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Taxonomic evidence that *Vibrio carchariae* Grimes *et al.* 1985 is a junior synonym of *Vibrio harveyi* (Johnson and Shunk 1936) Baumann *et al.* 1981

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A collection of 94 *Vibrio* isolates closely related to *Vibrio harveyi*, together with named reference and type strains, were investigated for phenotypic and genotypic properties. Using amplified fragment length polymorphism (AFLP), nine clusters were recognized. The largest cluster ($n = 36$), considered to be the bona fide *V. harveyi* group, contained the type strains of *V. harveyi* and *Vibrio carchariae* and most of the strains isolated from fish. The type strains of all other species, including *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio campbellii* and *Vibrio natriegens*, clustered outside this group. By ribotyping, *V. harveyi* and *V. carchariae* patterns were very similar, insofar as they shared most bands. The *V. campbellii* type strain had several bands in common with the type strains of both *V. harveyi* and *V. carchariae*, whereas the other species were clearly distinct from these three species. DNA–DNA hybridization experiments showed 88% DNA binding between the type strains of *V. harveyi* and *V. carchariae*, whereas the DNA binding between *V. harveyi* and *V. campbellii* was 40%. Although the delineation of the species *V. harveyi* is still uncertain, the authors propose, on the basis of a number of tests, to delineate a core of *V. harveyi* strains which contained the type strains of both *V. harveyi* and *V. carchariae*. It is concluded that *V. carchariae* is the junior synonym of *V. harveyi*.

Keywords: *Vibrio* species, AFLP, DNA–DNA hybridization, ribotyping

INTRODUCTION

Vibrio harveyi is a luminous, marine bacterium, first described by Johnson & Shunk (1936) as '*Achromobacter harveyi*'. Over the years, it has also been known under the synonyms *Beneckea harveyi* (AL, Reichelt & Baumann, 1973) and *Lucibacterium harveyi* (AL, Hendrie *et al.*, 1970). The name *V. harveyi* was then given by Baumann *et al.* (1980) and validated in 1981 (Anonymous, 1981). *V. harveyi* is ubiquitous in warm marine waters and sediment, and is also a common member of the natural intestinal microflora of marine animals – vertebrates as well as invertebrates (O'Brien & Sizemore, 1979; Ruby & Morin, 1979). It is usually not considered to be pathogenic to fish or other vertebrates, but may cause mortalities in various

Abbreviation: AFLP, amplified fragment length polymorphism.

Table 1. List of strains of *V. harveyi*- and *carchariae*-like strains and *Vibrio* type strains

