

16S ribosomal DNA sequencing confirms the synonymy of *Vibrio harveyi* and *V. carchariae*

Eric J. Gauger, Marta Gómez-Chiarri*

Department of Fisheries, Animal and Veterinary Science, 20A Woodward Hall, University of Rhode Island, Kingston, Rhode Island 02881, USA

ABSTRACT: Seventeen bacterial strains previously identified as *Vibrio harveyi* (Baumann et al. 1981) or *V. carchariae* (Grimes et al. 1984) and the type strains of *V. harveyi*, *V. carchariae* and *V. campbellii* were analyzed by 16S ribosomal DNA (rDNA) sequencing. Four clusters were identified in a phylogenetic analysis performed by comparing a 746 base pair fragment of the 16S rDNA and previously published sequences of other closely related *Vibrio* species. The type strains of *V. harveyi* and *V. carchariae* and about half of the strains identified as *V. harveyi* or *V. carchariae* formed a single, well-supported cluster designed as 'bona fide' *V. harveyi/carchariae*. A second more heterogeneous cluster included most other strains and the *V. campbellii* type strain. Two remaining strains are shown to be more closely related to *V. rumoiensis* and *V. mediterranei*. 16S rDNA sequencing has confirmed the homogeneity and synonymy of *V. harveyi* and *V. carchariae*. Analysis of API20E biochemical profiles revealed that they are insufficient by themselves to differentiate *V. harveyi* and *V. campbellii* strains. 16S rDNA sequencing, however, can be used in conjunction with biochemical techniques to provide a reliable method of distinguishing *V. harveyi* from other closely related species.

KEY WORDS: *Vibrio harveyi* · *Vibrio carchariae* · *Vibrio campbellii* · *Vibrio trachuri* · Ribosomal DNA · Biochemical characteristics · Diagnostic · API20E

Resale or republication not permitted without written consent of the publisher

INTRODUCTION

Vibrio harveyi (Baumann et al. 1981) and *V. carchariae* (Grimes et al. 1984) can cause disease in a large number of marine fish and shellfish species worldwide (Austin & Austin 1999). *V. harveyi*, first isolated from a dead amphipod (Johnson & Shunk 1936), can affect penaeid shrimp (Liu et al. 1996, Abraham et al. 1997, Alvarez et al. 1998, Robertson et al. 1998), rock lobster (Diggles et al. 2000) and salmonids (Zhang & Austin 2000). *V. carchariae*, first identified by Grimes et al. (1984) has been shown to cause disease in elasmobranch species (Grimes et al. 1985), cephalopods (Hanlon & Forsythe 1990), grouper *Epinephelus coioides* (Yii et al. 1997) and summer flounder *Paralichthys dentatus* (Soffientino et al. 1999). *V. harveyi* and *V. carchariae* are also ubiquitous in warm marine environments and have been isolated from a variety of

environmental sources (Ruby & Morin 1979, Orndorff & Colwell 1980, Feldman & Buck 1984, Grimes et al. 1985, Grisez et al. 1997).

Until recently, *Vibrio harveyi* and *V. carchariae* have been accepted as closely related species that could, nonetheless, be distinguished by biochemical characteristics (Grimes et al. 1993, Alsina & Blanch 1994) and 16S ribosomal RNA (rRNA) sequences (Kita-Tsukamoto et al. 1993, Ruimy et al. 1994, Soffientino et al. 1999). In contrast, a study by Pedersen et al. (1998) based on Amplified Fragment Length Polymorphisms (AFLP), DNA:DNA hybridization, and ribotyping found that the type strains of *V. harveyi* and *V. carchariae* were indistinguishable and should therefore be considered synonymous.

Here, we sought to investigate the discrepancies between classifications of *Vibrio harveyi* and *V. carchariae* based on different methods (16S rRNA sequencing, AFLP, ribotyping, DNA-DNA hybridization, biochemical characteristics). We describe the phyloge-

*Corresponding author. E-mail: gomezchi@uri.edu

netic relationship, based on 16S rDNA sequences, of the type strains of *V. harveyi* and *V. carchariae*, 17 putative *V. harveyi* and *V. carchariae* strains (Yii et al. 1997, Pedersen et al. 1998, Soffientino et al. 1999), and published 16S rDNA sequences of *V. harveyi*, *V. carchariae* and other closely related *Vibrio* spp. (Valle et al. 1990, Ruimy et al. 1994, Yumoto et al. 1999). Also, we compared 16S rDNA sequencing data to biochemical profiles. The implications for bacterial classification and diagnosis are discussed.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains are listed in Table 1. The strains designated VIB 286, 295, 350, 394, 400, 403, 411, 570, 573, 645, 651, 654, 655, 656, 657, 660, and 697 belong to a collection maintained by Dr. Dawn Austin of Heriot-Watt University (Edinburgh, UK) (Pedersen et al. 1998). Also included in this study are a strain of *Vibrio carchariae* from grouper in Taiwan (Yii et al. 1997), designated here as *V. carchariae* grouper, a strain from summer flounder in the USA (Soffientino et al. 1999), designated here as *V. carchariae* flounder, and the type strains (^T) of *V. harveyi* (American Type Culture Collection, ATCC 14126^T) and *V. campbellii* (ATCC 25920^T). VIB 286 is the designation given to

the *V. carchariae* type strain in Pedersen et al. (1998). Throughout the text, the names VIB 286 and *V. carchariae* type strain are used interchangeably and refer to the same strain. VIB 295 is the designation given to the *V. harveyi* type strain by Pedersen et al. (1998). An additional sample of the *V. harveyi* type strain was obtained directly from ATCC (ATCC 14126^T) and is referred to in the text as *V. harveyi* type strain.

