Phylogenetic Relationships of Marine Bacteria, Mainly Members of the Family *Vibrionaceae*, Determined on the Basis of 16S rRNA Sequences

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The phylogenetic relationships of 50 reference strains, mostly marine bacteria which require Na⁺ for growth, were determined on the basis of 600 16S rRNA nucleotides by using reverse transcriptase sequencing. Strains belonging to 10 genera were included (four genera of the family *Vibrionaceae*, the genus *Aeromonas* of the family *Aeromonadaceae*, and the genera *Alteromonas*, *Marinomonas*, *Shewanella*, *Pseudomonas*, and *Deleya*). The sequences were aligned, the similarity values and evolutionary distance values were determined, and a phylogenetic tree was constructed by using the neighbor-joining method. On the basis of our results, the family *Vibrionaceae* was separated into at least seven groups (genera and families). *Vibrio marinus* clearly was on a line of descent that was remote from other vibrios. As determined by the similarity and evolutionary distance values, *V. marinus* is more distantly related to the family *Vibrionaceae* than the members of the *Aeromonadaceae* are. Also, *Vibrio cholerae* strains formed a separate group with *Vibrio mimicus* at the genus level. Of 30 species of the *Vibrionaceae*, 17 formed a large phylogenetic cluster. The genus *Listonella* was found to be a heterogeneous group, and the species were distributed in various subgroups of the *Vibrionaceae*. The separation of the family *Aeromonadaceae* from the family *Vibrionaceae* and the separation of the genera *Marinomonas* and *Shewanella* from the genus *Alteromonas* were confirmed in this phylogenetic study. However, a marine *Pseudomonas* species, *Pseudomonas nautica*, was clearly separated from two terrestrial *Pseudomonas* species. Each group that was separated by the phylogenetic analysis had characteristic 16S rRNA sequence patterns that were common only to species in that group. Therefore, the characteristic sequences described in this paper may be useful for identification purposes.

Heterotrophic bacteria which are gram negative and motile by means of flagella are commonly isolated from marine environments and apparently are a major component of the bacterial flora of the sea. On the basis of their ability to ferment carbohydrates, these organisms can be divided into two groups. The fermentative strains have been assigned to the genera *Vibrio*, *Listonella*, *Photobacterium*, *Colwellia* (10), and *Aeromonas*, and the nonfermentative strains have been included in the genera *Alteromonas*, *Pseudomonas*, *Alcaligenes*, *Deleya*, *Marinomonas*, *Shewanella*, and *Flavobacterium* (1).

The family *Vibrionaceae* is one of the most important bacterial groups in marine environments. Members of this family often predominate in the bacterial flora of seawater, plankton, and fish. In a survey carried out in the West Pacific Ocean, vibrios accounted for nearly 80% of the bacterial population in surface seawater (31). Members of the *Vibrionaceae* are also the main organisms present in the intestinal flora of marine fish (30). In addition, some members of the *Vibrionaceae* are important pathogens for humans and animals.

The marine members of the *Vibrionaceae* have been the subject of many taxonomic studies, and this group is now the best-established marine bacterial taxon. The number of described species in the family *Vibrionaceae*, particularly the genus *Vibrio*, has been expanding rapidly. The number of *Vibrio* species increased from 5 in *Bergey's Manual of Determinative Bacteriology* in 1974 (29) to 20 in *Bergey's Manual of Systematic Bacteriology* in 1984 (2) and now exceeds 34 (3, 4, 9, 12–16, 20, 26, 28, 33, 34, 37). Because of

the expansion of the family *Vibrionaceae*, it is important to establish the phylogenetic relationships among its members.

Although methods based on molecular biology, including DNA-DNA hybridization and DNA-rRNA hybridization, have been used for bacterial taxonomy for a long time, the recent development of techniques for sequencing DNA and RNA is expected to lead to a fundamental revision of microbial systematics. MacDonell and Colwell have applied the results of a sequence analysis of 5S rRNAs to the taxonomy of marine bacteria, mainly members of the genus *Vibrio* (22).

Recently, many reports have been published on the 16S rRNA sequences of bacteria and the phylogenetic relationships deduced from analyses of these sequences (6, 7, 11, 19, 25). Most of the results indicate that phylogenetic relationships based on 16S rRNA sequences support the distinction among eubacteria, archaeobacteria, and eucaryota and are generally in accord with the present status of bacterial classification. Also, the phylogenetic significance of bacterial classification based on 16S rRNA sequences has been recognized by many workers, and an ad hoc committee has recently recommended changes in classification (36).

In this paper we describe the phylogenetic relationships among 50 reference strains that are representative of marine bacteria, including 34 strains of the family *Vibrionaceae*. Most of the marine bacterial strains which we studied require Na⁺ for growth.

MATERIALS AND METHODS

Strains and cultivation. The 50 reference strains used in this study are listed in Table 1. These organisms were obtained from several culture collections, including the

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TABLE 1. Strains used in this study

Taxon	Strain	Abbreviation ^a
<i>Aeromonas caviae</i>	JCM 1060 ^{Tb}	ACAV
<i>Aeromonas hydrophila</i> subsp. <i>anaerogenes</i>	IFO 13282 ^T	AHAN
<i>Aeromonas media</i>	JCM 2385 ^T	AMED
<i>Aeromonas salmonicida</i> subsp. <i>masoucida</i>	IFO 13784	ASMA
<i>Aeromonas veronii</i>	ATCC 35624 ^T	AVER
<i>Alteromonas espejiana</i>	IAM 12640	ALES
<i>Alteromonas haloplanktis</i>	ATCC 14393 ^T	ALHA
<i>Alteromonas nigrifaciens</i>	IAM 13010 ^T	ALNI
<i>Deleya marina</i>	IAM 12928	DMAR
<i>Escherichia coli</i>		ECOL
<i>Listonella anguillara</i>	IFO 13266 ^T	LANG
<i>Listonella damsela</i>	ATCC 33539 ^T	LDAM
<i>Listonella pelagia</i>	ATCC 25916 ^T	LPEL
<i>Marinomonas communis</i>	IAM 12914 ^T	MCOM
<i>Marinomonas vaga</i>	IAM 12923 ^T	MVAG
<i>Photobacterium angustum</i>		PANG
<i>Photobacterium leiognathi</i>	ATCC 25521 ^T	PLEI
<i>Photobacterium phosphoreum</i>	ATCC 11040 ^T	PPHO
<i>Plesiomonas shigelloides</i>	ATCC 14029 ^T	PLSH
<i>Pseudomonas aeruginosa</i>	IAM 1514 ^T	PSAE
<i>Pseudomonas fluorescens</i>	IAM 12022 ^T	PSFL
<i>Pseudomonas nautica</i>	IAM 12929 ^T	PSNA
<i>Shewanella putrefaciens</i>	IAM 12079	SPUT
<i>Vibrio aestuarianus</i>	ATCC 35048 ^T	VAES
<i>Vibrio alginolyticus</i>	NCMB 1903 ^T	VALG
<i>Vibrio campbellii</i>	ATCC 25920 ^T	VCAM
<i>Vibrio carchariae</i>	ATCC 35084 ^T	VCAR
<i>Vibrio cholerae</i> Eltor Ogawa	IID 936	VCHO1
<i>Vibrio cholerae</i> Classical Inaba	IID 467	VCHO2
<i>Vibrio cholerae</i> Eltor Inaba	IID 1655	VCHO3
<i>Vibrio cholerae</i> Classical Inaba	IID 935	VCHO4
<i>Vibrio cholerae</i> Classical Ogawa	IID 934	VCHO5
<i>Vibrio costicola</i>	NCMB 701 ^T	VCOS
<i>Vibrio diazotrophicus</i>	ATCC 33466 ^T	VDIA
<i>Vibrio fischeri</i>	ATCC 7744 ^T	VFIS
<i>Vibrio fluvialis</i>	NCTC 11328	VFLU
<i>Vibrio gazogenes</i>	ATCC 29988 ^T	VGAZ
<i>Vibrio harveyi</i>	ATCC 14126 ^T	VHAR
<i>Vibrio hollisae</i>	JCM 1283 ^T	VHOL
<i>Vibrio logei</i>	ATCC 15382	VLOG
<i>Vibrio marinus</i>	ATCC 15381 ^T	VMAR
<i>Vibrio metschnikovii</i>	NCTC 8443 ^T	VMET
<i>Vibrio mimicus</i>	ATCC 33655	VMIM
<i>Vibrio natriegens</i>	CCM 2575 ^T	VNAT
<i>Vibrio nereis</i>	ATCC 25917 ^T	VNER
<i>Vibrio nigrapulchritudo</i>	ATCC 27043 ^T	VNIG
<i>Vibrio ordalii</i>	ATCC 33509 ^T	VORD
<i>Vibrio orientalis</i>	ATCC 33934 ^T	VORI
<i>Vibrio parahaemolyticus</i>	ATCC 17802 ^T	VPAR
<i>Vibrio proteolyticus</i>	NCMB 1326 ^T	VPRO

^a Abbreviations are used Fig. 1 through 3.^b T = type strain.

