Phylogenetic Analysis and Assessment of the Genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* Deduced from Small-Subunit rRNA Sequences

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We sequenced nearly complete small-subunit rRNAs of 54 reference strains belonging to the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas*. We then performed a phylogenetic analysis by comparing the sequences which we obtained with all other known sequences for bacteria belonging to the gamma subgroup of the *Proteobacteria* (thus providing a data base consisting of 70 sequences for the genera investigated), using methods such as neighbor joining, maximum likelihood, and maximum parsimony, as well as bootstrap, to assess the robustness of each topology. Our results confirmed that the family *Vibrionaceae* should include only *Photobacterium* and *Vibrio* species (but not *Vibrio marinus*); that *Aeromonas* species deserve family rank; and that *Plesiomonas shigelloides* is linked to the family *Enterobacteriaceae*. The genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas*, together with the family *Enterobacteriaceae*, the family *Pasteurellaceae*, and probably the genus *Alteromonas*, form a robust monophyletic unit within the gamma 3 subgroup of the *Proteobacteria*.

rRNA oligomer cataloging (18, 37, 43), DNA-DNA and RNA-DNA hybridization (3, 6, 18, 32), and 5S rRNA sequencing (15, 27, 28) have been used previously to study the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas*. Small-subunit rRNA oligomer catalogs have revealed that these genera, along with the family *Enterobacteriaceae*, form a branch of the gamma 3 subdivision of the purple bacteria (43). More recently, inter- and intragenetic relationships have been determined by using small-subunit rRNA sequences of *Vibrio* strains (nearly complete sequences for 10 species [14] and partial sequences for *Vibrio* and *Photobacterium* species [23]) and *Aeromonas* strains (10 species), as well as *Plesiomonas shigelloides* (29). From a taxonomic point of view, broad general agreement has been reached between classification based on a molecular approach and classical classification derived from physiological comparisons and numerical taxonomy. The results of genetic studies suggested, however, that the family *Vibrionaceae* should include only the genera *Vibrio* and *Photobacterium* (9, 16, 41, 42), that the genus *Aeromonas* should be placed in a different family, the *Aeromonadaceae*, and that *Plesiomonas shigelloides* is more closely related to the family *Enterobacteriaceae* than to the family *Vibrionaceae* (6, 12, 18, 27, 43).

Comparisons of small-subunit rRNA sequences are more informative than either analyses of 5S rRNA sequences or analyses of small-subunit rRNA oligomer catalogs. However, the results of studies in which workers used small-subunit rRNA sequences, which for many species were either not determined or were only partially determined, have left some questions unanswered. Also, the phylogenetic methods used previously to analyze relationships often have not included any estimate of the robustness of the results. Under these conditions, the phylogenetic relationships of all of these species were uncertain. In this study, we sequenced 54 strains and combined our data with previously published sequence data; thus, in our study we included data for almost all of the type strains of

species in the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas*. The nearly complete small-subunit rRNA sequences were aligned by comparison with other eubacterial rRNA sequences, and phylogenetic relationships were determined by using different phylogenetic methods (maximum likelihood, parsimony, neighbor joining) to check the reliability of each topology. Each topology was then analyzed by bootstrap to assess robustness.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains belonging to the family *Vibrionaceae* for which small-subunit rRNA sequences are now available are listed in Table 1. Table 1 shows the strain designations, sources of strains, GenBank or EMBL accession numbers for small-subunit rRNA sequences, and methods used to sequence either small-subunit rRNAs or ribosomal DNAs (rDNAs) when the sequences were determined in our laboratory.

All of the bacterial strains were grown in liquid medium (100 ml) for 15 h at 22°C (±2°C). The culture medium used was the medium recommended in the *American Type Culture Collection Catalogue of Bacteria and Bacteriophages* (1).