Culture and biochemical profiles. Bacterial stock cultures were maintained in 15 to 20% (v/v) glycerol at -70°C. Active cultures were grown on Luria Bertani (LB) agar plates containing 20 g l⁻¹ of NaCl (LB 20) (Nelson et al. 1997) or LB 20 broth at 28°C. Biochemical profiles were analyzed using API 20E strips (bioMérieux Vitek Inc.). Each strain was evaluated at least twice. One to 3 colonies from 24 to 48 h plate cultures, depending on growth rate, were resuspended in 5 ml of 0.85% (w/v) NaCl solution and vortexed. Sufficient bacteria was used to make a solution that was visibly turbid but not opaque. Strips were incubated at 28°C; results were read at 48 h according to the manufacturer's instructions.

DNA extraction, amplification, and sequencing. Extraction of genomic DNA was accomplished by a modified version of a protocol by Medrano et al. (1990). Briefly, a single bacterial colony from a LB 20 plate was suspended in 550 µl lysis buffer (50 mM Tris HCl,

Table 1. *Vibrio* spp. Bacterial strains used for 16S rDNA sequencing and API20E analysis. LMG: Laboratorium voor Microbiologie, Rijksuniversiteit Gent, Belgium; CCUG: Culture Collection, University of Gothenburg, Göteborg, Sweden; ATCC: American Type Culture Collection, Rockville, MD, USA; NCIMB: National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, UK; HWU: Heriot-Watt University, Edinburgh, Scotland, UK; IP: Institut Pasteur, Paris, France; UB: Universitat de Barcelona, Spain; RVAU: Royal Veterinary and Agricultural University, Copenhagen, Denmark. ^T: type strain

Identification used in phylogenetic analysis	Original identification	Reference numbers	Location, year of isolation	Source of strain
VIB 286 (<i>V. carchariae</i> ^T)	<i>V. carchariae</i>	LMG 7890 ^T , CCUG19116, ATCC 35084 ^T	USA, 1982	Brown shark
VIB 295 (<i>V. harveyi</i> ^T)	<i>V. harveyi</i>	LMG 4044 ^T , NCIMB 1280, ATCC 14126 ^T	USA, 1936	Amphipod
VIB 350	<i>V. carchariae</i>	LMG 11754, ATCC 43515	Bahamas	Shark
VIB 394	<i>V. harveyi</i>	HWU F377	Australia	Barramundi
VIB 400	<i>V. harveyi</i>	LMG 11659, CCUG 16178	Hawaii	Seawater
VIB 403	<i>V. harveyi</i>	LMG 16828	Thailand	Black tiger prawn
VIB 411	<i>V. harveyi</i>	IP 3.86	South Africa, 1986	Fish ulcer
VIB 570	<i>V. harveyi</i>	UB A065	Spain, 1990	Sea bream
VIB 573	<i>V. harveyi?</i>	UB A072	Spain, 1990	Sea bass
VIB 645	<i>V. harveyi/carchariae</i>		Tunisia, 1993	Sea bass
VIB 651	<i>V. harveyi/carchariae</i>	RVAU 94-5-75	Denmark, 1994	Shark aquarium water
VIB 654	<i>V. harveyi/carchariae</i>	RVAU 94-3-46	Denmark, 1994	Shark aquarium water
VIB 655	<i>V. harveyi/carchariae</i>		France, 1989	Sea bass
VIB 656	<i>V. harveyi/carchariae</i>		Greece, 1990	Sea bass
VIB 657	<i>V. harveyi/carchariae</i>		France, 1990	Sea bass
VIB 660	<i>V. harveyi/carchariae</i>		Greece, 1990	Sea bass
VIB 697	<i>V. harveyi</i>	LMG (90M4)	Spain, 1989	Oyster
<i>V. carchariae</i> flounder	<i>V. carchariae</i>		USA, 1998	Flounder
<i>V. carchariae</i> grouper	<i>V. carchariae</i>		Taiwan, 1993	Grouper
<i>V. harveyi</i> ^T	<i>V. harveyi</i>	LMG 4044 ^T , NCIMB 1280, ATCC 14126 ^T	USA, 1936	Amphipod
<i>V. campbellii</i>	<i>V. campbellii</i>	ATCC 25920 ^T		Seawater

20 mM EDTA, 1% SDS, 200 mM NaCl, pH 8.0) and 0.4 mg ml⁻¹ Proteinase K in a microcentrifuge tube. This solution was incubated at 55°C until the cells lysed. NaCl was then added to a 2 M final concentration and the solution was centrifuged (13 400 × *g* for 30 min at room temperature). The aqueous portion was retained, DNA was ethanol precipitated, centrifuged (13 400 × *g* for 30 min at room temperature) and washed in 70% (v/v) ethanol. The resulting DNA was re-suspended in 50 µl of ddH₂O by incubation at 55°C. DNA stocks were held at -20°C. Polymerase Chain Reaction (PCR) amplification was accomplished using Ready-To-Go PCR beads (Amersham-Pharmacia Biotech), approximately 100 ng of genomic DNA, and 0.8 µM oligonucleotide primers 13F (5'-TTGATCATGGCTCAGATTGATGAAGCG-3') and 1443R (5'-AACTACCTACTTCTTTTGCAGCCCACT-3'). Primer designations correspond to the 5' *Escherichia coli* positions (GenBank accession no. J01859). The amplification program consisted of an initial denaturation step of 5 min at 94°C, followed by 36 cycles consisting of a 1 min denaturation step at 94°C, a 2 min annealing step at 54°C, and a 5 min elongation step at 72°C. Four reactions were performed per strain. PCR products were run on a 0.7% (w/v) agarose gel for 1 h along with a DNA molecular weight standard ladder to ensure a fragment of the proper length was observed. The fragments were cut from the gel and the DNA was extracted using a QIAquick column (Qiagen), following manufacturer's instructions. Prior to sequencing, quantification of the DNA was accomplished by an ethidium bromide spot method (Sambrook et al. 1989). Twenty-four µl sequencing reactions were prepared with approximately 100 ng of template DNA and a 0.33 µM concentration of either primer 637F (5'-TGAACTGGCAGACTAGAG-3') or 1443R. Sequencing was performed at the HHMI Biopolymer Laboratory & W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT, USA) using an Applied Biosystems 377 slab gel and 3700 and 3100 capillary instruments. The sequencing reactions used fluorescently labelled dideoxynucleotides and *Taq* FS DNA polymerase in a thermal cycling protocol. The region corresponding to *E. coli* positions 637-1443 was chosen because it contained the majority of the sequence variations found between *Vibrio harveyi* X74706 and *V. carchariae* X74693 and could be covered effectively by a single sequencing reaction. Both strands of DNA were sequenced so that nucleotide uncertainties could be resolved. Sequences were submitted to GenBank (see below for accession numbers).

