase, α -mannosidase, β -glucosidase, β -glucuronidase, lipase (C14), N-acetyl- β -glucosaminidase, and trypsin. All the strains could utilise α -D-glucose, cellobiose, dextrin, D-fructose, D-mannitol, D-trehalose, gentiobiose, inosine, L-asparagine, L-glutamic acid, L-serine, maltose, N-acetyl-D-glucosamine, sucrose, thymidine, and uridine as sole source of carbon. None of the strains could utilise 2,3-butanediol, 2-amino ethanol, acetic acid, adonitol, alaninamide, α -D-lactose lactulose, α -hydroxy butyric acid, α -keto butyric acid, α -keto glutaric acid, α -keto valeric acid, α -lactose, β -hydroxy butyric acid, bromo succinic acid, cis-aconitic acid, citric acid, D,L- α -glycerol phosphate, D,L-carnitine, D-alanine, D-arabitol, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, D-mannose, D-melibiose, D-raffinose, D-saccharic acid, D-serine, formic acid, γ -amino-butyric acid, γ -hydroxy butyric acid, glucose-1-phosphate, glucose-6-phosphate, glucuronamide, glycerol, glycyl-L-glutamic acid, hydroxy L-proline, i-erythritol, itaconic acid, L-arabinose, L-fucose, L-histidine, L-leucine, L-ornithine, L-phenyl alanine, L-proline, L-pyro glutamic acid, L-rhamnose, L-threonine, malonic acid, m-inositol, N-acetyl-D-galactosamine, phenyl ethylamine, p-hydroxy phenylacetic acid,

propionic acid, putrescine, quinic acid, sebacic acid, succinamic acid, turanose, Tween 40, Tween 80, urocanic acid, and xylitol. The following cellular fatty acids are present in decreasing order (percentage of the three strains analysed, maximum and minimum of the total fatty acid content): 16:0 (18.7, 25.5 – 14.1), 18:1 ω 7c (11.1, 13.4 – 9.9), 16:0 iso (9.0, 9.7 – 8.4, not detected in LMG 21514), 14:0 (6.9, 11.2 – 4.7), 12:0 (3.7, 5.2 – 1.9), 12:0 3-OH (3.1, 4.9 – 1.5), 14:0 iso 3-OH (1.9, 2.7 – 1.1, not detected in LMG 21514), and 17:0 iso (1.4, 2.0 – 0.90, not detected in LMG 21514). 16:1 ω 7c and/or 15 iso 2-OH (summed feature 3, 38.66, 39.21 – 37.78) and 14:0 3-OH and/or 16:1 iso I (summed feature 2, 2.9, 3.6 – 1.5). Eighteen other fatty acids are detected but in percentages below 1.0, these are: 18:0 iso, 14:0 iso, 16:1 ω 7c alcohol, 13:0 iso, 15:0 iso, 17:0 anteiso, 18:0, 15:0, 17:0, 11 methyl 18:1 ω 7c, 17:1 ω 8c, 13:0 iso 3-OH, 15:0 iso 3-OH, 12:0 iso, 12:0 2-OH, 15:0 anteiso, 15:1 ω 8c, and 16:1 ω 5c. Antibiotic susceptibility was observed for chloramphenicol (30 μ g), oxolinic acid (2 μ g), oxytetracycline (30 μ g), polymyxin B (300 U), tetracycline (30 μ g); resistance to gentamicin (10 μ g) except LMG 21514 which is intermediate, kanamycin (30 μ g), streptomycin (25 μ g), and to the vibriostatic agent 0/129 at 10 and 150 μ g. Additional phenotypic features are listed in Table 3.23. The G + C content of the DNA is 44.9 mol %. The type strain is LMG 19999^T (= CAIM 530^T) isolated shrimp larvae (*Penaeus chinensis*) in Dahua hatchery in Laizhou (Shandong Province, China). Reference strains are LMG 13245 (=CAIM 526) and strain LMG 21514 (=CAIM 466).

Table 3.23. Phenotypic differences among strains of *V. pacinii* sp. nov.

Test	LMG 19999 ¹	LMG 21514	LMG 13245
Voges-Proskauer	+	+	-
Utilization of:			
Citrate	w	w	+
β -methyl D-glucoside	+	-	-
D,L-lactic acid, D-galactose, psicose	+	-	+
D-sorbitol, glycogen, α -cyclodextrin	-	-	+
Glycyl-L-aspartic acid	w	+	-
L-alanine, L-alanyl-glycine	+	w	+
L-aspartic acid	+	+	-
Methyl pyruvate	+	-	-
Mono methyl succinate	-	+	-
Succinic acid	+	+	-
Activity of:			
β -galactosidase	+	-	+
cystine arylamidase	+	w	-
% of fatty acid content			
16:0 iso	8.41	nd	9.69
14:0 iso 3-OH	2.71	nd	1.08
16:0	14.07	25.55	16.62
Susceptibility to:			
ampicillin (30 μ g)	S	S	R
amikacin (30 μ g)	I	S	R

+, positive; -, negative; w, weak reaction; S, sensitive; I, intermediate; R, resistant; nd, not detected.

TABLE 1
 Statistical Analysis of the Data

Variable	Mean	Standard Deviation	Sample Size
1. Age	35.2	12.5	100
2. Sex	0.5	0.5	100
3. Education	12.5	2.5	100
4. Income	15000	5000	100
5. Health	4.5	1.5	100
6. Satisfaction	3.8	1.2	100
7. Stress	5.2	1.8	100
8. Quality of Life	4.1	1.4	100
9. Social Support	3.5	1.1	100
10. Coping Mechanisms	4.8	1.6	100
11. Resilience	4.3	1.3	100
12. Life Satisfaction	3.9	1.2	100
13. Psychological Well-being	4.6	1.5	100
14. Overall Health	4.4	1.4	100
15. Life Expectancy	75.5	5.5	100
16. Quality of Life	4.2	1.3	100
17. Life Satisfaction	3.7	1.1	100
18. Psychological Well-being	4.7	1.6	100
19. Overall Health	4.5	1.4	100
20. Life Expectancy	76.2	5.8	100

3.9. *Vibrio rotiferianus* sp. nov., isolated from cultures of the rotifer *Brachionus plicatilis*

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Int. J. Syst. Evol. Microbiol. (2003), 53, 239-243

Abstract

Five Gram-negative strains, oxidase positive, motile by means of more than one polar flagella, facultative anaerobe, arginine dihydrolase negative, lysine and ornithine decarboxylase positive, sensitive to the vibriostatic agent O/129, were isolated from a flow-through rotifers culture system in Gent, Belgium and previously characterized by FAFLP. Comparison of the 16S rDNA sequence of strain LMG 21460^T indicated close relationship (~ 99 % similarity) to *V. campbellii*, *V. harveyi*, *V. alginolyticus*, and *V. parahaemolyticus*. However, DNA hybridisation experiments revealed similarity values below 70 % with its closest species *V. campbellii* and *V. harveyi*. Additionally, the analysed strains differ from related *Vibrio* species by the utilization of melibiose, and production of acid from L-arabinose and amygdaline. Among the strains analysed, differences were observed in some phenotypic characters, particularly susceptibility to ampicillin, polymixin B, and amikacin, and urease activity. The major fatty acids identified were 16:0, 18:1 ω 7c, 14:0, 12:0 3-OH, and 18:0. The type strain is LMG 21460^T (= CAIM 577^T) with a DNA G+C content of 44.5 mol %. *Vibrio rotiferianus* is proposed as a new species. The Genbank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of *Vibrio rotiferianus* LMG 21460^T is AJ316187.

Introduction

Rotifers are an important nutritional source for the culture of many aquatic organisms larvae, specially fish and crustaceans. Bacteria present in rotifer cultures can reach high numbers and are transmitted to the target larvae with the rotifers at feeding (Munro *et al.*, 1994), and thus may cause poor survival and growth of the fish larvae (Gatesoupe, 1989). Other bacteria may enhance the growth of rotifers

(Douillet, 2000), and of fish larvae (Skjermo and Vadstein, 1999). The principal genera identified in rotifer cultures have been *Pseudomonas*, *Vibrio*, *Moraxella*, and *Flavobacterium* (Verdonck *et al.*, 1994, Verdonck *et al.*, 1997). *Vibrio* was the dominant genus in rotifer cultures, constituting up to 56 % of the bacterial community, with *V. anguillarum*, *V. alginolyticus*, *V. diazotrophicus*, *V. mediterranei*, and *V. tubiashii*-like as representative species (Verdonck *et al.*, 1997). Understanding the bacterial composition of rotifers and rotifer cultures is important for the aquaculture industry. Several bacteria have been isolated from rotifers and from the water of a flow-through rotifer system during August 1999 at the Artemia Reference Centre, University of Ghent, Belgium. Here we present a polyphasic characterisation of five dominant *Vibrio* isolates in this system. The isolates (LMG 21456, LMG 21457, LMG 21458, LMG 21459, and LMG 21460^T) were analysed by Thompson *et al.* (2001) by Fluorescent Amplified Fragment Length Polymorphism (FAFLP) and 16S rDNA sequencing. They showed: 1) that these strains formed a tight cluster, and 2) that no known *Vibrio* type species grouped into this cluster. Therefore, all five isolates were considered as potentially novel species of *Vibrio*.

Material and Methods

The rotifer rearing system and bacterial isolation procedures have been described by Suantika *et al.* (2001). Samples of rotifer culture water and from rotifers + water were homogenized and serially diluted in sterile saline solution (1.5 % w/v), plated onto marine agar (Difco, USA) and thiosulphate-citrate-bile salts-sucrose agar (TCBS; Difco, USA), and incubated for 24-48 h at 25 °C. The five strains were phenotypically analysed by API 20E, API ZYM (bioMeriux, France), and Biolog GN2 (Biolog, Hayward, USA) according to the manufactures instructions, except that sterile saline solution (SSS, 1.5 % NaCl w/v) was used to prepare the inocula. Other phenotypic tests were performed following the methodologies of Lanyi (1987). Presence of flagella was determined with Gray's stain (Murray *et al.*, 1994). Antibiotic sensitivity was estimated by the disk diffusion test (Bauer *et al.*, 1966) in Iso-sensitest agar (Oxoid, UK) + 1.5 % NaCl (w/v). Fatty acid analysis was performed as described by Osterhout *et al.* (1991), except that the cells were grown on Tryptone Soya Agar (TSA; Oxoid, UK) + 1.5 % NaCl (w/v) and incubated at 28 °C for 24 h. The 16S rDNA sequence of strain LMG 21460^T (EMBL accession no. AJ316187) was compared with sequences deposited in EMBL (FASTA, Pearson and Lipman,

1988) and in the Ribosomal Database Project (RDP; Maidak *et al.*, 1999) to specify the closest related species. Sequences of relevant taxa and of strain LMG 21460^T were aligned by means of Clustal X ver. 1.8 (Thompson *et al.*, 1997). Distance estimations (Jukes and Cantor, 1969), tree topology (Neighbour joining, Saitou and Nei, 1987 with 0.4 Gamma correction and pairwise deletion), and stability of groupings (Bootstrap analysis, 1000 replicates) were performed with the MEGA ver. 2.1 software (Kumar *et al.*, 2001) with *V. cholerae* as outlier. G+C content of the DNA of strain LMG 21460^T was determined as described by Mesbah *et al.* (1989) using the modifications proposed by Logan *et al.* (2000). DNA-DNA hybridisation analysis was carried out at stringent conditions (39 °C) following the methodology described by Willems *et al.* (2001).

Results and Discussion

All five isolates grew well on TCBS agar as bright non-luminescent yellow colonies and unpigmented translucent colonies in marine agar. Phenotypically, the five strains can be clearly assigned to the genus *Vibrio* (Alsina and Blanch, 1994a), and present many characters that clearly distinguish them from similar species (Table 3.24). Of particular interest is the capacity to utilize melibiose, a feature only observed in *V. nigripulchritudo*, *V. agarivorans*, and some strains of *V. natriegens*, but in none of the arginine dihydrolase negative, lysine and ornithine decarboxylase positive species. Differences were observed in the phenotypic characters among the five strains analysed (see Table 3.25 in the description of the species).

Table 3.24. Phenotypic characters differentiating *Vibrio rotiferianus* sp. nov. from related arginine negative, lysine and ornithine positive (A-, L+, O+) *Vibrio* species.*

Test	1	2	3	4	5	6	7	8	9	10	11	12
Growth in the presence of:												
0% NaCl (w/V)**	-	-	-	+	-	-	-	+	-	-	-	-
8% NaCl(w/V)**	-	+	v	-	-	(+)	-	-	+	v	v	-
Citrate**	-	+	v	v	-	v	-	+	+	d	d	+
Voges-Proskauer**	-	(+)	-	+	-	-	-	(-)	-	-	-	-
Utilization of:												
L-Arabinose**	+	-	-	-	-	v	-	-	-	-	-	-
D-Mannitol	-	+	(+)	+	v	+	+	nd	+	+	+	d
D-Mannose	+	v	+	v	-	+	v	+	-	-	-	-
Melibiose	+	-	-	-	-	-	-	-	-	-	-	-
Acid from:												
L-Arabinose	+	-	-	-	-	-	-	-	-	-	-	-
Amygdaline	+	-	-	-	-	-	+	-	-	-	-	-
Activity of:												
α-chymotrypsin	+	-	+	-	-	+	-	-	-	-	nd	-

*Data for related A-, L+ and O+ *Vibrio* species were taken from Alsina and Blanch (1994a) and Baumann and Schubert (1984). Percentages indicate positive results; +, positive for $\geq 90\%$; (+), positive for 75-89%; -, negative for $\leq 10\%$; (-), negative for 25-11%; v, variable for 26-74%; nd, no data; d, discrepancies between authors. 1, *V. rotiferianus* (n=5); 2, *V. alginolyticus*; 3, *V. campbellii*; 4, *V. cholerae*; 5, *V. fischeri*; 6, *V. harveyi*; 7, *V. logei*; 8, *V. mimicus*; 9, *V. parahaemolyticus*; 10, *V. splendidus* I; 11, *V. splendidus* II; 12, *V. vulnificus*. **test useful to differentiate A-, L+, O+ *Vibrio* species according to the scheme of Alsina and Blanch (1994).

Fatty acid analysis showed a distinctive pattern different from its closest phylogenetic neighbours, *V. harveyi* and *V. campbellii*. Percentage of the fatty acid 14:0 was 9.52 % in average (Max. 10.31 %, Min. 8.89 %), while in *V. harveyi* and *V. campbellii* was 4.88 and 4.28 % respectively; 16:0 was 25.40 % (Max. 28.47 %, Min. 21.18 %) compared to 13.94 % and 17.04 % respectively; and 18:1 w7c 10.79 % (Max. 12.34 %, Min. 9.13%) against 21.05 % and 22.55 % respectively. For other fatty acids see species description, but no clear differences with the other type strains were observed. In general, the identified fatty acids of strain LMG 21460^T were in agreement with the fatty acid signature of the genus *Vibrio*; only the fatty acid 14:0 was slightly above the maximum reported for the genus (8.63 %) (Bertone *et al.*, 1996).

The 16S rDNA sequence clearly classified strain LMG 21460^T in the genus *Vibrio*. The closest phylogenetic neighbours were *V. campbellii* (99.86 % FASTA and 99.2 % RDP) and *V. harveyi* (99.11 % and 96.7 %) (Figure 3.14). Phylogenetic little taxonomic change overtime; the last species described was *V. vulnificus* (Farmer, 1980).

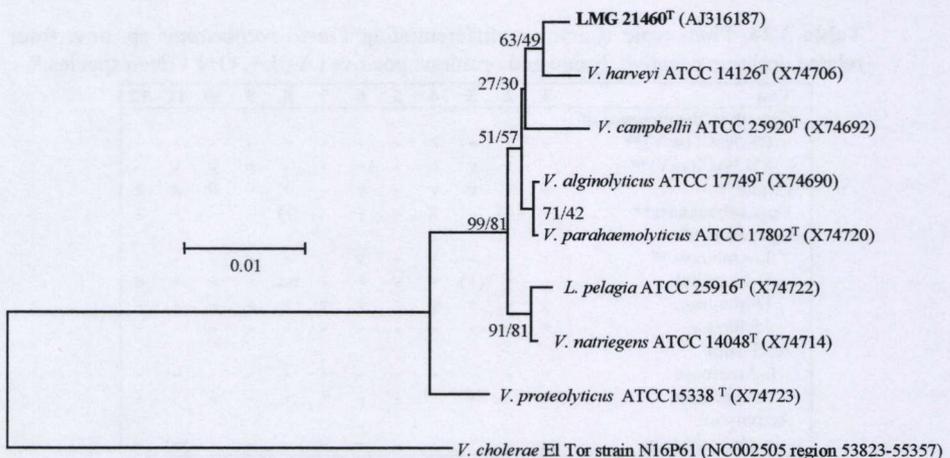


Figure 3.14. Phylogenetic dendrogram of strain LMG 21460^T (= CAIM 577^T) and the closest *Vibrio* species derived from the almost complete 16S rDNA sequence data. Neighbour-joining method, 0.4 gamma correction, pairwise deletion, with Jukes-Cantor correction. Numbers at nodes denote the level of bootstrap support based on 1000 replicates, neighbour joining/maximum likelihood. Bar = 1 % sequence divergence.

analysis with Maximum Likelihood and Maximum Parsimony treeing methods produced congruent results with the neighbour joining method regarding the positioning of the type strain LMG 21460^T. Strain LMG 21460^T clustered within the group of *Vibrio* species called the *V. harveyi* group (Reichelt *et al.*, 1976), and later called the core group of the *Vibrio* genus (Dorsch *et al.*, 1992).

The DNA G+C content determined was 44.5 ± 0.01 mol % ($n = 3$); this value is within the range of values reported for *Vibrio* (Baumann and Schubert, 1983). Strain LMG 21460^T was hybridised with its two closest neighbours (by 16S rDNA) *V. campbellii* (LMG 11216^T) and *V. harveyi* (LMG 4044^T) showing 65 and 66 % reassociation respectively. The DNA reassociation between *V. campbellii* and *V. harveyi* was 69 %, a similar result to the 65 % obtained by Reichelt *et al.* (1976). These results clearly showed that strain LMG 21460^T is closely related to *V. campbellii* and *V. harveyi*, but it can be differentiated from these taxa by means of FAFLP (Thompson *et al.*, 2001), rep-PCR (Gomez-Gil *et al.*, unpublished), DNA-DNA hybridisations, as well as by several phenotypic traits, i.e. utilization of melibiose, and acid formation of L-arabinose and amygdaline (Table 3.24).

Description of *Vibrio rotiferianus* sp. nov.

Vibrio rotiferianus (ro.ti.fe.ria'nus. English n. *rotifer*; L. suf. *-nus a um*; N.L. masc. adj. *rotiferianus*, pertaining to rotifers). Gram-negative curved rods (0.8-1.2 x 2.0-3.5 μ m), facultative anaerobic, motile by means of more than one polar flagella. Non-pigmented, translucent, non-luminescent colonies on marine agar with no swarming. Bright, round, 2-3 mm yellow colonies, with umbilicated growth in TCBS agar. No growth occurs without NaCl ions in the culture medium; growth occurs in the presence of 1.5, 3.0, and 6.0 % NaCl (w/v), but not at 8 or 10 %; grows at 28 - 40 °C, but not at 4 °C. Susceptible to chloramphenicol (30 μ g), tetracycline (30 μ g), oxolinic acid (2 μ g), oxytetracycline (30 μ g), and to the vibriostatic agent 0/129 at 10

and 150 µg; resistant to kanamycin (30 µg), streptomycin (25 µg), and gentamicin (10 µg). Arginine dihydrolase negative, lysine and ornithine decarboxylase positive, ferments glucose without producing gas; indole, oxidase, urease, tryptophane deaminase, gelatinase positive. Voges-Proskauer, H₂S, and citrate negative. Phenotypic differences are observed between the strains (Table 3.25). It utilizes the following substrates as sole carbon source: alaninamide, α -cyclodextrin, α -D-glucose, β -methyl D-glucoside, cellobiose, dextrin, D-fructose, D-galactose, D-gluconic acid, D-glucuronic acid, D-mannose, D-melibiose, D-raffinose, D-serine, D-trehalose, gentiobiose, glucose-6-phosphate, glycogen, glycyl-L-aspartic acid, inosine, L-alanine (LMG 21460^T and LMG 21458 weakly positive), L-alanine-glycine (LMG 21460^T and LMG 21458 weakly positive), L-arabinose, L-asparagine, L-aspartic acid, L-serine, maltose, n-acetyl-D-glucosamine, psicose, sucrose, thymidine, and uridine. None of the strains utilizes the following carbon sources: 2,3-butanediol, 2-amino ethanol, acetic acid, adonitol, α -D-lactose lactulose, α -hydroxy butyric acid, α -keto butyric acid, α -keto glutaric acid, α -keto valeric acid, Cis-aconitic acid, citric acid, D,L-alpha-glycerol phosphate, D,L-carnitine, D-alanine, D-arabitol, D-galactonic acido lactone, D-galacturonic acid, D-glucosaminic acid, D-mannitol, D-saccharic acid, D-sorbitol, formic acid, γ -amino-butyric acid, glucose-1-phosphate, glucuronamide, glycerol, glycyl-L-glutamic acid, hydroxy L-proline, i-erythritol, itaconic acid, L-fucose, L-histidine, L-leucine, L-ornithine, L-phenyl alanine, L-proline, L-pyro glutamic acid, L-rhamnose, malonic acid, methyl pyruvate, m-inositol, mono methyl succinate, n-acetyl-D-galactosamine, phenyl ethylamine, P-hydroxy phenylacetic acid, propionic acid, putrescine, quinic acid, sebacic acid, succinamic acid, succinic acid, turanose, Tween 40, Tween 80, urocanic acid, and xylitol. All are weakly positive for D, L-lactic acid (except LMG 21457, positive). All strains have activities of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, and naphthol-AS-BI-phosphohydrolase. None showed activity of lipase (C14), cystine arylamidase, β -galactosidase, α -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, n-acetyl- β -glucosaminidase, α -mannosidase, and α -fucosidase. The following cellular fatty acids are present in decreasing order (mean percentage of the five strains analysed, and maximum and minimum of the total fatty acid content): 16:0 (25.40, 28.47 - 21.18), 18:1 ω 7c (10.79, 12.34 - 9.13), 14:0 (9.52, 10.31 - 8.89), 12:0 3-OH (2.91, 3.84 - 2.33), 18:0 (1.10, 1.35 - 0.75). Undefined fatty acids are also

observed, summed feature 3 (16:1 ω 7c and/or 15 iso 2-OH; 37.14, 39.77-34.79), summed feature 2 (14:0 3-OH and/or 16:1 iso I; 7.05, 8.65 - 5.98), and one unknown (0.74, 0.98 - 5.98). The G + C content of the DNA is 44.5 mol %. The type strain is LMG 21460^T (= CAIM 577^T) isolated from a rotifer (*Brachionus plicatilis*) flow-through culture system.

Table 3.25. Phenotypic differences among the five strains of *Vibrio rotiferianus* sp. nov.

Test	1	2	3	4	5
γ -lactose	-	+	-	-	w
D,L-lactic acid	w	w	+	w	w
Bromo succinic acid	-	+	w	w	-
L-Glutamic acid	-	+	w	w	-
L-Threonine	-	+	-	w	w
Urease	+	+	+	+	-
Susceptibility to:					
Ampicillin (30 μ g)	R	I	S	R	I
Polymyxin B (300 U)	R	R	I	R	R
Amikacin (30 μ g)	I	R	R	R	R

w, weak reaction; R, resistant; I, intermediate; S, sensitive. 1, LMG 21460^T; 2, LMG 21459; 3, LMG 21457; 4, LMG 21456; 5, LMG 21458.

3.10. *Vibrio superstes* sp. nov., isolated from the gut of Australian abalones *Haliotis laevis* and *Haliotis rubra*

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Int. J. Syst. Evol. Microbiol. (2003), in press

Abstract

Five alginolytic, facultative anaerobic, non-motile bacteria were isolated from the gut of abalone *Haliotis laevis* and *H. rubra*. Phylogenetic analyses based on 16S rDNA data indicated that these strains are closely related to *Vibrio halioticoli* (98 % sequence similarity). DNA-DNA hybridization and AFLP fingerprinting demonstrated that the five strains constituted a single species different from all currently known vibrios. The name *Vibrio superstes* sp. nov. (LMG 21323^T=IAM15009^T=G3-29^T; mol% G+C of DNA is 48.0-48.9; EMBL accession no. is AY155585) is proposed to encompass this new taxon. Several phenotypic features were disclosed which discriminate *V. superstes* from other *Vibrio* species; *V. superstes* sp. nov. and *V. halioticoli* can be differentiated on the basis of 17 traits (indole production, β -galactosidase test and assimilations of 15 carbon compounds).

