Sequence Determination of rRNA Genes of Pathogenic *Vibrio* Species and Whole-Cell Identification of *Vibrio vulnificus* with rRNA-Targeted Oligonucleotide Probes

R. AZNAR,* W. LUDWIG, R. I. AMANN, AND K. H. SCHLEIFER

Lehrstuhl für Mikrobiologie, Technische Universität München, D-80290 Munich 2, Germany

A comparative analysis of seven new 16S rRNA gene sequences of pathogenic *Vibrio* species with previously published vibrio sequences confirmed that *Vibrio vulnificus* represents a group that is not closely related to the core organisms of the genus *Vibrio*. In addition, we found that *V. vulnificus*, *Listonella* (*Vibrio*) *anguillarum* and *Vibrio diazotrophicus* branch off separately from the core group. A comparison of the 16S rRNA gene sequences of *V. vulnificus* strains belonging to biotypes 1 and 2 revealed that the sequences of all but four biotype 1 strains were identical to each other but slightly different (17 bases) from the sequences of the rest of the *V. vulnificus* strains investigated. In addition, the sequences of variable regions of the 23S rRNA genes of *Vibrio fluvialis*, *Vibrio furnissii*, *Vibrio harveyi*, *Vibrio cholerae*, and *V. vulnificus* C7184 and TW1 were determined, aligned, and compared with all available bacterial 23S rRNA sequences in order to search for specific target sites. As a result, four oligonucleotide probes specific for *V. vulnificus* were synthesized, and the specificities of these probes were evaluated by dot blot hybridization to membrane-bound RNAs from 21 *V. vulnificus* strains, 13 strains belonging to other *Vibrio* species, 61 strains belonging to species that are members of the alpha, beta, and gamma subclasses of the *Proteobacteria*, and 3 eucaryotic microorganisms. Two probes hybridized with all of the *V. vulnificus* strains tested, and the other two probes distinguished *V. vulnificus* biotype 1 strains from all other organisms. In situ identification of *V. vulnificus* by using tetramethylrhodamine- or fluorescein-labelled oligonucleotides is now possible.

The first description of the taxon that is designated *Vibrio vulnificus* was published by Baumann et al. (5); in a taxonomic study of marine bacteria, these authors reported that a group designated C2 could be clearly distinguished phenotypically. Subsequently, in 1976 Reichelt et al. (25) performed DNA-DNA hybridization studies whose results showed that this taxon was a separate species, which was then named *Beneckeia vulnifica*. The present name, *V. vulnificus*, was revived by Farmer in 1980 (13) to describe a group of lactose-positive *Vibrio* strains which were phenotypically and genetically distinct from other *Vibrio* species (31). These organisms are common inhabitants of estuarine and marine waters, and they can be isolated under a wide range of salinity and temperature conditions from oysters, clams, mussels, and fish, as well as from sediment and plankton. Recent studies of the incidence of *V. vulnificus* in the United States have shown that it is present along the Gulf Coast, the Atlantic Coast, and the West Coast, and several cases of shellfish-associated *V. vulnificus* infections have been reported (23). The disease syndromes caused by *V. vulnificus* include septicemia and gastroenteritis following ingestion of shellfish and wound infections derived from contact with shellfish or the marine environment. In immunodepressed individuals the infections can be fatal. Stelma et al. (29) found no significant differences in virulence between clinical and environmental isolates. The species *V. vulnificus* also includes strains pathogenic for eels which have been responsible for severe recurrent epizootic outbreaks in cultured eels and are referred to as biotype 2 strains (7, 30).

Since *V. vulnificus* has become a public health problem, as well as a major obstacle in aquaculture, a rapid and effective detection method is needed in order to monitor its presence in

the environment, especially since biotype 2 strains have been isolated only from diseased eels.

Nucleic acid probes can be used for rapid and specific identification of microorganisms (28). In 1987, Morris et al. (21) described a DNA probe for the cytotoxin-hemolysin gene of *V. vulnificus*. Later, oligonucleotide primers directed to the gene sequence were used in a PCR in attempts to identify *V. vulnificus* in artificially contaminated oysters (16) and to detect culturable and nonculturable *V. vulnificus* cells (8). However, this approach was not successful, and the authors discussed the possibility that the cytotoxin-hemolysin gene was lost. Such losses do not occur when DNA probes directed against rRNAs are used. rRNA molecules are essential constituents of all living organisms and are present in growing cells in very high copy numbers. rRNA-targeted probes are excellent tools for rapid in situ identification of whole cells (2, 11) and for colony hybridization (6, 15).