VIB no.	Received as species*	Laboratory no.*†	Synonym†	Country and year of isolation	Source	AFLP cluster‡
291	*V. fischeri*^{T}	LMG 4414^T	ATCC 7744, NCMB 1281			UC
289	*V. damselae*^{T}	LMG 7892^T	CDC 2588-80, ATCC 33539	USA, 1980	Damselfish (*Chromis punctipinnis*)	UC
305	*V. pelagius*^{T}	LMG 3897^T	ATCC 25916	Hawaii	Sea water	UC
853	*Vibrio* sp.	D52a		Tasmania	Atlantic salmon (*Salmo salar*)	UC
398	*V. harveyi*	LMG 11225	NCIMB 993	Italy	Sea water	UC
392	*V. harveyi*	HWU F373		Australia	Skin ulcer, barramundi	1
574	*V. harveyi*?	UB A087		Spain, 1991	Sea bass (*Dicentrarchus labrax*)	1
402	*V. harveyi*	LMG 11661	ATCC 33843	USA	Sea water	1
571	*V. harveyi*	UB A067		Spain, 1990	Sea bass	1
413	*V. harveyi*	IP 10.88		France, 1988	Sea water	1
409	*V. harveyi*	LMG 16832	PmH 1193 St	Thailand	Black tiger prawn	1
812	*V. alginolyticus*	UB A039		Spain, 1988	Sea bass	1
572	*V. harveyi*?	UB A068		Spain, 1990	Sea bream (*Sparus aurata*)	1
295	*V. harveyi*^{T}	LMG 4044^T (ATCC 14126)	ATCC 14126, NCMB 1280	USA	Dead amphipod (*Talorchestia* sp.)	1
411	*V. harveyi*	IP 3.86		South Africa, 1986	Ulcer, fish	1
326	*V. alginolyticus*	LMG 12091		Belgium	Eel (*Anguilla anguilla*)	1
631	*Vibrio* sp.	HWU Ven.33		Venezuela	Bivalve mollusc	1
651	*V. harveyi/carchariae*	RVAU 94-5-75		Denmark, 1994	Water from aquarium with sharks in captivity	1
649	*V. harveyi/carchariae*	Nb 11608		Malta, 1993	Sea bream	1
789	*Vibrio* sp.	UB A030		Spain	Sea bream	1
351	*V. carchariae*	LMG 11755	ATCC 43516	Bahamas	Mouth of shark	1
393	*V. harveyi*	HWU F376		Australia	Heart, barramundi	1
286	*V. carchariae*^{T}	LMG 7890^T	CCUG 19116, ATCC 35084	USA, 1982	Brown shark (*Carcharhinus plumbeus*)	1
561	*V. alginolyticus*	UB A038		Spain, 1988	Sea bass	1
654	*V. harveyi/carchariae*	RVAU 94-3-46		Denmark, 1994	Water from aquarium with sharks in captivity	1
650	*V. harveyi/carchariae*	Nb 11649		Greece, 1993	Sea bass	1
23	*V. anguillarum*	HWU 58		Greece, 1991	Sea bass	1
24	*V. anguillarum*	HWU 59		Greece, 1991	Sea bream	1
647	*V. harveyi/carchariae*	Nb 11409		Greece, 1992	Sea bream	1
22	*V. anguillarum*	HWU 57		Greece, 1991	Sea bass	1
568	*V. harveyi*?	UB A060		Spain, 1990	Turbot (*Scophthalmus maximus*)	1
660	*V. harveyi/carchariae*	Nb 11310		Greece, 1990	Sea bass	1
645	*V. harveyi/carchariae*	Nb 11601		Tunisia, 1993	Sea bass	1
658	*V. harveyi/carchariae*	Nb 10949		France, 1990	Sea bream	1
573	*V. harveyi*?	UB A072		Spain, 1990	Sea bass	1
771	*Vibrio* sp.	HWU 87/0919-1		Tasmania	Blue tang	1
775	*Vibrio* sp.	HWU 90/4316		Tasmania	Atlantic salmon	1
643	*Vibrio* sp.	HWU O-3		Japan	Milkfish(*Chanos chanos*)	1
652	*V. harveyi/carchariae*	943/93		Italy, 1993	Sea bass	1
570	*V. harveyi*	UB A065		Spain, 1990	Sea bream	1
633	*Vibrio* sp.	HWU Ven.40		Venezuela	Bivalve mollusc	1
648	*V. harveyi/carchariae*	RVAU 93-5-157		Denmark, 1993	Liver from shark dead in captivity	UC
788	*Vibrio* sp.	UB A029		Spain	Sea bream	2
618	*Vibrio* sp.	HWU Ven.3		Venezuela	Penaeid shrimp	2
816	*Vibrio* sp.	HWU Ven.45		Venezuela	Sediment	2
811	*V. alginolyticus*	UB A017		Spain, 1986	Sea bass	2
646	*V. harveyi/carchariae*	RVAU 93-8-287		Denmark, 1993	Water from aquarium with sharks in captivity	2
296	*V. mediterranei*^{T}	LMG 11258^T	ATCC 43341	Spain	Coastal sediment	UC
641	*Vibrio* sp. Zoea	HWU 85Z1		Japan		3
642	*Vibrio* sp. Zoea	HWU 87Z1		Japan		3
622	*Vibrio* sp.	HWU Ven.16		Venezuela	Penaeid shrimp	3
397	*V. harveyi*	LMG 10948	NCIMB 1896, ATCC 29919, Baumann 74	Hawaii	Sea water	3
667	*Vibrio* sp.	94-5-74		Denmark, 1994	Water from aquarium with sharks in captivity	3
788	*Vibrio* sp.	UB A029		Spain	Sea bream	3
400	*V. harveyi*	LMG 11659	CCUG 16178, Baumann 130	Hawaii	Sea water	3
396	*V. harveyi*	LMG 10947	NCIMB 1847	Red Sea	Sea water	3
819	*V. vulnificus*	*V. vulnificus* 6.80		Senegal, 1980	Fish	4
822	*V. vulnificus*	*V. vulnificus* 8.80		Senegal, 1980	Shrimp	4
629	*Vibrio* sp.	HWU Ven.29		Venezuela	Bivalve mollusc	4
404	*V. harveyi*	LMG 16835	PmH 2193 SK	Thailand	Black tiger prawn	4
403	*V. harveyi*	LMG 16828	PmH 293 SK	Thailand	Black tiger prawn	4
406	*V. harveyi*	LMG 16830	PmH 593 STC	Thailand	Black tiger prawn	4
395	*V. harveyi*	LMG 10946	NCIMB 394	India?	Prawn	4
391	*V. harveyi*	LMG 13949		Thailand, 1990	Shrimp	4
538	*V. vulnificus*		AAHRI 06	Thailand, 1992	Shrimp	4
347	*V. campbellii*	LMG 11256	ATCC 25921	Hawaii	Sea water	5
285	*V. campbellii*^{T}	LMG 11216^T	ATCC 25920	Hawaii, 1971	Sea water	5
696	*V. harveyi*	LMG (80M8)		Spain, 1989	Oyster	5
697	*V. harveyi*	LMG (90M4)		Spain, 1989	Oyster	5
348	*V. campbellii*	LMG 11257	ATCC 33864	Hawaii	Sea water	5
401	*V. harveyi*	LMG 11660	ATCC 33842	USA	Sea water	UC

Table 1. *(cont.)*

VIB no.	Received as species*	Laboratory no.*†	Synonym†	Country and year of isolation	Source	AFLP cluster‡
585	*V. parahaemolyticus*	UB A084		Spain	Sea bream	6
584	*V. nereis*?	UB A108		Spain, 1993	Turbot	6
657	*V. harveyi/carchariae*	Nb 10732		France, 1990	Sea bass	UC
394	*V. harveyi*	HWU F377		Australia	Heart, barramundi	7
628	*Vibrio* sp.	HWU Ven.28		Venezuela	Penaeid shrimp	7
655	*V. harveyi/carchariae*	Nb 10324		France, 1989	Sea bass	7
214	*V. anguillarum*	LMG 13241		Greece, 1991	Sea water	7
661	*V. harveyi/carchariae*	Nb 11421		Tunisia, 1992	Sea bass	7
656	*V. harveyi/carchariae*	Nb 11406		Greece, 1990	Sea bass	7
569	*V. harveyi*?	UB A063		Spain, 1990	Turbot	7
304	*V. parahaemolyticus*^{T}	LMG 2850^T	ATCC 17802	Japan, 1965	Patient with food poisoning	8
803	*V. vulnificus*	LMG 16874	AAHRI 010	Thailand, 1992	Shrimp	8
306	*V. proteolyticus*^{T}	LMG 7893^T	ATCC 15338	USA, 1958	Intestine of isopod (*Imnoria tripunctata*)	UC
283	*V. alginolyticus*^{T}	LMG 4408^T	ATCC 17749, NCMB 1903	Japan	Spoiled horse macharel (*Trachuris trachuris*)	9
350	*V. carchariae*	LMG 11754	ATCC 43515	Bahamas	Mouth of shark	9
666	*Vibrio* sp.	93-12-344/1		Denmark, 1993	Water from aquarium with sharks in captivity	9
299	*V. natriegens*^{T}	LMG 10935^T	ATCC 14048	USA	Salt marsh mud	UC
293	*V. furnissii*^{T}	LMG 7910^T	ATCC 35016	Japan	Human faeces	UC
310	*V. vulnificus*^{T}	LMG 13545^T	ATCC 27562	USA	Human blood	UC
301	*V. nereis*^{T}	LMG 3895^T	ATCC 25917	Hawaii	Sea water	UC
308	*V. splendidus*^{T}	LMG 4042^T	ATCC 33125			UC
290	*V. diazotrophicus*^{T}	LMG 7893^T	ATCC 33466	Canada, 1981	Sea urchin (*Strongylocentrocus dreobrachiensis*)	UC
292	*V. fluvialis*^{T}	LMG 7894^T	ATCC 33809	Bangladesh	Human faeces	UC