Introduction

Vibrio halioticoli and genetically related species, which are alginolytic, non-motile fermentative marine bacteria, are abundant in the gut of *Haliotis* abalones in Japan and South Africa (Sawabe et al., 1995, 2002). The hypothesized roles of *V. halioticoli* are contribution in digesting alginate which is a major polysaccharide in Japanese kelps ingested by these animals, and in its conversion into volatile short chained fatty acids via fermentation (Sawabe et al., 2003). Nearly 80 species of abalones are known and they appear in offshore areas worldwide, but little is known about the presence of *V. halioticoli*-like bacteria in the gut of these molluscs. Recently we isolated a set of five strains which were phenotypically most similar to *V. halioticoli* from the gut of Australian abalones (*Haliotis laevis* and *H. rubra*). DNA-DNA

hybridization experiments, phenotypic characterizations, phylogenetic and genetic analyses demonstrated that these strains represent a so far unknown species of *Vibrio*.

Material and Methods

Five strains of *V. superstes* LMG 21319=IAM 15007 (B1-5), LMG 21320=IAM15008 (B2-3), LMG 21321 (G3-11), LMG 21322 (G3-15), LMG 21323^T= IAM 15009^T (G3-29) isolated from the gut of Australian abalones *H. laevigata* and *H. rubra*. These were collected at the coastal area of Criffton Springs (Victoria) by Scuba diving in December, 2000. Strains were cultured on ZoBell 2216E agar (Oppenheimer and ZoBell, 1952) and stored at -80°C in 10 % glycerol. A total of 78 phenotypical characteristics, including alginase activity were determined as described previously (Baumann and Schubert, 1984; Hidaka and Sakai, 1968; Holt et al., 1994; Leifson, 1963; Ostel and Holt, 1982; West et al., 1977). The phenotypic characterization was done at 20°C.

DNAs of bacterial strains were prepared by the procedures of Marmur (1961). Mol % G+C content of DNAs were determined by HPLC (Tamaoka and Komagata, 1984). DNA-DNA hybridization experiments were performed in microdilution wells using a fluorometric direct binding method previously described by Ezaki et al. (1988, 1989). A total of 1400 bp 16S rDNA gene sequences of strains LMG 21319, LMG 21320, LMG21321, LMG 21322 and LMG 21323^T were determined according to Sawabe et al. (1998) using six sequence primers (24F, 530F, 1100F, 520R, 920R, and 1540R). FAFLP analysis was performed as described previously (Sawabe et al., 2002; Thompson et al., 2001). Clustering of the patterns was done using Dice (SD) and Ward coefficients (Sneath and Sokal, 1973).

Results and Discussion

The results of our phylogenetic analysis clearly showed that the strains belong to the gamma-3 subgroup, phylum *Proteobacteria* (Garrity and Holt, 2000). The five strains of *V. superstes* had high levels of similarity i.e. 99.7 to 99.9 %. The closest phylogenetic neighbor of the five Australian abalone strains is *V. halioticoli* (98 % similarity) (Figure 3.15A). Similarity levels below 97 % were found with other *Vibrio* species.

The five strains had AFLP patterns consisting of 90 fragments (SD ± 10; max-min. 80-108) and mutual similarity of at least 64.6 % (Figure 3.15B). *V. superstes*

showed pattern similarities below 50 % towards other *Vibrio* species, clearly pointing out that this novel species is different from other vibrios (Thompson et al., 2002). The AFLP results are supported by our DNA-DNA hybridization experiments which showed that the five strains of *V. superstes* LMG 21319, LMG 21320, LMG 21321, LMG 21322 and LMG 21323^T were conspecific strains clearly apart from *V. haliotocoli* (Table 3.26).

The five Australian abalone strains have the main phenotypical features of the genus *Vibrio* (except for the absence of flagella). The strains are non-motile, gram-negative, fermentative (Sawabe et al., 1998). No flagellated cells were detected by TEM analyses, but short tubular projections were observed in *V. superstes* cells similar to those reported previously in *V. campbellii* (Allen and Baumann, 1971). Proper function of the

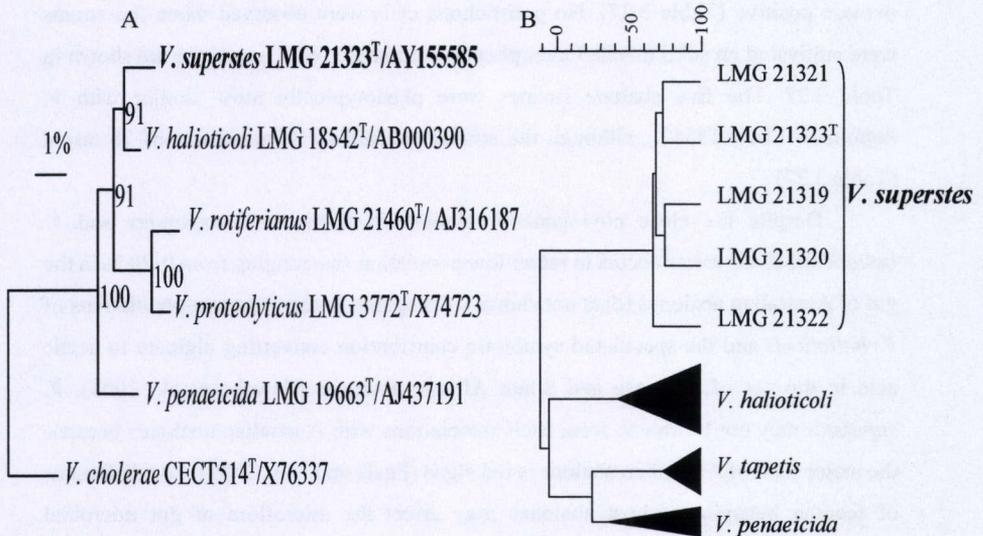


Figure 3.15. Phylogenetic relationships of *V. superstes* and other *Vibrio* species. A. Phylogenetic tree based on the almost complete 16S rDNA sequences and the neighbour joining methodology. Bar indicates 1 % divergence. B. Dendrogram of the FAFLP patterns of the five *V. superstes* from Australian abalone. *V. haliotocoli*, *V. tapetis* and *V. penaeicida* were included as out-groups.

Table 3.26. DNA-DNA binding values and mol % G+C of DNA of *Vibrio* strains

	Mol % G+C	Reassociation (%) with biotinylated DNA from:	
		1. LMG 21323 ^T	6. LMG 18542 ^T
<i>V. superstes</i>			
1. LMG 21323 ^T	48.6	100	22
2. LMG 21319	48.0	84	30
3. LMG 21320	48.9	75	28
4. LMG 21321	48.9	87	24
5. LMG 21322	48.3	94	26
<i>V. haliotocoli</i>			
6. LMG 18542 ^T	43.1	13	100

tubular projections has never been clarified (Allen and Baumann, 1971). This strains required salt for its growth, did not accumulate poly- β -hydroxybutyrate and was oxidase positive (Table 3.27). No peritrichous cells were observed when the strains were cultivated on solid media. Other phenotypic features of *V. superstes* are shown in Table 2.27. The five abalone isolates were phenotypically most similar with *V. haliotocoli* LMG 18542^T, although the strains differed by 17 traits out of 78 tested (Table 3.27).

Despite the close phylogenetic relationship between *V. superstes* and *V. haliotocoli*, *V. superstes* occurs in rather low population size ranging from 0-20 % in the gut of Australian abalones (data not shown). Compared to the abundant populations of *V. haliotocoli* and the speculated symbiotic contribution converting alginate to acetic acid in the gut of Japanese and South African abalones (Sawabe et al., 2003), *V. superstes* may not be able to form such associations with Australian abalones because the major food of Australian abalone is red algae (Foale and Day, 1989). The difference of feeding behavior of host abalones may affect the microflora of gut microbial ecosystem, and select the biochemical traits of symbiotic vibrios. The major phenotypic traits of *V. superstes* differed from those of *V. haliotocoli* in being positive for use of 14 carbon compounds (Table 3.27). *V. superstes* might have acquired the ability to assimilate multiple carbon compounds to survive in the gut of Australian abalone.

Table 3.27. Phenotypic characteristics for distinguishing *Vibrio superstes* from previously described alginolytic *Vibrio* species.

	<i>V. superstes</i>	<i>V. halioticoli</i>	<i>V. pelagius</i>
Motility	-	-	+
Growth at 37 °C	-	-	+
Production of:			
Lipase	-	-	+
Indole production	-	+	-
ONPG	-	+	+
Growth in:			
1 and 6 % NaCl	-	-	+
Acid from:			
sucrose, D-sorbitol	-	-	+
Utilization of:			
D-Mannose, sucrose, D-gluconate, D-galactose, γ -aminobutyrate, L-glutamate, acetate	+	-	+
Glycerol	-	+	+
Cellobiose, D-xylose, melibiose, lactose, D-glucuronate	+	-	-
Trehalose	v+	-	+
Putrescine	v-	-	+
Pyruvate, α -ketoglutarate	-	-	+
Propionate	v+	-	+

+, Positive; -, negative; v+, variable but type strain is positive; v-, variable but type strain is negative. All species are negative for: pigmentation, swarming, PHB accumulation, luminescence, growth at 4 and 40 °C, amylase, gelatinase, chitinase, agarase, gas production from D-glucose, acetoin production, lysine decarboxylase, arginine dehydrolase, ornithine decarboxylase, acid from L-arabinose, inositol, L-rhamnose, requirement for organic growth factors, utilization of D-sorbitol, L-tyrosine, meso-erythritol, L-arabinose, citrate, DL-malate, d-aminovavate, aconitate. All species are positive for: Na⁺ requirement, growth at 15 and 30°C, oxidase, catalase, alginase, nitrate reduction, O/129 sensitivity, methyl red test, growth on TCBS, growth in 3% NaCl, acid from D-glucose, D-mannitol, maltose, Utilization of alginate, D-fructose, D-glucose, maltose, D-mannitol, D-glucosamine, N acetylglucosamine, fumarate, succinate. All species are fermentative in OF test.

In conclusion, our polyphasic study clearly demonstrated that the five abalone isolates represent a new species of the genus *Vibrio*, for which we propose the name *Vibrio superstes*. The name *V. superstes*, which means survivor, has been chosen in this respect. Global whole genome analyses could clarify the evolutionary history of *V. superstes* and *V. halioticoli*. Studies on the ecology of *V. superstes* are underway in order to better understand its interactions in the gut of marine herbivores, particularly abalones.

Description of *Vibrio superstes* sp. nov.

Vibrio superstes (L. n. *su.per'.stes*. the survivor). The bacterium is Gram-negative, facultatively anaerobic, non-motile, non-flagellated. Cells on ZoBell 2216E broth are rod shaped, with rounded ends (0.6-0.8 x 1.2-1.3 μm). No endospores or capsules are formed. Flagellation is not observed when the organism is cultivated on solidified media and/or in liquid media. Colonies on ZoBell 2216E agar are beige, circular, and smooth and convex with entire edge. Sodium ions are essential for growth. The bacterium is a mesophilic and neutrophilic chemo-organotroph which grows at temperatures between 15 and 30 °C. No growth occurs at 40 °C. The bacterium is positive for acid production from glucose; nitrate reduction, hydrolyses of alginate; oxidase; catalase; and assimilation of D-mannose, sucrose, D-gluconate, D-galactose, cellobiose, melibiose, lactose, D-glucronate, trehalose, γ -aminobutyrate, acetate, propionate, L-glutamate, D-xylose, D-fructose, maltose, D-glucosamine, N-acetylglucosamine, D-mannitol, fumarate, succinate, D-glucose and alginate. The following tests are negative: gas production from glucose, acetoin production, lysine decarboxylase, arginine dehydrolase, ornithine decarboxylase, indole production, β -galactosidase test, luminescence, pigmentation; requirement for organic growth factors; hydrolysis of starch, gelatin, chitin, Tween 80 and agar; accumulation of poly- β -hydroxybutyrate; assimilation of, D-sorbitol, glycerol, citrate, meso-erythritol, DL-malate, α -ketoglutarate, putrescine, δ -aminovarate, pyruvate, L-tyrosine, aconitate, and L-arabinose. The G+C content of DNA is 48.0-48.9 mol %. The type strain is LMG 21323^T=IAM 15009^T. The bacterium is isolated from the gut of Australian abalone *Haliotis rubra* and *H. laevigata*. The nucleotide sequences of *V. superstes* were deposited in the DDBJ and EMBL and GenBank DNA databases with the accession number AF519806 (LMG 21319=strain B1-5), AY155582 (LMG 21320=strain B2-3), AY155583 (LMG 21321=strain G3-11), AY155584 (LMG 21322=strain G3-15) and AY155585 (LMG 21323^T=strain G3-29^T).

3.11. *Vibrio tasmaniensis* sp. nov. , isolated from Atlantic Salmon (*Salmo salar* L.)

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Syst. Appl. Microbiol. (2003), 26, 65-69

Abstract

We describe the polyphasic characterization of four *Vibrio* isolates which formed a tight AFLP group in a former study. The group was closely related to *V. cyclitrophicus*, *V. lentus* and *V. splendidus* (98.2-98.9 % similarity) on the basis of the 16S rDNA sequence analysis, but by DNA-DNA hybridisation experiments it had at maximum 61 % DNA similarity towards *V. splendidus*. Thus, we propose that the isolates represent a new *Vibrio* species i.e. *V. tasmaniensis* (LMG 20012^T; EMBL under the accession numbers AJ316192; mol % G+C of DNA of the type strain is 44.7). Useful phenotypical features for discrimination of *V. tasmaniensis* from other *Vibrio* species include gelatinase and β -galactosidase activity, fatty acid composition (particularly 14:0), utilisation and fermentation of different compounds (e.g. sucrose, melibiose and D-galactose) as sole carbon source.

Introduction

The description of bacterial diversity has attracted much attention in the last years, and an increasing number of new species has been proposed (Rosselló-Mora and Amann, 2001). Recent estimations of the bacterial diversity in marine ecosystems by means of 16S rDNA similarity have revealed the existence of about 1200 bacterial species (Hagström et al., 2002). It has also been concluded that much of the species richness in these systems is already sampled (Hagström et al., 2002). On the other hand, more detailed studies based on whole genome analyses and phenotypic characterisation clearly demonstrated that many of the culturable bacteria (including vibrios) isolated from the marine environment are yet to be characterised (Mccammon and Bowman, 2000; Thompson et al., 2001). Indeed novel bacterial species found in the environment and in association with marine animals have recently been proposed

(Thompson et al., 2002a; Ben-Haim et al., 2002). In this respect it is clear now that coevolved animal-bacterial partnerships have been happening throughout the evolution of life of marine organisms. Benign e.g. *V. fischeri*-squid or *V. haliotocoli*-abalone and pathogenic e.g. *V. corallyticus*-coral or *V. harveyi*-white shrimp interactions among animals and vibrios have been documented, but understanding of animal-bacterial interactions is yet to be established and may depend also on a better overview of the bacterial diversity associated with host animals (Mcfall-Ngai, 2002; Sawabe et al., 2002; Ben-Haim et al., 2002). The bacterial microflora associated with Atlantic salmon has been examined in several studies, but mainly with the aim of identifying known bacterial species which have presumptive probiotic properties for fish (Ringø and Holzapfel, 2000; Ringø et al., 2001).

In this study we report on the taxonomic characterisation AFLP cluster A45 consisting four isolates originated from Atlantic salmon reared in Tasmania (Thompson et al., 2001). We propose that these isolates represent a new *Vibrio* species i.e. *V. tasmaniensis*.

Material and Methods

Bacterial strains, growth conditions and DNA isolation

Strains characterised in this study were: *Vibrio tasmaniensis* LMG 20012^T (VIB 836^T) LMG 21574 (VIB 840), LMG 21575 (VIB 848) and LMG 21576 (VIB 842); *V. lentus* LMG 21355 (LMG 21355) and LMG 21356 (R-3912); *V. cyclitrophicus* LMG 21579 (R-14870), LMG 21580 (R-14874) and LMG 21581 (R-1556). Strains were grown aerobically on tryptone soy agar (TSA; Oxoid) supplemented with 2 % (w/v) NaCl for 24 h at 28 °C. DNA was extracted following the methodology described by Pitcher et al. (1989). All strains included in this study are deposited in the BCCMTM/LMG Bacteria Collection at Ghent University and in the CAIM collection of the Centre for Research on Nutrition and Development (CIAD) in Mazatlán, México.

Genotypic analyses

Sequencing of the almost complete 16S rDNA sequences were accomplished essentially as described previously (Thompson et al., 2001). Alignment of the 16S rDNA sequences, distance estimations (Jukes and Cantor, 1969), clustering by neighbour joining (Saitou and Nei, 1987), maximum likelihood and maximum

parsimony methods and stability of the clusters (Bootstrap analysis with 1000 replicates) were performed with the software BioNumerics 2.5 (Applied Maths). DNA-DNA hybridisation experiments using photobiotin-labelled DNAs were run at stringent conditions (39 °C) following the methodology described by Willems et al. (2001). Hybridisations were performed in four replicates. DNA binding values are the mean of reciprocal and non-reciprocal reactions. The mol % G+C of DNA was determined by HPLC (Mesbah et al., 1989).

Phenotypic analyses

Phenotypic characterisation of the isolates was performed using API20E and Biolog GN metabolic fingerprinting kits following the instructions of the manufacturers, with slight modifications (Thompson et al., 2002b). Classical phenotypic tests were performed as described previously (Baumann et al., 1984; Farmer III and Whickman-Brenner, 1992; Murray et al., 1994; Thompson et al., 2002b). Antibiograms were carried out using the disc diffusion methodology (Acar and Goldstein, 1996) using commercial antibiotic-impregnated discs (Oxoid). The inhibition zone of each antibiotic was determined on Iso-sensitest agar (Oxoid) supplemented with 1.5 % (w/v) NaCl for 24 hours at 28 °C. Fatty acid methyl esters (FAME) analysis was carried out as described by Huys et al. (1994). Isolates were grown on Trypticase Soy Broth (TSB; Becton Dickinson) supplemented with 1.5 % (w/v) Bacto agar (Becton Dickinson) and 1.5 % (w/v) NaCl at 28 °C for 24 hours. Approximately 50 mg of cells were harvested and the fatty acid were isolated following the recommendations of the manufacturer using the Microbial Identification System manual and software package, version 3.9 (Microbial ID).

Results and Discussion

Phylogenetic analysis of representative strains of each AFLP cluster (i.e. A45, A55, and A61) using almost complete 16S rDNA sequences clearly showed that these strains branch within the *V. splendidus* phylogenetic branch (Figure 3.16). Strain LMG 20012^T had 98.9, 98.8 and 98.2 % 16S rDNA similarity towards *V. cyclitrophicus*, *V. lentus* and *V. splendidus* type strains, respectively. Strains LMG 21581 and LMG 21355 had 99.1 and 98.7 % 16S rDNA similarity towards *V. cyclitrophicus* and *V. lentus*, respectively. The 16S rDNA similarity of the

representative strains towards known *Vibrio* species was above the level proposed recently as the intraspecies variability (i.e. $\geq 98\%$) (Stackebrandt and Embley, 2000) and thus the 16S rDNA did not provide discrimination between the strains at species level. Nevertheless, it is well known that the 16S rDNA similarity between certain *Vibrio* species (e.g. *V. harveyi* and *V. campbellii*, *V. anguillarum* and *V. ordalii*) is nearly 100 %, indicating a need for applying other molecular techniques in order to better discriminate such species.

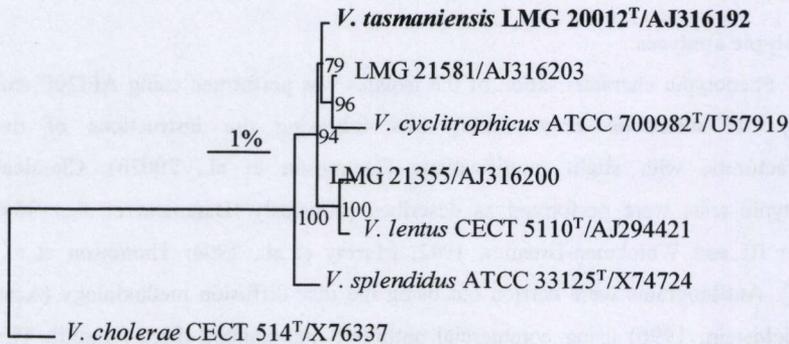


Figure 3.16. Phylogenetic tree with the estimated position *V. tasmaniensis*, using the neighbour joining method based on the almost complete 16S rDNA sequences. Bootstrap values ($> 50\%$) after 1000 simulations are shown. Bar, 1% estimated sequence divergence.

The results of DNA-DNA hybridisation experiments are summarised in the **Table 3.28**. Strain LMG 20012^T had at maximum 61 % DNA similarity towards *V. splendidus*. Additionally, DNA similarity towards the recently described species *V. kanaloaei*, *V. pomeroyi* and *V. chagassi* was below 60 %. Thus *V. tasmaniensis* can be considered a new species in the genus *Vibrio*. On the other hand A55 representative strains LMG 21356 and LMG 21355 clearly belong to *V. lentus* while A61 representative strains LMG 21579, LMG 21580 and LMG 20581 belong to *V. cyclitrophicus*. As already pointed out by Macián et al. (2001a), DNA-DNA similarity levels within this phylogenetic branch are high. These authors found 59 % DNA-DNA similarity between *V. lentus* and *V. splendidus*. The discrimination of different *Vibrio* species which are highly related on the basis of both 16S rDNA and DNA-DNA hybridisation seems to be more appropriate and reliable by applying genomic fingerprinting techniques e.g. AFLP and rep-PCR which can unambiguously identify

the different *Vibrio* species (Thompson et al., 2001). The A45 isolates were clearly distinguishable from other closely phylogenetic neighbours by means of AFLP analysis. Another elegant alternative would be the application of Multilocus sequence typing (MLST) to unravel the structure of the genus *Vibrio*. Attempts have been made in this respect, particularly on the study of the population structure of *V. cholerae* (Farfán et al., 2002). By analysing six house keeping genes spaced around the genome of *V. cholerae* strains these authors concluded that this bacterium forms a metapopulation which consists of several ecological populations of environmental and pathogenic strains.

Table 3.28. DNA-DNA binding values and mol % G+C of DNA of *Vibrio* strains

	1	2	3	4	5	6	7	8	9	10	Mol % G+C
<i>V. tasmaniensis</i> (A45)	100										44.7
1. LMG 20012 ^T											
<i>V. lentus</i> (A55)		100									44.5
2. LMG 21356											
3. LMG 21355		77	100								44.3
<i>V. ciclytrophicus</i> (A61)											
4. LMG 21580				100							44.3
5. LMG 20581				92	100						44.3
6. LMG 21579				94	99	100					44.1
<i>V. splendidus</i>											
7. LMG 19031 ^T	61	56	62	61	64	65	100				45.0
8. LMG 16751		61	63				70	100			44.5
<i>V. lentus</i>											
9. LMG 21034 ^T	55	78	81	52	55	56	57	62	100		45.2
<i>V. ciclytrophicus</i>											
10. LMG 21359 ^T	57	57	59	92	91	93	61	59	58	100	44.2

The novel *Vibrio* species represented by the four isolates of A45 examined in this study shared the main phenotypical and chemotaxonomic features of the genus *Vibrio* (Bertone et al., 1996; Farmer III and Hickman-Brenner, 1992; Lambert et al., 1983). The four isolates were facultative anaerobic, catalase and oxidase positive and showed prolific growth on thiosulfate-citrate-bile salts-sucrose agar (TCBS), forming green. Isolates were slightly curved rods, motile by at least a polar flagellum, susceptible to the vibriostatic agent 0/129, and did not grow without NaCl.

Genomic and phenotypic data described in this study clearly indicate that the four isolates should be accommodate in a new *Vibrio* species, namely *V. tasmaniensis*. Although the new species had the main phenotypical traits of the genus *Vibrio*, several useful differentiating features were disclosed which discriminate it from other related *Vibrio* species (Table 3.29).

