Classification of Fish-Pathogenic Vibrios Based on Comparative 16S rRNA Analysis

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No systematic classification of fish-pathogenic vibrios has been accomplished previously despite the use of serological, physiological, and genetical classification systems. In this study, a comparative 16S rRNA analysis of 34 strains (representing seven species) of fish-pathogenic vibrios was performed. The 16S rRNA sequences were obtained by using reverse transcriptase. Nearly complete sequences were obtained for nine strains. On the basis of the results of this analysis, the remaining strains were investigated by analyzing selected stretches containing a total of 560 nucleotides. With the exception of a few strains, including ATCC 43313 (serovar O9), our comparative 16S rRNA analysis confirmed that strains preliminarily identified as *Vibrio anguillarum* were phylogenetically closely related. Strains of *V. anguillarum* could be divided into groups, with the main group containing serotype O1 and O2 strains isolated from Atlantic salmon, rainbow trout, turbot, cod, and saithe. The other distinctive group was represented by type strain NCMB 6. This strain was nearly indistinguishable from the type strains of *Vibrio ordalii* and *Vibrio damsela* on the basis of the 16S rRNA stretches compared. The results of a comparative 16S rRNA analysis justified the status of *Vibrio salmonicida* as a distinct species. Originally, this species was characterized biochemically as a very homogeneous species. However, two strains, which were isolated from diseased halibut and from the intestines of healthy cod, could not be distinguished from *V. salmonicida* strains phylogenetically, although they differed from the original species description in several phenotypic traits. Our results indicate that *V. salmonicida* and *Vibrio fischeri* form a cluster that is clearly separated from the cluster that includes *V. anguillarum*.

Vibriosis is the name given to a group of related systemic infections in fish that are caused by marine vibrios (7, 12). Variants of vibriosis occur worldwide and affect a great variety of fish species (7, 13, 15, 40, 41, 47, 48, 51, 53), as well as crustaceans and molluscs (4, 5, 20). Although vibriosis has been reported in complete freshwater aquaculture systems (17), this group of diseases is primarily a marine problem.

Historically, the fish-pathogenic vibrios have been assigned to the species *Vibrio anguillarum* (1, 2). This classification, however, has turned out to be an oversimplification (27, 41, 48). Efforts have been made to bring order to the heterogeneous group of fish-pathogenic vibrios by establishing serological (43, 48) and physiological (18, 34) classification systems and by describing new *Vibrio* species (such as *Vibrio salmonicida* [13], *Vibrio damsela* [26], and *Vibrio ordalii* [41]) on the basis of both phenotypic and genotypic characteristics. Despite these efforts, a satisfactory systematic classification of the fish-pathogenic vibrios has not been accomplished previously. The unresolved taxonomic affiliation of the fish-pathogenic vibrios is a problem for epidemiological studies and vaccination programs.

The goal of this study was to investigate whether fish-pathogenic vibrios could be unambiguously classified on the basis of the results of an analysis of their 16S rRNA sequences. In recent years, comparative 16S rRNA sequence analysis has become a very valuable tool for both delineation of evolutionary relationships (16, 54) and taxon identification (45, 55).

MATERIALS AND METHODS

Bacterial strains. The strains which we examined, as well as their serotypes, origins, and nucleotide sequence accession numbers, are listed in Table 1. The serotypes of the *V. anguillarum* strains have been determined previously (23, 38). *V. anguillarum* strains of Norwegian origin were isolated and identified as described by Wiik et al. (53). The *V. salmonicida* strains were isolated and identified as described by Wiik et al. (51). *Aeromonas salmonicida* NCMB 1102^T (T = type strain) was used as a fish-pathogenic reference organism.

Growth conditions. The general growth substrates used in this study were tryptone soya broth (Oxoid, Ltd., London, United Kingdom) supplemented with NaCl at a final concentration of 1.5% and tryptone soya agar (Oxoid) supplemented with NaCl at a final concentration of 1.5%. The *V. salmonicida* cultures were incubated at 15°C for 48 h, while the remaining cultures were incubated at 22°C for 16 to 24 h. All cultures were incubated aerobically with continuous shaking. At the end of the incubation period each of the cultures had reached the late exponential phase. The agar plates were incubated for 1 to 3 days under the same temperature conditions as the broth cultures.

Extraction of RNA. The following three procedures for extraction of RNA were evaluated: (i) the original method of Chomczynski and Sacchi (6); (ii) a small-scale variation of the method of Chomczynski and Sacchi (6) (the original volumes were reduced by a factor of 20); and (iii) the alkaline lysis method of Birnboim and Doly (3), modified so that the nucleic acids were precipitated from the "high-salt" supernatant with 1 volume of isopropanol and the remaining high-molecular-weight RNA was selectively precipitated from the redissolved pellet in sterile, distilled water by adding 10 M ammonium acetate to a final concentration of 2.5 M. After 20 min on ice, the RNA was harvested by centrifugation at 15,800 rpm with an Eppendorf model 5402 centrifuge for 10 min at 4°C. The pellet was dried in a Savant Speedvac concentrator and then dissolved in sterile distilled water (50 to 100 µl) to an RNA concentration of 3 µg/µl.