American Type Culture Collection (ATCC), Rockville, Md.; the Czechoslovak Collection of Microorganisms (CCM); the Institute of Applied Microbiology (IAM), Tokyo, Japan; the Institute for Fermentation, Osaka (IFO), Osaka, Japan; The Institute of Medical Science (IID), Tokyo, Japan; the Japan Collection of Microorganisms (JCM), Tokyo, Japan; the National Collection of Marine Bacteria (NCMB), Aberdeen, Scotland; and the National Collection of Type Cultures (NCTC), London, England.

Strains were grown with shaking in half-strength ZoBell 2216E medium (38) at 20°C. Cells were harvested at approx-

imately early stationary phase by centrifugation, washed with sterile TMK buffer (10 mM magnesium acetate, 25 mM KCl, 50 mM Tris-HCl [pH 7.6]), and stored at -25°C.

Extraction and determination of 16S rRNA sequences. Cells were suspended in TMK buffer, and Macoloid solution (23)

TABLE 2. Accession numbers for partial 16S rRNA sequences of strains included in this study

Taxon	Accession no.		
	Primer R520	Primer R920	Primer R1400
<i>Aeromonas caviae</i> JCM 1060 ^T	D11169	D11218	D11267
<i>Aeromonas hydrophila</i> subsp. <i>anaerogenes</i> IFO 13282 ^T	D11170	D11219	D11268
<i>Aeromonas media</i> JCM 2385 ^T	D11174	D11223	D11272
<i>Aeromonas salmonicida</i> subsp. <i>masoucida</i> IFO 13784	D11175	D11124	D11273
<i>Aeromonas veronii</i> ATCC 35624 ^T	D11176	D11225	D11274
<i>Alteromonas espejiana</i> IAM 12640	D11171	D11220	D11269
<i>Alteromonas haloplanktis</i> ATCC 14393 ^T	D11172	D11221	D11270
<i>Alteromonas nigrifaciens</i> IAM 13010 ^T	D11173	D11222	D11271
<i>Deleya marina</i> IAM 12928	D11177	D11226	D11275
<i>Listonella anguillara</i> IFO 13266 ^T	D11178	D11227	D11276
<i>Listonella damsela</i> ATCC 33539 ^T	D11179	D11228	D11277
<i>Listonella pelagia</i> ATCC 25916 ^T	D11180	D11229	D11278
<i>Marinomonas communis</i> IAM 12914 ^T	D11181	D11230	D11279
<i>Marinomonas vaga</i> IAM 12923 ^T	D11182	D11231	D11280
<i>Photobacterium angustum</i>	D11183	D11232	D11281
<i>Photobacterium leiognathi</i> ATCC 25521 ^T	D11184	D11233	D11282
<i>Photobacterium phosphoreum</i> ATCC 11040 ^T	D11186	D11235	D11284
<i>Plesiomonas shigelloides</i> ATCC 14029 ^T	D11185	D11234	D11283
<i>Pseudomonas aeruginosa</i> IAM 514 ^T	D11187	D11236	D11285
<i>Pseudomonas fluorescens</i> IAM 12022 ^T	D11188	D11237	D11286
<i>Pseudomonas nautica</i> IAM 12929 ^T	D11189	D11238	D11287
<i>Shewanella putrefaciens</i> IAM 12079	D11190	D11239	D11288
<i>Vibrio aestuarianus</i> ATCC 35048 ^T	D11191	D11240	D11289
<i>Vibrio alginolyticus</i> NCMB 1903 ^T	D11192	D11241	D11290
<i>Vibrio campbellii</i> ATCC 25920 ^T	D11193	D11242	D11291
<i>Vibrio carchariae</i> ATCC 35084 ^T	D11194	D11243	D11292
<i>Vibrio cholerae</i> Eltor Ogawa IID 936	D11195	D11244	D11293
<i>Vibrio cholerae</i> Classical Inaba IID 467	D11196	D11245	D11294
<i>Vibrio cholerae</i> Eltor Inaba IID 1655	D11197	D11246	D11295
<i>Vibrio cholerae</i> Classical Inaba IID 935	D11198	D11247	D11296
<i>Vibrio cholerae</i> Classical Ogawa IID 934	D11199	D11248	D11297
<i>Vibrio costicola</i> NCMB 701 ^T	D11200	D11249	D11298
<i>Vibrio diazotrophicus</i> ATCC 33466 ^T	D11201	D11250	D11299
<i>Vibrio fischeri</i> ATCC 7744 ^T	D11202	D11251	D11300
<i>Vibrio fluvialis</i> NCTC 11328	D11203	D11252	D11301
<i>Vibrio gazogenes</i> ATCC 29988 ^T	D11204	D11253	D11302
<i>Vibrio harveyi</i> ATCC 14126 ^T	D11205	D11254	D11303
<i>Vibrio hollisae</i> JCM 1283 ^T	D11206	D11255	D11304
<i>Vibrio logei</i> ATCC 15382	D11207	D11256	D11305
<i>Vibrio marinus</i> ATCC 15381 ^T	D11208	D11257	D11306
<i>Vibrio metschnikovii</i> NCTC 8443 ^T	D11209	D11258	D11307
<i>Vibrio mimicus</i> ATCC 33655	D11210	D11259	D11308
<i>Vibrio natriegens</i> CCM 2575 ^T	D11211	D11260	D11309
<i>Vibrio nereis</i> ATCC 25917 ^T	D11212	D11261	D11310
<i>Vibrio nigrapulchritudo</i> ATCC 27043 ^T	D11213	D11262	D11311
<i>Vibrio ordalii</i> ATCC 33509 ^T	D11214	D11263	D11312
<i>Vibrio orientalis</i> ATCC 33934 ^T	D11215	D11264	D11313
<i>Vibrio parahaemolyticus</i> ATCC 17802 ^T	D11216	D11265	D11314
<i>Vibrio proteolyticus</i> NCMB 1326 ^T	D11217	D11266	D11315

	290	300	310	320	330	340	350	360	370	380	390	400
ECOL	CUGGUCUGAG	AGGAUGACCA	GCCACACUGG	AACUGAGACA	CGGUCCAGAC	UCCUACGGGA	GGCAGCAGUG	GGGAUAUUG	CACAAUGGGC	GCAAGCCUGA	UGCAGCCAUG	CCGCGUGUAU
VCH01	..	U				W						
VCH02	-K					W						
VCH03							-K					
VCH04							-K					
VCH05							-K					
VMIM		U			U	W						
VMAR	...	U	-K	U	W	U				A	K	A
VCOS				G	C	NN	U		D	G	AG	C
VHOL					U							U
LDAM									R	G	A	C
VFIS							G	A	W	W	NNN	
VLOG										A	N	
LANG	B	U	K	W		W	U	W	NNN		B	
VAES						W						
VGAZ												
VMET		U			N				R			Y
VORD		U										
VORI		U			U						R	
LPEL		U			W				W			
VALG		U			D	U		U				
VCAM	-K	U	W		W	U	W	UGU	N	D	K	U
VCAR		U			W						D	
VDIA		U	K		U						N	
VFLU		U									K	
VFIAR		U			W			U				
VNAT		U			W						N	
VNER		U			W			N			N	
VNIG		U										
VPAR		U			D	U						
VPRO	-K	U										
PPHO		U							W			
PANG	-K	U			Y		Y		W			
PLEI		U			W	W						
PLSH		A	U	U	U	W	KKKD	D	NNNN			
AHAN												
ACAV		U			W	U	U		K			
AMED					W	W						
ASMA		U										
AVER	-K	U			W	D	D		D			
ALES		U		G	NN	W						
ALHA		U		G	W	W						
ALNI	U	U		G	W	W						
SPUT							W	U				
MCOM		U		G	C	U	U					
MVAG		U		G	C	H	W					
PSAE		U										
PSFL		U										
PSNA		U										
DMAR												