The aims of this study were to investigate the phylogenetic relationships of some pathogenic *Vibrio* species and to design rRNA-targeted oligonucleotide probes specific for *V. vulnificus*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Twenty-one *V. vulnificus* biotype 1 and 2 strains were used in this study. The *V. vulnificus* biotype 1 strains included the type strain of the species, ATCC 27562, clinical isolates C7184, L-180, Vv11, UHM1, and 374, and four wild strains recovered either from gills of European eels (*Anguilla anguilla*) or from the tank water of an eel farm. The *V. vulnificus* biotype 2 strains included reference strains NCIMB 2136, NCIMB 2137, NCIMB 2138, and ATCC 33149 and six isolates recovered from internal organs of European eels (*A. anguilla*). All wild strains were isolated from an eel farm during different epizootic outbreaks of vibriosis in Spain (3, 7). We also studied

* Corresponding author. Present address: Departamento de Microbiología, Universitat de Valencia, Av. Dr. Moliner, 50, E-46100 Burjassot, Valencia, Spain.

the type strains of *Vibrio harveyi*, *Vibrio furnissii*, *Vibrio fluvialis*, and *Vibrio cholerae* and one *V. cholerae* non-O1 environmental isolate. In addition, 77 strains (13 strains belonging to other *Vibrio* species, 61 strains belonging to species that are members of the alpha, beta, and gamma subclasses of the *Proteobacteria*, and 3 eucaryotic microorganisms) were used to test the specificity of oligonucleotide probes. All of these strains and their sources are listed in Table 1.

The *Vibrio* strains were grown in brain heart infusion medium (Difco) supplemented with 0.5% (wt/vol) NaCl and 1.5% agar (for solid medium) for 24 h. The clinical strains were incubated at 37°C, and the environmental isolates were incubated at 25°C. For in situ hybridization experiments, in order to maximize the cellular rRNA content, cells were harvested at the mid-logarithmic phase by centrifugation in an Eppendorf Microfuge at 5000 × *g* for 5 min. The pelleted cells were washed once and then resuspended in phosphate-buffered saline (PBS) (130 mM sodium chloride, 10 mM sodium phosphate; pH 7.2).

Cell fixation. Cells were fixed in a paraformaldehyde solution and were stored in a 1:1 mixture of PBS and 96% ethanol at -20°C as described previously (1). The fixed cells were spotted onto gelatin-coated slides, dried at 37°C for 2 h, and dehydrated in 50, 80, and 96% (vol/vol) ethanol (3 min each).

PCR and sequence determination. In vitro amplification and direct sequencing of 16S and 23S rRNA genes were performed as described previously (19).

Purification of nucleic acids. Genomic DNA was extracted by the guanidium thiocyanate method of Pitcher et al. (24) and was further purified by RNase and proteinase K treatments as described by Sambrook et al. (27). RNA was isolated as follows. Cells from 2-ml overnight cultures were harvested by centrifugation and suspended in 400 µl of 0.01 M Tris-HCl-0.001 M EDTA (pH 8.0). A 50-µl portion of 25% sodium dodecyl sulfate (SDS) and 450 µl of a chloroform-phenol-isoamyl alcohol (24:25:1) mixture were added, and samples were incubated at 60°C for 10 min. After centrifugation at 12,000 × *g*, for 10 min, the supernatants were extracted with phenol. The nucleic acids were precipitated by adding 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate.

Immobilization of RNA onto membranes. RNA samples (1.5 µg) were denatured at 60°C for 10 min in 100 µl of a buffer containing 50% formamide, 4% formaldehyde, 0.02 M 3-(*N*-morpholino)propanesulfonic acid, 0.005 M sodium acetate, and 0.001 M EDTA. The denatured RNA samples were applied to Qiabran nylon membranes (Diagen, Düsseldorf, Germany) according to the manufacturer's specifications by using a Minifold dot blot apparatus (Schleicher & Schuell, Dassel, Germany). The membranes were air dried, and the nucleic acids were immobilized on the membranes by heating the preparations at 80°C for 30 min.

Dot blot hybridizations with nonradioactively labelled probes. Hybridizations and hybrid detection by chemiluminescence were carried out by using components of a DNA labelling kit, anti-digoxigenin antibodies, and AMPPD [3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)phenyl-1,2-dioxetane] obtained from Boehringer, Mannheim, Germany. Membranes were prehybridized in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0), 5% blocking reagent, 0.1% *N*-lauroyl-sarcosine, and 0.02% SDS for 1 h. The hybridization solution contained 0.9 M NaCl, 10 mM Tris-HCl (pH 7.2), 0.01% SDS, 0.1% *N*-lauroyl-sarcosine, 4% blocking reagent, and 50 ng of digoxigenin-labelled oligonucleotide. The hybridization mixtures were incubated overnight at 46°C. Subsequently, the membranes were washed twice in a solution containing 0.9 M NaCl, 10 mM

Tris-HCl, 0.01% SDS, and 0.1% *N*-lauroyl-sarcosine at the hybridization temperature. Hybridization was detected as described by the manufacturer.