Table 3.29. Differentiating features among *V. tasmaniensis* and closely related *Vibrio* species

	<i>V. tasmaniensis</i>	<i>V. cyclitrophicus</i>	<i>V. lentus</i>	<i>V. splendidus</i>
Growth at /in:				
35 °C	+	+	-	V
10 % NaCl	-	+	-	-
Susceptibility to 0/129	+	+	-	+
β-galactosidase	-	-	+	+
Gelatinase	-	+	+	+
Fermentation of:				
Sucrose	-	+	-	-
Melibiose	-	V	+	-
Utilisation of:				
Sucrose	-	+	-	-
Methyl pyruvate	-	V	V	+
L-proline	-	V	-	+
D-galactose	-	+	+	+
Fatty acid composition:				
14:0	9.5-12.4	6.2-8.2	4.8-8.8	6.3-8.7
Summed feature 3	34.4-36.1	37.1-38.3	33.3-41.7	39.4-40.4

Phenotypic data were obtained from Baumann et al. (1984); Farmer III and Hickman-Brenner (1992); Hedlund and Staley (2001) and Macián et al. (2001a). Fatty acids are minimum and maximum. Fatty acid profiles of known *Vibrio* species are from our own database. V, variable feature.

Description of *V. tasmaniensis*

Vibrio tasmaniensis (tas.ma.ni.en'sis N.L. fem. adj. *tasmaniensis* of Tasmania, where the organism was isolated). Cells are slightly curved, 1 µm in width and 2-3 µm in length. They form translucent, convex, non-swarming, smooth-rounded colonies with entire margin, beige in colour and of about 4 mm in diameter on TSA after 48 h incubation at 28 °C. Strains formed green, translucent, smooth-rounded colonies of 4-5 mm on TCBS. All strains have a facultative anaerobic metabolism and

ferment glucose, mannitol and amygdalin, but not inositol, sorbitol, rhamnose, sucrose, melibiose and arabinose. Growth occurs at 4 to 35 °C. No growth in the absence of NaCl or in media with $\geq 8\%$ (w/v) NaCl. Prolific growth occurs at 28 °C in media containing 2.5 % (w/v) NaCl. The following tests are positive for all strains: Oxidase, catalase, tryptophane deaminase, indole, NO₃ reduction and acetoin production. All strains utilise α -cyclodextrin, dextrin, N-acetyl-D-glucosamine, cellobiose, D-fructose, α -D-glucose, maltose, D-mannitol, D-mannose, D-trehalose, D-gluconic acid, L-alanine, L-alanyl-glycine, L-glutamic acid, L-serine, inosine and glycerol as sole carbon source. None of the strains utilise glycogen, Tween 40, Tween 80, N-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, i-erythritol, L-fucose, D-galactose, gentiobiose, M-inositol, α -lactose, α -D-lactose lactulose, D-melibiose, β -methyl D-glucoside, psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, turanose, xylitol, methyl pyruvate, mono methyl succinate, acetic acid, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, α -hydroxy butyric acid, β -hydroxy butyric acid, γ -hydroxy butyric acid, P-hydroxy phenylacetic acid, itaconic acid, α -keto butyric acid, α -keto glutaric acid, α -keto valeric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, bromo succinic acid, succinamic acid, glucuronamide, alaninamide, D-alanine, L-histidine, hydroxy L-proline, L-leucine, L-ornithine, L-phenyl alanine, L-proline, L-pyro glutamic acid, D-serine, D,L-carnitine, γ -amino-butyric acid, urocanic acid, phenyl ethylamine, putrescine, 2-amino ethanol, 2,3-butanediol, D,L- α -glycerol phosphate, glucose-1-phosphate and glucose-6-phosphate as sole carbon source. The following tests are negative for all strains: β -galactosidase, arginine dihydrolase, lysine and ornithine decarboxylases, H₂S production, urease, and gelatinase. The most abundant fatty acids are summed feature 3 (35.4 % \pm 0.9; comprising 16:1 ω 7c and/or 15 iso 2-OH), 16:0 (28.4 % \pm 1.8), 14:0 (11.0 % \pm 1.4), 12:0 (6.6 % \pm 0.7), 18:1 ω 7c (7.6 % \pm 4.4) , summed feature 2 (2.9 % \pm 0.3; comprising 14:0 3-OH and/or 16:1 iso I and/or unidentified fatty acid with equivalent chain length value of 10-928 and/or 12:0 ALDE), 12:0 3-OH (2.8 % \pm 0.3), 18:0 (1.5 % \pm 0.13), 16:1 ω 7c alcohol (0.4 % \pm 0.04), 15:0 (0.2 % \pm 0.1), 12:0 2-OH (0.1 % \pm 0.01), 15:0 anteiso (0.2 % \pm 0.4), 15:0 iso (0.2 % \pm 0.3), 16:1 ω 5c (0.1 % \pm 0.2), 16:0 3-OH (0.2 % \pm 0.4), 16:0 N alcohol (0.1 % \pm 0.2), and 20:1 ω 7c (0.1 % \pm 0.2). All strains are susceptible towards the vibriostatic agent 0/129

(10 and 150 µg), polymyxin (300 U), tetracycline (30 µg) and chloramphenicol (30 µg), but resistant to ampicillin (25 µg). The 16S rDNA sequences of strains LMG 20012^T and LMG 21574 are deposited in the EMBL under the accession numbers AJ316192 and AJ514912, respectively. The type strain of this species is LMG 20012^T (CAIM 634^T), isolated from Atlantic salmon (*Salmo salar*) in Tasmania. The mol % G+C of the type strain is 44.7.

3.12. Reclassification of *V. hollisae* as *Grimontia hollisae* gen. nov., comb. nov.

F. L. Thompson, B. Hoste, K. Vandemeulebroecke and J. Swings

Int. J. Syst. Evol. Microbiol. (2003), in press

Abstract

We investigated the taxonomic position of three representative strains of *V. hollisae* (LMG 17719^T, LMG 21416 and LMG 21538), by means of 16S rDNA sequences and phenotypic data. *V. hollisae* strains (EMBL accession no. AJ514909-AJ514911) shared 99.5 % 16S rDNA sequence similarity, but only 94.6 % towards its closest phylogenetic neighbour, *Enterovibrio norvegicus*. The 16S rDNA sequence similarity of *V. hollisae* and *V. cholerae* was only 91 %. Our results suggest that *V. hollisae* should be placed into a new genus for which we propose the name *Grimontia*.

Introduction

Vibrio hollisae was first described by Hickman et al. (1982). This organism produces a number of toxins and also invades host epithelial cells (Miliotis et al., 1995), causing both gastroenteritis and septicemia (Abbott and Janda, 1993). Phenotypically, *V. hollisae* strains are atypical as they are arginine dihydrolase, lysine and ornithine decarboxylase negative and cannot grow on Thiosulphate-Citrate-Bile salts-Sucrose (TCBS) agar. FAFLP fingerprinting revealed that *V. hollisae* is quite different from other vibrios as it remained unclustered when 506 strains were grouped by FAFLP pattern similarity (Thompson et al., 2001). Dorsch et al. (1992) analysed the phylogenetic position of several *Vibrio* species by means of 16S rRNA sequences and concluded that *V. hollisae* should be considered a new genus. Although these authors highlighted the need of more representative 16S rRNA sequences of diverse *Vibrio* branches in order to eventually figure out whether *V. hollisae* would be or not a new genus. In a comprehensive phylogenetic study on the families *Vibrionaceae*, *Aeromonadaceae* and *Plesiomonadaceae*, based on 16S rRNA sequences, Ruimy et al. (1994) showed that *V. hollisae* is in fact at the outskirts of the genus *Vibrio*, being

as related to this genus as to *Photobacterium* and *Salinivibrio*. Analyses of *toxR* and *gyrB* gene sequences have pointed out to the very low similarity between *V. hollisae* and other *Vibrio* species (Osorio and Klose, 2000; Vuddhakul et al., 2000). More recently, on the basis of 16S rDNA analysis we found *V. hollisae* branching between *Photobacterium* species and a newly described genus, *Enterovibrio*, rather than within other vibrios (Thompson et al., 2002).

Material and Methods

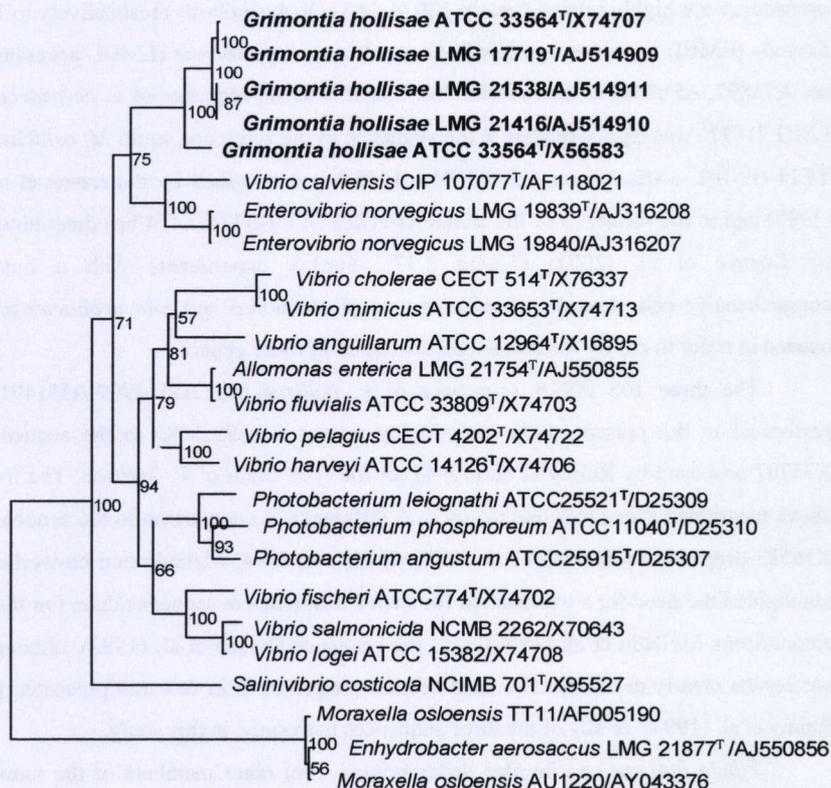
In this study we further analysed the phylogenetic position of three representative strains of *V. hollisae* (LMG 17719^T, LMG 21416, LMG 21538) associated with gastroenteritis cases in the USA. Strains LMG 17719^T (ATCC 33564^T) and LMG 21416 (ATCC 33565, JCM 1284) were analysed in detail in the original description of *V. hollisae* (Hickman et al., 1982), while strain LMG 21538 (CIP 104354) was isolated and studied by Carnahan et al. (1994). The 16S rDNA sequence analysis was performed as described previously (Thompson et al., 2001). Fatty acid methyl esters (FAME) analysis was carried out as described by Huys et al. (1994). Isolates were grown on Trypticase Soy Broth (Becton Dickinson) supplemented with 1.5 % (w/v) Bacto agar (Becton Dickinson) and 1.5 % (w/v) NaCl at 28 °C for 24 hours. Approximately 50 mg of cells were harvested and the fatty acid were isolated following the recommendations of the manufacturer using the Microbial Identification System manual (MIDI) and software package, version 3.9.

Results and Discussion

The results of our phylogenetic analysis are depicted in Figure 3.17. The three *Vibrio hollisae* strains shared 99.5 % 16S rDNA sequence similarity, but only 94.6 % towards its closest phylogenetic neighbour, *Enterovibrio norvegicus*. A maximum parsimony tree gave very similar branching as the neighbour joining tree (Figure 3.17). The 16S rDNA similarity of *V. hollisae* towards *Photobacterium* and *Salinivibrio* was 93 and 91.2 %, respectively. Whereas, the similarity of *V. hollisae* and *V. cholerae* was only 90.8 %, clearly indicating that *V. hollisae* is a different genus in the family *Vibrionaceae*.

In our phylogenetic analyses we included representative 16S rDNA sequences of all six genera i.e. *Allomonas*, *Enhydrobacter*, *Listonella*, *Photobacterium*,

Salinivibrio, and *Vibrio* currently assigned to the family *Vibrionaceae* (see the outline of Bergey's manual of systematic bacteriology, 2002, at <http://dx.doi.org/10.1007/bergeysoutline200210>). We



2%

Figure 3.17. Phylogenetic tree with the estimated position *V. hollisae*, *Allomonas enterica* and *Enhydrobacter aerosaccus* using the neighbour joining methodology based on the almost complete 16S rDNA sequences (positions 10 to 1415). Bootstrap percentages after 1000 simulations are shown. Bar, 2 % estimated sequence divergence.

also included representative sequences of the newly described genus, *Enterovibrio* (Thompson et al., 2002). The 16S rDNA sequences of *Allomonas enterica* LMG 21754^T (EMBL accession no. AJ550855, 1468 bp) and *E. aerosaccus* LMG 21877^T (EMBL accession no. AJ550856, 1484 bp) were performed in the course of this study. The genera *Allomonas* (Kalina et al., 1984) and *Enhydrobacter* (Staley et al.,

1987) were tentatively allocated to the family *Vibrionaceae* based on phenotypic features, but so far there was not a confirmation of their phylogenetic position based on 16S rDNA sequence data. According to our 16S rDNA data, *A. enterica* and *E. aerosaccus* are highly related (nearly 100 % 16S rDNA similarity) respectively to *V. fluvialis* (EMBL accession no. X74703) and *Moraxella osloensis* (EMBL accession no. X74897, AF005191, and AY043376). The 16S rDNA sequence of *E. aerosaccus* LMG 21877^T was highly related to the sequence of the reference strain *M. osloensis* TT11 (EMBL accession no. AF005191; 1448 bp) determined by Pettersson et al. (1998) and to the sequence of the isolate UA1220 (AY043376; 1454 bp) determined by Coenye et al. (2002) (Figure 3.17). Further experiments with a more comprehensive collection of reference strains of *Allomonas* and *Enhydrobacter* are needed in order to clarify the taxonomic affiliation of these genera.

The three 16S rDNA sequences of *V. hollisae* (i.e. AJ514909-AJ514911) performed in the present study were highly related (i.e. 99.3 %) to the sequence X74707 proposed by Ruimy et al. (1994) for the type strain of *V. hollisae*. The four above mentioned sequences had nearly 4 % difference in comparison to the sequence X56583 proposed by Dorsch et al. 1992. Already in 1996, Mellado and co-workers highlighted the need for an explanation for such a difference in sequence data. For their comparisons Mellado et al. (1996) used the sequence Dorsch et al. (1992), although our results clearly prove that the most accurate sequence is in fact that presented by Ruimy et al. (1994), or any of the three sequences performed in this study.

Vibrio hollisae can be also differentiated from other members of the family *Vibrionaceae* by several phenotypic features (Table 3.30). *V. hollisae* has higher levels of the fatty acids 18:1 ω 9c and 16:1 ω 9c and does not grow on TCBS as most vibrios do. *V. hollisae* is arginine dihydrolase negative and nitrate reduction positive, different from *Enterovibrio*, *Photobacterium*, and *Salinivibrio*.

On the basis of the phylogenetic and phenotypic data presented here we propose to accommodate *V. hollisae* in a new genus i.e. *Grimontia*. The type species of the new genus is *Grimontia hollisae*.

Description of *Grimontia* gen. nov. comb. nov.

Grimontia (Gri.mon'tia, N.L. gen. n. *Grimontia* of Grimont, after the French Microbiologist P. A. D. Grimont). Cells are Gram negative, motile by polar flagellum, and oxidase positive. *Grimontia* strains have a mol % G+C of DNA content ranging

from 48.5 to 51 %. The most abundant fatty acids are summed feature 3 (31 % ± 1; comprising 16:1 ω7c and/or 15 iso 2-OH), 18:1 ω7c (23 % ± 2), 16:00 (14 % ± 1), 12:00 (5 % ± 1), 18:1 ω9c (5 % ± 1), summed feature 2 (5 % ± 2; comprising 14:0 3-OH and/or 16:1 iso I and/or unidentified fatty acid with equivalent chain length value of 10.928 and/or 12:0 ALDE), 16:1 ω9c (4 % ± 0), 12:0 3-OH (4 % ± 2), 14:00 (3 % ± 0), 18:00 (2 % ± 1). Chemoheterotrophic, mesophilic and moderately halophilic. *Grimontia* strains are Voges-Proskauer, arginine dihydrolase, lysine and ornithine decarboxylase negative, but indole and nitrate reduction are positive. The 16S rDNA sequences of *Grimontia* strains have typical signatures at the positions 970-971 (TC instead of AG) and 1107-1108 (CG instead of AA) which differ from other members of the family *Vibrionaceae*. The type species is *Grimontia hollisae* (LMG 17719^T; EMBL accession number is AJ514909, mol % G+C of DNA is 48.5 %). The description of *Grimontia hollisae* comb. nov. are based on the original description of Hickman et al. (1982).

Table 3.30. Differentiating features between *Grimontia* and other genera of the *Vibrionaceae*.

	1	2	3	4	5
Growth on/in:					
TCBS	-	+	+	+	+
12 % NaCl	-	-	-	+	-
Acetoin production	-	-	+/-	+	_*
Indole production	+	+	-	-	+/-
Nitrate reduction	+	-	+/-	-	_*
Arginine dihydrolase	-	+	+	+	+/-
Fatty acids:					
18:1 ω9c	4-6	3	0	<1	0-5
16:1 ω9c	4	4	0	1-2	0-2

Taxa are indicated as: 1. *Grimontia*, 2. *Enterovibrio* (Thompson et al., 2002), 3. *Photobacterium* (Alsina and Blanch, 1994a), 4. *Salinivibrio* (Mellado et al., 1996), 5. *Vibrio* (Alsina and Blanch, 1994a). *_over 85 % of the species show this feature. Fatty acid data (%) are from our own database. TCBS medium obtained from Oxoid.

3.13. The coral bleaching *Vibrio shiloi* Kushmaro et al. 2001 is a later synonym of *Vibrio mediterranei* Pujalte and Garay 1986

F. L. Thompson, B. Hoste, C. C. Thompson, G. Huys and J. Swings

Syst. Appl. Microbiol. (2001), 24, 516-519

Abstract

The coral bleaching *Vibrio shiloi* LMG 19703^T was characterized by means of Fluorescent Amplified Fragment Length Polymorphism (FAFLP), DNA-DNA hybridisation, mol% G+C content, fatty acids methyl ester (FAME) analysis and phenotypical tests. Numerical analysis of the FAFLP band patterns indicated that the type strain of *V. shiloi* in fact belongs to the species *V. mediterranei*. The type strains of both species shared 77% DNA similarity, as determined by DNA-DNA hybridisation experiments at stringent conditions. Moreover, *V. shiloi* and *V. mediterranei* showed almost identical fatty acid composition and phenotypical features. Collectively, the genotypic and phenotypic data presented in this study suggest that *V. shiloi* Kushmaro et al. 2001 should be considered a later synonym of *V. mediterranei* Pujalte and Garay 1986. The involvement of *V. mediterranei* in coral bleaching was unknown until now. □

Introduction

Coral bleaching is the disruption of the symbiotic association between coral hosts and photosynthetic microalgal endosymbionts (i.e. zooxanthellae) (Rosenberg et al., 1999). *Vibrio shiloi*, a causative agent of bleaching of the coral *Oculina patagonica*, was isolated in the Mediterranean coast of Israel in 1995. Since then, it has been the subject of various ecological studies (Banin et al., 2000; Banin et al., 2001; Ben-Haim et al., 1999; Kushmaro et al., 1997; Kushmaro et al., 1998; Rosenberg et al., 1999). It was shown that infection and subsequent coral bleaching are temperature dependent (Kushmaro et al., 1998). Elevated temperatures (e.g. 29 °C) induce virulence in the coral pathogen, stimulating the synthesis of virulence factors, such as adhesins and toxins (Kushmaro et al., 1998). In fact, *V. shiloi*

produces an extracellular toxin that directly inhibits algal photosynthesis and bleach and lyse the zooxanthellae (Rosenberg et al., 1999).

The name *Vibrio shiloi* has recently been proposed for two strains, AK1^T and AK2, both obtained from bleached corals (Kushmaro et al., 2001). It was shown that *V. shiloi* have similar phenotypical features (Kushmaro et al., 1997; Rosenberg et al., 1999) and high DNA-DNA similarity with the species *V. mediterranei*. Moreover, the closest phylogenetic neighbour of *V. shiloi* is *V. mediterranei* (99.4% 16S rDNA similarity) (Kushmaro et al., 2001). The species *V. mediterranei* is indigenous to the marine environment and has been isolated from a wide range of environments and hosts (Ortigosa et al., 1994; Pujalte and Garay, 1986; Pujalte et al., 1992; Thompson et al., 2001), but it has never been reported as a disease agent. A recent study has shown that certain strains of *V. mediterranei* have probiotic properties for turbot larvae (*Scophthalmus maximus*) under rearing conditions (Huys et al., 2001). This study sought to determine the taxonomic affiliation of the type strain of *Vibrio shiloi* to *V. mediterranei* using a polyphasic combination of genomic and phenotypic techniques.

Material and Methods

Strains analysed in this study are described in table 1 (Annex). The eight *V. mediterranei* strains and *V. shiloi* LMG 19703^T represent the so-called cluster A6 described in a previous study (Thompson et al., 2001). In order to check the authenticity of *V. shiloi* LMG 19703^T, a partial 16S rDNA sequence was determined as described previously (Thompson et al., 2001). This sequence was compared with the complete sequence of *V. shiloi* (accession number AF007115) deposited in EMBL. The analyses showed that the sequences were identical. Fluorescent Amplified Fragment Length Polymorphisms (FAFLP) patterns were generated and analysed as described previously (Thompson et al., 2001). The mol% G+C content was determined by the HPLC methodology (Tamaoka and Komagata, 1984). DNA-DNA hybridisation was performed using the microplate technique with photobiotin-labelled DNA at the temperature of 36 °C for 3 hours as described previously (Willems et al., 2001). Phenotypical characterization was performed using the commercial kits API 20E (bioMérieux, France) and Biolog GN (Biolog Inc., USA) following the instructions of the manufacturers with slight modifications. Strains were grown on Marine Agar 2216 (Difco, USA) for 24 hours at 25 °C. Subsequently,

V. shiloi is a synonym of *V. mediterranei*

cells were suspended in 1.5% NaCl solution. These suspensions which had a standard opacity equivalent to McFarland No. 3 were inoculated into the API 20E strips which were incubated for 48 hours at 25°C. For Biolog GN metabolic fingerprinting, strains were grown on Blood Agar (Biolog Inc., USA) for 24 hours at 28°C. Subsequently, cells were suspended in inoculating fluid (1.5% NaCl, 0.03% Pluronic F-68, 0.01% Gellan Gum) and cell densities were photometrically standardised between 0.28 and 0.30 OD at 590 nm. The wells of the Biolog GN microplates were inoculated with the cell suspension and the microplates were incubated for 24 hours at 28 °C. Changes in colour were measured using a spectrophotometer (Biolog Inc., USA) at 550 and 750 nm. Fatty acid analysis was performed following the protocol of the Microbial Identification System (Microbial ID Inc, USA) as described previously (Huys et al., 1994). Strains were grown on Marine Broth (Difco, USA) supplemented with 1.8% Bacteriological Agar (Difco, USA) for 48 hours at 30 °C. For salt tolerance test, *Vibrio shiloi* LMG 19703^T was grown on Brain Heart Infusion (Difco, USA) supplemented with 2% agar no.1 (Oxoid, England) 5.5 and 6% NaCl for 48 hours at 28 °C.

Results and Discussion

FAFLP analysis clearly showed that *V. shiloi* LMG 19703^T and *V. mediterranei* have highly related genomes (Figure 3.18). These two species formed a tight FAFLP cluster which was distinguishable from all their closest phylogenetic neighbours (Fig. 3.18).

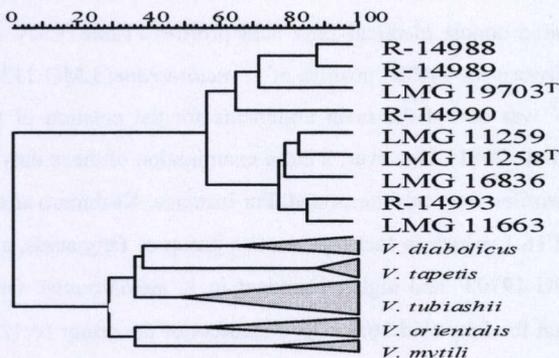


Figure 3.18. Dendrogram derived from a band based (Dice) cluster analysis (Ward) of the FAFLP patterns of *V. mediterranei* (n = 8), *V. shiloi* LMG 19703^T and their closest phylogenetic neighbours. Patterns consisted of 111 ± 24 bands.