Phylogenetic analysis. The following sequences were used in the phylogenetic analysis: *Vibrio harveyi* type strain AF426825, *V. campbellii* type strain AF426824,

V. carchariae flounder AF426805, *V. carchariae* grouper AF426806, VIB 286 AF426814, VIB 295 AF426811, VIB 350 AF426813, VIB 394 AF426821, VIB 400 AF426816, VIB 403 AF426820, VIB 411 AF426823, VIB 570 AF426822, VIB 573 AF426817, VIB 645 AF426812, VIB 651 AF426815, VIB 654 AF426819, VIB 655 AF426807, VIB 656 AF426810, VIB 657 AF426809, VIB 660 AF426808, and VIB 697 AF426818. The following previously published sequences used in the phylogenetic analyses were obtained from GenBank: *Aeromonas salmonicida* X74681, *V. alginolyticus* X74690, *V. campbellii* X74692, *V. carchariae* X74693, *V. cholerae* classical X74695, *V. cholerae* Eltor X74694, *V. mediterranei* X74710, *V. nereis* X74716, *V. ordalii* X74718, and *V. orientalis* X74719 (Ruimy et al. 1994), *Listonella* (= *Vibrio*) *anguillarum* X16895 (Valle et al. 1990), *V. ru-moiensis* AB013297 (Yumoto et al. 1999), and *V. campbellii* AY035896 (unpubl.). Sequences were aligned using the Clustal W program and pairwise genetic distances were determined using the ClustalDist program (Higgins et al. 1992) on the San Diego Super Computer Center's (SDSC) Biology Workbench (<http://workbench.sdsc.edu>). Alignments were checked manually. Groups of sequences that were found to be identical were treated as a single sequence for the remainder of the analysis. Aligned sequences were analyzed using PAUP version 4 (Swofford 1998). Bootstrap analysis (100 replicates) was performed using heuristic, neighbor-joining and Unweighted-Paired Grouping Mathematical Average (UPGMA) search algorithms, with the optimality criterion set to distance, codon positioning set to unknown, maximum number of trees saved set to 1000 and *A. salmonicida* X74681 set as the out group. Bootstrap analysis (100 replicates) was also performed with the optimality criterion set to maximum parsimony with all other settings identical to other search methods. Clusters found in this analysis were compared to the AFLP clusters found by Pedersen et al. (1998).

RESULTS

Biochemical profiles

API 20E strips were used to evaluate the biochemical profiles of the bacterial strains used in this study (Table 2). All strains were consistently positive for lysine decarboxylase, tryptophane deaminase, indole production, gelatinase, cytochrome-oxidase, and fermentation of glucose, mannitol, and amygdalin, and consistently negative for arginine dihydrolase, H₂S production, Vogues-Proskauer reaction, and fermentation of inositol, rhamnose, and arabinose. b-galactosidase, ornithine decarboxylase, citrate utilization, urease, and the fermentation of sorbitol, sucrose, and

Table 2. *Vibrio* spp. Selected API20E characteristics of bacterial strains. Strains are grouped according to sequence homology. V: variable result; V*: – by API 20E; + by Simmon's citrate slant. ONPG, ODC, CIT, URE, SOR, SAC, MEL correspond to b-galactosidase, ornithine decarboxylase, citrate utilization, urease, and fermentation of sorbitol, sucrose, and melibiose, respectively

Bacterial strain	API characteristic						
	ONPG	ODC	CIT	URE	SOR	SAC	MEL
<i>V. harveyi</i> ^T ATCC 14126	–	+	+	–	–	+	–
<i>V. carchariae</i> flounder	–	+	+	V	+	+	–
<i>V. carchariae</i> grouper	–	+	+	+	–	+	–
VIB 286 (<i>V. carchariae</i> ^T)	–	V	V	V	–	+	–
VIB 295 (<i>V. harveyi</i> ^T)	–	+	V	V	–	V	V
VIB 411	–	–	V	–	–	+	–
VIB 570	–	+	+	+	+	+	–
VIB 573	–	+	V	–	+	–	–
VIB 645	–	+	+	+	+	+	–
VIB 651	–	+	+	–	+	+	–
VIB 654	–	+	V	V	–	+	–
VIB 660	–	+	+	+	+	+	–
<i>V. campbellii</i> ^T ATCC 25920	–	–	–	–	–	–	–
VIB 655	–	+	V*	+	–	+	–
VIB 656	–	+	–	–	–	+	–
VIB 657	–	+	V*	–	–	–	–
VIB 403	+	+	V*	+	–	–	–
VIB 697	–	+	–	–	–	–	–
VIB 394	–	+	–	–	–	–	–
VIB 350	+	–	–	–	+	+	V
VIB 400	+	+	–	–	–	+	–

melibiose showed some degree of variability among the strains examined.