During the small-scale procedures, cells harvested from tryptone soya agar plates were examined to see if they were as well suited to be sources of 16S rRNA as cells harvested from tryptone soya broth were. About 50 mg (wet weight) of cells needed to be harvested from each agar plate.

A comparison of the RNA extraction methods showed that the small-scale variation of the method of Chomczynski and Sacchi (6) was as successful as the original method. Harvesting cells from plates was the strategy that saved the most time. Since the method of Birnboim and Doly (3) was used only rather late in this study, only a few 16S rRNAs were isolated by this procedure. This method, however, seemed to save even more time than the small-scale variation of the method of Chomczynski and Sacchi (6); the whole procedure from colony to purified RNA was completed in about 2 h. In addition, the RNA obtained by the

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TABLE 1. Bacterial strains used in this study

Strain	Source ^a	Serotype	Host	EMBL accession no.
<i>V. anguillarum</i>				
NCMB 6 ^T	NCMB	O2a	Cod (<i>Gadus morhua</i>)	X16895
NCMB 2130	NCMB	O2	Saithe (<i>Pollachius virens</i>)	X71818
HI 11340	HI	O2a	Cod (<i>G. morhua</i>)	X71830
HI 11351	HI	O2a	Cod (<i>G. morhua</i>)	X71824
HI 11423	HI	O2a	Cod (<i>G. morhua</i>)	X71822
HI 4791	HI	O2b	Cod (<i>G. morhua</i>)	X71816
HI 10424	HI	O2b	Cod (<i>G. morhua</i>)	X71833
HI 11336	HI	O2b	Cod (<i>G. morhua</i>)	X71831
HI 11343	HI	O2b	Cod (<i>G. morhua</i>)	X71827
HI 11349	HI	O2b	Cod (<i>G. morhua</i>)	X71825
NCMB 2129	NCMB	O1	Rainbow trout (<i>Oncorhynchus mykiss</i>)	X71819
775	UO	O1	Coho salmon (<i>Oncorhynchus kisutch</i>)	X71814
HI 7400	HI	O2	Atlantic salmon (<i>Salmo salar</i>)	X71815
HI 11331	HI	O2a	Turbot (<i>Scophthalmus maximus</i>)	X71832
HI 11341	HI	O1	Turbot (<i>Scophthalmus maximus</i>)	X71829
HI 11345	HI	O1	Turbot (<i>Scophthalmus maximus</i>)	X71826
HI 11421	HI	O1	Turbot (<i>Scophthalmus maximus</i>)	X71823
HI 11342	HI	NT ^b	Turbot (<i>Scophthalmus maximus</i>)	X71828
HI 11431	HI	NT	Turbot (<i>Scophthalmus maximus</i>)	X71821
HI 11446	HI	NT	Turbot (<i>Scophthalmus maximus</i>)	X71820
ATCC 43313	ATCC	O9	Cod (<i>G. morhua</i>)	X71817
<i>V. ordalii</i>				
ATCC 33509 ^T (= DF ₃ K ^T)	ATCC		Coho salmon (<i>O. kisutch</i>)	X70641
NCMB 2167 (= DF ₁ K)	NCMB		Coho salmon (<i>O. kisutch</i>)	X71812
MSC2-75	UO		Coho salmon (<i>O. kisutch</i>)	X71834
<i>V. salmonicida</i>				
NCMB 2262 ^T	NCMB		Atlantic salmon (<i>Salmo salar</i>)	X70643
HI 11366-2	HI		Cod (<i>G. morhua</i>)	X70638
HI 11391	HI		Atlantic salmon (<i>Salmo salar</i>)	X70639
HI 651	HI		Halibut (<i>Hippoglossus hippoglossus</i>)	X71811
PT2	UT		Cod (<i>G. morhua</i>)	X70637
<i>V. iliopiscarius</i> PS1	UT		Herring (<i>Clupea harengus</i>)	X70636
<i>V. fischeri</i>				
NCMB 1281 ^T	NCMB		Seawater	X70640
NCMB 1274	NCMB		Merluza (<i>Merluccius vulgaris</i>)	X71813
<i>V. damsela</i> NCMB 2184 ^T	NCMB		Damsel fish (<i>Chromis punctipinnis</i>)	X71835
<i>V. pelagius</i> NCMB 1900 ^T	NCMB		Seawater	X70642
<i>A. salmonicida</i> NCMB 1102 ^T	NCMB		Atlantic salmon (<i>Salmo salar</i>)	X71836

^a ATCC, American Type Culture Collection; HI, Havforskningssinstituttet; NCMB, National Collection of Marine Bacteria; UO, University of Oregon; UT, Universitetet i Tromsø.