* T, Type strain.

† LMG, Laboratorium voor Microbiologie, Rijksuniversiteit Gent, Belgium; HWU, Heriot-Watt University, Edinburgh, UK; UB, Universitat de Barcelona, Spain; IP, Institut Pasteur, Paris, France; ATCC, American Type Culture Collection, Rockville, MD, USA; RVAU, Royal Veterinary and Agricultural University, Copenhagen, Denmark; CDC, Centers for Disease Control and Prevention, Atlanta, USA; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, UK; NCMB, National Collection of Marine Bacteria, Aberdeen, UK; CCUG, Culture Collection, University of Gothenburg, Göteborg, Sweden.

‡ UC, Unclustered.

shellfish species (Karunasagar *et al.*, 1994; Lavilla-Pitogo *et al.*, 1990; Liu *et al.*, 1996; Pass *et al.*, 1987).

The first reports of *Vibrio carchariae* as a pathogen were published in 1984, after the organism had been isolated in 1982 from a brown shark (*Carcharhinus plumbeus*) found dead in captivity (Grimes *et al.*, 1984a, b). The biochemical properties were thoroughly described by Grimes *et al.* (1984a, b), who also showed that the organism was pathogenic to various elasmobranch fish and to mouse cell cultures. The name of the species was approved in 1985 (Anonymous, 1985). Grimes *et al.* (1985) described the bacterial flora of sharks caught off the Bahamas coast, and mainly found *Vibrio alginolyticus*, *V. harveyi*, *Vibrio furnissii*, *Vibrio damselae*, *V. carchariae* and some unidentified vibrios. This was the first report of the isolation of *V. carchariae* in nature, i.e. not associated with disease in sharks.

V. harveyi and *V. carchariae* were shown to be members of the normal flora of carcharinid sharks (Grimes *et al.*, 1993). Recently, we have studied bacterial strains isolated from outbreaks of disease among sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) in aquaculture, and from sharks held in captivity, as well as from water samples from the tanks in which they were kept. These bacteria were identified as *V. harveyi* or *V. carchariae*, but we were not able to distinguish the two species. In a study of Grimes *et al.* (1993), *V. harveyi* and *V. carchariae* clustered together on the basis of biochemical characters, and Alsina & Blanch (1994) could not separate the two species by biochemical properties. In addition, the type cultures of these two species showed almost identical 16S rRNA base sequences (Aznar *et al.*, 1994; Kita-Tsukamoto *et al.*, 1993; Ruimy *et al.*, 1994). These results leave no doubt that *V. harveyi* and *V. carchariae* are closely related. The purpose of the present study was to identify and characterize *V. harveyi*- and *V. carchariae*-like organisms and to compare type strains of the two species to evaluate more precisely the relationship between them by means of phenotypic and genotypic properties.

METHODS

Bacterial strains. A total of 94 *Vibrio* strains were examined, including cultures tentatively identified as *Vibrio harveyi* or *Vibrio carchariae*, together with the type strains of *V. harveyi*, *V. carchariae*, *Vibrio alginolyticus*, *Vibrio campbellii*, *Vibrio damselae*, *Vibrio diazotrophicus*, *Vibrio fischeri*, *Vibrio fluvialis*, *Vibrio furnissii*, *Vibrio mediterranei*, *Vibrio natriegens*, *Vibrio nereis*, *Vibrio parahaemolyticus*, *Vibrio pelagius*, *Vibrio proteolyticus*, *Vibrio splendidus* and *Vibrio vulnificus* (Table 1). The species names in Table 1 are the names received or which had been previously assigned on the basis of biochemical tests. All strains were maintained in 20–25% (v/v) glycerol at −70, −80 or −140 °C. Active cultures were maintained as bench cultures at room temperature on marine agar (Difco), on marine agar supplemented with 5% (w/v) sterile calf blood, or on Tryptone Soya Agar (Oxoid) with 1% (w/v) NaCl.

Amplified fragment length polymorphism (AFLP) fingerprinting of genomes

Extraction of genomic DNA. Cells were harvested from marine agar and washed in 500 µl RS buffer (150 mM NaCl, 10 mM EDTA; pH 8·0). After centrifugation, the cells were re-suspended in 100 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA; pH 7·6) and 15 µl RNase (250 µg ml^{-1}) was added to the suspensions. Lysis of the cells using Sarkosyl/guanidinium thiocyanate (Sigma) and further extraction of genomic DNA were as described by Pitcher *et al.* (1989). The DNA was finally dissolved in 100 µl TE buffer (10 mM Tris, 0·1 mM EDTA; pH 8·0). The DNA concentration was determined by measuring the A_{260} on a spectrophotometer (1 A_{260} unit = 50 µg DNA ml^{-1}). The integrity of the DNA was checked by electrophoresis in a submerged horizontal agarose gel (0·8–1·0 %, w/v) using an electrophoresis buffer (40 mM Tris-acetate, 1 mM EDTA; pH 8·0) containing 0·5 µg ethidium bromide ml^{-1}. DNA preparations were stored at −20 °C.

