Towards a Phylogeny of the Genus *Vibrio* Based on 16S rRNA Sequences

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The inter- and intragenetic relationships of the genus *Vibrio* were investigated by performing a comparative analysis of the 16S rRNAs of 10 species, including four pathogenic representatives. The results of immunological and 5S rRNA studies were confirmed in that the genus is a neighboring taxon of the family *Enterobacteriaceae*. With regard to the intragenetic structure, *Vibrio alginolyticus*, *Vibrio campbellii*, *Vibrio natriegens*, *Vibrio harveyi*, *Vibrio proteolyticus*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* form the core of the genus, while *Vibrio* (*Listonella*) *anguillarum*, *Vibrio diazotrophicus*, and *Vibrio hollisae* are placed on the outskirts of the genus. Variable regions around positions 80, 180, and 450 could be used as target sites for genus- and species-specific oligonucleotide probes and polymerase chain reaction primers to be used in molecular identification.

The relationships among members of the genus *Vibrio* have been thoroughly investigated by using phenotypic and genotypic analyses. Baumann and Baumann (3, 4), Baumann et al. (5, 6), and Colwell (11) have compiled the relevant approaches and publications of the early era. The more recent studies dealing with the inter- and intragenetic relationships of these organisms are studies in which numerical phenetic analyses (19, 32, 42, 43), chemotaxonomy (39), and genetic analyses (13, 27, 44, 46) were used. The results of all of the genetic studies are consistent in that the genera *Vibrio* and *Photobacterium* (family *Vibrionaceae* [41]) are phylogenetic neighbors of the families *Aeromonadaceae*, *Enterobacteriaceae*, and *Pasteurellaceae* and the genera *Alteromonas* and *Ruminobacter*; these taxa form one major subline of descent within the gamma subclass of the *Proteobacteria* (12, 46, 47). When a polyphasic approach was used, the genus *Vibrio* was subdivided, and former *Vibrio* species are now found in the genera *Listonella* (27), *Colwellia* (16), and "*Moritella*" (13).

However, the inconsistent levels at which members of the genus *Vibrio* are found to be related when different phylogenetic approaches are used are unsatisfactory. Several species that have been found to be closely related by DNA-DNA hybridization (i.e., *Vibrio harveyi*, *Vibrio campbellii*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio natriegens*, and *Vibrio vulnificus* [13, 34] [called the *Vibrio* core organisms below]) also appear to be closely related when immunological approaches (1a), rRNA cistron similarities (5), and 5S rRNA sequences (13, 28) are used. However, the levels at which these species are related vary. For example, as determined by DNA similarity, *V. harveyi* and *V. alginolyticus* are more closely related to each other (55%) than they are to *Vibrio proteolyticus* (average level of relatedness, 26%). While determinations of the immunological distances of the superoxide dismutases gave similar results (2, 5), *V. alginolyticus* clustered with *V. proteolyticus* to the exclusion of *V. harveyi* when the 5S rRNAs were compared (28, 30). In a more recent 5S rRNA study, in which the authors included additional outside reference sequences from *Shewanella* species, members of the family *Enterobacteriaceae*, and *Aeromonas* species, members of

the genus *Photobacterium* grouped within the radiation of the genus *Vibrio*, separating *V. harveyi*, *Vibrio cholerae*, and *Vibrio marinus* from the other vibrios (14). In contrast to other genera of the *Proteobacteria*, there are few 16S rRNA data available for *Vibrio* species (33, 40, 47). Therefore, in this work we analyzed data for almost complete 16S rRNA sequences from 10 *Vibrio* species. Similarities based upon full 16S rRNA sequences are less precise for determining relationships among highly related species than DNA similarities. Nevertheless, our data may help resolve questions about the composition of the core of the genus *Vibrio*, identify species-specific signatures, and design oligonucleotide probes that are suitable for rapid identification of members of the genus.

MATERIALS AND METHODS

Microorganisms. The strains which we investigated were *V. campbellii* ATCC 25920^T (T = type strain), *V. alginolyticus* ATCC 17749^T, *Vibrio diazotrophicus* ATCC 33466^T, *V. harveyi* ATCC 14126^T, *V. proteolyticus* ATCC 15338^T, *V. parahaemolyticus* ATCC 17802^T, *V. natriegens* ATCC 14048^T, *V. vulnificus* ATCC 27562^T, and *Vibrio hollisae* ATCC 33564^T.