Sequence alignments and genetic distances

Sequence variations were observed at positions 976, 1003–1009, 1015–1021, 1131–1140, 1256–1261, 1264, 1269, 1307, and 1318. The most variable region is

located from positions 1131 to 1140, where 1 of 2 sequence motifs is observed (Table 3). The first sequence motif, ACTTCGGGT-, is found in strains VIB 295, 411, 570, 573, 645, 651, 654, 660, *Vibrio carchariae* flounder, *V. carchariae* grouper, and the type strains of *V. harveyi* and *V. carchariae*. Among these strains there are only minor differences elsewhere, with the sequences for the type strains of *V. harveyi* (VIB 295 and ATCC 14126^T) and *V. carchariae* (VIB 286) being identical. These sequences were found to be distinctly different from the sequence reported by Ruimy et al. (1994) for the same *V. harveyi* strain, ATCC 14126^T (X74706).

The second sequence motif, GAGTAATGTC, is observed in strains VIB 350, 394, 400, 403, 655, 656, 657, 697, and the *Vibrio campbellii* type strain (ATCC 25920), differing from the first group in 7 of the 10 base pairs (bp) in this region. In this group a greater degree of sequence heterogeneity is seen in other regions that can be used as the basis of further sub-divisions.

Pairwise genetic distances for the 21 strains listed in Table 1 and the 13 reference strains listed previously are shown in Table 4. Strains VIB 411, 570, 573, 645, 651, 654, 660, *Vibrio carchariae* flounder and *V. carchariae* grouper were identical or nearly identical to our sequences of the type strains of *V. harveyi* (VIB 295 and ATCC 14126^T) and *V. carchariae* (VIB 286). Strains VIB 394, 403, 655, 656, 657, and 697 were more similar to previously reported sequences of *V. campbellii* (X74692, AY035896). VIB 350 and VIB 400 were nearly identical to the previously published sequences of *V. mediterranei* (X74710), and *V. rumoiensis* (AB013297) respectively.

Table 3. *Vibrio* spp. Abbreviated sequence alignments. Variations from *V. harveyi*/*V. carchariae* type strain sequences are in boldface. Strains listed in the same row have identical sequences in the region investigated

Bacterial strain	<i>Escherichia coli</i> position								
	976	1003–1009	1015–1021	1131–1140	1256–1261	1264	1269	1307	1318
<i>V. harveyi</i> ^T (ATCC 14126), VIB 286, 295, 645, 651, 654, 660	A	CTTTCCA	TGGATTG	ACTTCGGGT-	G-C-A	T	G	C	G
<i>V. carchariae</i> grouper, VIB 411, 570, 573	A	CTTTCCA	TGGATTG	ACTTCGGGT-	A -C-A	T	G	C	G
<i>V. carchariae</i> flounder	C	CTTTCCA	TGGATTG	ACTTCGGGT-	G-C-A	T	G	C	G
<i>V. campbellii</i> ^T (ATCC 25920), VIB 655, 656, 657	A	CTTTCCA	TGGATTG	GAGTAATGTC	A -C-A	T	G	C	G
VIB 403, 697	A	CTTTCCA	TGGATTG	GAGTAATGTC	G-C-A	T	G	C	G
VIB 394	A	CTTT T CA	T G AATTG	GAGTAATGTC	A -C-A	T	G	C	G
VIB 400	A	CTTTCCA	TGGATTG	GAGTAATGTC	A - A - G	A	T	T	A
VIB 350	A	GCCAGCG	C GCAGGT	GAGTAATGTC	A -C-A	T	A	T	A

Table 4. Uncorrected genetic distances (% difference) of *Vibrio* strains and reference strains. Boxed values represent clusters identified in phylogenetic analysis

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	
<i>Vibrio cholerae</i> classical (1)	-																			
<i>V. cholerae</i> Eitor (2)	0.000	-																		
<i>V. harveyi</i> ^T , VIB 286, 295, 645, 651, 654, 660 (3)	0.054	0.055	-																	
<i>V. carchariae</i> flounder (4)	0.055	0.057	0.001	-																
<i>V. carchariae</i> grouper, VIB 411, 570, 573 (5)	0.053	0.054	0.001	0.003	-															
<i>V. carchariae</i> X74693 (6)	0.053	0.054	0.000	0.001	0.000	-														
<i>V. campbellii</i> ^T , VIB 655, 656, 657 (7)	0.051	0.053	0.009	0.011	0.008	0.008	-													
VIB 394 (8)	0.051	0.053	0.012	0.013	0.011	0.011	0.003	-												
<i>V. campbellii</i> AY035896, VIB 403, 697 (9)	0.053	0.054	0.008	0.009	0.009	0.008	0.001	0.004	-											
<i>V. campbellii</i> X74692 (10)	0.051	0.053	0.009	0.011	0.008	0.008	0.000	0.003	0.001	-										
<i>V. nereis</i> X74716 (11)	0.053	0.054	0.015	0.016	0.016	0.015	0.008	0.011	0.007	0.008	-									
<i>V. alginolyticus</i> X74690 (12)	0.050	0.051	0.013	0.015	0.012	0.012	0.004	0.007	0.005	0.004	0.004	-								
<i>V. rumoiensis</i> AB013297 (13)	0.051	0.053	0.019	0.020	0.017	0.018	0.009	0.012	0.011	0.009	0.008	0.007	-							
VIB 400 (14)	0.050	0.051	0.017	0.019	0.016	0.016	0.008	0.011	0.009	0.008	0.009	0.005	0.001	-						
<i>V. mediterranei</i> X74710 (15)	0.036	0.038	0.032	0.034	0.031	0.031	0.023	0.023	0.024	0.023	0.023	0.019	0.026	0.024	-					
VIB 350 (16)	0.039	0.040	0.030	0.031	0.028	0.028	0.020	0.020	0.021	0.020	0.020	0.016	0.023	0.021	0.003	-				
<i>V. orientalis</i> X74719 (17)	0.046	0.047	0.030	0.031	0.028	0.028	0.020	0.020	0.022	0.020	0.020	0.016	0.023	0.022	0.012	0.009	-			
<i>V. ordalii</i> X74718 (18)	0.043	0.045	0.041	0.042	0.039	0.039	0.031	0.031	0.032	0.031	0.031	0.028	0.024	0.023	0.018	0.020	0.024	-		
<i>Listonella</i> (= <i>Vibrio</i>) <i>anguillarum</i> X16895 (19)	0.043	0.045	0.043	0.044	0.042	0.042	0.034	0.034	0.035	0.034	0.035	0.031	0.027	0.026	0.020	0.023	0.027	0.003	-	
<i>Aeromonas salmonicida</i> X74681 (20)	0.093	0.094	0.091	0.093	0.091	0.090	0.090	0.090	0.090	0.090	0.095	0.093	0.089	0.087	0.093	0.090	0.086	0.078	0.081	-