^b NT, not typeable.

method of Birnboim and Doly (3) was as good as the RNA obtained by the method of Chomczynski and Sacchi (6) as a template for the enzyme reverse transcriptase. It should be noted in this context that reverse transcriptase sequencing of rRNA has been almost completely replaced by the PCR method, which is often followed by automatic sequence analysis.

Reverse transcriptase sequencing of 16S rRNA. For the sequence analysis we used previously described methods (24, 46). Almost complete 16S rRNA sequences were determined for nine bacterial strains. Primers (14) were located around positions 350, 530, 700, 900, 1100, 1200, and 1400 (*Escherichia coli* nomenclature). In addition, we used two primers having the following sequences (*E. coli* 3' positions indicated): GGCCGAAGGTCCCCCT (position 215) and TTACGACTTCACCCCAGT (position 1500). On the basis of the results of this analysis, the remaining strains were investigated by using primers 215, 530, and 1100.

Terminal deoxynucleotidyl transferase (Boehringer Mannheim GmbH) was used as described previously (8). The products of the sequencing reactions were separated as described by Embley et al. (14) with an LKB sequencing apparatus.

16S rRNA data analysis. The 16S rRNA sequences were manually aligned with the sequences in a database containing data for *Vibrio* strains and related taxa (10, 32). The number of positions included in the analysis depended on the size of the shortest sequence in the database. Pairwise evolutionary distances (expressed as estimated number of changes per 100 nucleotides) were computed from percentages of similarity by using the correction of Jukes and Cantor (21). Phylogenetic trees were constructed from the distance matrices by the algorithm of De Soete (9).

Physiological and serological characterization. Only the phenotypic characteristics of *V. salmonicida* HI 651 are presented below, as the remaining strains which we studied have been described elsewhere. The growth of strain HI 651 in the presence of NaCl was determined in tryptone soya broth and on tryptone soya agar amended with NaCl at final concentrations of 0.5, 1.5, 4.0, 6.0, and 8.0%. The cultures were incubated at 15°C and observed for 7 days. The ability of strain HI 651 to grow at different temperatures was determined by incubating cultures at 4, 10, 15, 20, 25, and 30°C. The growth medium used was nutrient agar (Oxoid) supplemented with 5% citrated sheep blood and NaCl at a final con-

FIG. 1. Distance tree derived from the almost complete 16S rRNA sequences of fish-pathogenic vibrios and *Vibrio* reference strains. The tree was constructed from a distance matrix by using the algorithm of De Soete (9). The distance matrix was derived from the similarity matrix (Table 2) by using the correction of Jukes and Cantor (21). *A. salmonicida* was used as the root organism. Bar = 2.5% sequence divergence.

centration of 1.5% (blood agar). The agar plates were observed for 1 week. Hemolysis was determined by examining growth on blood agar plates. The fermentative ability of strain HI 651 was determined by using the API 50CH (API 50CHE medium) system (API Systems, S.A., La Balme Les Grottes,

Montalieu Vercieu, France); sterile NaCl was added to the fluid to a final concentration of 1.5%. Additional biochemical activities were determined with API 20E galleries (70% sterile seawater was used as the medium). The galleries were incubated at 15°C, and results were determined after 48 h of incubation. Rabbit polyclonal antisera against formalin-killed cells of strains of *V. salmonicida* and different serogroups of *V. anguillarum* were prepared by the methods of Oeding (29). Serotyping was performed by using an enzyme-linked immunosorbent assay as described by Wiik et al. (53).
Nucleotide sequence accession numbers. The nucleotide sequences of the strains included in this study have been deposited in the EMBL Data Library, Heidelberg, Germany, under the accession numbers shown in Table 1.

RESULTS

Phylogenetic relationships based on an analysis of almost complete 16S rRNA sequences. The almost complete sequences obtained from eight strains of four *Vibrio* species and the type strain of *A. salmonicida* were analyzed. A thorough phylogenetic analysis of aeromonads, including *A. salmonicida*, has been published recently (39), and the results of this analysis supported the finding that there is moderate degree of relatedness between members of the family *Vibrionaceae* and members of the family *Aeromonadaceae*. Only data for sequence positions that were known for all of the strains used in the analysis were included. Binary homology values, which were based on an analysis of 1,270 nucleotides, were determined for type strains of eight *Vibrio* species (10, 11, 50; this study) and six additional strains identified as either *V. salmonicida* or *V. anguillarum*. The dendrogram of relationships (Fig. 1) indicates that *Vibrio fischeri* and the two strains of *V. salmonicida* studied are very similar to one another. A second group is formed by strains of *V. anguillarum* and the type strain of *V. ordalii*, which is almost identical to the type strain of *V. anguillarum* (level of sequence similarity, 99.4%). Most *V. anguillarum* strains exhibit high levels of sequence similarity to one another (98.2 to 99.9%) (Tables 2 and 3); only *V. anguillarum* HI 11342, HI 11431, HI 11446, and ATCC 43313 are more distantly related (levels of sequence similarity, 95.2 to 97.6%). The other *Vibrio* species, including those whose sequences have been published previously (10) and *Vibrio chol-*