The usefulness of FAFLP technique in discriminating closely related species of the family Vibrionaceae (e.g. *Listonella anguillarum* and *V. ordalli*, *V. cholerae* and *V. mimicus*, *V. fluvialis* and *V. furnissii*) has been addressed in a previous study (Thompson et al., 2001). Those species share above 99% 16S rDNA similarity and about 65% DNA-DNA similarity with each other, but their FAFLP patterns show lower than 40% similarity. DNA-DNA similarity value between *V. shiloi* LMG 19703^T and *V. mediterranei* LMG 12258^T was 77%. The mol% G+C content of DNA of *V. shiloi* LMG 19703^T and *V. mediterranei* LMG 11258^T were 43.2 and 44.1, respectively.

Phenotypic features of LMG 19703^T listed in Table 3.31 are in close agreement with the description of *V. mediterranei* (Ortigosa et al., 1994; Pujalte and Garay, 1986; Pujalte et al., 1992). It is important to mention that *V. mediterranei* is a very heterogeneous species (Ortigosa et al., 1994; Pujalte et al., 1992) and that utilization of certain compounds e.g. N-acetyl-D-galactosamine, m-inositol, α -D-lactose, β -methyl-D-glucoside, L-rhamnose, mono-methyl succinate and β -hydroxybutyric acid, as sole carbon source by this organism is variable and thus of none taxonomic value for differentiating between *V. mediterranei* and *V. shiloi*. In contrast with the results reported previously (Kushmaro et al., 1997; Kushmaro et al., 2001), we found that *V. shiloi* LMG 19703^T utilised m-inositol and mono-methyl succinate (Table 3.31), produced indole, showed gelatinase activity and grew on 6-6.5 % salt. These discrepancies may be due to differences in the protocols used in both studies. *Vibrio shiloi* LMG 19703^T and a group of four *V. mediterranei* reference strains exhibited almost identical fatty acid profiles (Table 3.32). Interestingly, the difference between the FAME profiles of *V. mediterranei* LMG 11258^T and *V. shiloi* LMG 19703^T was one of the main arguments for the creation of the latter species (Kushmaro et al., 2001). However, a close examination of these data clearly indicated that FAME profiles were misinterpreted. For instance, Kushmaro et al. (2001) refer to the fatty acid 16:1, which in fact represents a group of fatty acids, as being absent in *V. shiloi* LMG 19703^T and highly abundant in *V. mediterranei*. On the other hand, they report that the fatty acid 16:1 ω 7c, a member of the group 16:1, is abundant in *V. shiloi* LMG 19703^T and absent in *V. mediterranei*.

Table 3.31. Phenotypical features of *V. mediterranei* and *V. shiloi* investigated by Kushmaro et al. (2001).

	<i>V. mediterranei</i> (n = 8)	<i>V. shiloi</i> LMG 19703 ^T
Utilization of:		
N-Acetyl-D-galactosamine	4	-
m-inositol	2	+*
α -D-lactose	+	+
β -Methyl-D-glucoside	3	+
L-rhamnose	7	+
Mono-methyl succinate	3	+*
β -Hydroxybutyric acid	1	+
Glucuronamide	+	+

+, all strains positive. -, all strains negative. Numbers indicate positive strains in each species. *, tests which gave opposite result in the original description of *V. shiloi*.

Table 3.32. Fatty acid composition of the *V. mediterranei* and *V. shiloi* strains.*

	<i>V. mediterranei</i> (n = 4)	<i>V. shiloi</i> LMG 19703 ^T
12:0	4.8-5.6	4.7
13:0 iso	1.8-4.4	1.9
12:0 3-OH	2.3-2.8	2.2
14:0 iso	1.8-2.7	3.5
14:0	7.1-8.6	7.1
13:0 iso 3-OH	0.0-1.3	0.3
15:0 iso	3.8-6.5	4.1
15:0 anteiso	1.7-2.9	1.2
15:0	0.9-1.2	0.9
Sum in feature 2	3.4-3.7	3.1
16:0 iso	3.9-5.2	8.3
Sum in feature 3	24.5-29.1	25.9
16:0	9.3-11.4	12.7
15:0 iso 3-OH	0.0-2.0	0.6
17:0 iso	2.6-6.6	2.7
17:0 anteiso	1.0-1.7	0.8
17:1 ω 8c	0.0-1.1	0.5
18:1 ω 7c	9.6-13.7	14.5
18:0	0.0-1.3	0.5
18:0 iso	0.0-0.5	1.2

*Range of values (%) of 4 *V. mediterranei* strains (LMG 11258^T, LMG 11663, R-14989 and R-14993) and *V. shiloi* LMG 19703^T. Sum in feature 2 comprises one or more of the following fatty acids: 14:0 3-OH and/or 16:1 iso I. Sum in feature 3 comprises one and/or more of the following fatty acids: 16:1 ω 7c or 15:0 iso 2-OH.

In our hands, *V. mediterranei* and *V. shiloi* LMG 19703^T showed very similar phenotypical features and highly related genomes. In our opinion, the virulence of *V. shiloi* LMG 19703^T as an argument for the proposal of a new species is not a reliable criterion because many currently known *Vibrio* species harbour virulent, non-virulent and even probiotic strains (Vandenberghe et al., 1998). Based on our polyphasic approach we therefore suggest that *V. shiloi* LMG 19703^T should be considered a later synonym of *V. mediterranei* Pujalte and Garay 1986.

**3.14. *Vibrio trachuri* Iwamoto et al. 1995 is a junior synonym of
Vibrio harveyi (Johnson and Shunk 1936) Baumann et al. 1981**

F L. Thompson, B. Hoste, K. Vandemeulebroecke, K. Engelbeen,
R. Denis and J. Swings

Int. J. Syst. Evol. Microbiol. (2002), 52, 973-976

Abstract

The taxonomic position of *Vibrio trachuri* was examined through a polyphasic approach using 16S rDNA sequencing, fluorescent amplified fragment length polymorphisms (FAFLP), DNA-DNA hybridisation experiments, mol % G+C content of DNA, and phenotypical tests. Phylogenetic analysis showed that *V. harveyi* is the closest neighbour of *V. trachuri* sharing about 98.8 % similarity for the 16S rDNA gene. Moreover, numerical analysis of the FAFLP patterns revealed that both species have highly related genomes, sharing 55 % pattern similarity. DNA-DNA hybridisation experiments and mol % G+C measurements reinforced these results since *V. trachuri* and *V. harveyi* had at least 74 % DNA similarity and 44.5 to 45.2 mol % G+C. Phenotypical features of both species were also very similar, except that *V. trachuri* utilised itaconic acid, whereas *V. harveyi* did not. Therefore, we propose that the species *V. trachuri* should be reclassified as *V. harveyi*.

Introduction

Vibrios are readily isolated from a wide range of marine and estuarine environments including rearing systems (Vandenberghé et al., 1999). Although some vibrios have been found as symbiotic (e.g. *V. halioticoli* and the abalone *Haliotis hannai hannai*) or probiotic (e.g. *V. alginolyticus* and the shrimp *Litopenaeus vannamei*), they are mostly known as pathogenic, particularly the species *L. anguillarum* and *V. harveyi*. Actually there have been numerous reports on the pathogenicity of *V. harveyi* for a wide range of fish and shellfish (Austin and Austin, 1999). *V. harveyi* is a well characterized bacterial species and has a clearly defined taxonomic position (Baumann et al. 1980; Dorsch et al., 1992; Johnson and Shunk,

1936; Pedersen et al., 1998). It belongs to the core group in the genus and its closest phylogenetic neighbours are *V. alginolyticus* and *V. campbellii* (Dorsch et al., 1992). The AFLP analysis of 36 *V. harveyi* strains revealed a high genotypic heterogeneity within this species and *V. carchariae* was considered its later synonym (Pedersen et al., 1998). Three *Vibrio trachuri* strains were isolated from diseased cultured Japanese horse mackerel (*Trachurus japonicus*) at Uchiura Bay, Numazu (Japan) in the 1990s. Based mainly on the relative low DNA homology values found and on its differential phenotypical features with *V. harveyi*, *L. anguillarum* and *V. parahaemolyticus*, it was proposed as being a new species (Iwamoto et al., 1995). Although the disease symptoms on fish and phenotypical characteristics of *V. trachuri* and *V. harveyi* were quite similar (Austin and Austin, 1999; Iwamoto et al., 1995).

Material and Methods

The 12 strains used in this study are listed in the Annex 1. Bacterial DNAs used in this study were isolated following the technique of Pitcher et al. (1989). Concentration and purity of the DNAs were estimated measuring optical densities at 260, 234 and 280 nm in an Uvicom 941+ spectrophotometer (Kontron Instruments, Italy). DNA integrity was verified on a 1 % agarose gel in 1 X TAE buffer (40 mM Tris/acetate, 1 mM EDTA, pH 8.0). 16S rDNA sequence and fluorescent amplified fragment length polymorphisms analyses were done following the protocols described previously (Thompson et al., in press). The mol % G+C of the genomic DNA and RNA content were determined by HPLC methodology (Tamaoka and Komagata, 1984). DNA-DNA hybridisation was performed using the microplate technique with photobiotin-labeled DNA at 36 °C for 3 hours as described by Willems et al. (2001). The temperature for optimal renaturation (T_{or}) was calculated as $[(0.51 \times \text{G+C content} + 47)] - 36$. In order to perform the hybridisation experiments in stringent conditions, it was chosen the temperature of 36 °C. Phenotypical characterization of the strains was performed using the commercial kits API 20E (bioMérieux, France) and Biolog GN microplates (Biolog Inc., USA) following the instructions of the manufacturers with slight modifications. For API20E tests, strains were grown on Marine Agar (Difco, USA) for 24 hours at 25 °C. Subsequently, cells were suspended in a 1.5 % NaCl solution. These suspensions which had a standard opacity equivalent to McFarland 3 were inoculated into the API 20E strips which

were incubated for 48 hours at 25 °C. For Biolog GN metabolic fingerprinting, strains were grown on Blood Agar (Biolog Inc., USA) for 24 hours at 28 °C. Subsequently, cells were suspended in inoculating fluid (1.5 % NaCl, 0.03 % Pluronic F-68, 0.01 % Gellan Gum) and cell densities were photometrically standardised between 0.28 and 0.30 OD at 590 nm. The wells of the Biolog GN microplates were inoculated with the cell suspension and the microplates were incubated for 24 hours at 28 °C. Changes in colour were measured using a spectrophotometer (Biolog Inc., USA) at 550 and 750 nm.

Results and Discussion

The 16S rDNA analysis revealed that the closest phylogenetic neighbours of *Vibrio trachuri* LMG 19643^T (EMBL accession number AJ312382) are *V. harveyi* LMG 4044^T (EMBL accession number X74706) and *V. alginolyticus* (EMBL accession number X56576) with 98.8 % and 98.2 % similarity, respectively. In order to confirm the authenticity of *V. trachuri* LMG 19714 used in this study, a partial 16S rDNA sequence (933 base pairs) was determined (EMBL accession number AJ312383). This partial sequence was compared with the one from *V. trachuri* LMG 19643^T (EMBL accession number AJ312382) and they were found to be identical.

Furthermore, numerical analysis of the FAFLP band patterns (114 ± 17 bands) of 10 *V. harveyi* and 2 *V. trachuri* strains revealed that both species have similar genomes (55 % pattern similarity) and formed a separated cluster which was distinguishable from all closest phylogenetic neighbours (Figure 3.19).

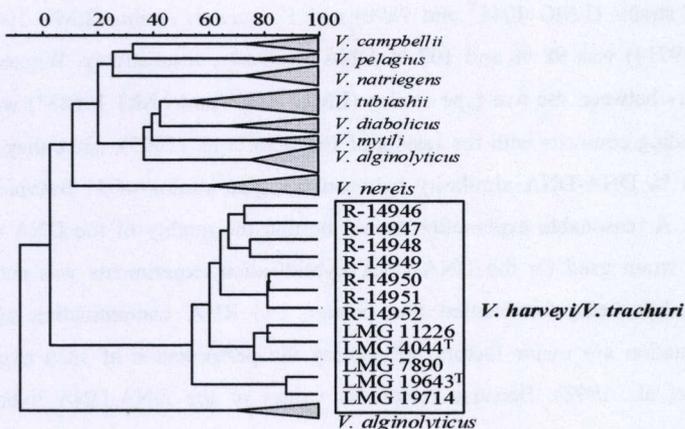


Figure 3.19. Dendrogram of the FAFLP patterns of *V. harveyi* and *V. trachuri* strains and their closest phylogenetic neighbours. A band based (Dice) cluster analysis (Ward) was used. The threshold for cluster delineation was 45%. *V. harveyi* and *V. trachuri* strains represent the FAFLP cluster A36 found in a previous study (Thompson et al., 2001). Patterns consisted of 114 ± 17 bands.

The clusters representing the closest phylogenetic neighbours of *V. trachuri* presented in Fig. 3.19 were described in a previous study (Thompson et al., 2001), and were included as out-groups in order to show the cut-off level for species delineation. Strains clustering at 45 % FAFLP pattern similarity should be considered to belong to the same species (Thompson et al., 2001). It is important to highlight that FAFLP is a fingerprinting technique which provides discrimination of strains beyond the species level. Recently, Jiang et al. (2000) have successfully used AFLP to discriminate *V. cholerae* serogroups O1 and O139. The two *V. trachuri* strains LMG 19643^T and LMG 19714 showed indistinguishable patterns, suggesting the occurrence of a particular clone, highly pathogenic for *Trachurus japonicus* (Iwamoto et al., 1995), clearly allocated to the species *V. harveyi*. Strains R-14946 and R-14949 were investigated in a previous study and were found in the so-called cluster 2 (Pedersen et al., 1998), but they could not be assigned to any of the species included in that study. Our results demonstrated that these strains belong to *V. harveyi*.

The mol % G+C of DNA and the DNA-DNA hybridisation results are presented in Table 3.33. *V. harveyi* and *V. trachuri* had a mol % G+C content of 44.9 to 45.2% and 44.5 to 44.6 %, respectively. DNA hybridisation performed with pure and high molecular weight DNA, revealed that internal DNA similarity between *V. harveyi* strains (LMG 4044^T and 7890) and *V. trachuri* strains (LMG 19643^T and LMG 19714) was 98 % and 103 % DNA similarity, respectively. Whereas, DNA similarity between the two type strains (LMG 4044^T and LMG 19643^T) was 80 %. This finding contrasts with the results of Iwamoto et al. (1995), since they obtained only 40 % DNA-DNA similarity between the type strains of *V. trachuri* and *V. harveyi*. A reasonable explanation would be that the quality of the DNA of the *V. harveyi* strain used for the DNA-DNA hybridisation experiments was not optimal since it has been documented that protein and RNA contamination and DNA fragmentation are major factors influencing the performance of such experiments (Goris et al., 1998). Because reciprocal values of the DNA-DNA hybridisation

experiments performed previously have not been shown (Iwamoto et al., 1995), it is difficult to have any evidence about the quality of the DNAs used.

Table 3.33. DNA similarity and G+C content (mol %) of *V. harveyi* and *V. trachuri*.

	1	2	3	4	Mol % G+C
<i>V. harveyi</i>					
1. LMG 4044 ^T	100				45.2
2. LMG 7890	98	100			44.9
<i>V. trachuri</i>					
3. LMG 19643 ^T	80	82	100		44.5
4. LMG 19714	79	74	103	100	44.6

The phenotypic features observed for *V. harveyi* and *V. trachuri* strains based on Biolog GN analysis are presented in the Table 3.34. All tests were not discriminatory between *V. harveyi* and *V. trachuri*, except utilization of itaconic acid, indicating that both species have similar phenotypes.

Table 3.34. Phenotypic features tested by Biolog. +, all strains positive. -, all strains negative. Numbers indicate positive strains in each species.

	<i>V. harveyi</i> (n = 10)	<i>V. trachuri</i> (n = 2)
Utilization of:		
Adonitol	1	-
D-mannose	8	1
D-melibiose	1	-
B-methyl-D-glucoside	3	1
D-sorbitol	3	+
Cis-aconitic acid	5	1
α -hydroxy butiric acid	1	-
Itaconic acid	-	+
Gluconamide	3	1
Glycerol	8	+
D,L- α -glycerol phosphate	9	+

In addition, several tests were found variable among strains of both species (Table 3.34). Arginine dihydrolase and gelatinase activity were positive for both *V. harveyi* LMG 4044^T and *V. trachuri* LMG 19643^T, but negative for *V. trachuri* LMG 19714. Whereas, tryptophane deaminase activity was found positive for *V. trachuri* LMG 19714 and negative for *V. harveyi* LMG 4044^T and *V. trachuri* LMG 19643^T. Besides, some results on the phenotype of *V. trachuri* obtained in this study are in

contrast with those of Iwamoto et al. (1995). It was reported that arginine dihydrolase and ornithine decarboxylase and citrate utilization were negative for *V. trachuri* LMG 19643^T (Iwamoto et al., 1995). However, we found for the same strain positive results for these features. One might relate these discrepancies between both results to differences in the protocols used in each study. The protocol used in this study was carefully established since its reproducibility and consistency have been analysed previously.

Based on our polyphasic approach combining phylogenetic, genotypic, and phenotypic data, we propose that *Vibrio trachuri* Iwamoto et al. 1995 should be considered a junior synonym of *Vibrio harveyi*.

3.15. Genotypic and phenotypic data of the *Vibrionaceae*

In this section we provide a synthesis of the new taxa described in this thesis. We also present a detailed comparison of the phenotypic identification of vibrios by Biolog (Vandenberghé et al., 2003) versus the FAFLP identification (Thompson et al., 2001). We further prove that FAFLP is an alternative to DNA-DNA hybridisations in the family *Vibrionaceae*.

We have examined 506 *Vibrionaceae* strains by FAFLP (Thompson et al., 2001). Sixty-nine clusters were delineated using an arbitrary cluster cut-off level of 45%. Initially, we concluded that the thirty-one clusters which did not harbour a type strain were new species. All subsequent studies presented in Chapter 3 (except the descriptions of *V. gallicus* and *V. superstes*) were sequels of our initial study, with the aim of analysing further, and wherever possible, describing new species. Although initially we assumed that each unidentified FAFLP cluster corresponded to a new species (Thompson et al., 2001), it became clear that this was not the case after the first DNA-DNA hybridisation experiments were finished. Certain FAFLP clusters e.g. A1 to A4 had DNA-DNA similarity values well above 70 % and were thus assigned to the same species. A list of all new species and respective FAFLP clusters is presented in Table 3.35, while the identification of FAFLP clusters to known species is presented in Table 3.36. Groups A30, A31, A32 and A37 were phylogenetically related to *V. harveyi* and *V. campbellii*, but also to *V. rotiferianus* (99-100 % 16S rDNA similarity). Clusters A55 and A61 were highly to *V. splendidus*, *V. lentus*, *V. cyclitrophicus* and *V. tasmaniensis* (99-100 % 16S rDNA similarity). This suggests that the identification of strains related to these groups will probably require FAFLP or rep-PCR analyses.

According to our comparison between FAFLP and Biolog identification, **different *Vibrio* species appear within the same Biolog group** (see Figure 1, Annex). For instance, strains misidentified as *V. harveyi* by Biolog were later correctly identified as *V. campbellii* or classified as a new species i.e. *V. rotiferianus* by FAFLP and DNA-DNA hybridisations. Indeed, *V. campbellii*, *V. harveyi* and *V. rotiferianus* have nearly indistinguishable phenotypes. Several strains phenotypically misidentified as *Vibrio harveyi* turned out to be *V. campbellii* (Gomez-Gil et al., in prep.). For a long time *V. harveyi* was considered the main agent of luminous vibriosis in shrimps (Lavilla-Pitogo et al., 1998; Manefield et al., 2000). Strains

misidentified by Biolog as *V. campbellii* turned out to be *V. chagasii*, while strains supposed to be *V. splendidus* were classified into *V. kanaloaei*. On the other hand, many strains identified by FAFLP as e.g. *V. cincinnatiensis*, *V. splendidus* and *V. tubiashii* correspond to multiple Biolog groups.

To summarise the data depicted in Figure 1 (see Annex), the following possible relationships between FAFLP and Biolog can be distinguished: (i) a single genotype corresponds to a single phenotype (e.g. A8; *V. brasiliensis*); (ii) a single genotype corresponds to multiple phenotypes (e.g. A9; *V. fortis*) and (iii) multiple genotypes are found in a single phenotype (e.g. A1, A2, A3, A4 and A5; *V. coralliilyticus* and *V. neptunius*).

FAFLP analysis proved to be valuable for the discrimination of phylogenetically and phenotypically related *Vibrio* species e.g. the pairs *V. coralliilyticus* and *V. neptunius* and *V. harveyi* and *V. rotiferianus*. The discrimination of FAFLP is indeed very high and is based on the survey of the entire bacterial genome. In our experiments with FAFLP (Thompson et al., 2001), the average number of fragments obtained for the 506 strains analysed with the enzyme combination *Hind*III and *Taq*I and with the primer combination H01 (a adenine as selective base) and T03 (a guanine as selective base) was 107 ± 23 (minimum of 46 and maximum of 164 fragments). The number of fragments obtained corresponded well with 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)] suggesting that overall our FAFLP experiments generated reliable results. FAFLP proved to be a most valuable tool for the circumscription of new species. With few exceptions (see Tables 3.1 and 3.2), strains from the same species were found in a single FAFLP cluster. Indeed FAFLP screens a high 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 X 12 nucleotides (6 plus 4 of the restriction sites plus the two selective bases) X number of strains]. FAFLP has been validated as an alternative to DNA-DNA hybridisations in a number of bacterial genera, including *Aeromonas* (Huys et al., 1996), *Agrobacterium* (Xavier et al., 2000), *Burkholderia* (Coenye et al., 2000) and *Xanthomonas* (Rademaker et al., 2000). In the present study we compared the FAFLP band pairwise similarities found in the study of Thompson et al. (2001) with all subsequent DNA-DNA homology values obtained in

the course of the new species descriptions. 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 fitted well in a polynomial regression of second degree ($r=0.8$) (Figure 3.20). The data depicted in Figure 4.20 clearly shows 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.

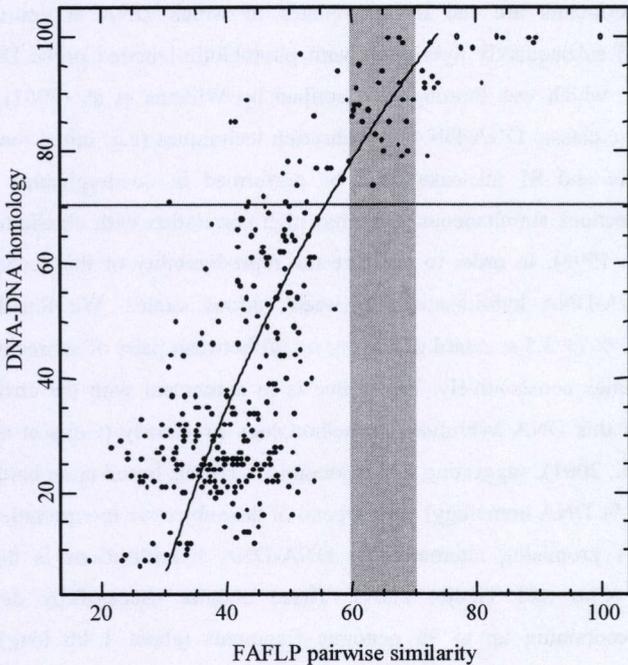


Figure 3.20. Polynomial regression (2nd degree) of FAFLP versus DNA-DNA homology data. FAFLP pattern pairwise similarities were calculated with Dice coefficient and 0.2 % band position tolerance was used to allow technical errors. The diagonal (i.e. 100 % theoretical values) of the DNA-DNA hybridisation 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.

(2000). Although, we initially delineated our FAFLP species clusters at a cut-off level of 45 % (Thompson et al., 2001), **it is now clear that a comparable identification using FAFLP as an alternative to DNA-DNA hybridisations is only achieved by adopting the FAFLP pattern similarity level of 60 to 70 % which corresponds to DNA-DNA similarity higher than 70 %.**

We therefore suggest that FAFLP 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, FAFLP is easy and fast to perform and is amenable to automation. FAFLP data can also be accumulated in databases.