Oligonucleotide adaptors and primers. The sequences of the *Hin*dIII and *Taq*I adaptors and primers used in this study are given in detail by Janssen *et al.* (1996).

Template preparation. Template preparation was as described by Vos *et al.* (1995) and Janssen *et al.* (1996). Thus in this study, 1 µg DNA was digested with 10 units *Hin*dIII and *Taq*I in a final volume of 30 µl. *Hin*dIII and *Taq*I adaptors were added to a final concentration of 0·04 and 0·4 µM, respectively, and ligated to the restriction fragments. The DNA was subsequently precipitated with 1·25 M ammonium acetate and 50 % (v/v) 2-propanol. Template DNAs were stored at −20 °C.

AFLP reactions. AFLP reactions employed two oligonucleotide primers, one corresponding to the *Hin*dIII ends and one corresponding to the *Taq*I ends. The *Hin*dIII primer was radioactively end-labelled using [γ-^{33}P]ATP and T4 polynucleotide kinase (Vos *et al.*, 1995). Selective amplification and PCR reactions were performed on a PE 9600 thermocycler (Perkin Elmer) and as described by Janssen *et al.* (1996).

Gel analysis. Prior to gel loading and electrophoresis, mixtures were heated for 3 min at 95 °C and then rapidly cooled on ice to prevent nucleic acid secondary structures from re-annealing. Each sample (1·8–2·0 µl) was loaded on a 5 % denaturing sequencing polyacrylamide gel (obtained as premixed SequaGel solutions from National Diagnostics). To 100 ml casting solution, 0·8 ml 10 % ammonium persulfate and 40 µl *N*,*N*,*N*′,*N*′-tetramethylethylenediamine were added, and gels were cast using a SequiGen 38 × 50 cm gel apparatus (Bio-Rad). TBE (100 mM Tris, 100 mM boric acid, 2 mM EDTA; pH 8·3) was used as electrophoresis buffer. Gels were run at a constant power of 110 W for approximately 150 min. After electrophoresis, gels were transferred to 3MM Whatman chromatography paper and vacuum-dried on a gel dryer (model 583; Bio-Rad) for 50 min at 80 °C. A sheet of Hyperfilm MP (Amersham) was exposed to the dried gel. Exposure times varied between 19 and 24 h, depending on the measured radioactivity of the gel. Films were developed using a Fuji RGII X-ray Film Processor. Autoradiograms were scanned by a RayVen RSU1 densitoscanner (X-Ray Scanner). Digitized optical densities were saved as TIFF files and further processed using the GelCompar 3.1 software (Applied Maths, Kortrijk, Belgium). Digital images were normalized and combined according to the methods described by Vauterin & Vauterin (1992). A similarity matrix was created using the Dice coefficient, S_{AB} (Sneath & Sokal, 1973). For band comparison, a band position tolerance value of 0·8 % was allowed to compensate for misalignment of homologous bands due to technical imperfections. The unweighted pair group method using average linkages was used to cluster the patterns (Priest & Austin, 1993; Sneath & Sokal, 1973; Vauterin & Vauterin, 1992). Each strain was tested only once.

DNA–DNA hybridization. DNA for DNA–DNA hybridization was extracted and purified as described by Marmur (1961). The levels of DNA binding were determined spectrophotometrically from the initial renaturation rates as described by De Ley *et al.* (1970). The experiments were carried out in 2 × SSC (SSC is 0·15 M NaCl, 0·015 M sodium citrate) at 70·9 °C, a temperature calculated from the G+C mol % as described by De Ley (1970). Each figure is the mean of triplicate experiments.

Ribotyping. Ribotyping was performed following the method described previously (Austin *et al.*, 1995). DNA was extracted from overnight cultures in tryptic casein soy broth supplemented with 2 % (w/v) NaCl, using the AutoGen automate. DNA (2–5 mg) was digested with *Mlu*I (Boehringer Mannheim). Restriction fragments were separated by electrophoresis in 0·8 % agarose gels (Appligene) in TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA; pH 8·3) buffer and subsequently transferred (VacuGene System; Pharmacia-LKB) and fixed (80 °C for 15 min) to nylon membranes (Hybond-N; Amersham International). Hybridization with an acetylaminofluorene-labelled rRNA probe (Eugentec) and subsequent immunoenzymic revelation of hybridized fragments by the application of mouse anti-acetylaminofluorene monoclonal antibodies followed by phosphatase-conjugated anti-mouse antibody, and using nitro blue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) as substrates, was performed as described by Grimont *et al.* (1989) and Austin *et al.* (1995). Alternatively, the method described by Pedersen & Larsen (1993) was used. The type cultures were tested several times in various combinations, always with identical results, apart from a few cases of incomplete digestion, whereas other strains tested were run only once.