Phylogenetic analysis

Phylogenetic analysis is based on a 746 bp region covering positions 642–1387 in the *Escherichia coli* sequence (J01859) for which sequence data was obtained for all 21 strains investigated for this study as well as the 13 reference sequences obtained from GenBank. An unrooted tree constructed using the UPGMA method is shown in Fig. 1. Other trees constructed using the neighbor-joining and heuristic search methods as well as the maximum parsimony method gave similar results (not shown). The bulk of the strains investigated for this study form a large, well-supported group that includes previously published sequences of *Vibrio carchariae*, *V. alginolyticus*, *V. campbellii*, *V. nereis*, and *V. rumoiensis* (Fig. 1). Within this group the type strains of *V. carchariae* and *V. harveyi*, the *V. carchariae* isolated from summer flounder in the USA, the *V. carchariae* from grouper in Taiwan and vibrios isolated from aquarium seawater in Denmark and disease outbreaks in fish from South Africa and the Mediterranean (VIB 411, 570, 573, 645, 651, 654), along with the reference strain *V. carchariae* X74693, form a strongly supported cluster (Cluster 1 in Fig. 1). This cluster contains all the strains belonging to AFLP cluster 1 (Pedersen et al. 1998) that were included in the present study and is considered the 'bona fide' *V. harveyi* cluster. Strains from prawn in Thailand, barramundi in Australia, oyster in Spain and sea bass in the Mediterranean (VIB 403, 394, 697, 655, and 656) belonging to AFLP Clusters 4, 5, and 7 and VIB 657 (AFLP unclustered) form a less well-supported cluster designated as Cluster 2 (Fig. 1) with the *V. campbellii* type strains (AF426824 and X74692) and a previously reported sequence of *V. campbellii* (AY035896, unpubl.). In fact, only the UPGMA method places all of these strains in 1 cluster (bootstrap value = 72%). Neighbor-joining and heuristic methods maintain the grouping of *V. campbellii* type strain (AF426824) and VIB 655, 656, and 657 with *V. campbellii* X74692 (average bootstrap value = 66%) but exclude VIB 394, 403, and 697 and *V. campbellii* AY035896. The maximum parsimony method does not group any of these strains together. Despite the low tendency of these strains to cluster together it should be noted that there are no more than 2 bp differences between any member of this group and the *V. campbellii* type strain. The 16S sequences of 2 other strains (VIB 350 and VIB 400), originally identified as *V. harveyi*, were also found to be distinctly different from the *V. harveyi* cluster (Cluster 1 Fig. 1), and match more closely with sequences corresponding to other *Vibrio* spp. VIB 400 (AFLP Cluster 3), a bacterium isolated from seawater in Hawaii, is shown in all phylogenetic analyses to be more closely related to *V. rumoiensis* (Cluster 3, Fig. 1). VIB 350 (AFLP Cluster 9), isolated from a shark from the Bahamas, was found to be most closely related