Currently, the DNA-DNA hybridisation experiments for delineating new species are performed mainly following the methodology described by Ezaki et al. (1989). Experiments are run in microplates in which DNA is non-covalently adsorbed and subsequently hybridised with photobiotin-labelled probe DNA. This methodology, which was thoroughly described by Willems et al. (2001), is much faster than the classic DNA-DNA hybridisation techniques (e.g. initial renaturation, hydroxyapatite and S1 nuclease), can be performed in quadruplicates and with reciprocal reactions simultaneously and has high correlation with classic techniques (Goris et al., 1998). In order to evaluate the reproducibility of this technique, we repeated DNA-DNA hybridisations between various strains. We found a mean variation of 5 % (\pm 3.5 standard deviation; n=24) between pairs of strains hybridised up to four times consecutively. This value is in agreement with the error of 7 % estimated for this DNA hybridisation methodology previously (Goris et al., 1998; Willems et al., 2001), suggesting that taxonomic decisions based upon border values (i.e. 65 to 75 % DNA homology) may depend of the subjective interpretation of each researcher. A promising alternative to DNA-DNA hybridisations is the use of microarrays (Cho and Tiedje, 2001). These authors 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 linearity between 50 to 100 % DNA-DNA homology and it was suggested that the chip could eventually contain 100 thousand genomic fragments which would allow the identification of most Gram negative bacteria (Cho and Tiedje, 2001).

Table 3.35. List of new species, their phylogenetic allocation, distribution and function.

	FAFLP Group	Distribution and function	Type strain	Closest Phylogenetic neighbour (%)	Refs*
<i>E. norvegicus</i>	A68 & A69	Gut of healthy turbot larvae (<i>Scophthalmus maximus</i>)	LMG 19839 ^T	<i>V. hollisiae</i> (94.6)	8
<i>V. brasiliensis</i>	A8	Bivalve larvae (<i>Nodipecten nodosus</i>)	LMG 20546 ^T	<i>V. hepatarius</i> (98.4)	9
<i>V. chagasii</i>	A52 & A53	<i>Artemia</i> sp., Sea bass (<i>Dicentrarchus labrax</i>), rotifer (<i>Brachionus plicatilis</i>), water	LMG 21353 ^T	<i>V. fortis</i> (98.2)	10
<i>V. coralliilyticus</i>	A1 to A4	Pathogen for bivalves (<i>Crassostera gigas</i> and <i>Nodipecten nodosus</i>) and corals (<i>Pocillopora damicornis</i>). Isolated also from <i>S. maximus</i> and <i>B. plicatilis</i>	LMG 20984 ^T	<i>V. neptunius</i> (98.2)	1
<i>V. fortis</i>	A9 & A60	Health and diseased <i>N. nodosus</i> larvae, Atlantic salmon (<i>Salmo salar</i>), <i>B. plicatilis</i> , <i>Litopenaeus vannamei</i> , seawater	LMG 21557 ^T	<i>V. pelagius</i> (98.8)	12
<i>V. gallicus</i>	-	Symbiont of French abalone (<i>Haliotis tuberculata</i>)	LMG 21330 ^T	<i>V. ezuriae</i> (97.6)	6
<i>V. hepatarius</i>	A26	Hepatopancreas of <i>L. vannamei</i>	LMG 20362 ^T	<i>V. xuii</i> (99.1)	12
<i>V. hispanicus</i>	A16	<i>Artemia</i> sp. and its culture water	LMG 13240 ^T	<i>V. proteolyticus</i> (97.4)	4
<i>V. kanaloaei</i>	A46	Diseased oyster larvae (<i>Ostrea edulis</i>), shrimp (<i>Penaeus chinensis</i>), seawater	LMG 20539 ^T	<i>V. tasmanienis</i> (98.8)	10
<i>V. neptunius</i>	A5	<i>B. plicatilis</i> , <i>N. nodosus</i> larvae, gut of <i>S. maximus</i> larvae, water	LMG 20536 ^T	<i>V. coralliilyticus</i> (98.2)	9
<i>V. pacinii</i>	A47	<i>D. labrax</i> , <i>S. salar</i> , <i>P. chinensis</i> larvae	LMG 19999 ^T	<i>V. kanaloaei</i> (96.8)	3
<i>V. pomeroyi</i>	A51	<i>N. nodosus</i> larvae, gut of <i>S. maximus</i> larvae	LMG 20537 ^T	<i>V. tasmanienis</i> (98.9)	10
<i>V. rotiferianus</i>	A33	<i>B. plicatilis</i>	LMG 21460 ^T	<i>V. harveyi</i> (99.5)	2
<i>V. superstes</i>	-	Australian Abalones (<i>H. laevigata</i> and <i>H. rubra</i>)	LMG 21323 ^T	<i>V. ezuriae</i> (98.1)	5
<i>V. tasmanienis</i>	A45	<i>S. salar</i>	LMG 20012 ^T	<i>V. pomeroyi</i> (98.9)	11
<i>V. xuii</i>	A23	<i>N. nodosus</i> larvae, <i>L. vannamei</i> , water	LMG 21346 ^T	<i>V. hepatarius</i> (99.1)	9
<i>V. halioticoli</i> subsp. <i>discus</i> ⁺	A64 & A65	Symbiont of Japanese abalone (<i>Haliotis discus discus</i>)	LMG 19972 ^T	<i>V. ezuriae</i> (99.2)	7
<i>V. ezuriae</i> sp. nov. ⁺	A66	Symbiont of Japanese abalone (<i>Haliotis diversicolor supertexta</i>)	LMG 19970 ^T	<i>V. hal.</i> subsp. <i>dis.</i> (99.2)	7

*References: 1, Ben-Haim et al. (2003); 2, Gomez-Gil et al. (2003a); 3, Gomez-Gil et al. (2003b); 4, Gomez-Gil et al. (2003c); 5, Hayashi et al. (2003a); 6, Hayashi et al. (2003b); 7, Sawabe et al. (in preparation); 8, Thompson et al. (2002); 9, Thompson et al. (2003a); 10, Thompson et al. (2003b); 11, Thompson et al. (2003c); 12, Thompson et al. (2003d). E., *Enterovibrio* and V., *Vibrio*.[†]*V. halioticoli* subspecies *discus* and *V. ezurae* are taxa to be proposed.

Table 3.36. Allocation of FAFLP clusters to known species by DNA-DNA hybridisation and 16S rDNA determinations.

FAFLP identification	Species allocation by DNA-DNA	16S rDNA similarity* (%)	Refs*
<i>Vibrio</i> sp. A30, A31 and A32	<i>V. harveyi</i>	98-99	1
<i>Vibrio</i> sp. A34	<i>V. diazotrophicus</i>	99	1
<i>Vibrio</i> sp. A37	<i>V. campbellii</i>	98	1
<i>Vibrio</i> sp. A55	<i>V. lentus</i>	99	2
<i>Vibrio</i> sp. A61	<i>V. cyclitrophicus</i>	99	2

*Almost complete sequences (1470 to 1507 bp) except for A30 (R-14913; 438 bp), A31 (LMG 20370; 1470 bp), A32 (R-14928; 441 bp) and A37 (LMG 20369; 828 bp). *References: 1. Gomez-Gil et al., in press; 2. Thompson et al., 2003.

CHAPTER 4. Conclusions and future perspectives

This thesis provided an extended taxonomic framework for the identification and classification of *Vibrionaceae* isolates abundantly found in aquatic environments. It is clear that these findings will have a positive impact in future studies on the taxonomy and ecology of *Vibrionaceae* species.

Our descriptions are in concordance with recommendations of the ad hoc committee for the re-evaluation of the species definition in bacteriology (Stackebrandt et al., 2002). We have applied and validated the genomic fingerprinting technique FAFLP and to some extent rep-PCR for the classification of the new taxa. We consider this to be the main contribution of the present thesis to the study of the biodiversity of *Vibrionaceae*. Our new classification of vibrios is based on genomic data rather than on more traditional phenotypic features.

4.1. Conclusions

According to Bergey's manual of determinative bacteriology (1994) *Vibrionaceae* strains are Gram negative, usually motile rods, chemoorganotrophic, which have a facultative fermentative metabolism and which are found in aquatic habitats. They are generally able to grow on Thiosulphate-Citrate-Bile salt-Sucrose agar (TCBS) and are often oxidase-positive. This phenotypic definition encompasses most of the currently known *Vibrionaceae* species. **Until now, 16S rDNA sequencing is considered as 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 (2002) (see <http://dx.doi.org/10.1007/bergeysoutline200210>) lists six genera i.e. *Allomonas*, *Enhydrobacter*, *Listonella*, *Photobacterium*, *Salinivibrio* and *Vibrio* within the family *Vibrionaceae*. The genera *Allomonas* (Kalina et al., 1984) and *Enhydrobacter* (Staley et al., 1987) were tentatively allocated to the family *Vibrionaceae* based on phenotypic features.

The genus *Vibrio* is the most important and the largest one, harbouring 60 species. The number of species increased from 20 in 1981 to 47 in 2002. New *Vibrio* species isolated from the marine environment have been described each year, *V. ruber* (Shieh et al., 2003) being one of the most recent ones. **In this work we have**

described 15 new *Vibrio* species and a novel genus, *Enterovibrio norvegicus* (Figure 4.1).

It is our intention to create three new families i.e. *Enterovibrionaceae*, *Photobacteriaceae* and *Salinivibrionaceae* based on phenotypic and genotypic data (Table 4.1 and Figure 4.1.; manuscript in preparation). This proposal is intended to facilitate further studies on vibrios. The creation of these new families results in a more compact *Vibrionaceae*. Allocation of strains into different families is obtained by 16S rDNA (Figure 4.1) and phenotypic analysis (Table 4.1), while allocation of strains into species is best achieved by using FAFLP or rep-PCR.

The genera *Enterovibrio* (*E. norvegicus*) and *Grimontia* (*G. hollisae*) clustered completely apart from other vibrios. *V. calviensis* probably belongs to the genus *Enterovibrio* (E. Denner et al., in prep.). **These two genera should be accommodated in the new family *Enterovibrionaceae*.**

The genus *Photobacterium*, which comprises 6 species, is not a tight group. **We propose the new family *Photobacteriaceae* to encompass these diverse species.** Clearly, *P. phosphoreum*, the type species of the genus, and *P. iliopiscarius* form a distinct branch within the genus. Glutamine synthetase and superoxide dismutase sequence divergence analyses readily proved that *Photobacterium* and *Vibrio* are two distinct phylogenetic groups (Baumann et al., 1980).

***V. cholerae* and *V. mimicus* appear together at the outskirts of this diverse taxon which we propose to name as *Vibrionaceae* (Figure 4.1).** Ruimy et al. (1994) compared the 16S rDNA sequences of several *Aeromonas*, *Grimontia*, *Photobacterium*, *Salinivibrio*, *Vibrio* and *Plesiomonas* representatives and concluded that the genus *Vibrio* is a distinct phylogenetic group which deserves the elevation to the family rank. Overall, the analyses of several chronometers e.g. 16S rDNA (Kita-Tsukamoto et al., 1993), 23S rDNA (Macián et al., 2001a, 2001b) recA (Stine et al., 2000), and gyrB (Kita-Tsukamoto et al., unpublished data) and phenotypic features (Baumann et al., 1983) indicate that *Vibrio cholerae* and *V. mimicus* should be given genus rank. **We consider that *V. cholerae* and *V. mimicus* are the bona fide members of the genus *Vibrio*.** Whether we should retain all the other named *Vibrio* species in the same taxon as *V. cholerae* and *V. mimicus* or split them in different genera remains to be determined in future studies. Many species of the genus *Vibrio*, particularly those of the core group, do not readily show conspicuous phenotypic

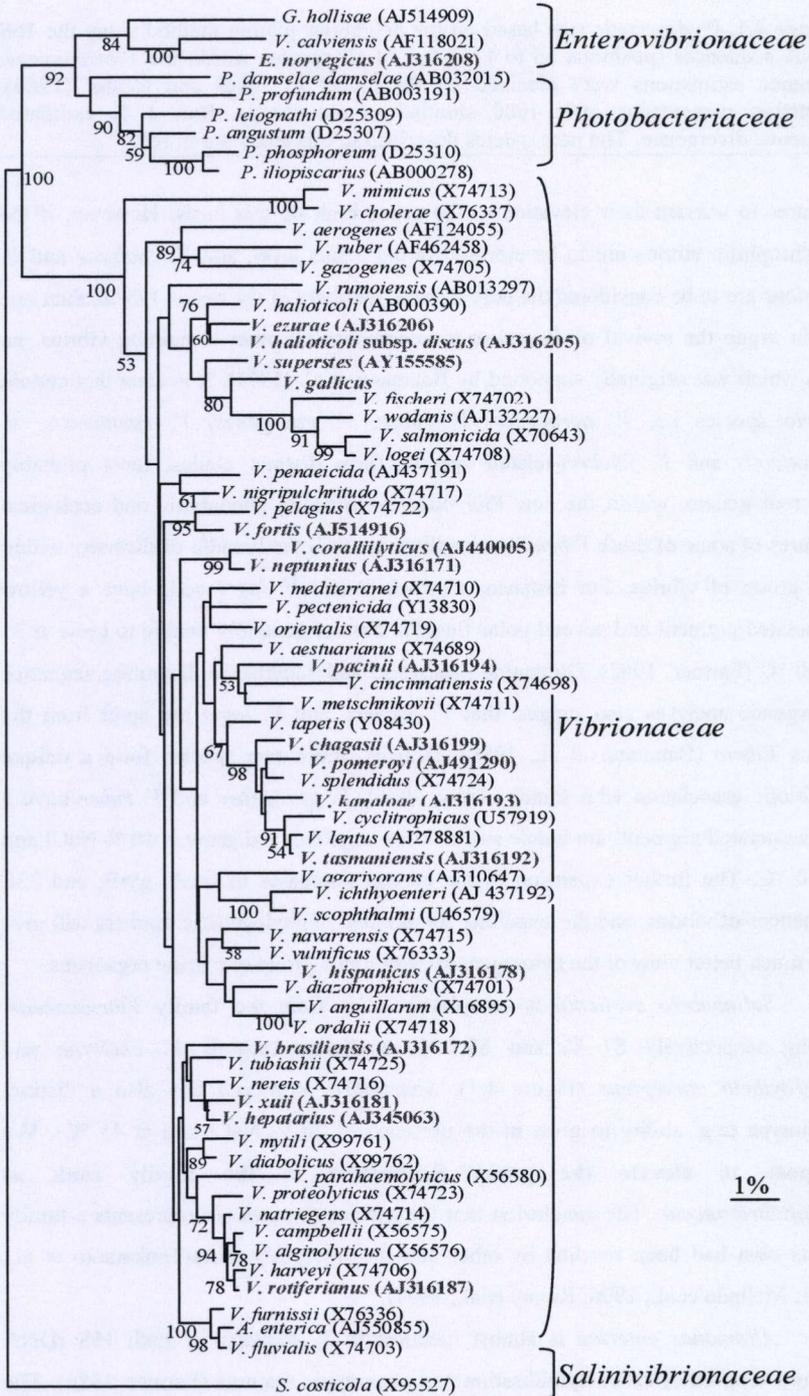


Figure 4.1. Phylogenetic tree based on the neighbour joining method using the 16S rDNA sequences (positions 65 to 1365 bp) of all species within the *Vibrionaceae*. Distance estimations were obtained by the model of Jukes and Cantor (1969). Bootstrap percentages after 1000 simulations are shown. Bar, 1 % estimated sequence divergence. The new species described in this study are in red.

features to warrant their elevation to the genus rank on this basis. However, if the psychrophilic vibrios are to be elevated to the genus level, and *V. cholerae* and *V. mimicus* are to be considered the only current members of the genus *Vibrio*, then one might argue the revival of *Beneckeia* to encompass all other remaining vibrios, an idea which was originally supported by Baumann et al. (1971). It is clear that certain *Vibrio* species i.e. *V. aerogenes*, *V. ruber*, *V. gazogenes*, *V. rumoiensis*, *V. haliotocoli*- and *V. fischeri*-related species form distinct clades, most probably different genera, within the new *Vibrionaceae*. Striking phenotypic and ecological features of some of these *Vibrio* species illustrate well the breadth of diversity within this group of vibrios. For instance, *V. fischeri* and *V. logei* cells have a yellow associated pigment and several polar flagella, but are generally unable to grow at 35 to 40 °C (Farmer, 1992). Glutamine synthetase and superoxide dismutase sequence divergence analyses also suggest that *V. fischeri*, and *V. logei* are apart from the genus *Vibrio* (Baumann et al., 1980). Besides, these two species form a unique symbiotic association with squids (Ruby, 1996). *V. gazogenes* and *V. ruber* have a red associated pigment, are indole and oxidase negative, and grow at 10 % NaCl and at 40 °C. The further expansion of the current databases of *recA*, *gyrB*, and 23S sequences of vibrios, and the searching for alternative phylogenetic markers will give us a much better view of the heterogeneity within this group of marine organisms.

Salinivibrio costicola is completely apart from the family *Vibrionaceae*, having respectively 87 % and 89.6 % similarity towards *V. cholerae* and *Enterovibrio norvegicus* (Figure 4.1). *Salinivibrio costicola* has also a distinct phenotype (e.g. ability to grow in the presence of 20 % NaCl and at 45 °C). **We propose to elevate the genus *Salinivibrio* to the family rank as *Salinivibrionaceae*.** The conclusion that the species *S. costicola* represents a family on its own had been reached by other authors previously (Kita-Tsukamoto et al., 1993; Mellado et al., 1996; Ruimy et al., 1994).

Allomonas enterica is almost identical to *V. fluvialis* by both 16S rDNA (Figure 4.1), DNA-DNA hybridisation and phenotypic features (Farmer, 1986). The

subcommittee on the taxonomy of *Vibrionaceae* had already concluded that *A. enterica* is a junior synonym of *V. fluvialis* (Farmer, 1986). Farmer (1992) suggested that the genus *Allomonas* could be proposed again in order to split the genus *Vibrio*. Although, current data do not support this hypothesis. *A. enterica*, *V. fluvialis*, and *V. furnissii* are phylogenetically related (97 to 98 % similarity) towards other vibrios, including *V. tubiashii*, *V. vulnificus*, and *V. proteolyticus* (Figure 4.1).

L. anguillarum and *L. pelagia*, currently within the genus *Listonella*, cannot be discriminated from other vibrios using 16S rDNA (Figure 4.1) or other methodologies (Austin et al., 1995b) and thus the two species should be called *V. anguillarum* and *V. pelagius*.

Enhydrobacter aerosaccus was tentatively allocated in the family *Vibrionaceae* based on its phenotypic similarity (oxidase and catalase positive, resistant to the vibriostatic agent 0/129, non-motile) towards *Aeromonas* (now in the family *Aeromonadaceae*). According to our 16S rDNA data, *E. aerosaccus* is within the family *Moraxellaceae*. *E. aerosaccus* is highly related to *Moraxella osloensis* (~100 % similarity) and we thus conclude that the genus *Enhydrobacter* should be transferred to the family *Moraxellaceae*.

The phenotypic identification of genera and species of the *Vibrionaceae* is problematic. The main reason for that is **the variability of several diagnostic phenotypic features** (e.g. arginine dihydrolase, lysine and ornithine decarboxylases, susceptibility to the vibriostatic agent 0/139, flagellation, indole production, growth at different salinities and temperatures, and carbon utilisation as revealed by Biolog) within currently recognised species. Traditionally used as clear-cut tests for identification, the latter should thus be interpreted with greatest care. Dichotomous keys (see e.g. Alsina and Blanch, 1994) are misleading for the identification of *Vibrionaceae* isolates.

FAFLP and rep-PCR analyses proved to be valuable for the discrimination of phylogenetically and phenotypically related *Vibrio* species e.g. *V. harveyi* and *V. campbellii*, *V. coralliilyticus* and *V. neptunius*. For identification purposes **it is now clear that FAFLP offers an alternative to DNA-DNA hybridisations by adopting the FAFLP pattern similarity levels of 60 to 70 % (which correspond to DNA-DNA similarity higher than 70 %).** FAFLP 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, FAFLP is easy and fast to perform and is amenable to automation. FAFLP data can also be accumulated in databases.

Table 4.1. Phenotypic differences among *Vibrionaceae*-related families

	<i>Enterovibrionaceae</i>	<i>Photobacteriaceae</i>	<i>Salinivibrionaceae</i>	<i>Vibrionaceae</i>
D-mannitol fermentation	-	-	V	V
PHB accumulation	-	+	-	V
Growth in/at: 20 % NaCl	-	-	+	-
45 °C	-	-	+	-
Voges-Proskauer	-	V	+	V
Indole	V	-	-	V
ADH	V	+	-	V
ODH	-	-	-	V
Presence of the fatty acids:				
16:1 ω 9c	+	-	+	V
18:1 ω 9c	+	-	-*	V

*trace amounts (< 1%).

4.2. Future perspectives

Research on other molecular chronometers e.g. *gyrB*, *recA*, *rpoB*, *rpoD* and 23S rDNA is urgently needed in order to complete the phylogeny of the *Vibrionaceae*, but also to refine the discrimination of closely related *Vibrionaceae* species. Analyses of sequences of these genes may also shed light on the role of recombination and mutation in the evolution of vibrios.

It is our conviction that the taxonomy and phylogeny of the family *Vibrionaceae* will be improved by multilocus sequence typing (MLST). **The delineation of clonal complexes within each recognised *Vibrio* species by means of MLST** may result in a unambiguous and direct way of identifying isolates in the future. According to the ecotype concept advanced by Cohan (2002) (see section 1.6), each clonal complex is a new species, and each currently recognised bacterial species represents in fact a genus. No more than 100 clonal complexes are expected to exist within a given species (M. Maiden, personal communication). 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 *Vibrionaceae* species

comprises 10 clonal complexes, we would expect a **multiplication of *Vibrionaceae* species from the current 60 to 600 species.**

The further exploration and description of the biodiversity amongst *Vibrionaceae* in the environment is also an important topic for future research. Although there are now already 60 validly described species within this family, the biodiversity of the *Vibrionaceae* has not been fully covered yet. If we assume that our original hypothesis (i.e. unknown phenotypic clusters or singletons correspond to unknown genotypes or new species) is correct, then we may conclude that several, if not all (n=106 unidentified groups, including 78 singletons), Biolog groups disclosed by Vandenberghe et al. (2003) represent potential new species. Future studies are needed to verify this hypothesis. We have proven that **several FAFLP genotypes correspond to a single Biolog phenotype (e.g. *V. campbellii*, *V. harveyi* and *V. rotiferianus* are found within the *V. harveyi* Biolog phenotype).** **One may thus anticipate, that many cryptic new species will be found within the identified Biolog groups.**

The ecological role, distribution and abundance of the new *Vibrionaceae* species is another important issue for further research. Several new species described were dominant on plates at the time of their isolation. 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. **Do these culturable microorganisms really represent the dominant microflora of those sites or are they just overestimated on marine agar plates?** Are there other dominant viable but non culturable (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 described here? Apparently, eutrophic environments e.g. certain estuarine ecosystems and aquacultural settings favour the growth of vibrios and one would expect that these organisms are really dominant over other groups, including VBNC bacteria, but this remains to be proven.

Summary

Our aim was the polyphasic taxonomic analysis of the family *Vibrionaceae*. The conceptual framework of this study and an introduction covering the foundation of the taxonomy, ecology and genomics of vibrios is presented in **chapter 1**. The genomic diversity of *Vibrionaceae* strains is addressed in **chapter 2**. Our analyses based on FAFLP fingerprinting and 16S rDNA sequences revealed that **many *Vibrio* species remained as yet to be described**: 31 groups (236 isolates in total) clustered apart from the type strains and were regarded to belong to new species.