FIG. 1. Partial 16S rRNA sequences of 50 reference strains, corresponding to positions 290 to 509, 690 to 904, and 1180 to 1379 of the *E. coli* 16S rRNA sequence (5). Abbreviations for nucleotides used in the alignment: R, purine (A or G); Y, pyrimidine (C or U); M, A or C; W, A or U; S, C or G; K, G or U; D, A, G, or U; H, A, C, or U; V, A, C, or G; B, C, G, or U; N, A, C, G, or U; the dots indicate padding. These abbreviations and symbol are currently under consideration by a commission of the International Union of Pure and Applied Chemistry-International Union of Biochemistry. For abbreviations of organism names, see Table 1. The portions enclosed in boxes are sequences that are common to the members of individual branching groups (Fig. 2).

was added as an RNase inhibitor. When cells which have particularly high levels of RNase activity were prepared, phenol was added along with the Macoloid solution as an additional RNase inhibitor. Nucleic acids were purified by phenol-chloroform extraction and cold isopropanol precipitation. DNA was digested with 60 U of RNase-free DNase I (Takara Shuzo Co., Ltd.). Each RNA preparation was dried and suspended in 10 mM Tris-HCl buffer (pH 8.5) at a concentration of 8 µg/µl.

16S rRNA sequences were determined by using the reverse transcriptase reaction and oligonucleotide primers specific to the 16S rRNAs. For sequencing we used the protocol described by Lane et al. (18), with some modifications. Three DNA primers were used in the sequencing reactions; primer R520 covered the sequence from position 517 to position 531 (*Escherichia coli* numbering) (5), primer R920 covered the sequence from position 907 to position

926, and primer R1400 covered the sequence from position 1392 to position 1406.

The reverse transcriptase sequencing reaction was stopped by adding 5 µl of stop mixture (15 mM EDTA [pH 8.0]), and the preparation was dried in vacuo. After a dye mixture (0.05% bromophenol blue and 0.05% xylene cyanol in formamide) was added, the reaction mixture was heated for 3 min at 97°C before it was loaded onto the sequencing gel. A nongradient 8% polyacrylamide gel (thickness, 0.35 mm; length, 50 cm) was used for electrophoresis.

Determination of phylogenetic relationships. Sequences were initially aligned on the basis of obvious primary structure homology. The evolutionary distance per site (*K*) was calculated as follows: $K = -(1/2) \ln [(1 - 2P - Q)(1 - 2Q)^{1/2}]$ (17), where *P* and *Q* were the fractions of nucleotide sites having transition and transversion types of substitu-

	410	420	430	440	450	460	470	480	490	500
ECOL	GAAGAAGGCC	UUCGGGUUGU	AAAGUACUUU	CAGCGGGGAG	GAAGGGAGUA	AAGUUAUAC	CUUUGCUCAU	UGACGUUACC	CGCAGAAGAA	GCACCGGCUA
VCH01	-----	-----	-----	-WUA-----	UG-U-----	-Y-----	AAUCAU-----	UA-----	-----	-----
VCH02	-----	-----	-----	UA-----	UG-U-----	-----	AAUCAU-----	UA-----	-----	-----
VCH03	-----	-----	-----	UA-----	UG-U-----	-Y-----	AAUCAU-----	UA-----	-----	-----
VCH04	-----	-----	-----	UA-----	UG-U-----	-----	AAUCAU-----	UA-----	-----	-----
VCH05	-----	-----	-----	UA-----	UG-U-----	-----	AAUCAU-----	UA-----	-----	-----
VMIM	-----	-----	-----	UA-----	UG-U-----	-----	AAUCAU-----	UA-----	-----	-----
VMAR	-----	-----	-----	-A-----	-A-U-GU-D-----	-----	U-AC-A-UG-----	U-----	-----	-----
VCOS	-----	-----	-C-----	-A-U-----	UC-GU-----	-----	G-GCA-GGC-----	G-U-----	-----	-----
VHOL	-----	-----	-C-----	-C-U-----	UC-GU-----	-----	GC-U-N-----	G-----	-----	-----
LDAM	-R-----	-DN-----	-C-----	-N-U-----	UC-GU-----	-----	GCAU-AU-----	-S-----	UDY-----	-N-----
VFIS	-----	-----	-----	-N-----	UGU-GU-----	-----	G-GCAGCAU-----	UA-----	-----	-----
VLOG	-----	-----	-----	NN-UC-U-----	UU-U-----	-----	G-GCAUAUC-----	G-----	-----	-----
LANG	-----	-C-----	-----	NNKKNN-----	-W-UN-G-N-----	-----	C-AGCCA-----	-U-CC-G-----	GA-S-----	-----
VAES	-----	-----	-----	UC-U-----	-G-----	UC-----	G-GG-AU-----	G-----	GA-----	-----
VGAZ	-----	-----	-----	U-U-----	AUA-----	GC-----	G-AG-AU-UU-----	G-----	U-----	-----
VMET	-R-----	-----	-----	UC-K-----	-K-----	UC-----	G-GG-AU-----	G-----	GA-----	-----
VORD	-----	-----	-----	A-U-----	-G-AU-----	GC-----	G-AUUCU-----	G-----	U-----	-----
VORI	-----	-----	-----	A-U-----	UG-AG-----	UC-----	G-GCCUAC-----	G-----	U-----	-----
LPOL	-----	-----	-----	N-U-----	-U-G-G-----	UC-----	G-GCAU-UC-----	G-----	U-----	-----
VALG	-----	-----	-C-----	UC-U-----	-U-G-UN-----	-----	G-SCAUAU-----	G-----	GA-N-----	-----
VCAM	-W-----	-----	-C-----	UC-B-----	-U-G-B-----	-----	G-GCAU-AU-----	G-----	GA-----	-----
VCAR	-----	-----	-C-----	UC-U-----	-U-G-----	-----	G-GCAU-AU-----	G-----	GA-----	CG-----
VDIA	-----	-----	-C-----	UC-U-----	UG-G-UU-----	-----	G-ACCAUYN-----	G-----	GA-----	CG-----
VFLU	-----	-----	-C-----	UCHU-----	-U-G-----	-----	G-NCAN-MU-----	G-----	GA-----	-----
VHAR	-----	-----	-C-----	UC-U-----	-U-G-----	-----	G-GCAU-AU-----	G-----	GA-----	-----
VNAT	-----	-----	N-C-----	UC-U-----	-U-AU-GC-----	-----	G-GKAAAGAA-----	G-----	GA-----	-----
VNER	-----	-----	-C-----	UC-U-----	-U-G-----	-----	G-GCAU-AU-----	G-----	GA-----	-----
VNIG	-----	-----	-C-----	VV-U-----	-M-----	U-G-----	G-A-GCAU-AU-----	G-----	US-----	-----
VPAR	-----	-----	-C-----	UC-Y-----	-U-G-----	U-----	G-GCAU-AU-----	G-----	GA-N-----	-----
VPRO	-----	-----	-C-----	UC-U-----	-U-G-----	UDU-----	G-A-GCAU-MU-----	G-----	GA-N-----	-----
PPHO	-----	-----	-----	UU-U-----	CU-U-----	G-----	G-CAGCGC-----	D-G-----	AA-----	-----
PANG	-----	-Y-----	-----	UUU-K-----	CG-----	G-----	A-YCA-Y-U-----	G-----	AA-----	-----
PLEI	-----	-----	-----	UA-----	-C-----	UC-----	G-GCAU-GU-----	-----	UA-----	-----
PLSH	-R-----	-----	-C-----	-K-----	CCAC-U-----	-----	-AGUGG-----	U-----	-----	-----
AHAN	-----	-----	-C-----	A-----	A-UCAG-U-----	-C-----	U-GCUGG-UG-----	U-----	-----	-----
ACAV	-K-----	-----	-C-----	A-----	A-YCAG-U-----	-C-----	U-GCUGG-UG-----	U-----	-----	-----
AMED	-----	-----	-C-----	A-----	A-UGG-UGCC-N-----	-----	GCA-CAG-UG-----	U-----	-----	-----
ASMA	-----	-----	-C-----	A-----	A-UGG CGCC-N-----	-----	G-G-YAA-UG-----	U-----	-----	-----
AVER	-----	-----	-C-----	D-A-----	A-UGG U-C-----	-----	A-GCCAG-UG-----	U-----	-----	-----
ALES	-----	-----	-C-----	U-----	A-UUAG U-----	-----	-GCUAG-UG-----	U-----	GA-----	-----
ALHA	-----	-----	-C-----	U-----	A-UUAG U-----	-----	-GCUAG-UG-----	U-----	GA-----	-----
ALNI	-----	-----	-C-----	U-----	A-UUAG U-----	-----	-GCUAG-UG-----	U-----	GA-----	-----
SPUT	-----	-----	-C-----	Y-UA-----	-A-GUGU-GY-----	-----	GC-AUA-UG-----	U-----	-G-----	-----
MCOM	-U-----	-A-----	-C-----	UU-U-----	-C-----	U-----	G-AU-UU-----	A-----	UC-----	-----
MVAG	-----	-----	-C-----	GA-U-----	UA-G-CC-----	-----	G-AU-UU-----	A-----	UC-----	-----
PSAE	-W-----	U-----A-----	-C-----	E-U-----	GA-U-----	-----	G-GUGUU-----	AA-----U-----	-----	-----
PSFL	-K-----	U-----A-----	-C-----	A-U-----	GA-U-----	CC-W-----	G-A-UGUU-----	GA-----U-----	-----	-----
PSNA	-----	-----	-C-----	UA-----	AA-CG-----	UG-C-----	CA-AGGC-----	UA-----	-----	-----
DMAR	-----	-----	-C-----	A-----	A-C-UUCG GGA-----	-----	UCCG-AGG-A A-----	A-C-----U-----	-----	-----