While our work was in progress, small-subunit rRNA sequences were published for some species whose sequences we had not determined at that time (14, 23, 29, 30). After these sequences were incorporated into our data base, alignment, and phylogenetic analyses, we found some phylogenetic discrepancies that let us to assume that some of the newly published sequences contained a few errors (for example, differences were observed in otherwise conserved domains in vibrios for the sequences of *Vibrio hollisae*, *Vibrio diazotrophicus*, *Vibrio vulnificus*, *Vibrio proteolyticus*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio campbellii*, and *Vibrio natriegens*). For confirmation, some sequences (in particular, the sequences of *V. alginolyticus*, *V. parahaemolyticus*, and *V. vulnificus*) were determined both by reverse transcription directly from the rRNAs and by PCR from the genes (direct sequencing of PCR products [see below]), and no differences between the results of the two methods were observed. Also,

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TABLE 1. Strains belonging to the *Vibrionaceae* for which complete or nearly complete small-subunit rRNA sequences are now available

Taxon	Source(s) (strain[s] used) ^a	ATCC no. ^a	Method ^b		EMBL accession no.
			rRNA	rDNA	
<i>Aeromonas allosaccharophila</i>	(CECT 4199) ^c				
<i>Aeromonas caviae</i>	CIP (CIP 76.16 ^T) ^d	ATCC 15468 ^T	++		X74674
<i>Aeromonas caviae</i>	NCIMB (NCIMB 13016 ^T)				X60408 ^e
<i>Aeromonas caviae</i>	ATCC	ATCC 15467			X60409 ^e
<i>Aeromonas eucrenophila</i>	CIP (CIP 76.17 ^T)	ATCC 23309 ^T	++		X74675
<i>Aeromonas eucrenophila</i>	NCIMB (NCIMB 74 ^T)				X60411 ^e
<i>Aeromonas hydrophila</i>	CIP (CIP 76.14 ^T)	ATCC 7966 ^T	++		X74676
<i>Aeromonas hydrophila</i>	NCIMB (NCIMB 9240 ^T)	ATCC 7966 ^T			X60404 ^e
<i>Aeromonas hydrophila</i>	bioM	ATCC 35654	++		X74677
<i>Aeromonas jandaei</i>	ATCC	ATCC 49568 ^T	++		X74678
<i>Aeromonas jandaei</i>	ATCC	ATCC 49568 ^T			X60413 ^e
<i>Aeromonas media</i>	bioM	ATCC 33907 ^T	++		X74679
<i>Aeromonas media</i>	ATCC	ATCC 33907 ^T			X60410 ^e
<i>Aeromonas salmonicida</i> subsp. <i>achromogenes</i>	NCIMB (NCIMB 1110 ^T)				X60405 ^e
<i>Aeromonas salmonicida</i> subsp. <i>masoucida</i>	CIP (CIP 103210 ^T)	ATCC 27013 ^T	++		X74680
<i>Aeromonas salmonicida</i> subsp. <i>masoucida</i>		ATCC 27013 ^T			X60406 ^e
<i>Aeromonas salmonicida</i> subsp. <i>salmonicida</i>	CIP (CIP 103209 ^T)	ATCC 33658 ^T	++		X74681
<i>Aeromonas salmonicida</i> subsp. <i>salmonicida</i>	NCIMB (NCIMB1102 ^T)				X60407 ^e
<i>Aeromonas shubertii</i>	bioM	ATCC 43700 ^T	++		X74682
<i>Aeromonas shubertii</i>	DSM (DSM 4882 ^T)				X60416 ^e
<i>Aeromonas sobria</i>	CIP (CIP 74.33 ^T)	ATCC 43979 ^T	++		X74683
<i>Aeromonas sobria</i>	CIP (CIP 74.33 ^T)	ATCC 43979 ^T			X60412 ^e