Colony hybridization. Vibrios were grown on Qiabran membranes (Diagen) layered on brain heart infusion agar under the conditions described above. To lyse the cells and to denature the DNA, the membranes were incubated for 10 min on filter paper saturated with a high-salt denaturation solution containing 0.5 M NaOH and 1.5 M NaCl with the colony side up. Then the membranes were placed briefly on dry filter paper to remove the excess fluid. The preparations were neutralized by placing the membranes on filter paper saturated with neutralizing solution containing 0.5 M Tris-HCl (pH 7.4) and 1.5 M NaCl for 5 min, and then the membranes were placed on filter paper saturated with 2× SSC for 5 min. The bacterial residues were removed by washing the membranes in 2× SSC. Then, the residues were air dried, and their DNAs were immobilized by incubating the preparations at 80°C for 30 min. Hybridization experiments were performed as described above.

In situ hybridization. The complete 23S rRNA sequence of *V. vulnificus* and partial sequences of the 23S rRNA genes of *V. vulnificus* biotype 1 strains TW1 and C7184, *V. fluvialis* NCTC 11327^T (T = type strain), *V. furnissii* ATCC 35016^T, *V. cholerae* CECT 514^T, and *V. harveyi* NCIMB 1280^T were aligned and compared with all available bacterial 23S rRNA sequences in order to search for specific target sites. Oligonucleotides complementary to selected regions were synthesized with a C6-TFA aminoink [6-(trifluoroacetyl-amino)-hexyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite] (MWG Biotech, Ebersberg, Germany) at the 5' end. The sequences and target sites used are shown in Table 2. In addition, probe EUB338, a oligonucleotide complementary to a region of 16S rRNA conserved in members of the domain *Bacteria*, was used as a positive control for hybridizations (1). Labelling with tetramethylrhodamine-5-isothiocyanate (TRITC; Molecular Probes, Eugene, Oreg.) or 5(6)-carboxy-fluorescein-*N*-hydroxysuccinimide ester (FLUOS; Boehringer) was performed as described previously (1). An aliquot (8 µl) of hybridization solution (0.9 M NaCl, 20 mM Tris-HCl [pH 7.2], 0.01% SDS, 25 ng of probe) was added to each well of a microscopic slide, and the preparation was incubated for 1.5 h at 46°C in an isotonicity equilibrated humidity chamber. The probe was removed from the slide with 2 ml of washing solution (0.9 M NaCl, 20 mM Tris-HCl [pH 7.2], 0.01% SDS) and immediately immersed in 50 ml of washing solution at 48°C for 20 min. The slides were rinsed briefly with distilled water, air dried, and mounted in Citifluor (Citifluor, Ltd., London, United Kingdom).

Fluorescence was detected with a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) fitted for epifluorescence microscopy with a 50-W mercury high-pressure bulb and Zeiss filter sets 09 and 15. Color micrographs were prepared by using Kodak Ektachrome P1600 color reversal film. The exposure times used were 0.01 to 0.03 s for phase-contrast micrographs and 10 to 60 s for epifluorescence micrographs.

Data analysis. The new 16S rRNA primary structures were introduced into an alignment of 1,200 homologous sequences on the basis of primary and higher-order structure similarities. Similarity values and phylogenetic distances (17) were calculated and alignment positions were selected on the basis of the degrees of evolutionary conservation by using the SEQDIS program (N. Stuckmann and W. Ludwig, unpublished data). Distance matrix, maximum-parsimony, and maximum-likelihood analyses were performed by using the NEIGHBOR (26) and DNAPARS programs of Felsenstein's PHYLIP package (14) and the fastDNAmI program of Olsen et al. (22), respec-