TABLE 2. Similarity matrix based on a comparison of almost complete 16S rRNA sequences

Strain	% Similarity to:																
	<i>V. anguillarum</i> NCMB 6 ^T	<i>V. ordalii</i> ATCC 33509 ^T	<i>V. anguillarum</i> ATCC 43313	<i>Vibrio</i> sp. strain HI 11431	<i>V. anguillarum</i> 775	<i>V. anguillarum</i> NCMB 2129	<i>V. anguillarum</i> NCMB 2130	<i>V. diazotrophicus</i> ATCC 33466 ^T	<i>V. hollisae</i> ATCC 33564 ^T	<i>V. parahaemolyticus</i> ATCC 17802 ^T	<i>V. campbellii</i> ATCC 25920 ^T	<i>V. alginolyticus</i> ATCC 17749 ^T	<i>V. proteolyticus</i> ATCC 15338 ^T	<i>V. cholerae</i> ATCC 14035 ^T	<i>V. salmonicida</i> NCMB 2262 ^T	<i>V. salmonicida</i> HI 651	<i>V. fischeri</i> NCMB 1281 ^T
<i>V. ordalii</i> ATCC 33509 ^T	99.4																
<i>V. anguillarum</i> ATCC 43313	98.6	98.2															
<i>Vibrio</i> sp. strain HI 11431	97.0	96.9	96.9														
<i>V. anguillarum</i> 775	99.3	98.7	98.0	97.5													
<i>V. anguillarum</i> NCMB 2129	99.2	98.8	98.1	97.6	99.9												
<i>V. anguillarum</i> NCMB 2130	99.2	98.8	98.2	97.8	99.7	99.8											
<i>V. diazotrophicus</i> ATCC 33466 ^T	97.3	97.4	96.1	95.5	97.0	97.1	97.1										
<i>V. hollisae</i> ATCC 33564	93.0	92.9	92.3	91.8	93.0	93.0	93.0	93.3									
<i>V. parahaemolyticus</i> ATCC 17802 ^T	96.8	96.6	96.0	95.5	96.8	96.9	96.9	93.5	93.5								
<i>V. campbellii</i> ATCC 25920 ^T	97.3	97.1	96.5	96.0	97.3	97.4	97.4	97.3	94.2	98.4							
<i>V. alginolyticus</i> ATCC 17749 ^T	97.3	97.1	96.5	95.9	97.3	97.4	97.4	97.4	94.2	98.3	99.9						
<i>V. proteolyticus</i> ATCC 15338 ^T	95.3	95.1	94.5	93.9	95.3	95.4	95.4	95.2	94.7	97.1	97.5	97.6					
<i>V. cholerae</i> ATCC 14035 ^T	94.3	94.5	93.8	93.6	94.4	94.5	94.3	93.9	92.2	94.4	95.0	95.0	93.2				
<i>V. salmonicida</i> NCMB 2262 ^T	96.4	96.3	96.0	96.3	95.8	95.9	95.9	94.6	91.9	94.5	95.3	95.3	94.0	94.7			
<i>V. salmonicida</i> HI 651	96.1	96.0	95.7	96.4	95.5	95.6	95.6	94.6	91.8	94.5	95.3	95.3	93.8	94.7	99.7		
<i>V. fischeri</i> NCMB 1281 ^T	95.7	95.6	95.5	95.8	95.7	95.8	96.0	94.2	91.3	94.7	95.4	95.4	93.9	94.7	97.0	97.3	
<i>A. salmonicida</i> NCMB 1102 ^T	90.1	90.4	90.0	89.7	89.6	89.7	89.7	89.6	88.0	90.0	90.2	90.1	89.0	89.6	91.2	91.1	90.7

TABLE 3. Similarity matrix based on partial 16S rRNA sequences comprising 560 nucleotides