Additionally, **the intraspecific polymorphism of four *Vibrio* species i.e. *V. campbellii*, *V. cholerae*, *V. haliotocoli* and *V. harveyi* is reported in more depth in chapter 2**. The study on 96 strains *V. cholerae* isolated in Brazil between 1991 and 2001 suggested the persistence of clones during several years in completely different geographical regions, while the analysis of 47 *V. haliotocoli* strains pointed out to a host-driven (host-dependent) polymorphism rather than a geographical or environmental one. Speciation has occurred within these isolates as revealed by fingerprinting (FAFLP and rep-PCR) and DNA-DNA hybridisation. Our analyses suggest that a new species (i.e. *V. ezurae*) and a new subspecies (*V. haliotocoli* subsp. *discus*) exist within the collection of *V. haliotocoli*-like isolates. Fourth-four *Vibrio* isolates identified phenotypically as *V. harveyi* and fifteen type and reference strains of *V. harveyi*, *V. campbellii*, *V. alginolyticus* and *V. parahaemolyticus* were analysed by FAFLP, rep-PCR and DNA-DNA hybridisations. Many of these *V. harveyi* isolates had been implicated in disease (e.g. luminous vibriosis) outbreaks in aquaculture settings. We found that presumptive *V. harveyi* isolates belong in fact to the species *V. campbellii*. We conclude that the fingerprinting methodologies used are most valuable tools for the study of closely related species of the *Vibrio* core group.

In chapter 3, we concentrated on the polyphasic taxonomic analysis of the potential new taxa disclosed in the previous chapter. The FAFLP and 16S rDNA analyses on the *Vibrionaceae* also led to some reclassifications: *V. shilonii* was transferred to the species *V. mediterranei*, *V. trachuri* to the species *V. harveyi* and *V. hollisae* to *Grimontia hollisae*. According to our 16S rDNA sequence analyses, the genera *Allomonas*, *Enhydrobacter* and *Listonella* should be abolished.

Some FAFLP groups disclosed were found to belong to known species as revealed by DNA-DNA hybridisation data. Clusters A30, A31 and A32 should be regarded as *V. harveyi*. Clusters A34, A37, A55 and A61 are respectively *V. diazotrophicus*, *V. campbellii*, *V. lentus* and *V. cyclitrophicus*. **Most unknown FAFLP groups turned out to be new species and thus in this chapter we present the description of the following new taxa: *Enterovibrio norvegicus*, *V. brasiliensis*, *V. chagasii*, *V. coralliilyticus*, *V. fortis*, *V. gallicus*, *V. hispanicus*, *V. kanaloaei*, *V. neptunius*, *V. pacinii*, *V. pomeroyi*, *V. probioticus*, *V. rotiferianus*, *V. superstes*, *V. tasmaniensis* and *V. xuii*.**

Our main conclusions and future perspectives are reported in Chapter 4. We propose the creation of three new families i.e. *Enterovibrionaceae*, *Photobacteriaceae* and *Salinivibrionaceae* based on phenotypic and genotypic data. This proposal is intended to facilitate further studies on vibrios. *V. cholerae* and *V. mimicus* appear together at the outskirts of this diverse taxon which we propose to name as *Vibrionaceae*. **We consider that *V. cholerae* and *V. mimicus* are the bona fide members of the genus *Vibrio*.** The creation of these new families results in a more compact *Vibrionaceae*. Allocation of strains into different families is obtained by 16S rDNA and phenotypic analysis, while allocation of strains into species is best achieved by using FAFLP or rep-PCR. According to our analyses, FAFLP correlates very well with DNA-DNA data and to a much lesser extent with phenotypic data (i.e. Biolog patterns). FAFLP offers an alternative to DNA-DNA hybridisation in the classification and identification of *Vibrionaceae* isolates.

It is our conviction that **this work provided a solid taxonomic framework for the identification and classification of *Vibrionaceae* isolates** abundantly found in aquatic environments.

Samenvatting

Het doel van deze doctoraatsthesis was de polyfasisch taxonomische analyse van de familie *Vibrionaceae*.

In hoofdstuk 1 wordt de conceptuele omkadering van deze studie en de inleiding voorgesteld; het omvat de fundamentele van de taxonomie, ecologie en genetica van vibrio's.

De genomische diversiteit van de *Vibrionaceae* stammen wordt beschreven in hoofdstuk 2. Mijn analyse, gebaseerd op FAFLP fingerprinting en 16S rDNA sequencing, toonde vele nog niet beschreven *Vibrio* species aan. In FAFLP clusterden 31 groepen (236 isolaten in totaal) afzonderlijk van de type stammen. Ze werden beschouwd als mogelijk nieuwe species.

In hoofdstuk 2 wordt er verder dieper ingegaan op het intraspecifiek polymorfisme van vier *Vibrio* species, nl. *V. campbellii*, *V. cholerae*, *V. haliotocoli* en *V. harveyi*.

De studie van 96 *Vibrio cholerae* stammen, geïsoleerd in Brazilië tussen 1991 en 2001, toonde de stabiliteit van bepaalde klonen aan, alhoewel ze gedurende verscheidene jaren in compleet verschillende geografische gebieden geïsoleerd werden. Bij 47 *Vibrio haliotocoli* stammen daarentegen, toonden de analyses eerder een gastheer-gebonden polymorfisme aan. Door de combinatie van fingerprinting (FAFLP en rep-PCR) en DNA-DNA hybridisaties, kon de speciatie binnen de onderzochte isolaten aangetoond worden.

Mijn analyses laten vermoeden dat een nieuw species, nl. *V. ezurae* en een nieuw subspecies nl. *V. haliotocoli* subsp. *discus* voorkomen binnen de collectie *V. haliotocoli*-achtige isolaten. Vierenveertig *Vibrio* isolaten, fenotypische geïdentificeerd als *V. harveyi*, en vijftien type- en referentiestammen van *V. harveyi*, *V. campbellii*, *V. alginolyticus*, werden eveneens geanalyseerd met behulp van FAFLP, rep-PCR en DNA-DNA hybridisaties. Veel van deze *V. harveyi* isolaten zijn betrokken bij ziekten zoals bijvoorbeeld lichtgevende vibriosis in de aquacultuur. Vermoedelijke *V. harveyi* isolaten behoren in feite tot het species *V. campbellii*. We kunnen dan ook besluiten dat de gebruikte genomische fingerprintingmethodologieën zeer geschikt zijn voor het bestuderen van de nauw verwante *Vibrio* species.

Hoofdstuk 3 bespreekt de verdere polyfasisch taxonomische analyse van de nieuwe taxa. De FAFLP en 16S rDNA analyses van de *Vibrionaceae* laten de volgende herclassificaties toe: *V. shilonii* werd getransfereerd naar *V. mediterranei*; *V. trachuri* naar het species *V. harveyi* en *V. hollisae* naar *Grimontia hollisae*. Volgens onze 16S rDNA analyses zouden volgende genera moeten verworpen worden: *Allomonas*, *Enhydrobacter* en *Listonella*.

Sommige FAFLP groepen worden toegeschreven aan gekende species en dit werd bevestigd met DNA-DNA hybridisaties: cluster A30, A31 en A32 worden beschouwd als *V. harveyi*. Cluster A34, A37, A55 en A61 zijn respectievelijk *V. diazotrophicus*, *V. campbellii*, *V. lentus* en *V. cyclitrophicus*.

De onbenoemde FAFLP groepen blijken nieuwe species te zijn en de beschrijving van de volgende nieuwe species wordt voorgesteld in dit hoofdstuk: *Enterovibrio norvegicus*, *V. brasiliensis*, *V. chagasii*, *V. coralliititicus*, *V. fortis*, *V. gallicus*, *V. hispanicus*, *V. kanaloaei*, *V. neptunius*, *V. pacinii*, *V. pomeroyi*, *V. probioticus*, *V. rotiferianus*, *V. superstes*, *V. tasmaniensis* en *V. xuii*.

De algemene besluiten en toekomstperspectieven zijn beschreven in hoofdstuk 4. Het is mijn overtuiging dat dit werk een nieuw verbeterd taxonomisch kader opleverde voor de identificatie en classificatie van *Vibrionaceae* isolaten die overvloedig voorkomen in waterige habitats. Volgens mijn analyses correleert FAFLP zeer goed met de DNA-DNA data en in veel minder mate met de fenotypische data (b.v. Biolog patronen). FAFLP biedt een alternatief voor DNA-DNA hybridisaties in de classificatie en de identificatie van *Vibrionaceae* isolaten.

Tenslotte wordt er een voorstel gedaan om voortaan deze organismen onder te brengen in de families *Enterovibrionaceae*, *Photobacteriaceae*, *Salinivibrionaceae* en *Vibrionaceae*.

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Table 1. List of strains used in this work

Strain no. (synonym no.)	Place and date of isolation	Source	Biolog Group*
Cluster A1			
LMG 10953 (NCIMB 2165)	Kent (UK)	Diseased oyster larvae (<i>Crassostrea gigas</i>)	STD3-348
LMG 20538 (INCO 83)	LCMM Florianópolis (Brazil), 1998	Bivalve larvae (<i>Nodipecten nodosus</i>)	STD3-348
R-14955 (TAR 15)	ARC Gent (Belgium), 1998	Gut of turbot larvae (<i>Scophthalmus maximus</i>)	STD3-348
R-14957 (RFT 15), R-14958 (RFT 31)	ARC Gent (Belgium), 1999	Rotifer in recirculation system (<i>Brachionus plicatilis</i>)	STD3-348
R-14959 (INCO 19), R-14960 (INCO 16), R-14961 (INCO 85), R-14962 (INCO 79)	LCMM Florianópolis (Brazil), 1998	Bivalve larvae (<i>Nodipecten nodosus</i>)	STD3-348
R-14963 (INCO 89)	LCMM Florianópolis (Brazil), 1998	Diseased bivalve larvae (<i>Nodipecten nodosus</i>)	STD3-348
Cluster A2			
LMG 20540 (R-14967), R-14966 (INCO 33), R-14968 (INCO 188)	Guernsey Sea Farm (UK), 1998	Oyster larvae (<i>Crassostrea gigas</i>)	STD3-348
R-14965 (INCO 38)	Guernsey Sea Farm (UK), 1998	Diseased oyster larvae (<i>Crassostrea gigas</i>)	STD3-348
R-14969 (INCO 47), R-14970 (INCO 39)	Guernsey Sea Farm (UK), 1998	Healthy oyster larvae (<i>Crassostrea gigas</i>)	STD3-348
Cluster A3			
LMG 19270 (INCO 222)	LCMM Florianópolis (Brazil), 1999	Diseased bivalve larvae (<i>Nodipecten nodosus</i>)	INCO 222
LMG 20541 (INCO 213), LMG 20543 (INCO 242), LMG 20544 (INCO 244),	LCMM Florianópolis (Brazil), 1999	Healthy bivalve larvae (<i>Nodipecten nodosus</i>)	INCO 222
LMG 20545 (INCO 246), R-14972 (INCO 243), R-14975 (INCO 214)	LCMM Florianópolis (Brazil), 1999	Healthy bivalve larvae (<i>Nodipecten nodosus</i>)	INCO 222
Cluster A4			

Table 1. (continued).

LMG 20548 (INCO 389), R-14978 (INCO 407), R-14979 (INCO 376), R-14981 (INCO 404)	LCMM Florianópolis (Brazil), 1999	Bivalve larvae (<i>Nodipecten nodosus</i>)	STD3-348
R-14982 (INCO 377), R-14983 (INCO 383), R-14984 (INCO 385), R-14985 (INCO 400)	LCMM Florianópolis (Brazil), 1999	Bivalve larvae (<i>Nodipecten nodosus</i>)	STD3-348
R-14986 (INCO 388), R-14987 (INCO 382)	LCMM Florianópolis (Brazil), 1999	Bivalve larvae (<i>Nodipecten nodosus</i>)	STD3-348
Cluster A5			
LMG 20536 (INCO 17)	LCMM Florianópolis (Brazil), 1998	Bivalve larvae (<i>Nodipecten nodosus</i>)	STD3-348
LMG 20610 (R-15124)	ARC Gent (Belgium), 1999	Culture water of rotifer	STD3-348
LMG 20611 (R-15122), R-15119 (RFT 48), R-15120 (RFT 49), R-15121 (RFT 26),	ARC Gent (Belgium), 1999	Rotifer in recirculation system (<i>Brachionus plicatilis</i>)	STD3-348
LMG 20612 (R-1529)	ARC Gent (Belgium), 1996	Gut of turbot larvae (<i>Scophthalmus maximus</i>)	STD3-348
LMG 20613 (R-15114), R-15113 (RFT 60), R-15116 (RFT 35), R-15117 (RFT 54), R-15118	ARC Gent (Belgium), 1999	Rotifer in recirculation system (<i>Brachionus plicatilis</i>)	STD3-348
LMG 20614 (R-15109), R-15108 (FKFO 126), R-15111 (RFT 55), R-15112 (RFT 59)	ARC Gent (Belgium), 1999	Rotifer in recirculation system (<i>Brachionus plicatilis</i>)	STD3-348
LMG 20615 (R-15110)	LCCM Florianópolis (Brazil), 1998	Diseased bivalve larvae (<i>Nodipecten nodosus</i>)	STD3-348
R-1575, R1579, R-1592	ARC Gent (Belgium), 1997	Gut of turbot larvae (<i>Scophthalmus maximus</i>)	STD3-348
R-15123 (FKFO 147)	ARC Gent (Belgium), 1999	Healthy rotifer (<i>Brachionus plicatilis</i>)	STD3-348
Cluster A6-Vibrio mediterranei/Vibrio shiloi			
<i>V. mediterranei</i> LMG 11258 ^T , LMG 11259 (VIB 416)	Valencia (Spain)	Coastal sea water	<i>V. mediterranei</i>

Table 1. (continued).

LMG 11663 (VIB 418)	Valencia (Spain), 1986	Sea water	<i>V. mediterranei</i>
LMG 16836 (VIB 429)	Spain, 1991	Sea bream larvae (<i>Sparus aurata</i>)	<i>V. mediterranei</i>
<i>V. shiloi</i> LMG 19703 ^T	Israel, 1995	Bleached coral	NA
R-14988 (TAR 1), R-14993 (TAR 2)	ARC Gent (Belgium), 1998	Gut of turbot larvae (<i>Scophthalmus maximus</i>)	<i>V. mediterranei</i>
R-14989 (FKFO 136)	ARC Gent (Belgium), 1999	Culture water of rotifers	<i>V. mediterranei</i>
R-14990 (VIB 419), R-14995 (VIB 420)	Mediterranean Sea	Sea water	<i>V. mediterranei</i>
Cluster A7-Vibrio wodanis			
<i>V. wodanis</i> NCIMB 13582 ^T	Norway, 1988	Salmon suffering of winter ulcer (<i>Salmo salar</i>)	NA
R-14996 (K 14)	North Iceland, 1989	Salmon suffering of winter ulcer (<i>Salmo salar</i>)	NA
R-14997 (K 26)	South-west Iceland, 1991	Salmon suffering of winter ulcer (<i>Salmo salar</i>)	NA
R-14998 (K 31)	North Iceland, 1990	Salmon suffering of winter ulcer (<i>Salmo salar</i>)	NA
R-14999 (K 32), R-15000 (K 16)	South-west Iceland, 1990	Salmon suffering of winter ulcer (<i>Salmo salar</i>)	NA
Cluster A8			
LMG 20010 (INCO 320), LMG 20546 (INCO 317), R-15002 (INCO 338), R-15003 (INCO 337), R-15004 (INCO 332), R-15005 (INCO 328)	LCMM Florianópolis (Brazil), 1999	Bivalve larvae (<i>Nodipecten nodosus</i>)	INCO 320
R-15007 (STD3-1028)	LCMM Florianópolis (Brazil), 1999	Bivalve larvae (<i>Nodipecten nodosus</i>)	INCO 320
	Feng Cheng (China), 1996	Diseased shrimp larvae (<i>Penaeus chinensis</i>)	INCO 320
Cluster A9			
R-15031 (INCO 297), R-15035 (INCO 305)	LCMM Florianópolis (Brazil), 1999	Healthy bivalve larvae (<i>Nodipecten nodosus</i>)	VIB 836
R-15032 (STD3-1247)	CENAIM (Ecuador), 1996	Shrimp larvae (<i>Litopenaeus vannamei</i>)	VIB 836

Table 1. (continued).

R-15033 (STD3-931)	Sinaloa (Mexico), 1994	Sea water	VIB 836
R-15034 (VIB 839)	MPL (Tasmania)	Atlantic salmon (<i>Salmo salar</i>)	<i>V. splendidus</i>
R-15036 (INCO 257)	LCMM Florianópolis (Brazil), 1999	Diseased bivalve larvae (<i>Nodipecten nodosus</i>)	INCO 254
R-15037 (INCO 31), R-15038 (INCO 35)	Guernsey Sea Farm (UK), 1998	Diseased oyster larvae (<i>Crassostrea gigas</i>)	VIB 836
Cluster A10-Vibrio pelagius			
<i>V. pelagius</i> LMG 3897 ^T	Hawaii (U.S.A.)	Sea water	<i>V. pelagius</i>
LMG 19995 (STD3-1008)	Feng Cheng (China), 1996	Shrimp larvae (<i>Penaeus Chinensis</i>)	STD3-1008
Cluster A11-Vibrio logei			
LMG 14011	Washington (U.S.A.)	Pacific cod	<i>V. logei</i>
<i>V. logei</i> LMG 19806 ^T	(U.S.A.)	Gut of arctic scallop	NA
LMG 19990 (STD3-996)	Sinaloa (Mexico), 1994	Diseased white shrimp juvenile (<i>Litopenaeus vannamei</i>)	STD3-996
LMG 20008 (FKFO 99)	ARC Gent (Belgium), 1998	Gut of turbot larvae (<i>Scophthalmus maximus</i>)	FKFO 99
R-15024 (VIB 523)	Sweden	Eel (<i>Anguilla anguilla</i>)	<i>V. vulnificus</i>
R-15025 (VIB 816)	Venezuela	Sediment	<i>V. vulnificus</i>
R-15028 (KV 1)	ARC Gent (Belgium), 2001	Turbot larvae (<i>Scophthalmus maximus</i>)	NA
Cluster A12-Vibrio cincinnatiensis			
LMG 7891 ^T	Ohio (U.S.A.)	Human blood and cerebrospinal fluid	<i>V. cincinnatiensis</i>
LMG 10950 (VIB 442)	Maryland (U.S.A.)	Oyster	<i>V. metschnikovii</i>
LMG 13217 (VIB 190)	Greece, 1990	Rotifer (<i>Brachionus plicatilis</i>)	<i>V. diazotrophicus</i>
LMG 19988 (STD3-932)	Sinaloa (Mexico), 1994	Diseased white shrimp juvenile (<i>Litopenaeus vannamei</i>)	STD3-932
R-15041 (STD3-938)		Turbot (<i>Scophthalmus maximus</i>)	<i>V. nigripulchritudo</i>
R-15042 (VIB 382)	Norway, 1992		<i>V. scopthalmi</i>
Cluster A13-Vibrio nigripulchritudo/Vibrio orientalis			

Table 1. (continued).

<i>V. nigripulchritudo</i> LMG 3896 ^T , LMG 11257 (VIB 348)	Hawaii (U.S.A.)	Sea water	<i>V. nigripulchritudo</i>
<i>V. orientalis</i> LMG 7897 ^T R-15045 (FKFO 70)	Yellow Sea (China) ARC Gent (Belgium), 1998	Sea water Gut of turbot larvae (<i>Scophthalmus maximus</i>)	<i>V. orientalis</i> <i>V. haliotocoli</i>
R-15047 (INCO 200) R-15048 (VIB 617) R-15049	Guernsey Sea Farm (UK), 1999 Venezuela Mediterranean coast, Spain	Oyster larvae (<i>Cassostrea gigas</i>) White shrimp (<i>Litopenaeus vannamei</i>) Plankton	<i>V. nigripulchritudo</i> STD3-1052 <i>V. mediterranei</i>
Cluster A14-Vibrio campbellii			
<i>V. campbellii</i> LMG 11216 ^T , LMG 11256 (VIB 347)	Hawaii (U.S.A.)	Sea water	<i>V. campbellii</i>
R-14908 (VIB 346)	Ivory Coast, 1988	Laguna water	NA
Cluster A15-Vibrio fischeri/Photobacterium iliopiscarius			
LMG 4412		Marine fish	<i>V. fischeri</i>
<i>V. fischeri</i> LMG 4414 ^T	Massachusetts (U.S.A.), 1933	Dead squid	<i>V. fischeri</i>
LMG 11653 (VIB 380)	Hawaii (U.S.A.)	Sea water	<i>V. fischeri</i>
<i>P. iliopiscarius</i> LMG 19543 ^T	Norway	Gut of fish	<i>P. iliopiscarius</i>
Cluster A16			
LMG 13211 (VIB 184), LMG 13213 (VIB 186)	Barcelona (Spain), 1990	<i>Artemia</i> sp.	<i>V. gazogenes</i>
LMG 13240 (VIB 213)	Barcelona (Spain), 1990	Culture water	<i>V. gazogenes</i>
Unclustered: R-1586	ARC Gent (Belgium), 1997	Gut of turbot larvae (<i>Scophthalmus maximus</i>)	<i>V. salmonicida</i>
Cluster A17-Vibrio scophthalmi			
<i>V. scophthalmi</i> LMG 19158 ^T	Spain	Turbot juvenile (<i>Scophthalmus maximus</i>)	<i>V. scophthalmi</i>

Table 1. (continued).

LMG 20023 (VIB 782)	Spain, 1988	Turbot (<i>Scophthalmus maximus</i>)	VIB 782
R-15029 (VIB 582)	Spain, 1991	Turbot (<i>Scophthalmus maximus</i>)	<i>V. scophthalmi</i>
Cluster A18-Vibrio navarrensis			
<i>V. navarrensis</i> LMG 15976 ^T	Villa Franca Navarra (Spain), 1982	Sewage	<i>V. navarrensis</i>
R-15013 (INCO 240)	LCMM Florianópolis (Brazil), 1999	Healthy bivalve larvae (<i>Nodipecten nodosus</i>)	NA
Cluster A19-Photobacterium phosphoreum			
<i>P. phosphoreum</i> LMG 4233 ^T	Hawaii (U.S.A.)	Sea water	<i>P. phosphoreum</i>
R-14856 (VIB 774)	Australia, 1990	Rainbow trout	<i>V. mimicus</i>
Cluster A20-Vibrio metschnikovii			
LMG 4416	India	Fowl	<i>V. metschnikovii</i>
LMG 4426 (VIB 478)	Tokio (Japan), 1884	Human feces	VIB 478
<i>V. metschnikovii</i> LMG 11664 ^T		Diseased fowl	<i>V. metschnikovii</i>
Cluster A21-Vibrio salmonicida			
<i>V. salmonicida</i> LMG 14010 ^T	Norway	Diseased atlantic salmon (<i>Salmo salar</i>)	<i>V. salmonicida</i>
<i>V. gazogenes</i> LMG 19540 ^T	Massachusetts (U.S.A.)	Mud from Saltmarsh	<i>V. gazogenes</i>
Unclustered: <i>Vibrio hollisae</i> LMG 17719 ^T	Maryland (U.S.A.)	Human feces	<i>V. hollisae</i>
Cluster A22-Salinivibrio costicola subsp. <i>costicola</i>			
<i>S. costicola</i> subsp. <i>costicola</i> LMG 11651 ^T		Bacon curing brine	<i>S. costicola</i>
R-15050 (VIB 479)		Turbot (<i>Scophthalmus maximus</i>)	<i>V. proteolyticus</i>
Cluster A23			

Table 1. (continued).

LMG 20011 (INCO 167)	LCMM Florianópolis (Brazil), 1998	Bivalve larvae (<i>Nodipecten nodosus</i>)	INCO 167
R-15052 (STD3-1071)	Dahua (China), 1995	Shrimp culture water	STD3-1008
R-15053 (STD3-1204)	CENAIM (Ecuador)	White shrimp (<i>Litopenaeus vannamei</i>)	STD3-1008
Unclustered: R-3681	AARS Austevoll (Norway), 1997	Gut of healthy turbot larvae (<i>Scophthalmus maximus</i>)	STD3-1052
Cluster A24-Vibrio vulnificus			
<i>V. vulnificus</i> LMG 13545 ^T	U.S.A.	Human wound infection	<i>V. vulnificus</i>
LMG 20015 (VIB 534), R-15059 (VIB 535), R-15062 (VIB 525), R-15063 (VIB 527)	Sweden	Eel (<i>Anguilla anguilla</i>)	<i>V. vulnificus</i>
Cluster A25-Vibrio proteolyticus			
<i>V. proteolyticus</i> LMG 3772 ^T	U.S.A.	Intestine of isopod (<i>Limnoria tipunctata</i>)	<i>V. proteolyticus</i>
LMG 16837 (VIB 445)	Spain, 1991	Sea bream larvae (<i>Sparus aurata</i>)	<i>V. metschnikovii</i>
R-15065 (FKFO 18)	Gent (Belgium), 1997	Diseased <i>Artemia</i> sp.	<i>V. proteolyticus</i>
Cluster A26			
LMG 20362 (1P), LMG 20366 (5P), LMG 20371 (2C)	CENAIM (Ecuador), 2000	Digestive gland of white shrimp (<i>Litopenaeus vannamei</i>)	NA
LMG 20378 (10D)	CENAIM (Ecuador), 2001	Digestive gland of white shrimp (<i>Litopenaeus vannamei</i>)	NA
Cluster A27-Vibrio fluvialis			
<i>V. fluvialis</i> LMG 7894 ^T , LMG 11654 (VIB 383)	Bangladesh	Human faeces	<i>V. fluvialis</i>
R-15088 (VIB 381)	Australia	Angelfish	<i>V. fluvialis</i>
R-15090 (VIB 387)	Australia	Fish	<i>V. fluvialis</i>
Cluster A28			

Table 1. (continued).