TABLE 1. Strains used in this study

Position ^a	Taxon	Strain ^b	Origin
A.1	<i>Rhodobacter capsulata</i>	LMG 2962	
A.2	<i>Paracoccus denitrificans</i>	LMG 4218	
A.3	<i>Pseudomonas diminuta</i>	LMG 2089	
A.4	<i>Rhodopseudomonas palustris</i>	DSM 123	
A.5	<i>Rhizobium meliloti</i>	DSM 30135	
A.6	<i>Magnetospirillum gryphiswaldense</i>	DSM 6361	
A.7	<i>Bradyrhizobium japonicum</i>	DSM 30131	
A.8	<i>Agrobacterium tumefaciens</i>	DSM 30205	
A.9	<i>Rhodospirillum rubrum</i>	DSM 467	
A.10	<i>Azospirillum halopreferens</i>	DSM 3675	
A.11	<i>Azospirillum brasiliense</i>	DSM 1690	
A.12	<i>Azospirillum amazonense</i>	DSM 2787	
B.1	<i>Leucothrix mucor</i>	DSM 2157	
B.2	<i>Shewanella putrefaciens</i>	LMG 2268	
B.3	<i>Acinetobacter calcoaceticus</i>	LMG 1046	
B.4	<i>Pseudomonas cepacia</i>	LMG 1222	
B.5	<i>Chromobacter violaceum</i>	LMG 1267	
B.6	<i>Alcaligenes faecalis</i>	LMG 1229	
B.7	<i>Alcaligenes eutrophus</i>	LMG 1199	
B.8	<i>Aquaspirillum metamorphum</i>	LMG 4338	
B.9	<i>Sphaerotilus natans</i>	LMG 7172	
B.10	<i>Comamonas testosteroni</i>	DSM 50244	
B.11	<i>Zoogloea ramigera</i>	LMG 4432	
B.12	<i>Thiobacillus acidophilus</i>	DSM 700	
C.1	<i>Pseudomonas pseudoalcaligenes</i>	LMG 1225	
C.3	<i>Pseudomonas alcaligenes</i>	LMG 1224	
C.5	<i>Serratia marcescens</i>	DSM 30121	
C.6	<i>Proteus vulgaris</i>	DSM 30118	
C.7	<i>Erwinia carotovora</i>	DSM 30168	
C.8	<i>Enterobacter cloacae</i>	DSM 30054	
C.9	<i>Enterobacter aerogenes</i>	DSM 30053	
C.10	<i>Escherichia coli</i>	DSM 30083	
C.11	<i>Listonella (Vibrio) anguillarum</i>	NCIMB 2129	
C.12	<i>Aeromonas hydrophila</i>	LMG 2844	
D.1	<i>Pseudomonas putida</i>	LMG 2257	
D.2	<i>Myxococcus virescens</i>	DSM 2260	
D.3	<i>Myxococcus fulvus</i>	DSM 434	
D.4	<i>Cytophaga johnsonae</i>	LMG 1341	
D.5	<i>Flavobacterium ferrugineum</i>	DSM 30193	
D.6	<i>Propionibacterium freudenreichii</i>	DSM 4902	
D.7	<i>Pimelobacter simplex</i>	DSM 20130	
D.8	<i>Brevibacterium ketoglutamicum</i>	DSM 20165	
D.9	<i>Brevibacterium linens</i>	DSM 20425	
D.10	<i>Rhodococcus rhodochrous</i>	LMG 5365	
D.11	<i>Micrococcus luteus</i>	DSM 20030	
D.12	<i>Corynebacterium glutamicum</i>	DSM 20300	
E.1	<i>Streptococcus salivarius</i>	DSM 20560	
E.2	<i>Staphylococcus aureus</i>	DSM 20231	
E.3	<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	DSM 20069	
E.4	<i>Lactococcus lactis</i> subsp. <i>lactis</i>	DSM 20481	
E.5	<i>Lactobacillus casei</i>	DSM 20011	
E.6	<i>Enterococcus faecalis</i>	DSM 20478	
E.7	<i>Enterococcus faecium</i>	DSM 20477	
E.8	<i>Pectinatus frisingensis</i>	ATCC 33332	
E.9	<i>Clostridium stercorearium</i>	NCIMB 11754 ^T	
E.10	<i>Clostridium acetobutylicum</i>	DSM 792	
E.11	<i>Bacillus subtilis</i>	DSM 10	
E.12	<i>Bacillus cereus</i>	DSM 31	
F.8	<i>Aquaspirillum (Magnetospirillum) magnetotacticum</i>	DSM 3856	
F.9	<i>Saccharomyces cerevisiae</i>	DSM 70449	
F.10	<i>Saccharomyces carlsbergensis</i> (formerly <i>Saccharomyces uvarum</i>)	66 ^c	
F.11	<i>Hansenula anomala</i>	DSM 70255	
F.12	<i>Staphylococcus carnosus</i>	DSM 20501	
G.2	<i>Vibrio tubiashii</i>	NCIMB 1340 ^T	
G.3	<i>Vibrio carchariae</i>	NCIMB 12705 ^T	
G.4	<i>Vibrio damsela</i>	CECT 626 ^T	
G.5	<i>Listonella (Vibrio) anguillarum</i>	NCIMB 6 ^T	
G.7	<i>Vibrio vulnificus</i> biotype 2	E39 ^d	European eel