Strain	% Similarity to:																				
	<i>V. anguillarum</i> NCMB 2130	<i>V. fischeri</i> NCMB 1274	<i>V. anguillarum</i> HI 11340	<i>V. anguillarum</i> NCMB 2129	<i>Vibrio</i> sp. strain HI 11342	<i>Vibrio</i> sp. strain HI 11446	<i>V. anguillarum</i> HI 4791	<i>V. salmonicida</i> HI 11391	<i>V. iliopiscarius</i> PS1 ^T	<i>V. ordalii</i> MSC2-75	<i>V. anguillarum</i> NCMB 6 ^T	<i>V. damsela</i> NCMB 2184 ^T	<i>V. anguillarum</i> ATCC 43313	<i>V. diazotrophicus</i> ATCC 33466 ^T	<i>V. hollisae</i> ATCC 33564 ^T	<i>V. parahaemolyticus</i> ATCC 17802 ^T	<i>V. campbelli</i> ATCC 25920 ^T	<i>V. pelagius</i> NCMB 1900 ^T	<i>V. proteolyticus</i> ATCC 15338 ^T	<i>V. cholerae</i> ATCC 14035 ^T	<i>V. salmonicida</i> NCMB 2262 ^T
<i>V. fischeri</i> NCMB 1274	94.1																				
<i>V. anguillarum</i> HI 11340	99.8	94.3																			
<i>V. anguillarum</i> NCMB 2129	99.8	93.8	99.6																		
<i>Vibrio</i> sp. strain HI 11342	96.0	93.6	95.8	95.8																	
<i>Vibrio</i> sp. strain HI 11446	95.2	93.6	94.9	94.9	98.7																
<i>V. anguillarum</i> HI 4791	99.3	93.4	99.1	99.1	95.4	94.5															
<i>V. salmonicida</i> HI 11391	93.6	99.1	93.8	93.4	93.2	93.2	93.0														
<i>V. iliopiscarius</i> PS1 ^T	93.0	95.8	93.2	92.7	93.0	91.6	92.3	95.4													
<i>V. ordalii</i> MSC2-75	93.8	92.1	93.6	93.6	93.4	93.4	93.2	91.6	91.4												
<i>V. anguillarum</i> NCMB 6 ^T	98.9	93.6	99.1	98.7	94.9	94.1	98.2	94.5	92.5	93.2											
<i>V. damsela</i> NCMB 2184 ^T	97.9	93.3	98.2	98.6	94.0	93.4	97.5	93.6	91.9	92.8	99.2										
<i>V. anguillarum</i> ATCC 43313	97.6	93.0	97.8	97.4	93.8	93.8	96.9	93.8	91.9	92.3	98.7	98.2									
<i>V. diazotrophicus</i> ATCC 33466 ^T	95.4	91.2	95.4	95.2	92.1	91.2	94.7	92.1	90.3	91.9	96.0	95.7	94.7								
<i>V. hollisae</i> ATCC 33564 ^T	93.6	91.9	93.4	93.4	92.7	93.2	93.0	92.3	89.9	94.1	93.6	93.1	93.2	92.7							
<i>V. parahaemolyticus</i> ATCC 17802 ^T	94.9	92.7	94.9	94.7	95.4	94.3	94.3	92.3	92.3	94.1	94.5	94.0	93.6	93.2	93.8						
<i>V. campbelli</i> ATCC 25920 ^T	95.4	93.4	95.4	95.2	94.5	94.1	94.7	93.0	92.3	94.1	94.9	94.6	94.1	93.6	94.1	97.4					
<i>V. pelagius</i> NCMB 1900 ^T	95.2	93.2	95.2	94.9	95.2	94.7	94.5	92.7	91.6	95.8	94.7	94.5	94.1	93.2	93.8	97.8	97.4				
<i>V. proteolyticus</i> ATCC 15338 ^T	94.5	92.3	94.5	94.3	94.3	94.1	93.8	91.9	91.2	93.4	94.1	93.7	93.2	92.7	94.7	97.6	98.9	96.9			
<i>V. cholerae</i> ATCC 14035 ^T	94.7	91.4	94.9	94.9	92.3	91.4	94.1	90.8	91.0	92.5	94.5	94.4	93.4	93.0	91.6	91.9	92.3	91.9	91.6		
<i>V. salmonicida</i> NCMB 2262 ^T	93.6	99.1	93.8	93.4	93.2	93.2	93.0	100.0	95.4	91.6	94.5	93.9	93.8	92.1	92.3	92.3	93.0	92.7	91.9	92.1	
<i>V. fischeri</i> NCMB 1281 ^T	93.2	95.2	93.4	93.0	93.6	92.7	92.5	94.3	93.8	94.5	92.7	92.3	91.9	91.0	91.9	93.6	93.6	94.7	93.0	92.3	94.3

FIG. 2. Distance tree derived from partial 16S rRNA sequences (560 nucleotides) of fish-pathogenic vibrios and *Vibrio* reference strains. The tree was constructed from a distance matrix by using the algorithm of De Soete (9). The distance matrix was computed from the similarity matrix (Table 3) by using the correction of Jukes and Cantor (21). *A. salmonicida* was used as the root organism. Bar = 2.5% sequence divergence.

erae (11), form several groups, and only *Vibrio campbellii* and *Vibrio alginolyticus* appear to be closely related (level of sequence similarity, 99.9%). A comparison of the dendrogram shown in Fig. 1 with the dendrogram published previously (10), for which a different treeing algorithm was used and which included fewer strains, showed that the trees are similar with respect to the composition of the *Vibrio* core species (*V. campbellii*, *V. alginolyticus*, *Vibrio parahaemolyticus*, and *Vibrio proteolyticus*) and with respect to the position of *Vibrio* outskirt organisms, such as *V. anguillarum* and *Vibrio diazotrophicus*. The major difference between the two trees is the position of *Vibrio hollisae*, which clustered with the *Vibrio* core organisms in our study. The positions of strains of *V. ordalii*, *V. salmonicida*, and *V. fischeri* indicate that none of these organisms belongs to the core group of the genus *Vibrio*.