FIG. 1—Continued.

tions in two sequences, respectively. A phylogenetic tree was constructed by using the neighbor-joining method (27).

Nucleotide sequence accession numbers. The partial 16S rRNA sequences reported in this paper have been deposited in the DDBJ (Mishima, Japan), EMBL (Heidelberg, Germany), and GenBank (Mountain View, Calif.) nucleotide sequence data bases under accession numbers D11169 to D11315 (Table 2).

RESULTS

Partial sequences consisting of 600 16S rRNA bases, covering base positions 290 to 509, 690 to 904, and 1180 to 1379 (*Escherichia coli* numbering) (5), were determined for 50 bacterial reference strains. The sequence alignments are shown in Fig. 1. An unrooted phylogenetic tree constructed from the *K* values is shown in Fig. 2.

According to our phylogenetic analysis, 7 of the 10 genera used in this study (the genera *Aeromonas*, *Alteromonas*, *Deleya*, *Marinomonas*, *Photobacterium*, *Plesiomonas*, and *Shewanella*) represent distinct isolated lines of descent. In contrast, the members of the genus *Listonella* were divided among several branches along with species of the genus

Vibrio. The members of the genus *Pseudomonas* were divided into two clearly diverse branches, and the members of the genus *Vibrio* occurred in five branches. A total of 30 *Vibrio* and *Listonella* strains were examined; 16 *Vibrio* strains and one *Listonella* strain constituted a large branch, and 13 strains occurred on branches that were distinct from the branch that contained the other 17 strains. In particular, *Vibrio marinus* was far removed from the other members of the genus *Vibrio*. The large cluster consisting of 17 strains could be further divided into two subgroups. One of these included *Vibrio aestuarianus*, *Vibrio gazogenes*, *Vibrio meschnikovii*, *Vibrio ordalii*, *Vibrio orientalis*, and *Listonella pelagia*; these organisms had similarity values and *K* values ranging from 96.39 to 98.85% and from 1.02 to 3.86, respectively. The members of the other subgroup, including 11 species belonging to the genus *Vibrio*, had similarity values and *K* values that ranged from 95.78 to 99.79% and from 0.22 to 3.81, respectively. When the data for all 17 strains were included, the similarity values and *K* values were more than 95.32% and less than 5.06, respectively. This finding indicates that the 17 strains constitute a fairly homogeneous group.

ECOL	690	700	710	720	730	740	750	760	770	780	790	800
	GGUGAAUUGC	GUAGAGAUUC	GGAGGAAUAC	CGGUGGCGAA	GGCGGCCCCC	UGGACGAAGA	CUGACGCUCA	GGUGCGAAAG	CGUGGGGAGC	AAACAGGAUU	AGAUACCCUG	GUAGUCCACG
VCHO1			-A-----	-K--K--K--	---Y---Y---	-N--AG-U--	-----A--	-A--W----	-----	-----	-----	-N-----
VCHO2			-A-----	-K--K--K--	---K-----	-N--AG-U--	-----A--	-A--W----	-----	-----	-----	-R-----
VCHO3					---UN-----	-U--AG-U--	-----A--	-A--KW----	-----	-----	-----	-----
VCHO4									-----	-----	-----	-----
VCHO5								-A--KW----	-----	-----	-----	-----
VMIM	-K-----		-A-----			-AG-U-----	-----A--	-A-----	-----	-----	-----	-----
VMAR			KA-----	-W-----	---YA-----	---CAAGUA--	-----Y--	-A--W----	-----U--	-----G--	-----C--	-----
VCOS			[A]-----	-A-----		[A]-----		[A]-----	-----U--	-----	-----	-----
VHOL			[A]-----	-A-----		[A]-----		[A]-----	-----	-----	-----	-K-----
LDAM			[A]-----	-A-----		[A]-----		[A]-----	-----	-----	-----	-D-----
VFIS			KC-----	-W-----K--	---K-YYYY-	-K--AG-C--	[A]-----	[A]-----	-----U--	-----	-----WY--K--	-----W--
VLOG					---YYYY--	-K--AG-C--	[A]-----	[A]-----	-----	-----	-----	-----K--
LANG			-A-----	-K-----	---K-YYYY-	-K--AG-U--	[A]-----	[A]-----	-----W--	-----	-----	-----W--
VAES	---N-----		-A-----			-N--AG-U--	-----A--	[A]-----	-----	-----	-----	-----
VGAZ			NA-----		---YYY--	-N--AG-U--	-----A--	[A]-----	-----W--	-----	-----	-----
VMET			-A-----		---N-YYYY-	-N--AG-U--	-----A--	[A]-----	-----	-----	-----	-----
VORD			-A-----	-A-----	---BBB--	-N--AG-U--	-----A--	[A]-----	-----	-----	-----	-----
VORI	---K-----				---Y--	-N--AG-U--	-----A--	[A]-----	-----W--	-----	-----	-N-----
LPEL			KA-----	-A-----	---KK-YY-	-N--AG-U--	-----A--	[A]-----	-----	-----	-----	-----
VALG			UC-----	---C-----	---YYY--	-K--AG-U--	-----A--	[A]-----	-----W--	-----	-----	-B-----D--
VCAM	---K-----		KA-----	-K--K--K--	---K-YYYY-	-B--AG-U--	-----A--	[A]-----	-----U--	-----K--	-----	-B-----
VCAR		---S--S--	-A--K-----		---N-YYYY-	-N--AG-U--	-----A--	[A]-----	-----	-----	-----	-----
VDIA			BA-----		---Y--	-N--AG-U--	-----A--	[A]-----	-----W--	-----	-----	-N-----
VFLU			-A-----	-K-----	---Y--	-K--AG-U--	-----A--	[A]-----	-----W--	-----	-----	-K-----
VHAR			U-----		---K--	-K--AG-U--	-----A--	[A]-----	-----	-----K--	-----W--	-----
VNAT	---K-----		-A-----	-D-----K--	---YYYY-	-K--AG-U--	-----A--	[A]-----	-----	-----	-----	-----
VNER				-K-----	---YYYY-	-K--AG-U--	-----A--	[A]-----	-----W--	-----K--	-----	-----
VNIG	---R-----		-A-----	---S-----		-K--AG-U--	-----A--	[A]-----	-----R--	-----	-----W--D--	-----W--
VPAR				-K-----	---YYY--	-N--AG-U--	-----A--	[A]-----	-----W--	-----	-----	-N-----
VPRO			-A-----		---YYY--	-N--AG-U--	-----A--	[A]-----	-----	-----	-----	-----
PPHO	---K-----		KA-----	-K--C-----	---K-YYYY-	[A]-----		[A]-----	-----W--	-----	-----	-----
PANG			KA-----	-K--K--K--	---YYY--	-AG-U-----	-----A--	[A]-----	-----U--	-----	-----	-K-----
PLEI				---U--	---YYYY-	[A]-----		[A]-----	-----Y--	-----	-----	-N-----
PLSH			K-----		---YY--	-A-----	---B-----	-----W--	-----	-----	-----D--	---B--
AHAN					---Y--	[A]-----		-----	-----	-----	-----	-N-----
ACAV	---K-----			-K-----		[A]-----		-----KW--	-----	-----	-----D--	-----
AMED			N-----	-K--S-----		[A]-----		-----W--	-----	-----	-----	-N-----
ASMA						[A]-----	---K-----	-----U--	-----	-----	-----	-N-----
AYER			---K-----	-K-----		[A]-----		-----KW--	-----	-----	-----	-----
ALES							---N--K--	[U--A--W--	-----	-----	-----	-----
ALHA			KA-----	-A-----	---A--A--	---GUC-C--		[U--A--U--	-----	-----	-----	-N-----W--
ALNI			KA-----	-A-----	---A--A--	---GUC-C--		[U--A--U--	-----	-----G--	-----CN--	-----W--
SPUT			K-----		---K-YYYY-	-A-----	---B-----	U-CA-KU--	-----	-----	-----	-N-----
MCOM									-----	-----	-----	-----
MVAG			U--AG--	-A--C--U--	-A--	-A--A--	-NAC--U--	-A--G--	-----	-----	-----	---N--
PSAE			U--AG--	-A--C--A--		[A]-----	-N--U--	[A]-----	-----	-----	-----	-----
PSFL			U--AG--	-A--C--AU--		[A]-----	-N--U--	[A]-----	-----	-----	-----	-----
PSNA			U--AG--	-A--C--A--		[A]-----	-K--C--C--	[A]-----	-----	-----	-----	-K-----
DMAR					---UUU--	-K--UG-C--	-----A--G--	-----W--	-----U--	-----	-----	-----