The strains were grown at GENE-TRAK Systems Corp., Framingham, Mass., by using the standard culture medium recommended in the American Type Culture Collection *Catalog of strains* (1).

Isolation, extraction, and analysis of 16S rRNA. 16S rRNA was isolated (23) and shipped lyophilized to the Department of Microbiology, Christian-Albrechts University, Kiel, Germany. The primary structures of the 16S rRNAs of the type strains of nine *Vibrio* species were determined by performing a sequence analysis of cDNAs during reverse transcription (15, 24, 37). The sequences consist of a continuous stretch that varies in length between 1,463 and 1,481 nucleotides, corresponding to about 95.5% of the complete *Escherichia coli* 16S rRNA sequence (10). The 16S rRNA sequence of *Vibrio anguillarum* (40) was included in all analyses. This sequence was corrected by including a cytosine residue at position 418 (43a). The sequences were aligned with the homologous regions of sequences from the following reference bacteria: *E. coli*, *Proteus vulgaris*, *Ruminobacter amy-*

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TABLE 1. *Vibrio* species investigated and the EMBL accession numbers for their 16S rRNA sequences

Species	Source	Accession no.
<i>V. anguillarum</i> ^a	NCMB 6 ^T	X16895
<i>V. campbellii</i>	ATCC 25920 ^T	X56575
<i>V. alginolyticus</i>	ATCC 17749 ^T	X56576
<i>V. diazotrophicus</i>	ATCC 33466 ^T	X56577
<i>V. harveyi</i>	ATCC 14126 ^T	X56578
<i>V. proteolyticus</i>	ATCC 15338 ^T	X56579
<i>V. parahaemolyticus</i>	ATCC 17802 ^T	X56580
<i>V. natriegens</i>	ATCC 14048 ^T	X56581
<i>V. vulnificus</i>	ATCC 27562 ^T	X56582
<i>V. hollisae</i>	ATCC 33564 ^T	X56583

^a The sequence for this organism has been published by Valle et al. (40).

lophilus, *Pseudomonas aeruginosa* (31), and two barophilic strains (26).

Nucleotide sequence accession numbers. The nucleotide sequences of the *Vibrio* species were deposited in the EMBL, Heidelberg, Germany, under accession numbers X56575 to X56583 (Table 1). The accession numbers for the sequences of barophilic strains 1 and 2 (see above) were X54744 and X54745, respectively (26). The phylogenetic analysis, which was based on pairwise distances (Hamming), was carried out by using the neighborliness method (17, 18, 35) with algorithms implemented as part of the SAGE program package (Technoma, Heidelberg, Germany) designed for the IBM XT/AT and compatible computers.

RESULTS AND DISCUSSION

Intergeneric relationships. To determine intergeneric relationships, the sequences were aligned with the homologous sequences of six members of the gamma subclass of the *Proteobacteria*. The root was determined by using *Comamonas testosteroni*, a member of the beta subclass, as a reference. Regions of alignment uncertainty (positions 76 through 111, 1008 through 1034, and 1446 through 1486; *E. coli* nomenclature [10]) were omitted from the analysis. The pairwise distances, expressed as numbers of nucleotide substitutions, are shown in Table 2. Within the tree that was derived from these values, all of the vibrios formed a coherent cluster (Fig. 1) that was adjacent to two other members of the core of the gamma-3 organisms as defined by Woese et al. (47) (i.e., *E. coli* and *Proteus vulgaris*). The barophilic isolates from the North Atlantic (26), *R. amylophilus*, and *Pseudomonas aeruginosa* (the latter two species are considered to be peripheral representatives of the gamma-3 subclass) were more remotely related. Although the data base for vibrios is small, the branching pattern nevertheless is in accord with the patterns derived from 16S rRNA cataloging (36, 47) and 5S rRNA sequences (27). As discussed previously for 5S rRNA data (28), the phylogenetic depth of the *Vibrio* cluster is comparable to that of the *Enterobacteriaceae* cluster, considering that the genera *Escherichia* and *Proteus* are phylogenetically diverse genera within this family (9, 28).