On the basis of variable 16S rRNA sequence regions which differentiated similar taxa, we selected three regions for further phylogenetic analysis. The isolates which we investigated had been identified previously by traditional taxonomic methods as strains of *V. anguillarum* and *V. salmonicida*. The results of an analysis of the selected regions, which contained a total of 560 nucleotides, were very similar to the results of the analysis based on almost complete sequences (Fig. 1 and 2). This was most obvious in the clustering of *V. fischeri* and *V. salmonicida*, in the clustering of *V. anguillarum* and *V. ordalii*, and in the composition of the *Vibrio* core species, which included the type strain of *Vibrio pelagius*. The results of our phylogenetic study

confirmed the finding that *V. anguillarum* and *V. pelagius* should not be placed in the same genus (22). When the results of this analysis were compared with the results of the analysis of almost complete sequences, *V. hollisae* changed its position and clustered with a strain identified as a member of *V. ordalii* (Fig. 2).

Our comparative 16S rRNA analysis confirmed that all of the strains preliminarily identified as *V. anguillarum* except strains HI 11342, HI 11431, HI 11446, and ATCC 43313 are closely related to each other and should be considered conspecific. These strains could be divided into groups. The main group consisted of serotype O1 and O2 strains isolated from Atlantic salmon, rainbow trout, turbot, cod, and saithe (Fig. 2 and Table 1). Serotype O2a strains isolated from cod formed one subgroup, while serotype O2b strains isolated from cod clustered with serotype O2a and O1 strains isolated from turbot and salmon. Thus, there was not an obvious correspondence among the subgroups based on host specificity, serotype, and phylogenetic position. The other distinct group was represented by the type strain, strain NCMB 6. On the basis of the 16S rRNA sequences compared, this strain could not be distinguished from strains identified as *V. ordalii* (ATCC 33509^T and NCMB 2167). An organism that is very closely related to these strains is *V. damsela* NCMB 2184^T (level of sequence similarity, 99.2%). Strain MSC2-75, which was previously identified as *V. ordalii* (40, 41), was far removed from the other *V. ordalii* strains (Fig. 2) and clustered with *V. hollisae*.

Three strains identified as *V. anguillarum* were only moderately related to the type strain of this species (levels of sequence difference, 3 to 5%). The levels of similarity between strain HI 11446 and strains HI 11342 and HI 11431 were as great as the levels of similarity between members of the subgroups containing the authentic *V. anguillarum* strains.

The results of our comparative 16S rRNA analysis justified the status of *V. salmonicida* as a distinct species. Despite diverging biochemically from the original species description (13) by being able to ferment galactose, cellobiose, and betagentiobiose, as well as by being able to produce lysine decarboxylase and to reduce nitrate to nitrite, strain HI 651, which was isolated from diseased halibut, exhibited a high level of sequence similarity to the type strain of *V. salmonicida* (Table 4). Except for the fact that strain HI 651 is able to grow in the presence of a maximum NaCl concentration of 6% and has a maximum growth temperature of 25°C (the corresponding values for NCMB 2262^T are 4% NaCl and 20°C), strains HI 651 and NCMB 2262^T exhibit similar growth patterns with respect to NaCl concentrations and temperatures. Strain HI 651 exhibited the same serological pattern as the type strain of *V. salmonicida*. Strain PT2, which was isolated from the intestines of healthy cod, is phylogenetically almost indistinguishable from *V. salmonicida* strains (33). This strain does not seem to have the same serological pattern as strains NCMB 2262^T and HI 651 (37). Biochemical and distant matrix data for strains *V. salmonicida* NCMB 2262^T, HI 651, and PT2 are shown in Table 4.

V. fischeri NCMB 1274 and *V. salmonicida* HI 11391 are closely related to the type strain of *V. salmonicida* and can be considered members of this species. The species *Vibrio iliopiscarius* has recently been described for a group of gut vibrios (33).