FIG. 1—Continued.

Figure 3 shows the similarity values and *K* values for the 16S rRNA sequences of the organisms used in this study. High similarity values and low *K* values were observed among the species in the separate groups (new genera) of the genus *Vibrio*. Five strains of *V. cholerae*, including two biotypes and two serotypes, formed one clearly distinguishable cluster along with *Vibrio mimicus*; the levels of similarity among the strains ranged from 99.06 to 100.00%, and the *K* values ranged from 0.00 to 0.78, indicating that these organisms are very closely related. On the other hand, the distances between the *V. cholerae*-*V. mimicus* group and other vibrio groups were quite large. Three species of the genus *Photobacterium* were members of one branch, which was located near the large compact cluster that included 17 species of the *Vibrionaceae*. *Vibrio fischeri*, *Vibrio logei*, and *Listonella anguillara* also were on one branch near the big cluster but were quite distant from each other. The similarity value and *K* value ranges for *Listonella* species were much greater than the ranges for most genera, showing that this group of organisms is heterogeneous. The similarity values and *K* values for the genus *Pseudomonas* also exhibited a great deal of variability, ranging from 88.48 to 96.15% and from 4.02 to 12.95, respectively. In this study, three

Pseudomonas species were used. Two species, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*, do not require Na⁺ for growth, while *Pseudomonas nautica* does. For the two terrestrial species the similarity and *K* values were 96.15% and 4.02, respectively, indicating a close relationship.

Plesiomonas shigelloides was far from the other members of the family *Vibrionaceae* (Fig. 2). The location of this species was rather close to the locations of family *Aeromonadaceae* and *E. coli*.

Another outstanding feature of the results of our 16S rRNA sequence analysis was that each bacterial genus had its own specific sequence pattern that was common to the species in that genus. These common sequences which characterized each genus are indicated in Fig. 1. For example, all three species of the genus *Photobacterium* had common sequences which were not identical to base sequences of other genera; they were U, G-A-C, U, C, G, CA, and A at positions 307, 379 to 384, 443, 455, 459, 473 to 474, and 491, respectively. The genus *Aeromonas* had a specific sequence pattern characterized by G-A-C, G, C, A-----A--U, C, and UG-----U at positions 379 to 384, 408, 434, 445 to 456, 463, and 478 to 489, respectively.

	810	820	830	840	850	860	870	880	890	900
ECOL	CCGUAACGA	UGUCGACUUG	GAGGUUGUC	CCUUGAGGCG	UGGCUUCCGG	AGCUAACGCG	UUAAGUCGAC	CGCCUGGGGA	GUACGGCCGC	AAGGU
VCH01		U		A	U	CG		A		UN
VCH02		U	R	A	U	CG		N		U
VCH03		U		A	U	CG		A		U
VCH04		U		A	U	CG		A		U
VCH05		U		A	U	CG		A		U
VMIM		U	A	A	U	CG		A		U
VMAR		UD-C	U-GUU	AAC	GC-VUUA	CG	A	A		
VCOS		U	A	UU-AGACUU	U		A		U	
VHOL	U	U	C	U-NK-AC	K-U	C-K		N		U
LDAM	U	U	G	KN-C		C-K		R	G	US
VFIS		U	N-U	A	U	CG		A		U
VLOG		U	G	Y	U	CG-N		A-N		U
LANG		U	G	C	U	CG-N		R		US
VAES		U	G	N	U	CG		A		U
VGAZ		U	G	C	U	CG		A	N	U
VMET	U	U	G	C	U	C-K		A	N	UN
VORD		U	G	C	U	CG		A		U
VORI		U	G	N	U	CG		A		U
LPEL		U	G	Y-NWUC	U	CG		A		U
VALG		U	G	B	C	CG		A		U
VCAM		U	G	C	U	CG		A		U
VCAR		U	G	N	C	CG		A		U
VDIA		U	N	N	C	CG		A		U
VFLU		U	G	Y	C-K	U		A		U
VHAR		U	G	C	N	CG		A		U
VNAT		U	G	C	U	CG		A		U
VNER	W	U	K	G	N	CG		A	N	U
VNIG		U	G	C	U	CG		A		U
VPAR		U	G	C	U	CG		A		U
VPRO		U	N	NN	C	CG		A		U
PPHO		U	A	G	N	CG		A		U
PANG		U	G	C	U	CG		A		U
PLEI		U	G	C	U	CG		A	YNYN	U
PLSH	U	U	D		YY	CG		A		
AHAN		U	C	U	NN	A		A		
ACAV		U	C	U		A		A		
AMED		U	C	U	NN	A		A		
ASMA		U	C	U		A		A		
AVER		U	C	U		A		A	NNN	
ALBS		U-A	A-C-C-GAG	C-CG	UUC	UU-U-AA	CG	A	A	
ALHA		U-A	A-C-C-GAA	C-CG	UUC	UU-U-AA	C-K	A	A	
ALNI		U-A	A-C-C-GAG	C-CG	UUC	UU-U-AA	C-K	A	A	
SPUT	W	UW-C	U-GUG	U-ACAC	GC-UCA	C-K	A	A	N	
MCOM		U-A	CC-U-G-G-A	U-AUUU	U-UAG-GG-C	CG	A	A	N	
MVAG		U-A	CC-U-G-G-A	U-AUUU	U-UAG-GG-C	CG	A	A		
PSAE		A	CC-U-G-GAG	AUC	UAG-GG-C	CG	A			
PSFL		A	CC-U-G-GAG	AUC	UAG-GG-C	CG	A			
PSNA		A	CC-U-G-GAG	AUC	UAG-GG-C	CG	A			
DMAR		A	CCUUGG-C	AC	UAG-GG-C	M-K	A	A	N	NN

FIG. 1—Continued.