R-15091 (VIB 555), R-15092 (VIB 554)	Spain, 1990	Turbot (<i>Scophthalmus maximus</i>)	<i>V. fluvialis</i>
Cluster A29-Vibrio fusnissii			
<i>V. fusnissii</i> LMG 7910 ^T	Japan	Human faeces	<i>V. fusnissii</i>
LMG 11655 (VIB 384), LMG 11757 (VIB 388)	UK	River water	<i>V. fusnissii</i>
LMG 11656 (VIB 385)			<i>V. fusnissii</i>
LMG 11758 (VIB 389)	UK	Pig faeces	<i>V. fusnissii</i>
Cluster A30			
LMG 4043			
LMG 10946 (VIB 395)	India	Prawn (<i>Penaeus monodon</i>)	<i>V. harveyi</i>
LMG 10947 (VIB 396)	Red Sea	Sea water	<i>V. harveyi</i>
LMG 10948 (VIB 397), LMG 11659 (VIB 400)	Hawaii (U.S.A.)	Sea water	<i>V. harveyi</i>
R-14912 (VIB 667)	Denmark, 1994	Water	<i>V. harveyi</i>
R-14913 (STD3-1259), R-14919 (STD3-1201)	CENAIM (Ecuador), 1996	White shrimp (<i>Litopenaeus vannamei</i>)	<i>V. harveyi</i>
R-14917 (INCO 245)	LCMM Florianópolis (Brazil), 1999	Healthy bivalve larvae (<i>Nodipecten nodosus</i>)	<i>V. harveyi</i>
R-14918 (VIB 618), R-14925 (VIB 622)	Venezuela	White shrimp (<i>Litopenaeus vannamei</i>)	<i>V. harveyi</i>
R-14920 (VIB 642), R-14922 (VIB 641)	Japan		<i>V. harveyi</i>
R-14921 (VIB 788)	Spain, 1988	Fish (<i>Sparus aurata</i>)	<i>V. harveyi</i>
Cluster A31			
LMG 20370 (1C), LMG 20373 (4C), LMG 20376 (7C)	CENAIM (Ecuador), 2000	Digestive gland of white shrimp (<i>Litopenaeus vannamei</i>)	NA
Cluster A32			
LMG 13949 (VIB 391)	Thailand, 1990	Shrimp	<i>V. harveyi</i>
LMG 13950 (VIB 477)			<i>V. harveyi</i>

Table 1. (continued).

R-14926 (VIB 661)	Tunisia, 1992	Sea bass (<i>Dicentrarchus labrax</i>)	<i>V. harveyi</i>
R-14927 (VIB 628)	Venezuela	<i>Paguara</i> sp.	<i>V. harveyi</i>
R-14928 (HPE-8-1)	Mexico	White shrimp (<i>Litopenaeus vannamei</i>)	<i>V. harveyi</i>
R-14930 (VIB 655)	France, 1989	Sea bass (<i>Dicentrarchus labrax</i>)	<i>V. harveyi</i>
R-14932 (VIB 656)	Greece, 1990	Sea bass (<i>Dicentrarchus labrax</i>)	<i>V. harveyi</i>
R-14933 (VIB 580)	Spain, 1989	Turbot (<i>Scophthalmus maximus</i>)	<i>V. harveyi</i>
R-14934 (VIB 585)	Spain, 1991	Sea bream (<i>Sparus aurata</i>)	<i>V. harveyi</i>
Cluster A33			
R-14935 (RFT 51), R-14936 (RFT 41), R-14937 (RFT 61), R-14938 (RFT 33), R-14939	ARC Gent (Belgium), 1999	Rotifer in recirculation system (<i>Brachionus plicatilis</i>)	<i>V. harveyi</i>
Cluster A34			
LMG 11217 (VIB 373), LMG 11218 (VIB 374)	Chetney Marshes Kent (UK)	Water	<i>V. diazotrophicus</i>
LMG 20033 (R-3666), R-3706	AARS Austevoll (Norway), 1997	Gut of turbot larvae (<i>Scophthalmus maximus</i>)	R-3666
R-14942 (VIB 584)	Spain, 1993	Turbot (<i>Scophthalmus maximus</i>)	<i>V. harveyi</i>
Cluster A35-Vibrio aestuariamus			
<i>V. aestuariamus</i> LMG 7909 ^T	Oregon (U.S.A.)	Oyster	<i>V. aestuariamus</i>
R-14943 (VIB 145), R-14944 (VIB 144)	Denmark	Water	<i>V. navarrensis</i>
R-14945 (VIB 142)	Denmark	Rainbow trout	<i>V. navarrensis</i>
Cluster A36-Vibrio harveyi/Vibrio trachuri			
<i>V. harveyi</i> LMG 4044 ^T	Massachusetts (U.S.A.), 1935	Dead amphipod (<i>Talorchestia</i> sp.)	<i>V. harveyi</i>
LMG 7890 (VIB 286)	Baltimore (U.S.A.)	Brown shark (<i>Carcharhinus plumbeus</i>)	<i>V. harveyi</i>
LMG 11226	Hawaii (U.S.A.)	Sea water	<i>V. harveyi</i>
<i>V. trachuri</i> LMG 19643 ^T , LMG 19714	Numazu (Japan)	Japanese horse mackerel (<i>Trachurus japonicus</i>)	NA

Table 1. (continued).

R-14946 (VIB 646), R-14947 (VIB 665)	Denmark, 1993	Water from tanks with sharks	<i>V. harveyi</i>
R-14948 (VIB 644)	Japan	Diseased milkfish (<i>Chamus chanus</i>)	<i>V. aestuarianus</i>
R-14949 (VIB 811)	Spain, 1986	Seabass (<i>Dicentrarchus labrax</i>)	<i>V. harveyi</i>
R-14950 (VIB 23)	Greece, 1991	Seabream (<i>Sparus aurata</i>)	<i>V. harveyi</i>
R-14951 (VIB 22)	Greece, 1991	Seabass (<i>Dicentrarchus labrax</i>)	<i>V. harveyi</i>
R-14952 (VIB 568)	Spain, 1990	Turbot (<i>Scophthalmus maximus</i>)	<i>V. harveyi</i>
Cluster A37			
LMG 13241 (VIB 214)	Greece, 1991	Water	<i>V. harveyi</i>
LMG 16828 (VIB 403), LMG 16830 (VIB 406), LMG 16835 (VIB 404)	Thailand	Black tiger prawn (<i>Penaeus monodon</i>)	<i>V. harveyi</i>
LMG 20369 (8P)	CENAIM (Ecuador), 2000	Digestive gland of white shrimp (<i>Litopenaeus vannamei</i>)	<i>V. harveyi</i>
R-14899 (VIB 629)	Venezuela	Bivalve	<i>V. harveyi</i>
R-14900 (VIB 819)	Senegal, 1980	Fish	<i>V. harveyi</i>
R-14901 (VIB 822)	Senegal, 1980	Shrimp	<i>V. harveyi</i>
R-14902 (STD3-946)	Mexico, 1994	White shrimp (<i>Litopenaeus vannamei</i>)	<i>V. harveyi</i>
R-14905 (VIB 394)	Australia	Barramundi heart	<i>V. harveyi</i>
Cluster A38-Vibrio anguillarum			
LMG 4411 (VIB 71)		Trout (<i>Salmo trutta</i>)	<i>V. anguillarum</i>
<i>V. anguillarum</i> LMG 4437 ^T	Norway	Diseased cod (<i>Gadus morhua</i>)	<i>V. anguillarum</i>
LMG 10861 (VIB 1)	Denmark	Rainbow trout (<i>Salmo gairdneri</i>)	<i>V. anguillarum</i>
LMG 10866 (VIB 6), LMG 10869 (VIB 9), LMG 10870 (VIB 10)	Denmark	Cod (<i>Gadus morhua</i>)	<i>V. anguillarum</i>
LMG 10867 (VIB 7)	Denmark	Eel (<i>Anguilla anguilla</i>)	<i>V. anguillarum</i>
R-15082 (VIB 18)	Denmark, 1991	Rainbow trout (<i>Salmo gairdneri</i>)	<i>V. anguillarum</i>
Cluster A39-Vibrio ordalii			
LMG 10951 (VIB 453)	Washington (U.S.A.)	Rainbow trout (<i>Oncorhynchus rhodurus</i>)	<i>V. ordalii</i>

Table 1. (continued).

<i>V. ordalii</i> LMG 13544 ^T	Washington (U.S.A.), 1973	Diseased coho salmon (<i>Oncorhynchus rhododurus</i>)	<i>V. ordalii</i>
R-15098 (VIB 715)	Hokkaido (Japan), 1985	Amago trout (<i>Oncorhynchus rhodurus</i>)	VIB 739
R-15099 (VIB 172)	U.S.A.	Salmon (<i>Salmo salar</i>)	<i>V. cholerae</i>
R-15101 (VIB 733),	Hokkaido (Japan), 1985	Rainbow trout (<i>Oncorhynchus mykiss</i>)	<i>V. ichthyoenteri</i>
R-15103 (VIB 738),			VIB 734
R-15104 (VIB 735), R-15105 (VIB 741),			<i>V. ichthyoenteri</i>
R-15106 (VIB 737),	Hokkaido (Japan), 1985	Rainbow trout (<i>Oncorhynchus mykiss</i>)	<i>V. ichthyoenteri</i>
R-15107 (VIB 714)			<i>V. ordalii</i>
Cluster A40-Vibrio cholerae			
<i>V. cholerae</i> LMG 4406	River Elbe (Germany)	Fish	<i>V. cholerae</i>
LMG 16743 (VIB 356)	China, 1983	Shrimp (<i>Penaeus orientalis</i>)	<i>V. cholerae</i>
LMG 19996 (STD3-988)	Mexico, 1994	White shrimp (<i>Litopenaeus vannamei</i>)	STD3-988
R-14847 (STD3-934)	Sinaloa (Mexico), 1994	Diseased white shrimp juvenile (<i>Litopenaeus vannamei</i>)	STD3-988
Cluster A41-Vibrio mimicus			
<i>V. mimicus</i> LMG 7896 ^T	North Carolina (U.S.A.)	Infected human ear	<i>V. mimicus</i>
R-14850 (VIB 440)	Peru	Shrimp	<i>V. orientalis</i>
Cluster A42-Vibrio parahaemolyticus			
<i>V. parahaemolyticus</i> LMG 2850 ^T , R-14855 (ATCC 17803)	Japan	Diseased human	<i>V. parahaemolyticus</i>
LMG 11670	Yellow Sea (China)	Sea water	<i>V. orientalis</i>
LMG 16842 (VIB 462),	Thailand, 1992	Shrimp	<i>V. parahaemolyticus</i>
LMG 16874 (VIB 803)			<i>V. harveyi</i>
R-14854	Mexico, 1994	White shrimp (<i>Litopenaeus vannamei</i>)	<i>V. parahaemolyticus</i>
Cluster A43-Vibrio pectenicida			
<i>V. pectenicida</i> LMG 19642 ^T	Brittany (France), 1991	Diseased bivalve larvae (<i>Pecten maximus</i>)	<i>V. pectenicida</i>

Table 1. (continued).

LMG 20549 (CIP 105229)	Brittany (France), 1992	Diseased bivalve larvae (<i>Pecten maximus</i>)	<i>V. pectenica</i>
LMG 20550 (A601)	Brittany (France), 1993	Diseased bivalve larvae (<i>Pecten maximus</i>)	<i>V. pectenica</i>
LMG 20551 (A700)	Brittany (France), 1995	Diseased bivalve larvae (<i>Pecten maximus</i>)	<i>V. pectenica</i>
R-14860 (VIB 415)	Norway, 1992	Halibut	<i>V. pectenica</i>
Cluster A44-Vibrio nereis			
<i>V. nereis</i> LMG 3895 ^T	Hawaii (U.S.A.)	Seawater	<i>V. nereis</i>
R-14838 (VIB 374)	Chetney Marshes Kent (UK)	Ditch water	<i>V. logei</i>
R-14839 (STD3-9)	Japan	Penaeid shrimp	<i>V. penaeicida</i>
R-14840 (VIB 514)	Milford Connecticut (U.S.A.)	Hard clam larvae (<i>Mercenaria mercenaria</i>)	<i>V. tubiashii</i>
Cluster A45			
LMG 20012 (VIB 836), R-14841 (VIB 828), R-14842 (VIB 840), R-14843 (VIB 848)	MPL (Tasmania)	Atlantic salmon (<i>Salmo salar</i>)	VIB 836
R-14845 (VIB 842), R-14846 (VIB 846)	MPL (Tasmania)	Atlantic salmon (<i>Salmo salar</i>)	VIB 836
Cluster A46			
LMG 11753 (VIB 470)	Hawaii (U.S.A.)	Sea water	<i>V. pelagius</i>
LMG 20539 (INCO 191), R-15012 (INCO 192)	IFREMER (France), 1998	Diseased oyster larvae (<i>Ostrea edulis</i>)	<i>V. splendidus</i>
R-15009 (STD3-1036)	Dahua (China), 1996	Shrimp (<i>Penaeus chinensis</i>)	<i>V. splendidus</i>
R-15010 (STD3-1085)	Feng Cheng (China), 1996	Shrimp (<i>Penaeus chinensis</i>)	<i>V. splendidus</i>
Cluster A47			
LMG 13245 (VIB 218)	Spain, 1991	Seabass (<i>Dicentrarchus labrax</i>)	STD3-1057
LMG 19999 (STD3-1057)	Dahua (China), 1996	Healthy shrimp larvae (<i>Penaeus chinensis</i>)	STD3-1057
R-15016 (VIB 847)	MPL (Tasmania)	Atlantic salmon (<i>Salmo salar</i>)	<i>V. ordalii</i>
Cluster A48-Vibrio natriegens			
LMG 2225			<i>V. navarrensis</i>
<i>V. natriegens</i> LMG 10935 ^T	Sapelo Island (U.S.A.)	Salt marsh mud	<i>V. natriegens</i>

Table 1. (continued).

R-15054 (VIB 834), R-15055 (VIB 841)	MPL (Tasmania)	Atlantic salmon (<i>Salmo salar</i>)	<i>V. splendidus</i>
R-15058 (VIB 240)	Marroco, 1990	Fish	<i>V. splendidus</i>
Cluster A49-Vibrio diabolicus			
<i>V. diabolicus</i> LMG 19805 ^T , R-14784 (HE 799)	East pacific rise, 1991	Dorsal integument of polychaete (<i>Alvinella pompejana</i>)	NA
LMG 20368 (7P)	CENAIM (Ecuador), 2000	Digestive gland of white shrimp (<i>Litopenaeus vannamei</i>)	NA
R-14785 (FKFO 1)	France, 1998	Diseased turbot larvae (<i>Scophthalmus maximus</i>)	<i>V. alginolyticus</i>
R-14786 (STD3-799)	Playaespec hatcheries (Ecuador), 1996	Probiont culture	<i>V. alginolyticus</i>
R-14787 (STD3-827)	Playaespec hatcheries (Ecuador), 1997	Probiont culture	<i>V. alginolyticus</i>
Cluster A50-Vibrio splendidus			
LMG 13242 (VIB 215)	Spain, 1991	Sea bass (<i>Dicentrarchus labrax</i>)	<i>V. harveyi</i>
LMG 16748 (20M1), LMG 16751 (30M2), LMG 16752 (30M19)	Spain, 1990	Oyster (<i>Ostrea edulis</i>)	<i>V. splendidus</i>
<i>V. splendidus</i> LMG 19031 ^T			
R-3734	AARS Austevoll (Norway), 1998	Marine fish Gut of turbot larvae (<i>Scophthalmus maximus</i>)	<i>V. splendidus</i> <i>V. harveyi</i>
R-3874	AARS Austevoll (Norway), 1997	Gut of turbot larvae (<i>Scophthalmus maximus</i>)	
R-14788 (VIB 783), R-14789 (VIB 784)	Spain, 1986	Sea bass (<i>Dicentrarchus labrax</i>)	STD3-1052 FKFO 99
R-14790 (VIB 239)	Greece, 1990	Sea perch	<i>V. harveyi</i>
R-14792 (VIB 507)	Norway	Turbot (<i>Scophthalmus maximus</i>)	FKFO 99
R-14796 (VIB 502)	Norway	Salmon (<i>Salmo salar</i>)	<i>V. orientalis</i>
R-14797 (VIB 504)	Norway	Atlantic salmon (<i>Salmo salar</i>)	<i>V. harveyi</i>

Table 1. (continued).

R-14798 (KV 2)	ARC Gent (Belgium), 2001	Diseased turbot larvae (<i>Scophthalmus maximus</i>)	NA
R-14799 (VIB 498)	Australia	Rainbow trout (<i>Oncorhynchus mykiss</i>)	STD3-1018
R-14800 (VIB 830)	MPL (Tasmania)	Atlantic salmon (<i>Salmo salar</i>)	STD3-1018
Cluster A51			
LMG 20537 (INCO 62)	LCMM Florianópolis (Brazil),	Healthy bivalve larvae (<i>Nodipecten</i>	FKFO 99
LMG 19269 (INCO 175), R-14801 (INCO 67), R-14802 (INCO 63)	1998	<i>nodosus</i>)	STD3-1052
R-14805 (VIB 575)	Spain, 1988	Turbot (<i>Scophthalmus maximus</i>)	STD3-1052
R-14806 (VIB 556)	Spain, 1992	Turbot (<i>Scophthalmus maximus</i>)	STD3-1052
Cluster A52			
LMG 13219 (VIB 192)	Greece, 1991	Rotifer (<i>Brachionus plicatilis</i>)	<i>V. harveyi</i>
LMG 13220 (VIB 193), LMG 13237 (VIB 210), LMG 13238 (VIB 211)	Greece, 1991	Water	<i>V. harveyi</i>
LMG 13222 (VIB 195)	Spain, 1990	<i>Artemia</i> sp.	<i>V. harveyi</i>
LMG 13239 (VIB 212), LMG 13251 (VIB 224)	Greece, 1991	Sea bass (<i>Dicentrarchus labrax</i>)	<i>V. harveyi</i>
Cluster A53			
R-3712, R-3718, R-3722, R-3765, R-3803	AARS Austevoll (Norway), 1997	Gut of turbot larvae (<i>Scophthalmus maximus</i>)	<i>V. campbellii</i>
Cluster A54-Photobacterium			
<i>leiognathi/Vibrio mytili</i>			
<i>P. leiognathi</i> LMG 4228 ^T	Thailand	Leiognathidae fish (Family Leiognathidae)	<i>P. leiognathi</i>
LMG 10944 (VIB 377)		Sardine	VIB 377
LMG 11221		Fish	<i>V. fischeri</i>
<i>V. mytili</i> LMG 19157 ^T	Valencia (Spain)	Bivalve (<i>Mytilus edulis</i>)	<i>V. mytili</i>
LMG 19980 (TC3-2)	Izu Ohshima (Japan), 1999	Gut of turban shell (<i>Turbo cornutus</i>)	NA

Table 1. (continued).

Cluster A55 R-3743, R-3841, R-3884, R-3893, R-3895, R-3912, R-3916	AARS Austevoll (Norway), 1997	Gut of turbot larvae (<i>Scophthalmus maximus</i>)	<i>V. harveyi</i>
Cluster A56-Photobacterium angustum/Photobacterium damselae subsp. <i>damselae</i> <i>P. damselae</i> subsp. <i>damselae</i> LMG 7892 ^T <i>P. angustum</i> LMG 8455 ^T LMG 10940 LMG 19445 LMG 19994 (STD3-348) R-14807 (VIB 787) R-14808 (INCO 48) R-14809 (STD3-1001)	U.S.A. Florida (USA) Kanagawa (Japan), 1987 China, 1995 Spain, 1988 Chile, 1988 China, 1996	Diseased damsel fish (<i>Chromis punctipinnis</i>) Sea water Sea water Poisoning fish (<i>Labracoglassa argentiventris</i>) Tank water Turbot (<i>Scophthalmus maximus</i>) Bivalve larvae (<i>Argopecten purpuratus</i>) Shrimp (<i>Penaeus chinensis</i>)	<i>P. damselae</i> <i>P. angustum</i> <i>P. damselae</i> STD3-348 STD3-1088 FKFO 99 STD3-348
Cluster A57-Vibrio diazotrophicus <i>V. diazotrophicus</i> LMG 7893 ^T LMG 13218 (VIB 191) R-15757 (HDV 1-2), R-15758 (HDV 2-1)	Nova Scotia (Canada) Greece, 1990 Izu Ohshima (Japan), 1999	Sea urchin (<i>Strongylocentrotus</i>) Rotifer (<i>Brachionus plicatilis</i>) Gut of abalone (<i>Haliotis diversicolor diversicolor</i>)	<i>V. diazotrophicus</i> <i>V. diazotrophicus</i> NA
Cluster A58-Vibrio penaeicida/V. rumoiensis/Vibrio tapetis LMG 11229 (VIB 515) LMG 16749 (VIB 686) <i>V. penaeicida</i> LMG 19663 ^T LMG 19704 (B 2.3), LMG 19705 (B 9.3), <i>V. tapetis</i> LMG 19706 ^T	Milford Connecticut, U.S.A Spain, 1990 Kagoshima (Japan) Landeda (France)	Oyster (<i>Crassostrea virginica</i>) Oyster Diseased kuruma prawn (<i>Penaeus japonicus</i>) Clam (<i>Tapes philippinarum</i>)	<i>V. tubiashii</i> <i>V. penaeicida</i> NA

Table 1. (continued).

<i>V. rumoiensis</i> LMG 20038 ¹ , LMG 20039 (S4)	Japan	Drain pool of a fish processing plant	NA
R-1491	ARC Gent (Belgium), 1996	Gut of turbot larvae (<i>Scophthalmus maximus</i>)	NA
R-3700	AARS Astevoll (Norway), 1997	Gut of turbot larvae (<i>Scophthalmus maximus</i>)	NA
R-14816 (INCO 41)	Guernsey sea farm (UK), 1998	Healthy oyster larvae (<i>Crassostrea gigas</i>)	<i>V. alginolyticus</i>
R-14817 (INCO 29)	LCMM Florianópolis (Brazil), 1998	Healthy bivalve larvae (<i>Nodipecten nodosus</i>)	NA
R-14818 (STD3-164)	ARC Gent (Belgium), 1995	Tank water	<i>V. fischeri</i>
R-14819 (STD3-1202)	CENAIM (Ecuador), 1995	Shrimp (<i>Litopenaeus vannamei</i>)	NA
R-14820 (INCO 293)	Japan, 1992	Healthy sea urchin (<i>Strongylocentrotus nudus</i>)	NA
R-14821 (KV 3), R-14822 (KV 4)	ARC Gent (Belgium), 2001	Diseased turbot larvae (<i>Scophthalmus maximus</i>)	NA
Cluster A59-Vibrio tubiashii			
<i>V. tubiashii</i> LMG 10936 ^T	Milford Connecticut (U.S.A.)	Hard clam (<i>Mercenaria mercenaria</i>)	<i>V. tubiashii</i>
LMG 11658 (VIB 399), LMG 10952,	Hawaii (U.S.A.)	Sea water	<i>V. nigripulchritudo</i>
LMG 11668 (VIB 447)			NA
LMG 11665 (VIB 432)		Mollusc	NA
LMG 13221 (VIB 194)	Greece, 1991	Water	<i>V. metschnikovii</i>
LMG 16851 (VIB 518)	Greece, 1991	Sea bream (<i>Sparus aurata</i>)	<i>V. campbellii</i>
LMG 19998 (STD3-338)	Aquatecsa hatcheries (Ecuador), 1995	Diseased white shrimp larvae (<i>Litopenaeus vannamei</i>)	NA
LMG 20542 (INCO 241)	LCMM Florianópolis (Brazil), 1999	Healthy bivalve larvae (<i>Nodipecten nodosus</i>)	STD3-338
			NA

Table 1. (continued).