DISCUSSION

In this study, we performed a comparative 16S rRNA analysis with selected fish-pathogenic vibrios and some nonpathogenic vibrios. The bacterial strains were selected with care on

TABLE 4. Differentiating biochemical characteristics and distance matrix data for *V. salmonicida* and *V. salmonicida*-like strains

Taxon	Lysine decarboxylase activity	Reduction of NO ₃ to NO ₂	Utilization of:			Maximum NaCl concn (%)	Maximum temp (°C)	% Similarity to:	
			Galactose	Cellobiose	β -Gentiobiose			Strain NCMB 2262 ^T	Strain HI 651
Strain NCMB 2262 ^T	— ^a	—	—	—	—	4	20		
Strain HI 651	+	+	+	+	+	6	25	99.4	
Strains represented by PT2 ^b	+	+	d	d	d	5	22	99.4	99.8

^a —, negative; +, positive; d, 11 to 89% of the strains are positive.

^b Data from reference 33.

the basis of their biochemical (38, 51, 53), serological (23, 38), genetic (51–53), and host-specific (38) properties. Such a strategy made it possible to compare the results of the 16S rRNA analysis with results based on other classification systems. The evolutionary relationships determined in this study agree in many respects with the current taxonomy of fish-pathogenic vibrios. However, our 16S rRNA analysis also revealed a few unexpected phylogenetic relationships.

The three 16S rRNA regions sequenced by using primers 215 (positions 1 to 186), 530 (positions 301 to 504), and 1100 (positions 925 to 1088) turned out to be specifically suitable for determining phylogenetic relationships among the vibrios. The region from position 925 to position 1088 (the most variable portion was between positions 987 and 1041) allowed us to differentiate *Vibrio* species from each other and from members of other genera. The region from position 301 to position 504 (the most variable portion was between positions 451 and 477) allowed us to differentiate *Vibrio* species from each other and to some degree to differentiate strains belonging to the same species. The region from position 1 to position 186 (the most variable portion was between positions 60 and 100) was found to be useful for intraspecies differentiation. The region from position 330 to position 500 has been used previously in phylogenetic studies of the genus *Vibrio* (10, 22, 35). Because of the limited overlap between the 16S rRNA regions used for analysis (restricted to the region from position 308 to position 504), the recently published 16S rRNA sequences of *Vibrio* species (22) were not included for comparison in this study. In addition, the sequences described by Kita-Tsukamoto et al. (22) contain many positions with ambiguous bases, and the omission of these positions from the analysis would have reduced the number of comparable nucleotides to an unacceptably low number.

In spite of being preliminarily identified as *V. anguillarum*, strains HI 11342, HI 11431, HI 11446, and ATCC 43313 diverge evolutionarily from the other *V. anguillarum* strains. This finding is consistent with the fact that these strains can be separated clearly from the other strains identified as *V. anguillarum* both serologically (Table 1) and biochemically (37).

The 16S rRNAs of strains NCMB 6^T (= ATCC 19264^T) and ATCC 43313 (= 1247/1) have been partially sequenced (position 413 through position 515) previously by Rehnstam et al. (35). However, since *V. anguillarum* was the only *Vibrio* species included in the study of Rehnstam et al. (35) and because only about 100 nucleotides were compared, an accurate systematic relationship between strains ATCC 43313 and NCMB 6^T could not be determined. According to the results of our study, strain ATCC 43313, which was identified as a *V. anguillarum* serotype O9 strain, (43), is as different from NCMB 6^T (level of sequence similarity, 89%) as type strain NCMB 2262 of *V. salmonicida* is (level of sequence similarity, 89%). The distant relationship between strains ATCC 43313 and NCMB 6^T has

been confirmed by phenotypic comparisons (37). Our results agree with those of Martinez-Picado et al. (28), who showed that *V. anguillarum* ATCC 43313 (serovar O9) is not recognized by an oligonucleotide probe for *V. anguillarum* 16S rRNA. Serovar O9 and O10 strains were found to have sequences in the V3 region which clearly differed from the sequences of strains belonging to the other serovars (28). In conclusion, the classification of strain ATCC 43313 as a strain of *V. anguillarum* should be confirmed by DNA-DNA hybridization experiments.

According to our 16S rRNA sequence data, type strain NCMB 6 (= ATCC 19264) paradoxically does not seem to be typical of the species *V. anguillarum*. This result is consistent with the results of our previous DNA-DNA homology studies (52) and also with the results of studies which showed that strain NCMB 6^T differs from the majority of the *V. anguillarum* strains that have been characterized with respect to biochemical characteristics and also with respect to plasmid pattern (53).

A fish-pathogenic vibrio designated *V. ordalii* (41) has been isolated in North America and Japan (19, 30, 31). The results of our 16S rRNA analysis show that the type strain of *V. ordalii*, strain ATCC 33509^T (= DF₃K^T), and strain NCMB 2167 (= DF₁K) are phylogenetically almost indistinguishable from type strain NCMB 6 of *V. anguillarum*. Strains ATCC 33509^T, NCMB 2167, and NCMB 6^T have nearly identical DNA G+C contents (44 mol% according to Schiewe et al. [41] and 47 mol% according to Wiik and Egidius [52]). Recently, Larsen et al. (25) found that all *V. ordalii* strains included in their study reacted with O2a antiserum prepared against *V. anguillarum*. On the basis of these results the validity of the species *V. ordalii* (41) needs to be reconfirmed by DNA-DNA hybridization.