Likewise, the separate groups (genera) of the genus *Vibrio* and the genera *Alteromonas*, *Marinomonas*, and *Pseudomonas* had their own characteristic sequence patterns. If the vibrio groups were not separated, the common sequence patterns were less clear; in particular, the group had no common sequence at base positions 290 to 509, where all of the other genera had one or more common sequences. This result confirms the validity of separating the members of the genus *Vibrio* into several new genera. Also, our results suggest that the region of 16S rRNA from base positions 290 to 509 is evolutionarily more variable than the other two regions which we studied.

DISCUSSION

The phylogenetic relationships which we determined on the basis of a 16S rRNA sequence analysis of 50 reference strains conform to the current taxonomy of marine bacteria in many respects. For example, the genera *Marinomonas*, *Alteromonas*, and *Shewanella* are clearly distinguished from each other. The members of these three genera were placed together in the genus *Alteromonas* until 1983. The genus *Shewanella* was separated from the genus *Alteromonas* on

the basis of the results of a 5S rRNA sequence analysis (22), and in 1983 the genus *Marinomonas* was distinguished from the genus *Alteromonas* as a result of data from a nucleic acid analysis (35). The genus-specific common sequence patterns (Fig. 1), as well as the similarity and *K* values (Fig. 3), support the separation of these three genera.

The genus *Aeromonas* was once placed in the family *Vibrionaceae*. In 1986, Colwell et al. proposed a newly created family *Aeromonadaceae* on the basis of the results of 5S rRNA sequencing; a member of the genus *Aeromonas* was the type species of this family (8). Figure 2 shows that species belonging to the genus *Aeromonas* form a branch that is clearly distinct from the other species of family *Vibrionaceae*. This result supports the proposal of Colwell et al.

On the other hand, our phylogenetic data also suggest that the current taxonomic system should be revised in many respects. Our 16S rRNA sequence analysis revealed the clear phylogenetic divergence of the marine bacteria from their terrestrial counterparts. Three *Pseudomonas* species were used in this study, including one marine species, *Pseudomonas nautica*, which requires Na^+ for growth, and two terrestrial species, *Pseudomonas fluorescens* and

ECOL	1180	1190	1200	1210	1220	1230	1240	1250	1260	1270	1280	1290
	AGGUGGGGAU	GACGUCAAGU	CAUACAUGGCC	CUUACGACCA	GGGCUACACA	CGUGCUACAA	UGGCGCAUAC	AAAGAGAAGC	GACCUCGCGA	GAGCAAGCGG	ACCUCAUAAA	GUGCGUCGUA
VCH01		[G]	-----U--	-----GU	-----	-----	-----	U--G-G-C-CG	[UAC]	-----GUGG--A-U--C--A	-----	-----
VCH02	K	[G]	-----	-----GU	-----	-----	-----	U--G-G-C-CG	[UAC]	-----GUGG--A-U--C--A	-----	-----
VCH03		[G]	-----	-----GU	-----	-----	-----	U--G-G-Y-CG	[UAC]	-----GUGG--A-U--C--A	-----	-----
VCH04		[G]	-----	-----GU	-----	-----	-----	U--G-G-Y-CG	[UAC]	-----GUGG--A-U--C--A	-----	-----
VCH05		[G]	-----	-----GU	-----	-----	-----	U--G-G-Y-CG	[UAC]	-----GUGG--A-U--C--A	-----	-----
VMIM		[G]	-----	-----GU	-----	-----	-----	U--G-G-Y-CG	[UAC]	-----GUGG--A-U--C--A	-----	-----
VMAR	C	-----	-----Y--	-----GU	-----	-----	-----	-----G-CU--	A-A-CA	UG-UN--A-U-C	-----	-----
VCOS		[G]	-----	-----GU	-----	-----	-----AG--	[G-G-C]	[GAC]	K-UGUU--A-U-C-U	-----UU--	-----
VHOL		[G]	-----	-----GU	-----	-----	-----U--	[G-G-C]	[GAC]	-----A-U-C-G	-----A--	-----
LDAM		[G]	-----Y--	Y--GU	-----	-----	-----AU--	[G-G-C]	A-[GAC]	-----A-U-C-G	-----AU--	-----
VFIS		[G]	-----	-----GU	-----	-----	-----	[G-G-CU]	A-G-A--	U--UG--A-U-C-A	-----	-----
VLOG		[G]	-----	-----GU	-----	-----	-----	[G-G-CU]	A-G-A--	U--UG--A-U-C-A	-----	-----
LANG		[G]	-----	-----GU	-----	-----	-----	[G-G-C]	A-G-A--	U--UG--A-U-C-A	-----	-----
VAES		[G]	-----NN--	N--GU	-----	-----	-----	[G-G-C]	C-A-G--	A--UG--A-U-C-A	-----A--	-----
VGAZ		[G]	-----Y--	-----GU	-----	-----	-----U--	[G-G-YW]	S-A-U--	U--UG--A-U-C-A	-----	-----
VMET		[G]	-----	-----GU	-----	-----	-----	[G-G-CG]	--G-A--	U--UG--A-U-C-A	-----	-----
VORD		[G]	-----	-----GU	-----	-----	-----	[G-G-CG]	C-A-U--	U--UG--A-U-C-A	-----	-----
VORI		[G]	-----Y--	-----GU	-----	-----	-----	[G-G-C]	C-A-U--	R--UG--A-U-C-A	-----	-----
LPEL		[G]	-----	-----GU	-----	-----	-----	[G-G-C]	C-A--	U--UG--A-U-C-A	-----	-----
VALG	C	-----	-----	-----GU	-----	-----	-----	[G-G-CG]	C-A--U--	A--UG--A-U-C-A	-----	-----
VCAR		[G]	-----U--	-----GU	-----	-----	-----	[G-G-C]	C-A--U--	U--UG--A-U-C-A	-----	-----
VDIA		[G]	-----	-----GU	-----	-----	-----	[G-G-CK]	C-A--U--	U--UG--A-U-C-A	-----	-----
VFLU		[G]	-----	-----GU	-----	-----	-----	[G-G-C]	--A--U--	U--UG--A-U-C-A	-----	-----
VHAR		[G]	-----	-----GU	-----	-----	-----	[G-G-CR]	C-A--U--	U--UG--A-U-C-A	-----	-----
VNAT		[G]	-----	-----GU	-----	-----	-----	[G-G-C]	C-A--U--	U--UG--A-U-C-A	-----	-----
VNER		[G]	-----	-----GU	-----	-----	-----	[G-G-CU]	C-A--U--	A--UG--A-U-C-A	-----	-----
VNIG		[G]	-----S--	M--GU	-----	-----	-----	[G-G-CG]	C-A--UR-R	A--UG--A-U-C-A	-----	-----
VPAR		[G]	-----	-----GU	-----	-----	-----	[G-G-CG]	C-A--U--K	A--UG--A-U-C-A	-----	-----
VPRO		[G]	-----	-----GU	-----	-----	-----	[G-G-CG]	C-A--U--	A--UG--A-U-C-A	-----	-----
PPHO		[G]	-----	-----GU	-----	-----	-----	U--G-G-CU	A-G-A--	U--UG--A-U-C-C	-----A--	-----
PANG		[G]	-----	-----GU	-----	-----	-----	U--G-G-CU	C-A--A--	UG--UG--A-U-C-G	-----A--	-----
PLEI		[G]	-----	-----GU	-----	-----	-----	U--G-G-CU	C-A--A--	UG--UG--A-U-C-G	-----A--	-----
PLSH	-----	-----	-----	-----GU	-----	-----	-----AU--	K--G-CG--	A-G--A--	U--UG--A-U-C	-----AU--	-----
AHAN	-----	-----	-----NN--	N--GU	-----	-----	-----	[G--G-G-CU--	A-G--A--	U--UG--A-U-C-A--	-----C--	-----
ACAV	-----	-----	-----NNNN	N--GU	-----	-----	-----	[G--G-G-CU--	A-G--A--	U--UG--A-U-C-A--	-----C--	-----
AMED	-----	-----	-----NNNN	N--GU	-----	-----	-----	[G--G-G-CU--	A-G--A--	U--UG--A-U-C-A--	-----C--	-----
ASMA	-----	-----	-----NNNN	N--GU	-----	-----	-----	[G--G-G-CU--	A-G--A--	U--UG--A-U-C-A--	-----C--	-----
AVER	-----	-----	-----NNNN	N--GU	-----	-----	-----	[G--G-G-CU--	A-G--A--	U--UG--A-U-C-A--	-----C--	-----
ALES		[G]	-----	-----UGU	-----	-----	-----	[G-U-CU--A]	-----	U--UG--A-U-A-U	-----	-----
ALHA		[G]	-----	-----UGU	-----	-----	-----	[G-U-CU--A]	-----	U--UG--A-U-A-U	-----	-----
ALNI		[G]	-----	-----UGU	-----	-----	-----	[G-U-CU--A]	-----	U--UG--A-U-A-U	-----	-----
SPUT	C	-----N--	-----	-----GU	-----	-----	-----AG--	G-G-G-UU--	A-AGC--	GUGG--UA-U--C--CU	-----	-----
MCOM		[G]	-----Y--	-----GU	-----	-----	-----	U--G-G-C--A	-----	G--UG--AA-U-C-A--A	-----	-----
MVAG		[G]	-----Y--	-----GU	-----	-----	-----	U--G-G-C--A	-----	G--UG--AA-U-C-A--A	-----	-----
PSAE		-----	-----	-----G-U	-----	-----	-----UCCG	[G-UU]	Y-AGC	GUGG--UA-U-C--ACCGA	-----	-----
PSFL		-----	-----	-----G-U	-----	-----	-----UCCG	[G-UU]	Y-AGCUU	GUGG--UA-U-C--ACCGA	-----	-----
PSNA		-----	-----Y--	-----G-U	-----	-----	-----	[G-G-CU]	C-A--	UG--UG--A-U-C-U	-----AC	-----
DMAR	C	-----	-----	-----GGU	-----	-----	-----AAG--	G-UU--	A-UACG	CGUGG--CA-U-C--CUU-C--C-	-----	-----