R-3691, R-3696, R-3787, R-3788	AARS Austevoll (Norway), 1997	Gut of turbot larvae (<i>Scophthalmus maximus</i>)	NA R-3696 <i>V. salmonicida</i> <i>V. alginolyticus</i>
R-14825 (STD3-866) R-14827 (RFT 1) R-14831 (TAR 9)	ARC Gent (Belgium) ARC Gent (Belgium), 1999 ARC Gent (Belgium), 1998	White shrimp (<i>Litopenaeus vannamei</i>) Healthy rotifer (<i>Brachionus plicatilis</i>) Gut of turbot larvae (<i>Scophthalmus maximus</i>)	<i>V. harveyi</i> NA
R-14835 (INCO 40) R-14836 (VIB 853)	Guernsey sea farm (UK), 1998 MPL (Tasmania)	Healthy oyster larvae (<i>Crassostrea gigas</i>) Atlantic salmon (<i>Salmo salar</i>)	<i>V. harveyi</i> <i>V. harveyi</i>
Cluster A60			
LMG 20547 (INCO 318), R-14861 (INCO 336), R-14862 (INCO 409), R-14863 (INCO 386)	LCMM Florianópolis (Brazil), 1999	Bivalve larvae (<i>Nodipecten nodosus</i>)	VIB 836
R-14865 (INCO 312), R-14866 (INCO 311), R-14867 (INCO 406), R-14868 (384)	LCMM Florianópolis (Brazil), 1999	Bivalve larvae (<i>Nodipecten nodosus</i>)	VIB 836
Cluster A61			
LMG 20001 (STD3-1018) R-1556, R-1563, R-1572, R-1578	Feng Cheng (China), 1996 ARC Gent (Belgium), 1997	Healthy shrimp larvae (<i>Penaeus chinensis</i>) Gut of turbot larvae (<i>Scophthalmus maximus</i>)	STD3-1052 STD3-1052
R-3761, R-3781, R-3784, R-3891	AARS Austevoll (Norway), 1997	Gut of turbot larvae (<i>Scophthalmus maximus</i>)	STD3-1052
R-14869 (VIB 505)	Norway	Sea bass (<i>Dicentrarchus labrax</i>)	STD3-1052
R-14870 (STD3-1059)	Dahua (China), 1996	Healthy shrimp larvae (<i>Penaeus chinensis</i>)	STD3-1052
R-14872 (INCO 296)	Japan, 1992	Healthy sea urchin (<i>Strongylocentrotus</i>)	STD3-1052
R-14873 (FKFO 117)	ARC Gent (Belgium), 1999	Tank water	STD3-1052
R-14874 (CHILI 3)	Chile, 1998	Bivalve larvae (<i>Argopecten purpuratus</i>)	STD3-1052
R-14875 (STD3-1070)	Dahua (China), 1996	Tank water	STD3-1052

Table 1. (continued).

<i>Cluster A62-Vibrio alginolyticus</i>			
LMG 2174	Port Hueneme (U.S.A.)	<i>Pinus palustris</i>	<i>V. alginolyticus</i>
LMG 4407	Japan, 1971	Human faeces	<i>V. alginolyticus</i>
<i>V. alginolyticus</i> LMG 4409 ^T , R-14888 (VIB 614)	Japan	Spoiled horse mackerel (<i>Trachurus trachurus</i>)	<i>V. alginolyticus</i>
LMG 19993 (STD3-331)	Aquatecsa hatcheries (Ecuador), 1997	Probiot culture	STD3-331
R-14876 (STD3-1234)	Aquatecsa hatcheries (Ecuador), 1993	Diseased white shrimp larvae (<i>Litopenaeus vannamei</i>)	<i>V. alginolyticus</i>
R-14878 (VIB 566)	Spain, 1991	Turbot (<i>Scophthalmus maximus</i>)	<i>V. alginolyticus</i>
R-14879 (STD3-336)	Aquatecsa hatcheries (Ecuador), 1995	Diseased white shrimp larvae (<i>Litopenaeus vannamei</i>)	<i>V. alginolyticus</i>
R-14880 (STD3-86)	Playaespec hatcheries (Ecuador), 1994	Probiot culture	<i>V. alginolyticus</i>
R-14881 (VIB 333)	Madagascar, 1987	Shrimp	<i>V. alginolyticus</i>
R-14882 (VIB 325)	Norway	Turbot (<i>Scophthalmus maximus</i>)	<i>V. alginolyticus</i>
R-14883 (VIB 320)	China, 1993	Shrimp (<i>Penaeus orientalis</i>)	<i>V. alginolyticus</i>
R-14884 (VIB 323)	Norway	Turbot	<i>V. alginolyticus</i>
R-14885 (VIB 565)	Spain, 1991	Water	<i>V. alginolyticus</i>
R-14886 (VIB 562)	Spain, 1990	Turbot (<i>Scophthalmus maximus</i>)	<i>V. alginolyticus</i>
R-14887 (VIB 563)	Spain, 1990	<i>Artemia</i> sp.	<i>V. alginolyticus</i>
R-14889 (INCO 51)	Chile, 1998	Bivalve larvae (<i>Ardopecten purpuratus</i>)	<i>V. campbellii</i>
R-14891 (STD3-1208)	CENAIM (Ecuador)	White shrimp larvae (<i>Litopenaeus vannamei</i>)	<i>V. alginolyticus</i>
R-14892 (INCO 81)	LCMM Florianópolis (Brazil), 1998	Healthy bivalve larvae (<i>Nodipecten nodosus</i>)	<i>V. alginolyticus</i>
R-14893 (INCO 32)	Gernsey sea farm (UK), 1998	Healthy bivalve larvae (<i>Crassostrea gigas</i>)	<i>V. alginolyticus</i>
R-14894 (FKFO 154)		<i>Artemia</i> sp.	NA
R-14896 (VIB 342)	France, 1991	Crab	<i>V. alginolyticus</i>

Table 1. (continued).

Cluster A63-Vibrio ichthyoenteri			
<i>V. ichthyoenteri</i> LMG 19664 ^T , R-14897 (VIB 639), R-14898 (VIB 640) R-3774, R-3789, R-3815, R-3854, R-3858, R-3882, R-3885, R-3904, R-3911	Hiroshima (Japan) AARS Austevoll (Norway), 1997	Gut of diseased Japanese flounder (<i>Paralichthys olivaceus</i>) Gut of turbot larvae (<i>Scophthalmus</i> <i>maximus</i>)	<i>V. ichthyoenteri</i> STD3-1088
Cluster A64			
LMG 19971 (HDD 2-1), LMG 19972 (HDD 1-1), LMG 19973 (HDD 3-1), R-15759 (HDD 2-2)	Kanagawa (Japan), 1999	Gut of abalone (<i>Haliotis discus discus</i>)	NA
R-15762 (HDD 1-2), R-15763 (HDD 3-2)	Kanagawa (Japan), 1999	Gut of abalone (<i>Haliotis discus discus</i>)	NA
LMG 19978 (HDD 7-2), R-15760 (HDD 7- 1)	Izu Ohshima (Japan), 1999	Gut of abalone (<i>Haliotis discus discus</i>)	NA
R-15761	Izu Ohshima (Japan), 1999	Gut of turban shell (<i>Turbo cornutus</i>)	NA
Cluster A65			
LMG 19976 (HDD 6-2), LMG 19977 (HDD 6-1)	Izu Ohshima (Japan), 1999	Gut of abalone (<i>Haliotis discus discus</i>)	NA
Unclustered: <i>Vibrio aerogenes</i> LMG 19650 ^T	Seagrass bed in Nanwan bay (Taiwan)	Sediment	NA
Cluster A66			
LMG 19966 (HDS 5-1), LMG 19967 (HDS 4-1), LMG 19968 (HDS 3-1), LMG 19969 (HDS 2-1),	Kanagawa (Japan), 1999	Gut of abalone (<i>Haliotis diversicolor</i> <i>supertexta</i>)	NA
LMG 19970 (HDS 1-1), R-15764 (HDS 2- 2), R-15765 (HDS 1-2),	Kanagawa (Japan), 1999	Gut of abalone (<i>Haliotis diversicolor</i> <i>supertexta</i>)	NA
R-15766 (HDS 3-2), R-15767 (HDS 5-2), R- 15768 (HDS 4-2)	Kanagawa (Japan), 1999	Gut of abalone (<i>Haliotis diversicolor</i> <i>supertexta</i>)	NA

Table 1. (continued).

LMG 19979 (HDV 1-1)	Izu Ohshima (Japan), 1999	Gut of abalone (<i>Haliotis diversicolor diversicolor</i>)	NA
R-15769 (TC 2-1)	Izu Ohshima (Japan), 1999	Gut of turban shell (<i>Turbo cornutus</i>)	NA
Cluster A67-Vibrio halioticoli			
<i>V. halioticoli</i> LMG 18542 ^T	Kumaishi (Japan), 1991	Gut of abalone (<i>Haliotis discus hanai</i>)	<i>V. halioticoli</i>
LMG 19701 (IAM 14598), LMG 19702 (IAM 14599)	Shiriuchi (Japan), 1994	Gut of abalone (<i>Haliotis discus hanai</i>)	<i>V. halioticoli</i>
LMG 19700 (IAM 14597)	Taisei (Japan), 1993	Abalone (<i>Haliotis discus hanai</i>)	<i>V. halioticoli</i>
LMG 19963 (TC 4-2)	Izu Ohshima (Japan), 1999	Gut of turban shell (<i>Turbo cornutus</i>)	NA
LMG 19964 (1Y2-26), LMG 19965 (2Y1-13), R-15770 (1Y2-20),	Kumaishi (Japan), 1997	Gut of abalone (<i>Haliotis discus hanai</i>)	NA
R-15779 (25Y1-10), R-15780 (25Y2-25)	Kumaishi (Japan), 1997	Gut of abalone (<i>Haliotis discus hanai</i>)	NA
LMG 19974 (HDD 4-1), LMG 19975 (HDD 5-1), R-15772 (HDD 5-2), R-15784 (HDD 4-2)	Kanagawa (Japan), 1999	Gut of abalone (<i>Haliotis discus discus</i>)	NA
R-15771 (COSW-2)	Kanagawa (Japan), 1999	Sea water	NA
R-15773 (KSW-5)	Kumaishi (Japan), 1997	Sea water	NA
R-15774 (2Y2-23)	Kumaishi (Japan), 1998	Gut of abalone (<i>Haliotis discus hanai</i>)	NA
R-15775 (KL1Y1-12), R-15776 (KLA1Y1-10), R-15777 (KL1Y2-10), R-15778 (KL1Y1-18)	Ofunato (Japan), 1997	Gut of abalone (<i>Haliotis discus hanai</i>)	NA
R-15781 (KN1Y2-9), R-15782 (KN1Y1-9), R-15783 (KLA1Y2-23)	Ofunato (Japan), 1997	Gut of abalone (<i>Haliotis discus hanai</i>)	NA
Cluster A68			
LMG 19840 (R-3690), LMG 19842 (R-3822), R-3717, R-3759, R-3929	AARS Austevoll (Norway), 1997	Gut of turbot larvae (<i>Scophthalmus maximus</i>)	R-3690
R-3731, R-3749, R-3764, R-3773, R-3792, R-3814, R-3819, R-3847, R-3668, R-3678,	AARS Austevoll (Norway), 1997	Gut of turbot larvae (<i>Scophthalmus maximus</i>)	R-3710

Table 1. (continued).

R-3692, R-3727, R-3729			R-3710
Cluster A69			
LMG 19839 (R-3710), R-3719	AARS Austevoll (Norway),	Gut of turbot larvae (<i>Scophthalmus</i>	R-3710
LMG 19841 (R-3725),	1997	<i>maximus</i>)	<i>V. campbellii</i>
R-3708			R-3690

AARS Austevoll, Aquaculture Research Station of Austevoll, Austevoll, Norway. ATCC, American Type Culture Collection, Manassas, USA. ARC Gent, Artemia Reference Center, Gent, Belgium. CIP, Institute Pasteur Collection, Paris, France. LMG, BCCMTM/LMG Bacteria Collection, Laboratory for Microbiology, Ghent University, Ghent, Belgium. IFREMER, French Institute for Exploitation of the Sea, Brittany, France. LCMM Florianópolis, Laboratory for Culture of Marine Molluscs, Florianópolis, Brazil. MPL, Mount Pleseant Laboratories in Tasmania. CENAIM, National Center for Marine and Aquaculture Research, Guayaquil, Ecuador. NCIMB, National Collection of Industrial Marine and Food Bacteria, Aberdeen, UK. R-, Research Collection at LMG. *Strain allocation according to Biolog (Vandenberghe et al., 2003). NA, not analysed by Biolog (Vandenberghe et al., 2003), but available as *Vibrio* reference strains in the LMG and/or R collections.

Table 2. Unidentified Biolog groups examined in the present work and remainder groups to be examined in further taxonomic studies.

Examined	Examined	Not examined	Not examined
FKFO 99	STD3-996	C6	STD3-674
INCO 167	STD3-988	FKFO 140	STD3-943
INCO 222	STD3-1008	FT1	STD3-1267
INCO 254	STD3-1018	IM10	TAR L8
INCO 320	STD3-1052	IM25	TR36
R-3666	STD3-1057	LMG 10942	VIB 377
R-3690	STD3-1088	MJPXOM26	VIB 386
R-3710	VIB 449	MJP3OM16	VIB 433
STD3-331	VIB 478	MS1	VIB 437
STD3-348	VIB 782	R-3716	VIB 510
STD3-932	VIB 836	R-3733	VIB 534
		R-3810	VIB 583
		STD3-338	VIB 637
		STD3-341	VIB 717
		STD3-536	VIB 734
		STD3-541	VIB 739
		STD3-565	VIB 791

Table 3. Additional list of *V. cholerae* strains used in this work.

Strain no. (synonym no.)	Place, date of isolation and source		Serogroup
LMG 21701 (CCUG 9123)		1979 Clinical	O1/Ogawa
LMG 21707 (CCUG 45392)		2001	non-O1
LMG 21697 (CCUG 537)			O13
LMG 21698T (CCUG 9118T)			O1/Ogawa
LMG 21699 (CCUG 9121)			autoagglutinable
LMG 21700 (CCUG 9122)	Mecca	1931	O1/Inaba
LMG 21702 (CCUG 19445)	Tunesia	1986 Clinical	O1/Ogawa
R-18239 (IOC 157)	Amazonas	1991 Clinical	non-O1/non-139
R-18240 (IOC 3874), R-18281 (IOC 4482)	Paraíba	1992 Clinical	non-O1/non-139
R-18241 (IOC 3905), R-18268 (IOC 3472)	Pernambuco	1992 Clinical	non-O1/non-139
R-18242 (IOC 14776)	Ceará	1995 Clinical	non-O1/non-139
R-18244 (IOC 3962)	Pernambuco	1992 Environmental	non-O1/non-139
R-18248 (IOC 14798)	Amapá	1995 Clinical	non-O1/non-139
R-18249 (IOC 15251), R-18251 (IOC 15380)	Ceará	1997 Clinical	non-O1/non-139
R-18252 (IOC 15673), R-18288 (IOC 15955)	Pernambuco	1998 Clinical	non-O1/non-139
R-18253 (IOC 16353), R-18266 (IOC 16352)	Ceará	1999 Clinical	non-O1/non-139
R-18256 (IOC 12861)	Rio Grande do Sul	1994 Environmental	non-O1/non-139
R-18258 (IOC 7915)	Santa Catarina	1993 Environmental	non-O1/non-139
R-18261 (IOC 6958)	Ceará	1992 Clinical	non-O1/non-139
R-18262 (IOC 10626), R-18283 (IOC 7695)	Bahia	1993 Clinical	non-O1/non-139
R-18263 (IOC 11159)	Pernambuco	1993 Clinical	non-O1/non-139
R-18265 (IOC 13663), R-18284 (IOC 13154)	Pernambuco	1994 Clinical	non-O1/non-139
R-18267 (IOC 2494)	Bahia	1992 Environmental	non-O1/non-139
R-18269 (IOC 10854)	Pernambuco	1993 Clinical	non-O1/non-139
R-18270 (IOC 13430)	Bahia	1994 Clinical	non-O1/non-139
R-18272 (IOC 14713)	Piauí	1995 Clinical	non-O1/non-139
R-18275 (IOC 4993), R-18274 (IOC 4992)	Amazonas	1992 Environmental	non-O1/non-139
R-18276 (IOC 12885)	Rio Grande do Sul	1994 Environmental	non-O1/non-139
R-18282 (IOC 7455)	Bahia	1992 Clinical	non-O1/non-139
R-18286 (IOC 14889)	Pernambuco	1995 Clinical	non-O1/non-139

Table 3. (continued).

R-18289 (IOC 16002)	Paraná	1998	Clinical	non-O1/non-139
R-18290 (IOC 16580), R-18340 (IOC 16838)	Rio Grande do Norte	1999	Clinical	non-O1/non-139
R-18292 (IOC 15290)	Pernambuco	1997	Environmental	non-O1/non-139
R-18293 (IOC 11121)	Rio Grande do Norte	1993	Clinical	non-O1/non-139
R-18295 (IOC 1), R-18297 (IOC 12), R-18299 (IOC 28),	Maranhão	2001	Environmental	non-O1/non-139
R-18301 (IOC 60), R-18303 (IOC 77)	Maranhão	2001	Environmental	non-O1/non-139
R-18304 (V 020)	Ceará	1993	Clinical	O1
R-18305 (V 060), R-18307 (V 87/01), R-18316 (V 450)	Rio de Janeiro	1993	Clinical	O1
R-18306 (V 85/01), R-18310 (V 139/1)	Pernambuco	2000	Clinical	O1
R-18308 (V 121)	India	1973	Clinical	O1/EI Tor
R-18309 (V 135)	Rio Grande do Sul	1993	Clinical	O1
R-18311 (V 146)	Bahia	1993	Clinical	O1
R-18312 (V 350)	Amazonas	1991	Clinical	O1
R-18313 (V 373), R-18317 (V 454), R-18318 (V 469)	Rio de Janeiro	1994	Clinical	O1
R-18314 (V 398), R-18357 (V 385)	Alagoas	1996	Clinical	O1
R-18315 (V 404)	Bahia	1994	Clinical	O1
R-18319 (V 484)	Amazonas	1995	Clinical	O1
R-18320 (V 502), R-18321 (V 505)	Amazonas	1995	Clinical	O1
R-18322 (V 564)	Amazonas		Clinical	O1
R-18324 (V 584)	Bahia	1998	Clinical	O1
R-18325 (V 585)	Bahia	1999	Clinical	O1
R-18326 (V 590), R-18327 (V 591), R-18358 (V 588)	Paraná	1999	Clinical	O1
R-18328 (V 640), R-18323 (V 566)	Amazonas	1991	Clinical	O1
R-18329 (V 726), R-18330 (V 732)	Pará	1992	Clinical	O1
R-18331 (V 3439), R-18332 (V 3503)	Amazonas	1992	Clinical	O1/Amazonian var.
R-18333 (V 3504), R-18359 (V 3218)	Amazonas	1991	Clinical	O1/Amazonian var.
R-18334 (V 3505), R-18335 (V 3506),	Amazonas	1992	Clinical	O1/Amazonian var.

Table 3. (continued).

R-18336 (V 3732), R-18361 (V 3508)	Amazonas	1992	Clinical	O1/Amazonian var.
R-18337 (V 4132), R-18362 (V 3731)	Amazonas	1992	Clinical	O1/Amazonian var.
R-18338 (IOC 6954), R-18246 (IOC 6952)	Ceará	1992	Clinical	non-O1/non-139
R-18339 (IOC 13644)	Mato Grosso	1994	Clinical	non-O1/non-139
R-18341 (IOC 5009)	Rio Grande do Norte	1992	Clinical	non-O1/non-139
R-18343 (IOC 2735)	Tocantins	1992	Clinical	non-O1/non-139
R-18344 (IOC 13488)	Espírito Santo	1994	Clinical	non-O1/non-139
R-18345 (IOC 4994), R-18273 (IOC 2766),	Amazonas	1992	Environmental	non-O1/non-139
R-18348 (IOC 16988)	Pernambuco	1999	Clinical	non-O1/non-139
R-18350 (IOC 10412)	Espírito Santo	1993	Environmental	non-O1/non-139
R-18354 (IOC 23)	Manaus	2001	Environmental	non-O1/non-139
R-18355 (V L34A), R-18356 (V L34B)	Amazonas	1991	Clinical	O1/Amazonian var.
R-18360 (V 3501)	Amazonas	1992	Clinical	O1/Amazonian var.
R-18364 (V 4010), R-18363 (V 4008)	Amazonas	1992	Clinical	O1/Amazonian var.

IOC, Oswaldo Cruz Institute (RJ, Brazil). R, Research collection at LMG (Gent, Belgium).

Table 4. Additional list of *V. campbellii*, *V. harveyi* and *V. parahaemolyticus* strains used in this work.

Strain no. (synonym no.)	Source of isolation	Place
<i>V. harveyi</i>		
LMG 20977 (Former <i>V. shilonii</i> AK2)	Diseased coral <i>Oculina patagonica</i>	Israel
R-16605 (Ea)	<i>Litopenaeus stylirostris</i> larvae	Hatchery in Santa Clara Gulf, Mexico
R-16607 (10Mz), R-16608 (11Mz)	<i>L. stylirostris</i> nauplii	Hatchery in Mazatlán, Sin., Mexico
<i>V. campbellii</i>		
2Mz (2Mz)	<i>Litopenaeus vannamei</i>	Mexico
LMG 21361 (R-16602, Z1), LMG 21362a	Seawater from <i>L. stylirostris</i> broodstock tank	Hatchery in Santa Clara Gulf, Mexico
LMG 21363 (R-16618, PN9801)	Diseased <i>P. monodon</i> juveniles	Philippines
LMG 21364 (R-16620, HL135), LMG 21365	Hemolymph from diseased <i>L. vannamei</i> juvenile	Sinaloa, Mexico
R-16603 (Z2), R-16604 (Z3)	Seawater from <i>L. stylirostris</i> broodstock tank	Hatchery in Santa Clara Gulf, Mexico
R-16606 (1A-1)	Near shore seawater	Santa Barbara Bay, Mexico
R-16609 (Na-1, Na-2, Na)	<i>L. stylirostris</i> nauplii	Hatchery in Santa Clara Gulf, Mexico
R-16610 (ML)	<i>L. vannamei</i>	Mexico
R-16616 (IPL8)	<i>P. monodon</i> postlarvae with luminescent vibriosis	Philippines
R-16617 (PL96-11-6)	Diseased <i>P. monodon</i> postlarvae	Philippines
R-16619 (HL34)	Hemolymph from diseased <i>L. vannamei</i> juvenile	Guasave, Sinaloa, Mexico
R-16621 (HL136)	Hemolymph from diseased <i>L. vannamei</i> juvenile	Sinaloa, Mexico
R-16622 (HL148)	Hemolymph from diseased <i>L. vannamei</i> juvenile	Huatabampo, Sonora, Mexico
R-16624 (HL151), R-16625 (HL152),	Hemolymph from diseased <i>L. vannamei</i> juvenile	La Brecha, Mexico
R-16627 (HL157), R-16628 (HL158),	Hemolymph from diseased <i>L. vannamei</i> juvenile	La Brecha, Mexico
R-16629 (HL159)	Hemolymph from diseased <i>L. vannamei</i> juvenile	La Brecha, Mexico
R-16630 (HP130)	Hepatopancreas from diseased <i>L. vannamei</i> juvenile	Guamuchil, Sinaloa, Mexico
R-16631 (SW-9702)	Seawater	Iloilo, Philippines
R-16695 (HL115)	Hemolymph from diseased <i>L. vannamei</i> juvenile	Acuac. Ind. del Matatipac S.A, Nayarit
<i>V. parahaemolyticus</i>		
R-14854 (STD3-948, HL54)	Diseased <i>L. vannamei</i> juvenile	Sinaloa, Mexico