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TABLE 1—Continued

Position ^a	Taxon	Strain ^b	Origin
		E22 ^d	European eel
		E58 ^d	European eel
		E64 ^d	European eel
		E86 ^d	European eel
		E105 ^d	European eel
G.8		ATCC 33149	Japanese eel
G.9		NCIMB 2138	Japanese eel
		NCIMB 2137	Japanese eel
		NCIMB 2136	Japanese eel
G.10	<i>Vibrio vulnificus</i> biotype 1	TW1 ^d	Tank water
		E63 ^d	European eel
		E114 ^d	European eel
		E335 ^d	Glass eels
		E647 ^d	European eel
G.11		C7184 ^e	Human blood
G.12		ATCC 27562 ^T	Human wound infection
		L-180 ^e	Septicemia case
		VvL1 ^c	Fatal wound infection
		374 ^e	Septicemia case
		UMH1 ^c	Fatal wound infection
H.1	<i>Shewanella putrefaciens</i>	CECT 331 ^T	
H.2	<i>Aeromonas salmonicida</i> subsp. <i>salmonicida</i>	CECT 894	
H.4	<i>Vibrio mimicus</i>	NCTC 11435 ^T	
H.5	<i>Vibrio cholerae</i> O1	CECT 514 ^T	
	<i>Vibrio cholerae</i> non-O1	137 ^f	Freshwater
H.6	<i>Vibrio parahaemolyticus</i>	CECT 511 ^T	
H.7	<i>Vibrio pelagius</i>	ATCC 25916 ^T	
H.8	<i>Vibrio alginolyticus</i>	NCIMB 1903 ^T	
H.9	<i>Vibrio cincinnatiensis</i>	NCTC 12012 ^T	
H.10	<i>Vibrio furnissii</i>	ATCC 35016 ^T	
H.11	<i>Vibrio fluvialis</i>	NCTC 11327 ^T	
H.12	<i>Vibrio harveyi</i>	NCIMB 1280 ^T	

^a Position on the membrane used for the dot blot hybridization experiment (see Fig. 2).

^b Abbreviations: ATCC, American Type Culture Collection, Rockville, Md; CECT, Colección Española de Cultivos Tipo, Valencia, Spain; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; LMG, Belgian Coordinated Collections of Microorganisms, Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium; NCIMB, National Collection of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, Scotland; NCTC, National Collection of Type Cultures, London, United Kingdom.

^c Obtained from the Institut für Brauereitechnologie und Mikrobiologie, Technische Universität, Munich, Germany.

^d Environmental isolate kindly supplied by E. G. Biosca and M. Oritigosa, University of Valencia, Burjasot, Valencia, Spain.

^e Clinical isolate kindly supplied by J. D. Oliver, University of North Carolina, Charlotte.

^f Environmental isolate kindly supplied by C. Amaro, University of Valencia, Burjasot, Valencia, Spain.

tively. The topologies of the trees were evaluated by using different treeing methods and various data sets that differed with respect to selection of reference sequences and alignment positions.

Nucleotide sequence accession numbers. The 16S rRNA

sequences determined in this study have been deposited in the EMBL data base under accession numbers X76333, X76334, X76335, X76336, and X76337.

RESULTS

Phylogenetic relatedness of *V. vulnificus* biotypes and other *Vibrio* species. A comparison of the complete sequences of PCR-amplified 23S and 16S rRNA genes of *V. vulnificus* biotype 1 type strain ATCC 27562 and biotype 2 reference strain NCIMB 2138 revealed no differences between the sequences of the two strains. The partial 23S rRNA and complete 16S rRNA sequences of *V. vulnificus* biotype 1 strains TW1 and C7184 were identical (rRNA type B), but were slightly different (2 of 600 bases in the 23S rRNA and 17 bases in the complete 16S rRNA sequence) from the corresponding sequences (rRNA type A) of the two reference strains mentioned above. The complete sequences of the 16S rRNA genes of the *V. vulnificus* strains, *V. fluvialis* NCTC 11327^T, *V. furnissii* ATCC 35016^T, and *V. cholerae* CECT 514^T were determined and compared with previously published sequences of 10 reference species of the genus *Vibrio* (12).