The distinct separation of the vibrios from moderately closely related genera was also shown by the occurrence of genus-specific signatures in the secondary structures of the

TABLE 2. Equally weighted (Hamming) distances between 16S rRNA sequence pairs from *Vibrio* species and other members of the gamma subclass of the *Proteobacteria*^a

Organism	Hamming distance															
	<i>V. parahaemolyticus</i>	<i>V. vulnificus</i>	<i>V. proteolyticus</i>	<i>V. natriegens</i>	<i>V. campbellii</i>	<i>V. alginolyticus</i>	<i>V. harveyi</i>	<i>V. anguillarum</i>	<i>V. diazotrophicus</i>	<i>V. hollisae</i>	<i>E. coli</i>	<i>Proteus vulgaris</i>	Barophilic strain 1	Barophilic strain 2	<i>R. amylophilus</i>	<i>Pseudomonas aeruginosa</i>
<i>V. parahaemolyticus</i>	0	77	101	94	94	93	96	113	108	136	207	220	221	252	262	281
<i>V. vulnificus</i>	51	0	105	91	94	93	95	109	110	137	203	213	216	243	256	279
<i>V. proteolyticus</i>	61	66	0	94	97	94	99	119	123	135	196	212	214	247	266	282
<i>V. natriegens</i>	64	65	53	0	71	68	71	103	104	135	201	215	217	249	246	271
<i>V. campbellii</i>	64	68	56	43	0	66	73	105	105	133	200	216	215	247	254	270
<i>V. alginolyticus</i>	63	67	53	40	38	0	70	103	102	131	198	214	213	246	251	268
<i>V. harveyi</i>	66	69	59	43	35	42	0	106	106	133	203	218	213	243	255	267
<i>V. anguillarum</i>	88	77	81	76	78	76	79	0	115	153	212	215	228	250	266	285
<i>V. diazotrophicus</i>	84	82	88	78	79	76	80	85	0	144	197	218	220	248	254	277
<i>V. hollisae</i>	111	107	107	109	107	105	107	124	115	0	207	228	216	252	284	301
<i>E. coli</i> ^b	186	184	182	178	177	175	180	190	176	184	0	155	220	272	258	273
<i>Proteus vulgaris</i> ^b												0	226	266	272	296
Barophilic strain 1													0	157	263	278
Barophilic strain 2														0	284	301
<i>R. amylophilus</i> ^b															0	266
<i>Pseudomonas aeruginosa</i> ^b																0
<i>Comamonas testosteroni</i> ^b																0

^a *Comamonas testosteroni* was included as an outgroup reference organism; this species is a member of the beta subclass of the *Proteobacteria*. The values above the diagonal are the values obtained with the full data set (Fig. 1); the values below the diagonal are the values for intrageneric relationships (Fig. 2). Regions of alignment uncertainty (see text) were omitted before the analyses.

^b Data from reference 31.

FIG. 1. Phylogenetic positions of the *Vibrio* species investigated in this study. Bar = 20 nucleotide exchanges.

16S rRNAs (Table 3). However, it should be kept in mind that the data base of previously published 16S rRNA sequences for gamma-3 subclass organisms is small and so-called specific features may be blurred when additional sequences become available.

Intragenetic relationships. To determine the intragenetic branching pattern, the *E. coli* sequence was used as an outgroup reference sequence. The pairwise distances shown in Table 2 were used to generate a tree (Fig. 2). The overall level of similarity among the 10 *Vibrio* sequences was 85.6%. Seven species appeared to be closely related, sharing an overall level of sequence similarity of 91.3%. Of these seven species, *V. alginolyticus*, *V. campbellii*, *V. harveyi*, and *V. natriegens*, as well as *V. parahaemolyticus* and *V. vulnificus* formed even phylogenetically tighter groups, with *V. proteolyticus* branching off between these two groups. *V. (Listonella) anguillarum*, *V. diazotrophicus*, and *V. hollisae* represent individual sublines of descent, with the latter species representing the earliest branch within the genus *Vibrio*.