Strain MSC2-75, which originally was identified as *V. ordalii*, is only distantly related to strains ATCC 33509^T and NCMB 2167, as shown by the 16S rRNA analysis results. These results are consistent with the results of our previous DNA-DNA hybridization studies which revealed a low level of DNA homology between strains NCMB 2167 and MSC2-75 (52). However, strains NCMB 2167 and MSC2-75 produce the same plasmid pattern (40, 51). The pathogenicity of these bacterial strains may be encoded by genes on the plasmid, and this may be the reason why they have been associated with the same fish disease. According to the 16S rRNA sequence data, strain MSC2-75 cannot be placed in any of the other *Vibrio* species included in this study.

Both *V. ordalii* and type strain NCMB 2184 of *V. damsela* (26) seem to be phylogenetically very closely related to the type strain of *V. anguillarum*. On the basis of our 16S rRNA results these strains can be considered members of the same species. On the basis of the results of the partial 16S rRNA analysis of Kita-Tsukamoto et al. (22), however, the type strains of *V. anguillarum* and *V. damsela* are not similar enough to justify

placement in the same species. The results of comparative 5S rRNA analysis have shown that the type strains of *V. anguillarum* and *V. damsela* are fairly closely related (27). However, these strains were not included in the same species by MacDonell and Colwell but rather were placed in the new genus *Listonella*, which includes the species *Listonella anguillara* (*V. anguillarum*), *Listonella damsela* (*V. damsela*), and *Listonella pelagia* (*V. pelagius*) (27). *V. damsela* and *V. anguillarum* have similar biochemical properties (36, 53). For both *V. damsela* and *V. anguillarum*, the main clinical symptoms of affected moribund fish are lethargy, hemorrhages at the base of the tail, and extensive ulcerative lesions (36). These taxa also have several host species in common. The G+C contents of *V. anguillarum* and *V. damsela* have been determined to be 46 to 47 mol% (52) and 43 mol% (26), respectively. Different methods were used to determine the G+C contents of *V. anguillarum* and *V. damsela*, and this may explain the disparity in the G+C content values.

Until now, *V. salmonicida* has been considered a biochemically very homogeneous species that consists of strains which are isolated from salmonid fish suffering from cold-water vibriosis (13, 44, 52). Occasionally, this bacterium, which has been assigned a maximum growth temperature of 20°C, has also caused disease in cod (44). By performing a comparative 16S rRNA sequence analysis, we found that strain HI 651, which was isolated from diseased halibut fry, is closely related to traditional *V. salmonicida* strains, which is consistent with the identical serotypes of these organisms. Strain HI 651, however, differs biochemically from other isolates of *V. salmonicida*.

In addition to strain HI 651, the apparently nonpathogenic strain PT2, which is indigenous to the intestines of marine fish, exhibits a close phylogenetic relationship to the *V. salmonicida* group (33). Strain PT2 clearly differs from typical *V. salmonicida* strains in its biochemical properties and also by the fact that it is able to grow at higher temperatures (33). This strain, however, has a DNA G+C content which is nearly identical to that of typical *V. salmonicida* strains (33, 52). On the basis of our new data for *V. salmonicida*, we suggest that the species description should be extended to include strains represented by strains HI 651 and PT2.

In this work, two groups of bacteria indigenous to the intestines of marine fish were represented by strains PT2 and PS1^T. While strain PT2, which was isolated from cod, should probably be included in the species *V. salmonicida*, strain PS1, which was isolated from herring, has recently been designated the type strain of the new species *V. iliopiscarius* (33). This new species is quite closely related to *V. salmonicida* and *V. fischeri* (NCMB 1281^T) (Fig. 2). The DNA base compositions of these three species range from 39 to 42 mol% G+C.

Although strain NCMB 1274 has been identified as *V. fischeri*, our 16S rRNA analysis results indicate that it does not belong to *V. fischeri* as represented by type strain NCMB 1281. On the other hand, strain NCMB 1274 is more closely related phylogenetically to the *V. salmonicida* group.

In conclusion, comparative 16S rRNA sequence analysis seems to be suitable for identification and differentiation of fish-pathogenic vibrios. The evolutionary relationships determined in our study in many respects are consistent with the current taxonomy, but interesting unexpected phylogenetic relationships were also revealed. Further studies should be performed in order to compare systematically results based on different classification systems.

ACKNOWLEDGMENTS

We thank Kjell Arne Hoff, Vigdis Torsvik, and Rogaland Research (RF) for support.

This work was supported by a grant from the Norwegian Council of Fisheries Research (NFFR).

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