A matrix of overall similarity values for the new and the

TABLE 2. Sequences of the oligonucleotide probes, specificities, and hybridization conditions^a

Probe	Sequence	Specificity (16S rRNA type)	Hybridization conditions	
			Temp (°C)	Formamide concn (%)
Vvu1	5'-CATAGAACATTGCCGAG-3'	A	46	40
Vvu2	5'-ACTCAATGATACTGGCTTA-3'	A, B	46	0
Vvu3	5'-ACCGTTCGTCTAACACAT-3'	A, B	46	25
Vvu4	5'-TCAAAGAACATTGCCGCA-3'	B	46	40

^a All probes were 23S rRNA targeted and were specific for *V. vulnificus*. They were able to separate biotype 1 strains into the following two groups corresponding to two different 16S rRNA sequences: type A, 16S rRNAs corresponding to *V. vulnificus* type strain ATCC 27562 (biotype 1) and *V. vulnificus* NCIMB 2138 (biotype 2) rRNAs; type B, rRNAs corresponding to *V. vulnificus* biotype 1 strains C7184 and TW1.

TABLE 3. Levels of 16S rRNA sequence similarity between *Vibrio* species

Taxon	% Similarity to:													
	<i>V. proteolyticus</i>	<i>V. parahaemolyticus</i>	<i>V. diazotrophicus</i>	<i>V. campbellii</i>	<i>V. alginolyticus</i>	<i>V. harveyi</i>	<i>V. natriegens</i>	<i>V. fluvialis</i>	<i>V. furnissii</i>	<i>V. vulnificus</i> (16S rRNA type A)	<i>V. vulnificus</i> (16S rRNA type B)	<i>V. anguillarum</i>	<i>V. cholerae</i>	<i>V. hollisae</i>
<i>E. coli</i>	89.32	88.96	90.61	89.46	89.52	89.82	90.20	90.09	89.95	89.82	89.96	89.32	90.28	89.04
<i>V. proteolyticus</i>		97.62		97.01	97.14	97.64	97.84	95.74	95.49	95.67	96.15	95.21	92.85	93.19
<i>V. parahaemolyticus</i>			95.20	96.41	96.47	97.10	97.43	96.44	96.13	96.51	95.83	94.82	92.47	93.07
<i>V. diazotrophicus</i>				95.41	95.54	95.88	96.08	96.29	95.04	96.70	96.77	96.37	94.29	93.12
<i>V. campbellii</i>					96.60	98.90	98.55	96.04	95.66	95.97	96.45	95.44	92.83	93.33
<i>V. alginolyticus</i>						99.17	98.75	96.11	95.72	96.04	96.45	95.51	93.03	93.46
<i>V. harveyi</i>							98.69	96.53	96.19	96.45	96.66	95.91	93.18	93.54
<i>V. natriegens</i>								96.86	96.66	96.72	97.21	96.04	93.30	93.80
<i>V. fluvialis</i>									98.50	97.98	97.59	95.58	94.98	93.33
<i>V. furnissii</i>										96.94	96.55	94.84	94.00	93.78
<i>V. vulnificus</i> (16S rRNA type A)											98.83	96.98	95.05	93.54
<i>V. vulnificus</i> (16S rRNA type B)												97.32	94.85	93.61
<i>V. anguillarum</i>													94.17	92.47
<i>V. cholerae</i>														90.85

previously published (12) vibrio sequences is shown in Table 3. The phylogenetic tree in Fig. 1 was based on the results of a maximum-likelihood analysis and was corrected by taking into account the results of distance and maximum-parsimony analyses of different data sets. In general, the topology of the tree was supported by the results of the majority of the different analyses. However, the short internode branches connecting the *V. fluvialis*-*V. furnissii*, *V. hollisae*, *V. diazotrophicus*, and *L. anguillarum*-*V. vulnificus* lines indicate that the relative branching order in this part of the tree is less significant.

In addition, partial sequences of the 23S rRNA genes of *V. vulnificus* biotype 1 strains TW1 and C7184, *V. fluvialis* NCTC 11327^T, *V. furnissii* ATCC 35016^T, *V. cholerae* CECT 514^T, and *V. harveyi* NCIMB 1280^T were determined. The fragments sequenced were homologous to positions 0 to 650, 1560 to 2040, and 2440 to 2750 of the *Escherichia coli* 23S rRNA (9) and included variable regions. The sequences were aligned and compared with all available bacterial 23S rRNA sequences in order to search for specific target sites.

Identification of *V. vulnificus* with rRNA-targeted oligonu-

FIG. 1. Phylogenetic tree showing the phylogenetic relationships of *V. vulnificus* and other *Vibrio* species.

cleotide probes. The sequences and designations of the oligonucleotide probes used and the hybridization conditions used are summarized in Table 2.