Phylogeny of the *Vibrio* core organisms. The clustering of the core organisms of the genus *Vibrio* correlated by and large with the results of DNA reassociation experiments. The grouping of *V. harveyi*, *V. campbellii*, and *V. alginolyticus* was confirmed, as was the more isolated position of *V. vulnificus* and *V. proteolyticus* (5, 34). The position of *V. parahaemolyticus* was not as clearcut. While this species

TABLE 3. Sequence signatures that distinguish members of the genus *Vibrio* from other members of the gamma-3 subclass of the *Proteobacteria*

Position(s)	Composition in:	
	<i>Vibrios</i>	<i>E. coli</i>
132	U	U
154-167	C-G	U-A
446-488	U-G	G-C
580-761	U-A	C-G
614-626	G-C	C-G
896-903	U-A	C-G
1123-1149	G-C	U-A

was shown to be highly related to *V. alginolyticus* (65% DNA similarity according to Reichelt et al. [34]), it clustered with *V. vulnificus* as determined by 16S rRNA similarity. However, in a more recent DNA-DNA hybridization study (29), *V. parahaemolyticus* and *V. alginolyticus* were found to be less closely related (viz., about 45 and 22% under optimal and stringent reassociation conditions, respectively). A cluster analysis of the immunological relationships among superoxide dismutases clustered all of the core organisms of the genus *Vibrio* investigated by 16S rRNA analysis except *V. vulnificus*, which was in a group by itself (2).

A comparison of the 16S and 5S rRNA branching patterns of the *Vibrio* core organisms revealed more significant differences than the differences found in the comparisons of the results from DNA pairing and 16S rRNA analyses. The topography of the 5S rRNA tree generated by MacDonell et al. (28) shows that *V. parahaemolyticus*, *V. alginolyticus*, *V. natriegens*, *V. proteolyticus*, and *V. diazotrophicus* form a tight cluster. When the similarity values were used for a principal-component analysis, the relationships changed in that *V. diazotrophicus* appeared to be as unrelated to the core species as *V. harveyi* and *V. vulnificus* are. In a more recent 5S rRNA tree (13), *V. harveyi* did cluster with the core organisms, as in the 16S rRNA analysis.

Outskirt organisms. A consistent finding in molecular systematic studies has been the lack of a close relationship between *V. anguillarum* and the *Vibrio* core organisms. Principal-component analysis (28) and DNA reassociation

FIG. 2. Phylogenetic relationships among the 10 *Vibrio* species investigated. The root was determined by using *E. coli* as a reference organism. Bar = 10 nucleotide exchanges.

experiments have revealed the isolated position of this species, which led to the description of the new genus *Listonella* to harbor *L. anguillarum* and related species (i.e., *Listonella damsela* and *Listonella pelagia*) (27). However, *V. hollisae*, which has been described as a probable cause of human diarrhea (21), is even more remotely related to the core organisms than *V. anguillarum* is, as indicated by the lack of significant levels of DNA similarity (21) to other *Vibrio* species and by the results of this study. On the basis of the data presented here, and assuming that diagnostic phenotypic properties are available, *V. hollisae* should be elevated to genus rank. However, if *V. hollisae* remains a valid member of the genus *Vibrio*, the genus *Listonella* should lose its rank of genus and *L. anguillarum* and related species should be returned to the genus *Vibrio*. At present, the data base provided by this study is too small to make definite proposals. The decision about whether to retain *V. hollisae* in the genus *Vibrio* depends upon the branching points of the *Vibrio* species not included in this study.

The position of *V. diazotrophicus* in the 16S rRNA tree is a moot point. When the Fitch-Margoliash (18) algorithm or UPGMA (28) was used with 5S rRNA similarity data, this species appeared to be closely related to *V. proteolyticus*, whereas it grouped outside the boundaries of the *Vibrio* core organisms (as defined here) in the principal-component analysis (28). Low DNA similarity values (20) and the results of numerical phenetic analyses (42, 43) confirmed the isolated position of *V. diazotrophicus*. Support for the conclusion that this species does not belong to the *Vibrio* core organisms comes from an analysis of fatty acid composition. Urdaci et al. (39) showed that *V. diazotrophicus*, like *V. anguillarum*, *Vibrio fischeri*, *Vibrio gazogenes*, *Vibrio damsela*, and *Vibrio ordalii*, lacks fatty acids of the 12:0, 17:0, and 17:1 types, which are present, either alone or in combination, in the *Vibrio* core organisms.

Diagnostic potential of 16S rRNA. The need for rapid diagnostic methods to identify water-, fish-, mussel-, and oyster-borne human pathogens (7, 22) makes the variable 16S rRNA regions attractive targets for synthetic oligonucleotide probes and polymerase chain reaction primers. A short stretch covering positions 137 through 141 (CUGAU), which forms part of a helical structure along with a stretch located between positions 222 through 226, shows promising potential for the development of genus-specific probes, especially when it is combined with the two *Vibrio*-specific nucleotides at positions 132 and 154 (Table 3).