<i>Aeromonas</i> sp.		ATCC 35941			X60417 ^e
<i>Aeromonas trota</i>		ATCC 49657 ^T			X60415 ^e
<i>Aeromonas veronii</i>	bioM	ATCC 35624 ^T	++		X74684
<i>Aeromonas veronii</i>	ATCC	ATCC 35624 ^T			X60414 ^e
<i>Photobacterium angustum</i>	CIP (CIP 75.10 ^T)	ATCC 25915 ^T	++	+	X74685
<i>Photobacterium leiognathi</i>	CIP (CIP 66.5 ^T)	ATCC 25521 ^T	++	+	X74686
<i>Photobacterium phosphoreum</i>	ATCC	ATCC 11040 ^T		++	X74687
<i>Plesiomonas shigelloides</i>	CIP (CIP 63.5 ^T)	ATCC 14029 ^T	++		X74688
<i>Plesiomonas shigelloides</i>	NCIMB (NCIMB 9242 ^T)				X60418 ^e
<i>Vibrio aestuarianus</i>	CIP (CIP 102971 ^T)	ATCC 35048 ^T	++	+	X74689
<i>Vibrio alginolyticus</i>	CIP (CIP 17749 ^T)	ATCC 17749 ^T	++	++	X74690
<i>Vibrio alginolyticus</i>		ATCC 17749 ^T			X56576 ^f
<i>Vibrio alginolyticus</i>	CIP (CIP 7065)		++	++	X74691
<i>Vibrio (Listonella) anguillarum</i>		ATCC 19264 ^T			X16895 ^g
<i>Vibrio campbellii</i>	CIP (CIP 75.01 ^T)	ATCC 25920 ^T		++	X74692
<i>Vibrio campbellii</i>		ATCC 25920 ^T			X56575 ^f
<i>Vibrio carchariae</i>	ATCC	ATCC 35084 ^T		++	X74693
<i>Vibrio cholerae</i> serovar O1	CIP (CIP 62.13 ^T)	ATCC 14035 ^T	++	+	X74694
<i>Vibrio cholerae</i> biotype Eltor, type inaba	INSERM U303	ATCC 14033	++	+	X74695
<i>Vibrio cholerae</i> non O:1 Nanking	CIP (CIP 63.40)	ATCC 14730	++	+	X74696
<i>Vibrio cholerae</i> non O:1 El Tor	CIP (CIP 63.41)	ATCC 14731	++	+	X74697
<i>Vibrio cincinnatiensis</i>	ATCC	ATCC 35912 ^T		++	X74698
<i>Vibrio costicola</i>	NCIMB (NCIMB 701)	ATCC 33508 ^T	++	+	X74699
<i>Vibrio (Listonella) damsela</i>	CIP (CIP 102761 ^T)	ATCC 33539 ^T	++	+	X74700
<i>Vibrio diazotrophicus</i>	CIP (CIP 103191 ^T)	ATCC 33466 ^T		++	X74701
<i>Vibrio diazotrophicus</i>		ATCC 33466 ^T			X56577 ^f
<i>Vibrio fischeri</i>	NCIMB (NCIMB 1281)	ATCC 7744 ^T	++	+	X74702
<i>Vibrio fluvialis</i>	NCIMB (NCIMB 2249)	ATCC 33809 ^T	++	+	X74703
<i>Vibrio furnisii</i>	CIP (CIP 102972 ^T)	ATCC 35016 ^T	++	+	X74704
<i>Vibrio gazogenes</i>	CIP (CIP 103173 ^T)	ATCC 29988 ^T	++	+	X74705
<i>Vibrio harveyi</i>	CIP (CIP 103192 ^T)	ATCC 14126 ^T		+	X74706
<i>Vibrio harveyi</i>		ATCC 14126 ^T			X56578 ^f
<i>Vibrio hollisae</i>	CIP (CIP 101886 ^T)	ATCC 33564 ^T		++	X74707
<i>Vibrio hollisae</i>		ATCC 33564 ^T			X56583 ^f
<i>Vibrio logei</i>	CIP (CIP 103204 ^T)	ATCC 15382	++	+	X74708
<i>Vibrio marinus</i>	CIP (CIP 102861 ^T)	ATCC 15381 ^T		++	X74709
<i>Vibrio mediterranei</i>	CIP (CIP 103203 ^T)		++		X74710
<i>Vibrio metschnikovii</i>	bioM (NCTC 11170)		++	+	X74711
<i>Vibrio metschnikovii</i>	CIP (CIP 69.14 ^T)		++	+	X74712
<i>Vibrio mimicus</i>	CIP (CIP 101888 ^T)	ATCC 33653 ^T	++	+	X74713
<i>Vibrio natriegens</i>	CIP (CIP 103193 ^T)	ATCC 14048 ^T		++	X74714
<i>Vibrio natriegens</i>		ATCC 14048 ^T			X56581 ^f
<i>Vibrio navarrensis</i>	CIP (CIP 103381 ^T)		++	+	X74715
<i>Vibrio nereis</i>	CIP (CIP 103194 ^T)	ATCC 25917 ^T	++	+	X74716