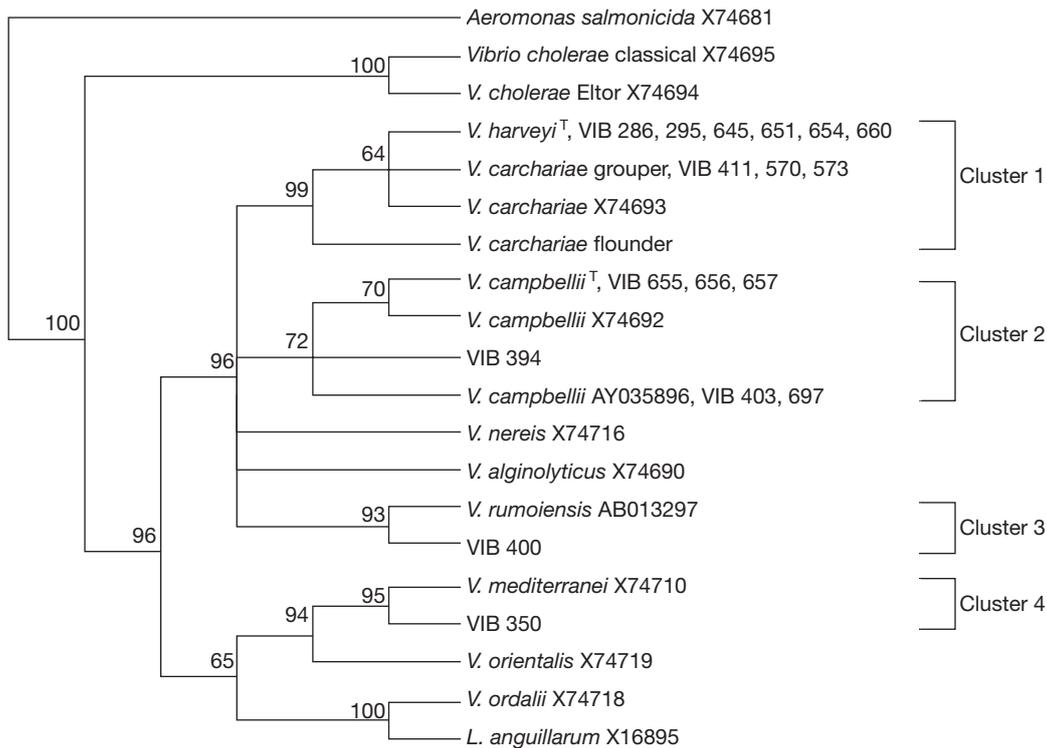


Fig. 1. UPGMA tree formed based on 16S rDNA sequences showing phylogenetic relationship between isolates identified as *Vibrio harveyi* or *V. carchariae* by biochemical characteristics and other *Vibrio* species. The numbers above the bar at each node are the percentages of bootstrap replications (only values over 50% are shown). *A. salmonicida*: *Aeromonas salmonicida*, *L. anguillarum*: *Listonella (=Vibrio) anguillarum*

to *V. mediterranei* (X74710) and also clusters closely with *V. orientalis* (X74719) (Cluster 4, Fig. 1).

DISCUSSION

Pedersen et al. (1998) found that *Vibrio harveyi* and *V. carchariae* could not be distinguished by molecular means (AFLP, DNA-DNA hybridization, ribotyping) and should therefore be considered synonymous. Previously published 16S rDNA sequences of *V. harveyi* and *V. carchariae*, however, had substantial differences (Ruimy et al. 1994, Soffientino et al. 1999), contradicting the molecular evidence found by Pedersen et al. (1998). Our analysis of 16S rDNA sequences focused on a 746 bp region that covers the area in which the majority of differences in the sequences of *V. harveyi* and *V. carchariae* were previously found (Ruimy et al. 1994). Our findings indicate that the partial 16S rDNA sequences for the type strains of *V. harveyi* and *V. carchariae* that were shown to be different by Ruimy et al. (1994) are in fact identical. Furthermore, sequences for all isolates belonging to the 'bona fide' *V. harveyi* AFLP cluster (Pedersen et al. 1998), as well as the *V. carchariae* grouper and *V. carchariae* flounder isolates and reference strain *V. carchariae* X74693, were identical or nearly identical to sequences for the *V. harveyi* and *V. carchariae* type strains. In our phylogenetic analysis, these isolates clustered together with high bootstrap values. We find, therefore, that 16S rDNA sequencing

confirms the finding by Pedersen et al. (1998) that *V. harveyi* and *V. carchariae* are synonymous.

Recently, Thompson et al. (2002) showed that *Vibrio trachuri*, a pathogen of the Japanese horse mackerel *Trachurus japonicus*, is also a synonym of *V. harveyi* based on DNA:DNA hybridization, AFLP, biochemical tests, and mol % G+C content. The 16S rDNA sequence of *V. trachuri* reported in Thompson et al. (2002) (AJ312382) is 100% similar to the sequence of the *V. harveyi* type strain reported here (AF426825). This further strengthens the conclusion of Thompson et al. (2002) that *V. trachuri* is a synonym of *V. harveyi*.

Vibrio harveyi and *V. carchariae* show a high degree of heterogeneity in their biochemical characteristics (Grimes et al. 1993, Alsina & Blanch 1994). This is not unexpected; biochemical properties are often highly variable for environmental and pathogenic strains (Alsina & Blanch 1994). When the strains included in the current study are grouped according to sequence homology (see Table 1), no API 20E characteristic can be used to effectively differentiate strains corresponding to all the clusters found in Fig. 1. Although more complete biochemical characterizations have been proposed to differentiate closely related *Vibrio* spp. (Bryant et al. 1986, Grimes et al. 1993, Alsina & Blanch 1994) they may be unreliable due to variability in biochemical traits and the possibility of errors in the interpretation of test results. The high number of tests required may also make them impractical. The weakness of using biochemical profiles as the sole means of iden-

tifying bacterial isolates is evidenced by our finding that 8 of 15 putative *V. harveyi* isolates in the Pedersen study had 16S rDNA sequences that match more closely to other *Vibrio* species. This suggests that biochemical analysis, by itself, may not be the most reliable method of distinguishing *V. harveyi* from other closely related species, particularly *V. campbellii*.

Molecular tools such as AFLP, DNA:DNA hybridization, and 16S rDNA sequencing have proven to be useful tools for classification and identification of bacterial species (Busse et al. 1996, Kolbert & Persing 1999). One advantage of 16S rDNA sequencing is that it is accessible to any laboratory with technical skills in PCR because, thanks to the Human Genome Project, fast and reliable automated sequencing is now available to researchers at a relatively affordable price. Computer applications such as BLAST (Altschul et al. 1990) can be used to search databases like GenBank and EMBL, allowing preliminary identifications without needing to include large numbers of reference strains in the study. With AFLP and DNA:DNA hybridization, comparisons can only be made with the bacterial strains on hand. Among the limits of 16S rDNA sequencing are the potential for DNA contamination in PCR reactions and the presence of conflicting or erroneous sequences deposited in the databases (Kolbert & Persing 1999, Macián et al. 2000), as is apparently the case for the 16S rDNA sequence of the *Vibrio harveyi* type strain (X74706) reported by Ruimy et al. (1994). Errors such as this can be caused by the difficulties of older (manual) methods of sequencing or simply by assigning a sequence to a strain incorrectly. The exponential increase in accurate sequencing information should help minimize these problems. Another significant weakness of 16S sequencing as a diagnostic method is the possibility of 2 distinct species sharing identical or nearly identical 16S rDNA sequences (Kolbert & Persing 1999).