Plasmid profiling and restriction analysis of plasmids. Bacteria were propagated overnight at 20 °C in Luria–Bertani broth (Gibco) or veal infusion broth (Difco) supplemented with 0·5 % (w/v) NaCl. Plasmid DNA was extracted by the method of Kado & Liu (1981), separated by gel electrophoresis in 0·6–1·0 % agarose gels (SeaKem GTG; FMC BioProducts) in TAE buffer, pH 8·0 (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA), stained with ethidium bromide (2 mg ml^{-1}) and photographed under UV light. Restriction analysis was carried out as described by Pedersen *et al.* (1996a). Briefly, plasmid DNA was extracted by the method of Olsen (1990) and digested with *Hin*dIII, *Bam*HI or *Eco*RI according to the manufacturer's instructions (Promega). Fragments were separated on 1·0 % agarose gels and stained with ethidium bromide or SYBR Green DNA Gel Stain I (FMC BioProducts). *Hin*dIII-digested lambda DNA was used as molecular mass markers. All plasmid experiments were run at least in triplicate.

RESULTS

AFLP genomic fingerprinting

After numerical analysis of the AFLP banding patterns of the strains, using S_{AB}, nine clusters were delineated

at 50% (Fig. 1). At this level, the *Vibrio* type strains belonged to separate clusters, except *V. harveyi* and *V. carchariae*, which clustered together.

The type strains of *V. fischeri*, *V. damselae*, *V. pelagius*, *V. mediterranei*, *V. proteolyticus*, *V. natriegens*, *V. furnissii*, *V. vulnificus*, *V. nereis*, *V. splendidus*, *V. diazotrophicus* and *V. fluvialis* each constituted single strain groups.

Cluster 1 ($n = 36$) contained both the type strains of *V. harveyi* (VIB 295^T) and *V. carchariae* (VIB 286^T). This cluster was considered to contain the bona fide *V. harveyi* strains. However, only 52% (24/46) of the strains that were received as *V. harveyi* or *V. carchariae* clustered with these type strains. The other strains received as *V. harveyi* or *V. carchariae* grouped in clusters 2, 3, 4 and 7, or together with the type strains of *V. campbellii* (cluster 5) or *V. alginolyticus* (cluster 9). Strains in cluster 1 were isolated worldwide and originated from fish (teleost and elasmobranch fish) ($n = 27$), shellfish ($n = 4$) and environmental sources ($n = 5$). Cluster 1 also contained misidentified *V. alginolyticus* and *Vibrio anguillarum* strains and strains received as unidentified *Vibrio* sp.

Cluster 2 ($n = 5$), containing strains from South America and Europe, originated from fish, shellfish and environmental sources. These strains were received as *V. harveyi*/*V. carchariae*, *V. alginolyticus* or *Vibrio* sp.

Cluster 3 ($n = 8$) contained three strains received as *V. harveyi*, two as *Vibrio* sp. Zoea and three as *Vibrio* sp. One strain was from sea bass, whereas remaining strains were from environmental sources ($n = 4$), shrimp ($n = 1$) or unknown ($n = 2$). The strains came from South America, Hawaii, Asia and Europe.

The strains in cluster 4 originated from shellfish ($n = 8$) and fish ($n = 1$). They had been isolated in South America, Asia and Africa. Three of the strains in this cluster were originally identified as *V. vulnificus*. However, the type strains of *V. vulnificus* clustered separately from these three strains and had a clearly different AFLP banding pattern.

Cluster 5 ($n = 5$) contained the *V. campbellii* type strain (VIB 285^T) and two other *V. campbellii* strains from Hawaii, together with two *V. harveyi*-like strains originating from oysters in Spain, whereas cluster 6 included only two Spanish fish isolates, previously identified as *V. parahaemolyticus* and *V. nereis*.

Strains in cluster 7 ($n = 7$) came from Australia, South America and the Mediterranean Sea, and included isolates previously identified as *V. harveyi*, *V. carchariae* or *V. anguillarum*. Strains in this cluster originated from fish, shellfish and water.

Clusters 8 ($n = 2$) and 9 ($n = 3$) contained the type strain of *V. parahaemolyticus* and *V. alginolyticus*, respectively, together with strains received as *V. vulnificus*, *V. carchariae* and Vibrio sp. The isolates came from different parts of the world.

Unnamed *Vibrio* strains were scattered over clusters 1, 2, 3, 4, 7 and 9.

DNA–DNA hybridization

By DNA–DNA hybridization, the DNA binding between the *V. harveyi* and *V. carchariae* type strains was calculated to be 88%, whereas the DNA binding between the *V. harveyi* and *V. campbellii* type strains was 40%.

Ribotyping

The ribotypes of the *V. harveyi* and *V. carchariae* type strains were almost identical, sharing most of the bands (Fig. 2). A second strain received as *V. carchariae*, VIB 351, had a ribotype pattern that was very similar but not quite identical to those of these type cultures. Also, the type strain of *V. campbellii* shared a number of bands with those of *V. harveyi* and *V. carchariae*. In contrast, the ribotype patterns of the other closely related species, *V. parahaemolyticus*, *V. alginolyticus*, *V. proteolyticus* and *V. natriegens*, were clearly distinct from the pattern of *V. harveyi* and *V. carchariae*. The ribotype patterns of the tested strains belonging to AFLP cluster 1 showed some diversity. The unclustered strain VIB 648, between AFLP clusters 1 and 2, had a ribotype pattern that was indistinguishable from that of VIB 660 from AFLP cluster 1 and, likewise, the ribotype pattern of strain VIB 646 from AFLP cluster 2 was almost identical to that of VIB 651 from AFLP cluster 1.