The specificity of the oligonucleotide probes was evaluated by performing dot blot hybridization experiments with membrane-bound RNAs from 21 strains of *V. vulnificus*, 13 strains belonging to other *Vibrio* species, 61 strains belonging to species that are members of the alpha, beta, and gamma subclasses of the *Proteobacteria*, and 3 eucaryotic microorganisms. The results of the dot blot hybridization experiments performed with the specific probes and with genomic DNAs from vibrios and other reference bacteria are shown in Fig. 2 and Table 4.

The oligonucleotide probes were also used in colony hybridization experiments. *V. vulnificus* strains, *V. fluvialis* NCTC 11327^T, *V. furnissii* ATCC 35016^T, *V. cholerae* CECT 514^T, and *V. harveyi* NCIMB 1280^T were grown on Qiabrane membranes layered on brain heart infusion agar as described above. Hybridization experiments performed with the *V. vulnificus*-specific probes labelled with digoxigenin produced results that were the same as the results obtained with radioactively labelled probes.

In situ identification of whole cells was performed by using the tetramethylrhodamine-labelled *V. vulnificus*-specific probes. Three *V. vulnificus*-specific probes (Vvu1, Vvu2, Vvu3) and fluorescein-labelled universal probe EUB338 (2) were tested by performing in situ hybridization experiments. Twenty-one *V. vulnificus* biotype 1 and 2 strains and strains of other *Vibrio* species were used in situ identification experiments. *Listonella (Vibrio) anguillarum* NCIMB 2129 was used as a control strain for hybridization experiments; this organism was negative for specific probes and positive for the universal probe. Mixtures containing *Listonella (Vibrio) anguillarum* (basonym, *Listonella anguillarum*) NCIMB 2129 and one of the *V. vulnificus* strains were in situ hybridized to every probe mentioned above and also simultaneously to EUB338 and every specific probe. When the fluorescein-specific filter set was used, both *V. vulnificus* and *Listonella (Vibrio) anguillarum* were green, but when the rhodamine-specific filter set was used, only *V. vulnificus* was red. Figure 3 and Table 4 show the results of the

FIG. 2. Dot blot hybridization experiments performed with crude RNAs from members of the alpha, beta, and gamma subdivisions of the *Proteobacteria* and three eucaryotic microorganisms, universal probe EUB338, and probes reacting with all *V. vulnificus* strains (probes Vvu2 and Vvu3) and with some of the strains (rRNA type A; see Table 2) (probe Vvu1). RNAs from 84 organisms were immobilized on membranes and arranged as indicated in Table 1. Positions C.2, C.4, G.1, G.6, and H.3 were empty.

color differential visualization experiments. Probes Vvu1 and Vvu2 hybridized poorly in situ and produced weak signals. In contrast, probe Vvu3 hybridized specifically to every *V. vulnificus* strain tested and produced strong hybridization signals (Fig. 3). The weak hybridization signals obtained with probes Vvu1 and Vvu2 may indicate that the target sites were not fully

accessible to the probes when the in situ cell hybridization technique was used. Nevertheless, these probes are excellent tools for analyzing extracted nucleic acids.

DISCUSSION

Seven different vibrio 16S rRNA gene sequences were determined. A careful comparative sequence analysis revealed that most of the *Vibrio* species formed a phylogenetically tight group. However, *V. vulnificus* and *Listonella (Vibrio) anguillarum* branched off separately from the main cluster. This finding is not in complete agreement with a phylogenetic tree inferred from a comparative analysis of 16S rRNA sequences obtained by reverse transcription of cDNAs from *Vibrio* species (12). According to the data of Dorsch et al. (12), *V. vulnificus* is more closely related to *V. parahaemolyticus* and belongs to the main cluster of *Vibrio* species, whereas *Listonella (Vibrio) anguillarum* represents an individual subline of descent. Our results are in good agreement with the results of comparative immunological studies of superoxide dismutases of vibrios (4). According to the superoxide dismutase data, *V. vulnificus* represents a group that is not closely related to the core organisms of the genus *Vibrio*. Our data show that *V. vulnificus* groups together with *Listonella (Vibrio) anguillarum* and *V. diazotrophicus*. The closer relationship of *V. vulnificus* and *V. parahaemolyticus* reported by Dorsch et al. (12) is not supported by the extended data set. Additional taxonomic studies will be necessary to decide whether the genus *Listonella* should be conserved and whether *V. vulnificus* should be transferred to this genus.

V. furnissii was originally described as a gas-producing biogroup of *V. fluvialis*. The two species are indistinguishable by conventional serological methods. This close relationship is confirmed by the high level of 16S rRNA similarity of the two species.