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TABLE 1—Continued

Taxon	Source(s) (strain[s] used) ^a	ATCC no. ^a	Method ^b		EMBL accession no.
			rRNA	rDNA	
<i>Vibrio nigripulchritudo</i>	CIP (CIP 103195 ^T)	ATCC 27043 ^T	++	+	X74717
<i>Vibrio ordalii</i>	CIP (CIP 103205 ^T)	ATCC 33509 ^T	++	+	X74718
<i>Vibrio orientalis</i>	CIP (CIP 102891 ^T)	ATCC 33934 ^T	++	+	X74719
<i>Vibrio (Listonella) pelagius</i>	CIP (CIP 102762 ^T)	ATCC 25916 ^T	++	+	X74720
<i>Vibrio parahaemolyticus</i>	CIP (CIP 75.02 ^T)	ATCC 17802 ^T	++	++	X74721
<i>Vibrio parahaemolyticus</i>		ATCC 17802 ^T			X56580 ^f
<i>Vibrio parahaemolyticus</i>	CIP (CIP 73.30)		++	++	X74722
<i>Vibrio proteolyticus</i>	CIP (CIP 102892 ^T)	ATCC 15338 ^T		++	X74723
<i>Vibrio proteolyticus</i>		ATCC 15338 ^T			X56579 ^f
<i>Vibrio splendidus</i>	CIP (CIP 102893 ^T)	ATCC 33125 ^T	++	+	X74724
<i>Vibrio tubiashii</i>	CIP (CIP 102760 ^T)	ATCC 19109 ^T	++	+	X74725
<i>Vibrio vulnificus</i>	CIP (CIP 75.04 ^T)	ATCC 27562 ^T	++	++	X74726
<i>Vibrio vulnificus</i>		ATCC 27562 ^T			X56582 ^f
<i>Vibrio vulnificus</i>	bioM	ATCC 29307	++	++	X74727

^a The sources are the collections from which the bacteria were obtained: ATCC, American Type Culture Collection, Rockville, Md.; CIP, Collection Institut Pasteur, Paris, France; bioM, Institut bioMerieux, Marcy l'Etoile, France; NCTC, National Collection of Type Cultures, London, United Kingdom; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom.

^b For sequences determined in our laboratory, the method used for sequencing is indicated. rRNA, sequence obtained directly from rRNA; rDNA, sequence obtained from amplification products; +, partially sequenced, ++, nearly completely sequenced.

^c Sequence obtained from reference 30.

^d T = type strain.

^e Sequence obtained from references 29 and 30.

^f Sequence obtained from reference 14.

^g Sequence obtained from reference 40.

no differences were observed for several strains of the same species (for example, *V. alginolyticus* and *V. parahaemolyticus* strains) (Table 1). Thus, because of the precautions which we took, we feel confident that our sequence data were more accurate than the data published previously.

Sequencing. We determined almost the entire small-subunit rRNA sequence (corresponding to positions 4 to 1425 in the *Escherichia coli* rRNA sequence for direct RNA sequences and to positions 29 to 1425 for PCR products) for each representative of the family *Vibrionaceae*. *Vibrio anguillarum* and some *Aeromonas* species were not sequenced because their sequences had been previously published (29, 30, 40). For *Vibrio harveyi*, previously described partial sequence was completed (positions 88 to 118 in the *E. coli* sequence).

Direct small-subunit rRNA sequencing. Bacteria were pelleted by centrifugation for 10 min at 5,000 rpm. The pellet was treated with lysozyme (2 mg/ml) for 15 min at 37°C in a buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 20 mg of sucrose per ml. After centrifugation for 10 min at 2,500 rpm, the resulting pellet was homogenized with an Ultra-turax homogenizer in 5 ml of a solution containing 4 M guanidinium thiocyanate, 50 mM Tris-HCl (pH 7.6), 4 mM EDTA, and 2% *N*-lauryl-sarcosyl. Total RNA was separated from protein by phenol extraction; this procedure was repeated three times and was followed by two chloroform washes. The total RNA was ethanol precipitated, suspended in sterile distilled water to measure the ratio of A_{260} to A_{280} , precipitated with sodium acetate and ethanol, and finally suspended in sterile water to a concentration of 2 µg/µl. The quality of the RNA extract was determined by staining 1% agarose gels with ethidium bromide.

RNA sequencing was carried out by using the method of Sanger et al. (34), which was modified to accommodate reverse transcriptase in place of DNA polymerase (31) and was also modified as follows: DNA synthesis was carried out in two steps, a labeling step involving primer extension with limited concentrations of ³⁵S-dATP, followed by a classical chain termination step in which dideoxynucleotides were used. The

synthetic primers used were complementary to conserved eubacterial domains identified previously (25).