Plasmids

In general, profiles were heterogeneous, with plasmid sizes ranging from 3·0 to approximately 150 kb. However, some plasmids showed considerable homology, as judged from the restriction analysis using three different restriction enzymes, *Bam*HI, *Hin*dIII and *Eco*RI. Some *V. harveyi* strains isolated from the Mediterranean area carried a plasmid varying in size from 70 to approximately 95 kb. This plasmid was detected in 16 strains belonging to the bona fide *V. harveyi* AFLP cluster 1. Plasmids of similar size were found outside this cluster, but these plasmids were all unrelated to the 70–95 kb plasmid found in AFLP cluster 1, as judged from their restriction patterns. Likewise, six of these strains of the AFLP cluster 1 additionally carried a plasmid of 40–50 kb with a high degree of homology, as judged from their restriction patterns, whereas plasmids of similar size in other strains both in and outside AFLP cluster 1 were unrelated to this plasmid. Finally, the 135 kb plasmid of strain 651 (AFLP cluster 1), the 150 kb plasmid of strain VIB 648 (unclustered strain between AFLP clusters 1 and 2) and the 130 kb plasmid of VIB 646

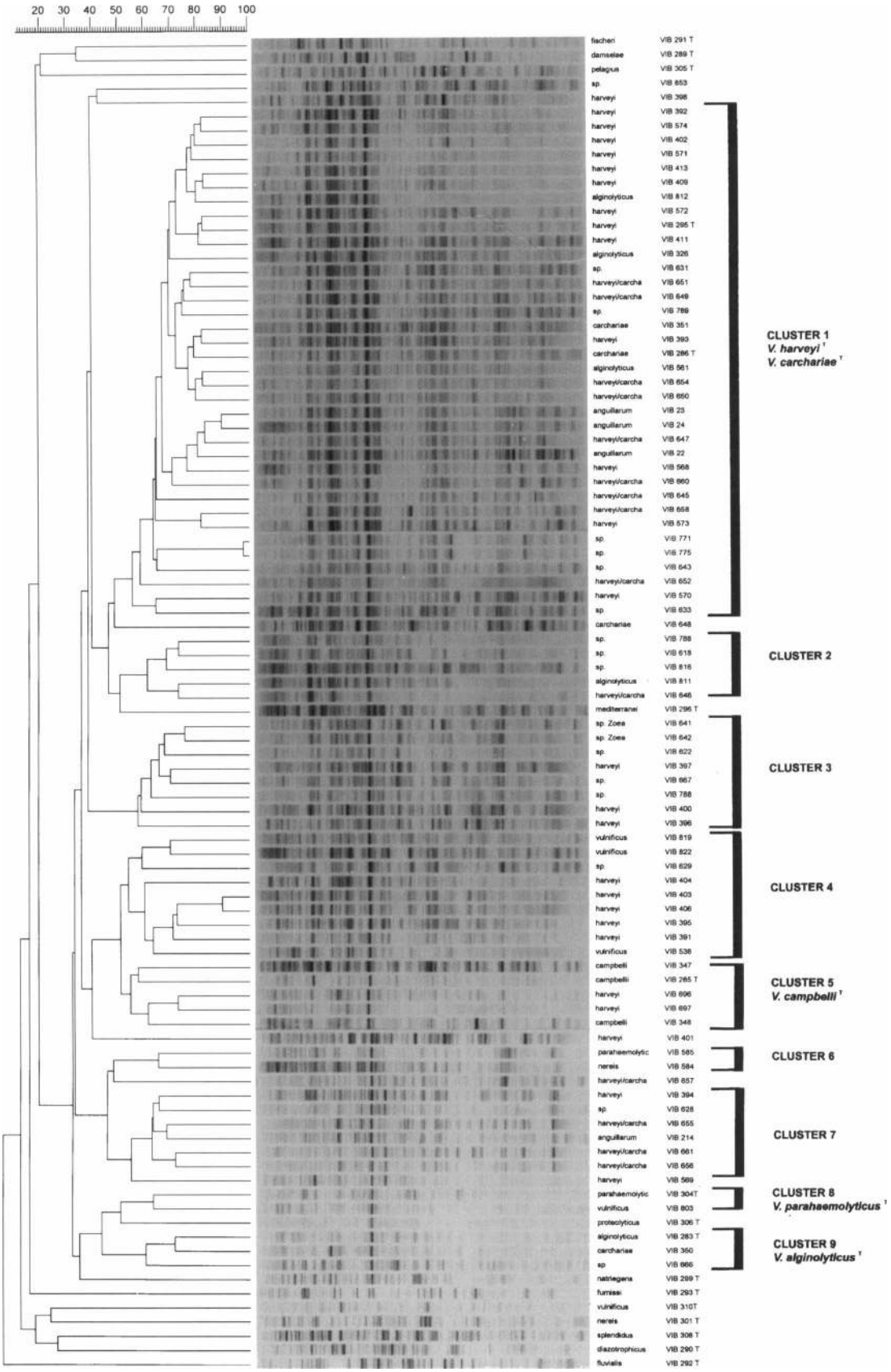

Fig. 1. AFLP patterns and the derived dendrogram of *V. harveyi* strains, related organisms and *Vibrio* type strains.