In this study, we find agreement between data from 16S rDNA sequencing and other molecular means when identifying *Vibrio harveyi* and *V. carchariae* isolates. Isolates belonging to the 'bona fide' *V. harveyi* AFLP cluster also clustered together on the basis of 16S rDNA sequence homology. The delineation of other clusters based on 16S rDNA also generally agreed with AFLP clusters; however, certain discrepancies were apparent. Strains that clustered with *V. campbellii* on the basis of 16S rDNA sequences (VIB 394, 403, 655, 656, 657, and 697) were spread among 3 AFLP clusters with only 1 strain (VIB 697) located in the same AFLP cluster as the *V. campbellii* type strain (see Fig. 1). It is uncertain if these strains represent distinct species with very similar 16S rDNA sequences or if AFLP analysis simply detected heterogeneities beyond the species level. One other anomalous result was that VIB 350 clustered with the

type strain of *V. alginolyticus* on the basis of AFLP patterns but was shown to be more similar to *V. mediterranei* by 16S rDNA sequencing. Pedersen et al. (1998) noted that this strain clearly clustered with other *V. harveyi* and *V. carchariae* strains in its original description (Grimes et al. 1993) and therefore doubted the authenticity of their subculture of this strain (Pedersen et al. 1998). Based on our analysis of current sequence databases, and general agreement with other molecular methods of identification, 16S rDNA sequencing appears to be a reliable method of identifying *V. harveyi*.

As suggested by Busse et al. (1996), a good strategy for the accurate identification of bacteria is to use a 'polyphasic' approach that might begin with sequencing a fragment of the 16S rRNA gene. Information obtained by the sequence analysis will provide clues on which genotypic and phenotypic methods will be most suitable to confirm the identification of a previously described species or for a more complete description of a new species. In the case of *Vibrio harveyi*, 16S rDNA sequencing can provide a relatively certain identification. A standard biochemical profile system such as the API 20E can be used to confirm the sequence-based identification and prevent the incorrect identification of unknown or not previously sequenced species that share highly similar 16S rRNA sequences.

In conclusion, 16S rDNA sequencing has confirmed the homogeneity and synonymy of *Vibrio harveyi*, *V. carchariae*, and *V. trachuri*, and indicated errors in the GenBank database with regard to *V. harveyi* (X74706). *V. harveyi* therefore emerges as a major bacterial pathogen, capable of infecting a wide range of species including penaeids (Liu et al. 1996, Abraham et al. 1997, Alvarez et al. 1998, Robertson et al. 1998), bivalves (Pass et al. 1987), cephalopods (Hanlon & Forsythe 1990), teleosts (Yii et al. 1997, Soffientino et al. 1999, Zhang & Austin 2000, Thompson et al. 2002) and elasmobranchs (Grimes et al. 1985). As such, it is likely that *V. harveyi* will be isolated from an increasing number of fish and shellfish species. The use of 16S rDNA sequencing in conjunction with the biochemical characteristics should provide a more accurate and efficient method of diagnosing *V. harveyi* and other related species than extensive biochemical profiling alone.

Acknowledgements. The authors wish to thank Dr. Dawn Austin for providing most of the bacterial strains used in this study and Dr. Brian Austin for his extremely useful comments in the preparation of the manuscript. We thank Dr. Yii for providing the *V. carchariae* strain isolated from grouper in Taiwan. Finally we wish to acknowledge HHMI Biopolymer Laboratory & W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University for performing the DNA sequencing for this study. This research was supported by the Rhode Island Agriculture Experiment Station, grant number H401 (Contribution number 3952).