A previous comparative sequence analysis of 5S rRNAs of members of the family *Vibrionaceae* revealed a close relationship between *V. cholerae* and *V. vulnificus* (20). However, this

TABLE 4. Probe specificities for vibrios as determined by dot blot and/or in situ hybridization experiments

Strain(s)	Reactions with the following probes:			
	Vvu1	Vvu2	Vvu3	Vvu4
<i>V. vulnificus</i> biotype 1 strains				
ATCC 27562 ^T , VvL1, UMH1, 63, 335, 647	+	+	+	—
L-180, 374, C7184, TW1, E114	—	+	+	+
<i>V. vulnificus</i> biotype 2 strains	+	+	+	—
NCIMB 2138, NCIMB 2137, NCIMB 2136, ATCC 33149, E22, E39, E58, E64, E86, and E105				
<i>V. cholerae</i> O1 strain CECT 514 ^T	—	—	—	—
<i>V. cholerae</i> non-O1 strain 137	—	—	NT ^a	—
<i>V. fluvialis</i> NCTC 11327 ^T	—	—	—	—
<i>V. furnissii</i> ATCC 35016 ^T	—	—	—	—
<i>V. harveyi</i> NCIMB 1280 ^T	—	—	—	—
<i>Listonella (Vibrio) anguillarum</i>	—	—	—	NT
NCIMB 2129 and NCIMB 6 ^{Tb}				
8 other <i>Vibrio</i> strains ^b	—	—	—	NT
61 strains belonging to species that are members of the alpha, beta, and gamma subclasses of the <i>Proteobacteria</i> ^b	—	—	—	NT
3 eucaryotic microorganisms ^b	—	—	—	NT

^a NT, not tested.

^b The probe specificities of these microorganisms were determined by performing dot blot hybridization experiments.

FIG. 3. Whole-cell hybridization. Cells of *V. vulnificus* ATCC 27562^T and *Listonella (Vibrio) anguillarum* NCIMB 2129 were simultaneously hybridized with fluorescein-labelled probe Vvu3 and a tetramethylrhodamine-labelled probe specific for *L. anguillarum* (32). Identical fields were viewed by phase-contrast microscopy (A) and epifluorescence microscopy (B and C), using fluorescein-specific (B) and rhodamine-specific (C) filter sets. Bar = 10 μ m.

finding is not consistent with the phylogenetic relationship deduced from 16S rRNA data (12, 18; this study) and from a more recent 5S rRNA analysis (10). Both the 16S rRNA data and the more recent 5S rRNA data show that *V. cholerae* is not closely related to *V. vulnificus* and does not belong to the core group of vibrios, but is only distantly related to this group. On the basis of these results, it has been suggested that the species *V. cholerae* should be elevated to genus rank (18).

The phylogenetic position of *V. hollisae* has to be regarded as preliminary. The remarkable length of the *V. hollisae* branch may indicate that the genes of this organism evolved more

rapidly. Whereas the overall tree topology shown in Fig. 1 was stable as determined by the majority of analyses in which different treeing methods and a variety of data sets were used, the branching point of *V. hollisae* was rather unstable. In particular, distance matrix methods tend to place the *V. hollisae* branch at a lower position between *V. cholerae* and the other vibrios.

The topology of a phylogenetic tree is influenced by the composition of the underlying data set. The addition of new sequences obviously results in more phylogenetic information and in improved, often restructured phylogenetic trees. There are some significant differences between the phylogenetic tree inferred from a limited data set by Dorsch et al. (12) and our new tree (see above). Very recently, Kita-Tsukamoto et al. (18) determined the partial sequences of 16S rRNAs (600 of about 1,500 bases) from 50 marine bacteria, mainly members of the family *Vibrionaceae*. The phylogenetic tree constructed from their data differed remarkably from our tree. The deep branching of *V. cholerae* is supported by the partial sequence data, but the moderately closely related species cannot be distinguished from the more closely related species. For instance, the partial sequence data are not sufficient to show the close relationship of *Listonella (Vibrio) anguillarum* and *V. diazotrophicus* and their separation from the core group of vibrios. This example demonstrates that the full resolution power of comparative 16S rRNA analyses can be achieved only when complete or almost complete sequences are compared. Comparisons of partial sequences are not sufficient for fine resolution and may indicate wrong relationships.

The assignment of *V. vulnificus* strains to two biotypes (biotypes 1 and 2) defined by biochemical and immunological properties does not reflect the natural relationships of the strains. The close relationship of the type strain of the species (a biotype 1 strain) and the biotype 2 strains is corroborated by the identical 16S and 23S rRNA sequences of these organisms (rRNA type A), whereas the minor sequence differences (1.1%) among biotype 1 strains indicate that there is a higher degree of heterogeneity within this biotype.

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