Small-subunit rDNA sequencing after PCR amplification. A pellet obtained after centrifugation for 10 min at 5,000 rpm was resuspended in 100 µl of a lysis solution (10 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100; pH 8), heated for 3 min at 100°C, and then transferred on ice. After a single chloroform extraction, 8 µl of supernatant was used in a PCR to amplify the small-subunit rRNA genes. The initial denaturation step consisted of heating the reaction mixture at 95°C for 180 s, and this was followed by an annealing step (52°C for 60 s) and an extension step (72°C for 90 s). The thermal profile then consisted of 25 cycles of annealing at 52°C for 60 s, extension at 72°C for 90 s, and denaturation at 94°C for 30 s. A final extension step was carried out at 72°C for 5 min. The PCR products were analyzed on a 1% low-melting-point agarose gel that included a molecular weight standard for quantification of the PCR yield. DNA sequencing was performed by using a protocol described by Anderson et al. (2), with slight modifications. The amplification primers used gave a PCR product that was 1.5 kb long and corresponded to positions 8 to 1508 in the *E. coli* sequence. The primers used for sequencing were identical to those used for direct sequencing of small-subunit rRNAs.

Primer positions. Eleven DNA primers were used in the sequencing reactions. These primers corresponded to the following positions in the *E. coli* sequence: primer S2, positions 99 to 119; primer S3, positions 242 to 262; primer S4, positions 342 to 356; primer S6, positions 518 to 534; primer S8, positions 684 to 702; primer S10, positions 906 to 925; primer S12, positions 1099 to 1114; primer S14, positions 1223 to 1240; primer S15, positions 1384 to 1400; and primer S17, positions 1493 to 1509. A reverse primer (positions 8 to 28) was used in conjunction with primer S17 to amplify the small-subunit rDNAs.

Phylogenetic analysis and alignment: general procedure. The phylogenetic data described below were obtained (i) by using successive alignment and phylogeny procedures, and (ii)

by reinvestigating deep branching patterns after close relationships were determined.

In each phylogenetic analysis, we restricted the comparisons to nucleotide positions that were aligned without doubt. Some analyses were performed several times, with or without small domains that could have reached the point of saturation for mutations. Although this approach was probably not as efficient as carefully weighting each position independently, it was easier to use and was probably a reasonable compromise considering the possible problems of crossing-over that might have affected some of the sequences investigated (36).

We also performed some analyses several times by including and excluding particular species that had high rates of mutation or that were distant outgroups. Indeed, a model study in which rRNA sequences of echinoderms were used showed that the presence of distantly related outgroups brings random noise into an analysis and does not allow workers to resolve relationships among the more closely related species (35). Long branches for ingroups have the same effect as distant outgroups (that is, they bring noise into a phylogenetic analysis). Thus, monophyletic units containing such species are unstable because of highly variable sequences, and their presence can also affect neighboring taxa. In order to alleviate this problem, we performed phylogenetic analyses successively with all outgroups or with a set of outgroups restricted to the closest relatives, with or without ingroups that were characterized by long branches.

For each phylogenetic analysis, in order to keep computation time within reasonable limits, it was not possible to include all representatives of outgroups and ingroups in maximum-parsimony and maximum-likelihood analyses. This problem was alleviated by performing multiple analyses with different outgroups and different ingroups (as determined from the neighbor-joining analysis). All sequence alignment and species selection operations were done by using computer programs developed by us and available on request from R. Christen.

Phylogenetic methods. (i) **Neighbor joining.** A neighbor-joining algorithm like that developed by Saitou and Nei (33) was used. The program was rewritten to include inputs and outputs compatible with the ribosomal data base and other programs developed in our laboratory (running on 386-compatible personal computers and above).

(ii) **Maximum parsimony.** The PAUP program (38) for MacIntosh computers was used. All topologies were first obtained by using the heuristic options. According to the time used, a branch and bound search was then undertaken by using the full data set or a restricted set of species selected on the basis of the results of the heuristic search. When several most-parsimonious trees were obtained, a 100% consensus tree was constructed and treated as the most-parsimonious tree for constructing figures. Finally, a bootstrap analysis was performed (heuristic option) to check each topology for robustness. We favored a large number of analyses with many different bacterial representatives and 100 replications for each analysis rather than a high number (1,000) of bootstrap replications, since our experience showed that increasing the number of bootstrap replications above 100 usually had only a small influence on the results, compared with the choice of species.