FIG. 1—Continued.

Pseudomonas aeruginosa, which do not require Na^+ . According to our phylogenetic analysis, *Pseudomonas nautica* is on a branch that is distinct from the branch containing the two terrestrial *Pseudomonas* species. *Deleya marina*, which was classified previously as *Pseudomonas marina* (24), requires Na^+ for growth, like *Pseudomonas nautica*. This species was also on a branch that was distinct from the terrestrial *Pseudomonas* species branch. Although additional study is needed, our results suggest that the halophilic, marine pseudomonads should be separated from the genus *Pseudomonas* and may be placed in newly created groups. The three species of the genus *Pseudomonas* had common, genus-specific sequence patterns. However, the two non-halophilic strains shared far more and clearer common sequence patterns than all three species shared.

Subdivision of the family Vibrionaceae. A comparison of the phylogenetic relationships among 34 strains of the family Vibrionaceae suggested that the family should be separated into at least seven groups, which correspond to genera or families.

The first distinct group is *V. marinus*. MacDonell and Colwell have also suggested that *V. marinus* is significantly different from other vibrio species on the basis of its 5S

rRNA sequence (21). In 1990, Steven proposed that *V. marinus* should be renamed *Moritella marinus* on the basis of the results of molecular genetic methods, including 5S rRNA sequencing and DNA-DNA hybridization (32). Our results based on 16S rRNA sequencing data support this proposal.

The second group comprises *V. cholerae* and *V. mimicus*. Whereas most species of the genus *Vibrio* require Na^+ , having optimal Na^+ concentrations of 2 to 3%, *V. cholerae*, the type species of the genus *Vibrio*, is able to grow without Na^+ in the medium (2), although growth is stimulated by a small amount of Na^+ . In addition to the salt requirement, *V. cholerae* has many characteristics that distinguish it from other vibrio species. In this study, various strains of *V. cholerae* formed a tight cluster and were clearly separated from other species of the genus *Vibrio*. *V. mimicus* was proposed as an atypical strain of *V. cholerae*; these two taxa are very closely related both phenotypically and genotypically (DNA homology) (9).

The third group is composed of *Vibrio costicola*, *Vibrio hollisae*, and *Listonella damsela*. The members of this group are different from members of other groups and do not form a tight cluster. The similarity values and *K* values for these

ECOL	1300 GUCCGGAUUG	1310 GAGUCUGCAA	1320 CUCGACUCCA	1330 UGAAGUCGGA	1340 AUCGCUAGUA	1350 AUCGUGGAUC	1360 AGAAUGCCAC	1370 GGUGAAUACG
VCH01	-----	-----	-----	-----	-----	CAA-----UUG	-----	-----
VCH02	-----	-----	-----	-----	-----	CAA-----UUG	-----	-----
VCH03	-----	-----	-----	-----	-----	CAA-----UUG	-----	-----
VCH04	-----	-----	-----	-----	-----	CAA-----UUG	-----	-----
VCH05	-----	-----	-----	-----	-----	CAA-----UUG	-----	-----
VMIM	-----	-----	-----	-----	-----	CAA-----UUG	-----	-----
VMAR	-----	-G-----	-----C--	-----	-----	-----A--	-----U--	-----
VCOS	-----	-----	-----	-----	-----	-----	-----	-----
VHOL	-----	-----	-----	-----	-----	-----	-----	-----
LDAM	-----C-	-----	-----G	-----	-----	-----	-----	-----
VFIS	-----	-----	-----	-----	-----	-----A--	-----U--	-----
VLOG	-----C-	-----	-----G	-----	-----	-----A--	-----U--	-----
LANG	-----	-----	-----	-----	-----	-----A--	-----U--	-----
VAES	-----	-----	-----	-----	-----	-----	-----	-----
VGAZ	-----	-----	-----	-----	-----	-----	-----	-----
VMET	-----	-----	-----	-----	-----	-----	-----	-----
VORD	-----	-----	-----	-----	-----	-----	-----	-----
VORI	-----	-----	-----	-----	-----	-----	-----	-----
LPEL	-----	-----	-----	-----	-----	-----A--	-----U--	-----
VALG	-----	-----	-----	-----	-----	-----	-----	-----
VCAM	-----	-----	-----R	-----	-----	-----	-----	-----
VCAR	-----C-	-----	-----G	-----	-----	-----	-----	-----
VDIA	-----	-----	-----	-----	-----	-----A--	-----U--	-----
VFLU	-----C-	-----	-----G	-----	-----	-----	-----	-----
VHAR	-----C-	-----	-----G	-----	-----	-----	-----	-----
VNAT	-----	-----	-----	-----	-----	-----	-----	-----
VNER	-----	-----	-----	-----	-----	-----	-----	-----
VNIG	-----	-----	-----M--	-----	-----	-----	-----	-----N--
VPAR	-----	-----	-----	-----	-----	-----	-----	-----
VPRO	-----	-----	-----	-----	-----	-----	-----	-----S--
PPHO	-----	-----	-----	-----	-----	-----A--	-----U--	-----
PANG	-----	-----	-----	-----	-----	-----A--	-----U--	-----
PLEI	-----	-----	-----	-----	-----	-----A--	-----U--	-----
PLSH	-----	-----	-----	-----	-----	-----C--	-----G--	-----
AHAN	-----	-----	-----	-----	-----	-----CAA--	-----UUU	-----
ACAV	-----	-----	-----	-----	-----	-----CAA--	-----UUU	-----
AMED	-----C-	-----	-----G	-----	-----	-----CAA--	-----UUU	-----
ASMA	-----C-	-----	-----G	-----	-----	-----CAA--	-----UUU	-----
AVER	-----C-	-----	-----G	-----	-----	-----CAA--	-----UUU	-----
ALES	-----	-----	-----	-----	-----	-----C-U--	-----A-G	-----
ALHA	-----	-----	-----	-----	-----	-----C-U--	-----A-G	-----
ALNI	-----	-----	-----	-----	-----	-----C-U--	-----A-G	-----
SPUT	-----	-----	-----	-----	-----	-----	-----	-----
MCOM	-----	-----	-----	-----	-----	-----A--	-----U--	-----
MVAG	-----	-----	-----	-----	-----	-----A--	-----U--	-----
PSAE	-----C--C--	-----	-----G--G--	-----	-----	-----A--	-----U--	-----
PSFL	-----C--C--	-----	-----G--G--	-----	-----	-----C-A--	-----U-G	-----
PSNA	-----C--C--	-----	-----G--G--	-----	-----	-----C-A--	-----U-G	-----
DMAR	-----	-----	-----	-----	-----	-----	-----	-----