The first species-specific 16S rRNA-based probe for vibrios was designed for *V. anguillarum* (33); this probe targets a stretch between positions 431 and 455 (*E. coli* nomenclature). Compared with the homologous regions of other *Vibrio* species, the only difference is the presence of a uracil residue at position 434 in the *V. anguillarum* sequence. All other *Vibrio* species possess a cytosine residue (transition from G-C to A-U at positions 408 and 434). This probe has not been tested with any of the species included in this study, and it remains to be determined whether the position of the altered nucleotide near the 3' terminus of the 25-mer probe allows discrimination of *V. anguillarum* from other *Vibrio* species. Three more highly variable regions are found at positions 73 through 100, 180 through 196, and 455 through 477. Although certain pairs have identical or highly similar sequences (Table 4), these regions offer the potential to discriminate among all of the species investigated in this study. The differences appear to be sufficiently significant that even without testing their selectivity can be predicted (a single nucleotide difference within probes up to 20 nucle-

TABLE 4. Variable 16S rRNA stretches that could be used as targets for oligonucleotide probing and polymerase chain reaction diagnosis

Organism	Sequence at:		
	Positions 73 to 100	Positions 180 to 196	Positions 455 to 477
<i>V. alginolyticus</i>	CGAGUUAACUGGAACUUGGGGAACGUAUACGGCGUUG	AATGCCUACGGGGCCAA	UAGUGTAGUUAUAGGUCACUUA
<i>V. campbellii</i>	CGAGUUAACUGGAACUUGGGGAACGUAUACGGCGUUG	AATGCCUACGGGGCCAA	UAGUGTAGUUAUAGGUCACUUA
<i>V. proteolyticus</i>	CGAGUUAUCUGAACUUCGGGGGAACGUAUACGGCGUUG	AATGCCUACGGGGCCAA	UAGUGTAGUUAUAGGUCACUUA
<i>V. parahaemolyticus</i>	CGAGUUAUCUGAACUUCGGGGGAACGUAUACGGCGUUG	AATGCCUACGGGGCCAA	UAGUGTAGUUAUAGGUCACUUA
<i>V. hollisae</i>	CGACAUAGAACAUCUUCGGGGGUCGUCUACUUGGGCGCG	AATGCCUACGGGGCCAA	UAGUGTAGUUAUAGGUCACUUA
<i>V. anguillarum</i>	CACAGAGGAACUUCUUCUUCUUGGGGUGGCG	AATGCCUACGGGGCCAA	UAGUGTAGUUAUAGGUCACUUA
<i>V. diazotrophicus</i>	CACAGAGGAACUUCUUCUUCUUGGGGUGGCG	AATGCCUACGGGGCCAA	UAGUGTAGUUAUAGGUCACUUA
<i>V. vulnificus</i>	CACAGAGGAACUUCUUCUUCUUGGGGUGGCG	AATGCCUACGGGGCCAA	UAGUGTAGUUAUAGGUCACUUA
<i>V. harveyi</i>	CGAGUUAUCUGGAAXXX ^a	AATGCCUACGGGGCCAA	UAGUGTAGUUAUAGGUCACUUA
<i>V. natriegens</i>	CGAGUUAACGXXX	AATGCCUACGGGGCCAA	UAGUGTAGUUAUAGGUCACUUA

^a X, not determined.

tides long has been used to selectively detect streptomycetes [45] and *Mycobacterium leprae* [25, 38] among closely related species). However, the specificity of each species-specific probe must be investigated with a larger number of strains. Interestingly, one of the most highly variable regions in eubacterial 16S rRNA (i.e., around position 465, *E. coli* nomenclature) is identical in *V. campbellii*, *V. alginolyticus*, *V. harveyi*, and *V. natriegens* (Table 4).

The distances between the three variable regions (100, 180, or 280 nucleotides) are large enough to use polymerase chain reaction diagnosis (8) when two of the regions serve as target sites for polymerase chain reaction primers and amplification. At least for certain strains, the differences in the target regions are sufficiently large to encourage use of this modern identification tool. The ease with which DNA can be extracted from gram-negative bacteria should facilitate rapid detection of pathogenic *Vibrio* species directly in the environment.

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