(iii) **Maximum likelihood.** Routinely, the fDNAmI program rewritten by G. J. Olsen (University of Illinois, Urbana) was used with a Hewlett-Packard model 700 workstation. All analyses were performed by using the global option (in fact, F Y G options), and each analysis was performed several times with species in a different order. For some topologies, the results were compared with the results of the DNAML pro-

gram of Felsenstein (17). Interestingly, the two programs sometimes led to alternative, but very similar topologies, in particular when the results of maximum-likelihood, maximum-parsimony, and neighbor-joining analyses were not in perfect agreement. Since these topologies were considered not resolved (intrageneric relationships within *Vibrio* species), we did not attempt to solve this problem in this study.

Finally, all trees were plotted by using a MacIntosh computer and a program developed by M. Gouy (University of Lyon, Villeurbanne, France) that allows transformation of a formal tree representation (Newick's format) into MacDraw drawings.

Only topologies that were found to be similar by all three methods were retained as "true trees." Recent theoretical works have indeed demonstrated that convergence of the results of all three methods is a very robust indication that the correct phylogeny has been determined (20, 22).

Domains used. Different parts of the small-subunit rRNA sequences were used for the different trees shown in this paper. For the tree shown in Fig. 1 we used positions 29 to 67, 100 to 153, 226 to 441, 484 to 589, 658 to 832, 860 to 997, 1035 to 1131, 1138 to 1266, and 1295 to 1357 (*E. coli* numbering). For the tree shown in Fig. 2 we used positions 9 to 69, 100 to 180, 226 to 441, 479 to 832, 859 to 928, 932 to 1004, 1028 to 1132, and 1137 to 1426. For the tree shown in Fig. 3 we used positions 29 to 74, 100 to 189, 218 to 453, 479 to 831, 859 to 928, 932 to 1004, 1028 to 1132, and 1137 to 1368. To determine intrageneric relationships among *Vibrio* species (Table 2) we used positions 34 to 74, 100 to 184, and 185 to 1352.

RESULTS

Phylogenetic positions of the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* within the *Proteobacteria*. A broad phylogenetic analysis clearly confirmed that all species belonging to the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* branch in the *Proteobacteria* subgroup of the *Eubacteria* (data not shown), a result in agreement with the results of all previous studies. The positions of these taxa within this major lineage are shown in Fig. 1, an unrooted tree that combines the results of a neighbor-joining analysis (shown in Fig. 1) with the topologies obtained by maximum-likelihood and parsimony analyses, as described in the legend to Fig. 1. This analysis revealed the major subgroups previously identified among the *Proteobacteria* (the alpha, beta, delta, gamma, and epsilon subgroups).

The genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* clearly belonged to the gamma subgroup and were strongly associated with the *Enterobacteriaceae*, the *Pasteurellaceae* and related species, *Alteromonas haloplanktis*, and, to a lesser extent, *Marinomonas vaga* and some other bacteria. These taxa form a monophyletic branch within the gamma 3 subgroup defined previously (43). This result was always obtained by all three methods (maximum likelihood, neighbor joining, and maximum parsimony) and was observed even when different species were chosen as outgroups or ingroups (data not shown).

Plesiomonas shigelloides appeared to be closely related to the *Enterobacteriaceae*, while the genera *Vibrio* plus *Photobacterium* and the genus *Aeromonas* formed two well-separated taxa with deep roots. Other internal branches were less robust (the percentages obtained in a bootstrap analysis are indicated in Fig. 1). The phylogenetic relationships among these taxa were difficult to resolve in our analyses, in part because the topology of the internal branches changed according to which species were chosen as representatives of distant outgroups (the alpha,

FIG. 1. Phylogenetic positions of the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* within the *Proteobacteria*. We constructed unrooted trees showing the topologies obtained when we used the neighbor-joining method (the tree shown), the maximum-parsimony method, and maximum-likelihood method. The most parsimonious tree had an identical topology. The double asterisks indicate branches that connect taxa that were also found to be related by the maximum-likelihood method ($P < 0.01$). Scale bar = 0.011 accumulated change per 100 nucleotides. A bootstrap analysis in which parsimony was used to assess the robustness of the topology (heuristic search, 100 replications) gave the values (expressed as percentages) above the lines (only values greater than 50% are shown).

beta, delta, and epsilon subgroups) and also because the branching orders were not supported by bootstrap data and were different depending on the method of phylogenetic analysis used. Low bootstrap values can indicate a weakly supported relationship, but they can also result from peculiar rates of mutation in some species or from the inclusion of outgroups that are too distantly related compared with the phylogenetic relationship being investigated. Thus, detailed

phylogenies were investigated by using a restricted set of outgroups (see Materials and Methods).