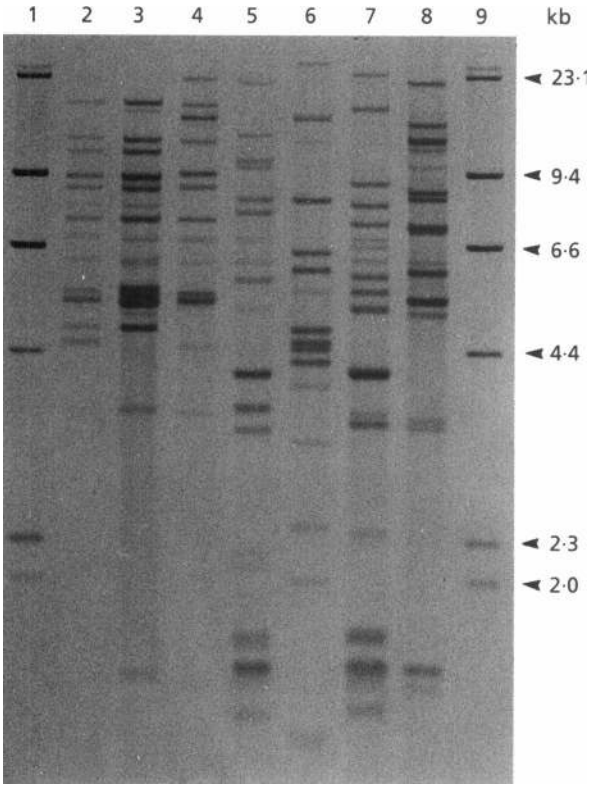

Fig. 2. Ribotype patterns of the type strains of *V. harveyi* (lane 2), *V. carchariae* (lane 3), *V. campbellii* (lane 4), *V. parahaemolyticus* (lane 5), *V. proteolyticus* (lane 6), *V. alginolyticus* (lane 7) and *V. natriegens* (lane 8). Lanes 1 and 9, *Hin*dIII-digested λ DNA as molecular mass markers.

(AFLP cluster 2) had similar, although not identical, restriction patterns.

DISCUSSION

This study has sought to re-examine the phenotypic and genotypic heterogeneity within, and relationship between, *V. harveyi* and *V. carchariae*. For this purpose, bacterial isolates were collected from a range of geographical locations, hosts and environmental sources, and subjected to investigation.

AFLP

AFLP is a reliable and reproducible genomic fingerprinting technique that has been applied in recent taxonomic studies (Huys *et al.*, 1996; Janssen *et al.*, 1996; Verdonck *et al.*, 1995). In these studies, numerical analyses of the AFLP banding patterns not only confirmed the genomic groups in *Xanthomonas*, *Aeromonas* and *Vibrio* which were formed on the basis of DNA–DNA hybridization and/or ribotyping, but also allowed the differentiation of individual strains of a species. In the present study, AFLP fingerprinting was used successfully to differentiate between strains in the collection. Strains that were biochemically characterized as *V. harveyi* or *V. carchariae* showed a marked genotypic heterogeneity. Some of the isolates were reidentified on the basis of their AFLP banding patterns as *V. alginolyticus*, *V. parahaemolyticus* or *V. campbellii*. The type strains of *V. harveyi* and *V. carchariae* were both found in AFLP cluster 1. This observation indicates that these two species cannot be distinguished genotypically and consequently are subjective synonyms. The AFLP banding patterns of the type strains of *V. mediterranei*, *V. campbellii*, *V. alginolyticus*, *V. proteolyticus*, *V. natriegens* and *V. parahaemolyticus* were more similar to the *V. harveyi* group than those of the other type strains included. In the AFLP dendrogram, *V. mediterranei* and *V. campbellii* had the highest similarity with the *V. harveyi* cluster. The type strain of *V. mediterranei* was positioned as an unclustered single strain between clusters 2 and 3, whereas the *V. campbellii* type strain was found in cluster 5 together with other *V. campbellii* strains. Thus cluster 1 is considered to be *V. harveyi sensu stricto*, cluster 5 *V. campbellii*, cluster 8 *V. parahaemolyticus* and cluster 9 *V. alginolyticus*, whereas the taxonomic position of clusters 2, 3, 4, 6 and 7, which did not contain any type strains, at present remains unclear.

DNA–DNA hybridization

The results obtained by AFLP were supported by the DNA–DNA hybridization studies. The DNA–DNA hybridization results in the present study showed 88% DNA binding between the *V. harveyi* and *V. carchariae* type strains, indicating that these two strains should be considered to belong to the same species. The homology between the *V. harveyi* and *V. campbellii* type strains was considerably lower, 40%. Reichelt *et al.* (1976) found a close relationship between *V. harveyi* and *V. campbellii* (65%) and *V. alginolyticus* and *V. parahaemolyticus* (65%). The relationship between these two groups was approximately 55%, and the relationship of the two groups to *V. natriegens* was approximately 50 and 40%, respectively. *V. vulnificus* was more distant: the genetic relationship of *V. vulnificus* to these five species was 30–35%. These DNA–DNA homologies perfectly reflect the AFLP dendrogram (Fig. 1) in the present study. Pujalte *et al.* (1992) found 0% relative DNA binding of *V. mediterranei* to *V. campbellii* using the S1 nuclease method, 0 and 14% relative binding to *V. carchariae* using the S1 nuclease and the hydroxyapatite methods, respectively, and 15% relative binding to *V. harveyi* using the hydroxyapatite method. These three species therefore seem to have the same level of genetic distance from *V. mediterranei*. In a very recent report by Ishimaru & Muroga (1997), the DNA–DNA homology between *V. harveyi* and *V. carchariae* was found to be 87–93%, whereas the DNA homology of

these species to *V. campbellii* was 45–50%. These results are very close to those of the present study. The *Vibrio* sp. Zoea, described as the cause of mortality of the zoeal state of swimming crab (*Portunus trituberculatus*), showed 46–51% homology to *V. harveyi*, *V. carchariae* and *V. campbellii* but was concluded to belong to a new species (Ishimaru & Muroga, 1997). In the present study, the two *Vibrio* sp. Zoea strains 86Z-1 and 87Z-1 both belonged to the AFLP cluster 3 with uncertain taxonomic position.

Ribotyping

The ribotyping data supported the AFLP and DNA–DNA hybridization results. The *V. harveyi* and *V. carchariae* type strains shared most bands and did not deviate more from each other than other strains tested from AFLP cluster 1. The type strain of *V. campbellii* shared some bands with the *V. harveyi* and *V. carchariae* type strains, whereas the patterns of the closely related species *V. alginolyticus*, *V. parahaemolyticus* and *V. natriegens* were clearly distinct from this pattern (Fig. 2). Also, the pattern of the *V. mediterranei* type strain deviated very clearly from that of *V. harveyi* and *V. carchariae*.