LITERATURE CITED

- Abraham TJ, Manley R, Palanippan R, Dhevendaran K (1997) Pathogenicity and antibiotic sensitivity of luminous *V. harveyi* isolated from diseased penaeid shrimp. *J Aquacult Trop* 12:1–8
- Alsina M, Blanch AR (1994) A set of keys for biochemical identification of environmental *Vibrio* species. *J Appl Bacteriol* 76:79–85
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Molec Biol* 215:403–410
- Alvarez JD, Austin B, Alvarez AM, Reyes H (1998) *Vibrio harveyi*: a pathogen of penaeid shrimps and fish in Venezuela. *J Fish Dis* 21:313–316
- Austin B, Austin DA (1999) Characteristics of the pathogens: Gram-negative bacteria, Vibrionaceae representatives. In: Laird S, Stead S (eds) *Bacterial fish pathogens: disease of farmed and wild fish*, 3rd rev. edn. Springer-Praxis, Chichester, p 102–118
- Baumann P, Baumann L, Bang SS, Woolkallis MJ (1981) Re-evaluation of the taxonomy of *Vibrio*, *Beneckeia* and *Photobacterium*: abolition of the genus *Beneckeia*. *Curr Microbiol* 4:127–132
- Bryant TN, Lee JV, West PA, Colwell RR (1986) A probability matrix for the identification of species of *Vibrio* and related genera. *J Appl Bacteriol* 61:469–480
- Busse HJ, Denner, EB, Lubitz W (1996) Classification and identification of bacteria: current approaches to an old problem. Overview of methods used in bacterial systematics. *J Biotechnol* 47:3–38
- Diggles BK, Moss GA, Carson J, Anerson CD (2000) Luminous vibriosis in rock lobster *Jasus verreauxi* (Decapoda: Palinuridae) phyllosoma larvae associated with infection by *Vibrio harveyi*. *Dis Aquat Org* 43:127–137
- Feldman KA, Buck JD (1984) Distribution and characterization of luminescent bacteria in a temperate estuary. *Estuaries* 7:93–97
- Grimes DJ, Colwell RR, Stemmler J, Hada H and 5 others (1984) *Vibrio* species as agents of elasmobranch disease. *Helgol Wiss Meeresunters* 37:309–315
- Grimes DJ, Brayton P, Colwell RR, Ruber SH (1985) *Vibriosis* as autochthonous flora of neritic sharks. *Syst Appl Microbiol* 6:221–226
- Grimes DJ, Jacobs D, Swartz DG, Brayton P, Colwell RR (1993) Numerical taxonomy of Gram-negative, oxidase-positive rods from carcharid sharks. *Int J Syst Bacteriol* 43:88–98
- Grisez L, Reyniers J, Verdonck L, Swings J, Ollevier F (1997) Dominant intestinal microflora of sea bream and sea bass larvae, from two hatcheries, during larval development. *Aquaculture* 155:391–403
- Hanlon RT, Forsythe JW (1990) Diseases of mollusca: Cephalopoda. In: Kinne O (ed) *Diseases of marine mammals*, Vol III. Biologische Anstalt Helgoland, Hamburg, p 21–43
- Higgins DG, Bleasby AJ, Fuchs R (1992) CLUSTAL V: improved software for multiple sequence alignment. *Comput Appl Biosci* 8:189–192
- Johnson FH, Shunk IV (1936) An interesting new species of luminous bacteria. *J Bacteriol* 31:585–592
- Kita-Tsukamoto K, Oyaizu H, Nanba K, Simidu U (1993) Phylogenetic relationships of marine bacteria, mainly members of the family Vibrionaceae, determined on the basis of 16S rRNA sequences. *Int J Syst Bacteriol* 43:8–19
- Kolbert CP, Persing DH (1999) Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. *Curr Opin Microbiol* 2:299–305
- Liu PC, Lee KK, Chen SN (1996) Pathogenicity of different isolates of *V. harveyi* to tiger prawns, *Penaeus monodon*. *Lett Appl Microbiol* 22:413–416
- Macián, MC, Wolfgang L, Schleifer KH, Esperanza G, Pujalte MJ (2000) *Vibrio pelagius*: differences of the type strain deposited at various culture collections. *Syst Appl Microbiol* 23:373–375
- Medrano JF, Aasen E, Sharrow L (1990) DNA extraction from nucleated red blood cells. *Biotechniques* 8:43–43
- Nelson DR, Sadlowski Y, Eguchi M, Kjelleberg S (1997) The starvation-stress response of *Vibrio (Listonella) anguillarum*. *Microbiology* 143:2305–2312
- Orndorff SA, Colwell RR (1980) Distribution and identification of luminous bacteria from the Sargasso Sea. *Appl Environ Microbiol* 39:983–987
- Pass DA, Dybdahl R, Mannion MM (1987) Investigations into the causes of mortality of the pearl oyster, *Pinctada maxima* (Jamson), in Western Australia. *Aquaculture* 65:149–169
- Pedersen K, Verdonck L, Austin B, Austin DA and 8 others (1998) Taxonomic evidence that *Vibrio carchariae* Grimes et al. 1985 is a junior synonym of *Vibrio harveyi* (Johnson and Shunk 1936) Baumann et al 1981. *Int J Syst Bacteriol* 48:749–758
- Robertson PAW, Calderon J, Carrera L, Stark JR, Zherdmant M, Austin B (1998) Experimental *Vibrio harveyi* infections in *Penaeus vannamei* larvae. *Dis Aquat Org* 32:151–155
- Ruby EG, Morin JG (1979) Luminous enteric bacteria of marine fishes: a study of their distribution, densities, and dispersion. *Appl Environ Microbiol* 38:406–411
- Ruimy R, Breittmayer V, Elbaze P, Lafay B, Boussemart O, Gauthier M, Christen R (1994) Phylogenetic analysis and assessment of the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* deduced from small-subunit rRNA sequences. *Int J Syst Bacteriol* 44:416–426
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning. A laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, p E.5–E.7
- Soffientino B, Gwaltney T, Nelson DR, Specker JL, Mauel M, Gómez-Chiarri M (1999) Infectious necrotizing enteritis and mortality caused by *Vibrio carchariae* in summer flounder *Paralichthys dentatus* during intensive culture. *Dis Aquat Org* 38:201–210
- Swofford DL (1998) PAUP* phylogenetic analysis using parsimony (* and other methods) Ver 4. Sinauer Associates, Sunderland
- Thompson FL, Hoste B, Vandemeulebroecke K, Denys R, Swings J (2002) *Vibrio trachuri* Iwamoto et al. 1995 is a junior synonym of *Vibrio harveyi* (Johnson and Shunk 1936) Baumann et al. 1981. *Int J Syst Evol Microbiol* 52:973–976
- Valle O, Dorsch M, Wiik R, Stackebrandt E (1990) Nucleotide sequence of the 16S rRNA from *Vibrio anguillarum*. *Syst Appl Microbiol* 13:257–257
- Yii KC, Yang TI, Lee KK (1997) Isolation and characterization of *Vibrio carchariae*, a causative agent of gastroenteritis in the groupers, *Epinephelus coioides*. *Curr Microbiol* 35:109–115
- Yumoto I, Iwata H, Sawabe T, Ueno K, Ichise N, Matsuyama H, Okuyama H, Kawasaki K (1999) Characterization of a facultatively psychrophilic bacterium, *Vibrio rumoiensis* sp. nov., that exhibits high catalase activity. *Appl Environ Microbiol* 65:67–72
- Zhang XH, Austin B (2000) Pathogenicity of *Vibrio harveyi* to salmonids. *J Fish Dis* 23:93–102