FIG. 1—Continued.

species range from 92.95 to 95.08% and from 5.37 to 7.40, respectively. The similarity values are much lower than the values for species in other branches. We suggest that this group may be divided into two or three genera on the basis of phylogenetic relationships. Dorsch et al. also suggested that *V. hollisae* should be elevated to genus rank (11).

The fourth group, which also does not form a compact cluster, consists of three species, *V. fischeri*, *V. logei*, and *L. anguillara*. This group should be divided into different genera. Dorsch et al. suggested that *L. anguillara* should be separated from the other vibrio species (11). Two of these organisms, *V. fischeri* and *V. logei*, have the ability to luminesce. The other luminescent vibrio species, *Vibrio harveyi* and *V. orientalis*, fall into another branch, suggesting that luminescence is not a key criterion for distinguishing vibrio groups.

The fifth group consists of the genus *Plesiomonas*. Like the species of the family *Aeromonadaceae*, the genus *Plesiomonas* is placed by itself on a distinct branch. The levels of similarity between the genus *Plesiomonas* and groups belonging to the family *Vibrionaceae* are much lower than the levels of similarity between this genus and members of the family *Aeromonadaceae* or even *E. coli*. Our results support

the suggestion of MacDonell and Colwell (22) that the genus *Plesiomonas* should be separated from the family *Vibrionaceae*. It seems that evolutionarily, *Plesiomonas shigelloides* is closely related to the family *Aeromonadaceae* and the family *Enterobacteriaceae*.

The sixth group comprises three species of the genus *Photobacterium*. The genus *Vibrio* and the genus *Photobacterium* have many common characteristics, but they can be separated on the basis of accumulation of poly-β-hydroxybutyrate and utilization of D-mannitol by *Photobacterium* species. These two genera are closely related, and the distinction between them based on phenotypic characteristics has often been a source of confusion. According to our data, a fairly clear separation of the two genera occurs on the phylogenetic tree (Fig. 2) and in similarity and distance matrices (Fig. 3).

The seventh group includes the remaining species of the family *Vibrionaceae*. Excluding *V. cholerae*, *V. mimicus*, *V. costicola*, *V. hollisae*, *L. damsela*, *V. marinus*, *V. fischeri*, *V. logei*, *L. anguillara*, and *Plesiomonas shigelloides*, the other 17 species of the *Vibrionaceae* form one large cluster. This group seems to be the main group of the genus *Vibrio*. Although the genus *Listonella* was separated from the genus

FIG. 2. Unrooted phylogenetic tree based on 16S rRNA sequences, showing the relationships among the 50 reference strains which we used. For abbreviations of organism names, see Table 1.

Vibrio on the basis of 5S rRNA sequences (22), according to the phylogenetic relationships based on a 16S rRNA sequence analysis the species of the genus *Listonella* occur on three separate branches. A comparison of the common sequence patterns of the genus *Vibrio* and the genus *Listonella* showed that species of both genera have almost identical sequence patterns. According to our results, the genus *Listonella* is a rather heterogeneous group. Although further study, including a detailed comparison of 5S rRNA data, is needed, some species of the genus *Listonella* may be combined into genera which include species of the current genus *Vibrio*.

Criteria that separate taxa. In this study a phylogenetic analysis was performed by using 600 16S rRNA nucleotides and three universal primers (18) that have been used very frequently. Therefore, the similarity values and *K* values should change when they are recalculated with the complete nucleotide sequences of the 16S rRNAs. However, by comparing similarity values and *K* values in this study, we arrived at a concept for distinguishing the taxonomic hierarchy on the basis of a 16S rRNA sequence analysis.

Within the seven groups on our phylogenetic tree, the similarity values and *K* values ranged from 92.95 to 99.79% and from 0.22 to 7.40, respectively. The lowest similarity value (92.95%) and the highest *K* value (7.40) were obtained with the species *V. costicola*, *V. hollisae*, and *L. damsela*. When the branch containing these three species was excluded, the similarity values among the species of each group were more than 95% and the *K* values were less than 5.00, with one exception.

Our clustering, including subdivision of the current genus *Vibrio*, is in accord with the sequence patterns of the 16S rRNAs. Each separate group on the phylogenetic tree has a common, characteristic sequence pattern. This is particularly clear when the grouped and ungrouped members of the genus *Vibrio* are compared. Our results indicate that the members of the genus *Vibrio* should be separated into at least five groups at the genus and family levels. From our observations of levels of interspecies similarity and distances among the genera, we concluded that the similarity values and *K* values which separate different genera are 95 to 96% and 5.0 to 4.0, respectively. If these values are adopted, the group consisting of *V. costicola*, *V. hollisae*, and *L. damsela* should be divided into two or three genera. Lower similarity values or higher distance values may indicate that the species should be separated into different genera, although other characteristics (e.g., phenotypic characteristics) of the species should also be taken into consideration before a final decision is made.

On the basis of a comparison of the distances among various families in Fig. 3, the similarity value which distinguishes different families is 90 to 91%, and the K value is 9 to 10. The similarity values and K values among species within the current family *Vibrionaceae* range from 86.74 to 99.79% and from 0.22 to 14.71, respectively. This means that the species of the *Vibrionaceae* are too diverse to be included in a single family. On the basis of our results, three species of the *Vibrionaceae*, *V. marinus*, *V. costicola*, and *Plesiomonas shigelloides*, are distant enough to create new families.

With respect to similarity values and K values at the

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88												

[illegible]

species level, multiple strains of only one species, *V. cholerae*, were compared in this study. The similarity values and *K* values for these organisms were 99.23 to 100.00% and 0.00 to 0.60, respectively. Therefore, we concluded that the 16S rRNA similarity value and *K* value that define a species are 99.2% and 0.6, respectively. However, these values are valid only in this present study, since the values were obtained from a limited number of 16S rRNA nucleotides. Also, the criteria for distinguishing species may vary in different taxonomic groups. Kawasaki (16a) has set a similarity value of 99.3% as the criterion for separating species on the basis of the results of a 16S rRNA sequence analysis for methanogens which belong to the archaeobacteria. For *V. mimicus*, which was a member of a compact cluster along with five strains of *V. cholerae*, the similarity values and *K* values in comparisons with the *V. cholerae* strains ranged from 99.06 to 99.60% and 0.40 to 0.78, respectively. According to DNA-DNA relatedness data, *V. mimicus* is not related to *V. cholerae* at the species level (9). More data are needed to determine more accurate values for species differentiation.

The results of our 16S rRNA sequence analysis showed that molecular genetic data are very valuable for establishing clear-cut definitions of various levels of bacterial taxa. However, more detailed studies will be needed to determine the exact similarity values or *K* values which distinguish these taxa and to define species, subspecies, and biotypes that are very closely related phenetically and phylogenetically.

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