Other authors have compared rRNA sequences of different *Vibrio* species. Kita-Tsukamoto *et al.* (1993) found the partial 16S rRNA sequences of *V. harveyi* and *V. carchariae* to be more than 99% identical, and the type strains of *V. harveyi* and *V. carchariae*, *V. parahaemolyticus*, *V. alginolyticus* and *V. campbellii* all had sequences that were very closely related. Aznar *et al.* (1994), Dorsch *et al.* (1992), Ruimy *et al.* (1994) and Wiik *et al.* (1995) found a high degree of homology of 16S rRNA sequences of *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. campbellii*, *V. natriegens* and *V. proteolyticus*, thus forming the core of the genus *Vibrio* together with *V. vulnificus* (Dorsch *et al.*, 1992). The highest homology was found between *V. harveyi* and *V. alginolyticus* (99·17%) (Aznar *et al.*, 1994) and between *V. alginolyticus* and *V. campbellii* (99·9%) (Wiik *et al.*, 1995). These sequence similarities almost correspond to that found between *V. anguillarum* serogroups O1 and O2 (Wiik *et al.*, 1995). *V. harveyi* and *V. carchariae* were not included in these studies. In their study of small-subunit rRNA sequences, Ruimy *et al.* (1994) found a 0·77% dissimilarity (genetic distance) between *V. harveyi* and *V. carchariae*, 0·93% between *V. carchariae* and *V. campbellii*, but only 0·54% between *V. harveyi* and *V. campbellii*. This seemed to indicate that *V. harveyi* is genetically more closely related to *V. campbellii* than to *V. carchariae* (Ruimy *et al.*, 1994).

V. carchariae and *V. harveyi* are frequently isolated together from sharks or from the environment (Grimes *et al.*, 1985, 1993), and numerical taxonomy based on biochemical characters has shown that they cluster together with a high similarity. However, in the literature, a set of characters by which they can be distinguished has never been published. In a study by Grimes *et al.* (1993), 21 *V. harveyi* and three *V. carchariae* strains clustered together. However, these authors were able to distinguish between the two species at a high level of similarity and it was indicated that there may be differences between them on the basis of their utilization of certain compounds as sole carbon sources. Alsina & Blanch (1994) also recorded that *V. harveyi* and *V. carchariae* were different in their utilization of melibiose and arabinose as sole carbon sources. Urease production is mentioned as an important trait for virulence of both *V. carchariae* and *V. damselae* but some *V. harveyi* isolates have also been described as urease-positive (Bryant *et al.*, 1986a, b; Grimes *et al.*, 1985, 1993).

On the basis of well-chosen characters, Ortigosa *et al.* (1989) found two groups within *V. harveyi* – one group clustering close to *V. alginolyticus* and *V. parahaemolyticus* and a second clearly distinct from these. This second group mainly differed by being positive for luminescence, arbutin and alginate, negative for ornithine decarboxylase and sucrose, and variable for lactose and urease. Unfortunately, the *V. carchariae* type strain was not included in the study.

The close phenotypic relationship between *V. harveyi*, *V. carchariae*, *V. alginolyticus*, *V. parahaemolyticus* and *V. proteolyticus* was also found by Baumann *et al.* (1984), Bryant *et al.* (1986a, b) and West *et al.* (1986). These authors concluded that biochemical criteria are not always sufficient to distinguish between these species because they have too many variable characters. In a very comprehensive study by Bryant *et al.* (1986a, b), the type strains of *V. harveyi*, *V. campbellii*, *V. alginolyticus* and *V. parahaemolyticus* grouped close together, *V. harveyi* and *V. campbellii* forming a close group and *V. alginolyticus* and *V. parahaemolyticus* another. Unfortunately, *V. harveyi* or *V. carchariae* strains were not included in any of these studies.

One strain received as *V. carchariae* (VIB 350) was reidentified as *V. alginolyticus*. This strain clearly clustered together with the other *V. harveyi* and *V. carchariae* strains in the original description of this isolate (Grimes *et al.*, 1993). We therefore doubt the authenticity of our subculture of VIB 350.

Plasmids

Some of the *V. harveyi* isolates contained one or two plasmids, approximately 40–50 and 70–80 kb, respectively, showing homology by restriction analysis. These isolates all fell into the AFLP cluster 1. They were all isolated from diseased fish (sea bass, sea bream or turbot). Additionally, they were all isolated from the Mediterranean area. Such plasmids that, within a species or a sub-group of a species, display homology have also been detected in other *Vibrio* species, e.g. *V. vulnificus* (Biosca *et al.*, 1997), *Vibrio ordalii* (Pedersen *et al.*, 1996a) and *V. anguillarum* (additional to the pJM1 plasmid) (Pedersen *et al.*, 1996b). However, other strains also carried plasmids. In general, there

was considerable diversity in the size of these plasmids. Only a single report on the presence of plasmids in *V. harveyi* has appeared so far: McCall & Sizemore (1979) found that some strains carried a plasmid of 38 MDa that encoded a bacteriocin. Whether this plasmid was related to the ones described in the present study is unknown. An important observation was that three other strains, VIB 651, VIB 648 and VIB 646, shared a plasmid with some homology. These three strains all came from the same aquarium, but while two of the strains were from water samples, the third was isolated in pure culture from the liver of a dead shark, and thus had caused septicaemia in the shark. VIB 651 belonged to AFLP cluster 1, VIB 646 to AFLP cluster 2, and the third, VIB 648, was the unclustered strain between clusters 1 and 2. Additionally, these three strains had almost identical ribotype patterns. These results strongly indicate that AFLP clusters 1 and 2 and the unclustered VIB 648 should be considered to be *V. harveyi*.

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