Relative phylogenetic positions of the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* within the gamma subgroup. Since we had too many sequences to include them all in a single phylogenetic analysis (particularly for maximum-likelihood or maximum-parsimony analyses), a large number of analyses were performed by using different representatives

FIG. 2. Phylogenetic analysis restricted to the gamma subgroup of the *Proteobacteria*: unrooted tree obtained by the maximum-likelihood method. Double plus signs indicate that a branch is significantly positive ($P < 0.01$). Boldface lines indicate monophyletic taxa also found in the neighbor-joining analysis. The asterisks indicate taxa also found on the most-parsimonious tree. The numbers above the branches are the percentages determined in a bootstrap analysis. For additional explanations, see Materials and Methods.

of the four genera belonging to the *Vibrionaceae* or of the outgroups. All of the analyses gave similar results, and the results of two of the analyses are shown in Fig. 2 and 3.

Figure 2 is an unrooted tree that combines the results of a maximum-likelihood analysis (which are shown) with the results of neighbor-joining and maximum-parsimony analyses. A comparison of the different methods, as well as bootstrap replications, revealed a solid group consisting of the following four robust monophyletic units: the *Aeromonadaceae*, the *Enterobacteriaceae* plus the genus *Plesiomonas*, the *Pasteurellaceae*, and the genus *Photobacterium* plus the genus *Vibrio*.

The outgroups closest to these four taxa were *Alteromonas haloplanktis* and, to lesser extents, *M. vaga* and *Halomonas elongata*. Surprisingly, one of the *Vibrio* species, *Vibrio marinus*, clustered very tightly with *Alteromonas haloplanktis* (but see the Discussion). The *Photobacterium-Vibrio* group appeared to be a sister group of the families *Pasteurellaceae*, *Aeromonadaceae*, and *Enterobacteriaceae*. The exact relationships among the latter taxa were more difficult to determine and, in particular, were not well supported by bootstrap data in the presence of distant taxa (see Fig. 2) or changed when different outgroups were used.

FIG. 3. Phylogenetic relationships among the *Enterobacteriaceae*, the *Pasteurellaceae*, and the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas*. An unrooted tree was drawn as described in the legend to Fig. 2. The numbers in parentheses are the percentages of bootstrap replications when *Pasteurella* strains were omitted from the analysis. For the *Vibrio-Photobacterium* taxon, the number on the right is the value obtained when *V. costicola* was omitted from the analysis. For additional explanations, see Materials and Methods.

To resolve this problem, in another analysis we excluded the least closely related outgroups (Fig. 3). An association supported by the results of all three methods was then obtained between the *Pasteurellaceae* and the *Enterobacteriaceae*. In this analysis, some of the low bootstrap values may have resulted not from the presence of distant outgroups, but from the presence of species with unusually high rates of mutation (see Materials and Methods), such as all of the members of the *Pasteurellaceae*. The deleterious effect of the presence of long branches is demonstrated in Fig. 3. When species on long branches were removed, (i) the removal of *Vibrio costicola* increased the bootstrap value from 69 to 90% for the branch uniting all species belonging to the genera *Photobacterium* and

Vibrio and (ii) the removal of the members of the *Pasteurellaceae* increased the bootstrap value from 70 to 96% for the branch uniting *Plesiomonas shigelloides* and the *Enterobacteriaceae*. In fact, removing the members of the *Pasteurellaceae* from the analysis increased the significance of almost all of the internal branches (numbers in parentheses in Fig. 3).

Intragenetic relationships among *Vibrio* and *Photobacterium* species. The relationships among all of the *Vibrio* and *Photobacterium* species for which the small-subunit rRNA sequences were known (Table 1) were investigated by all of the methods described above. The phylogenetic relationships of these species proved to be difficult to resolve (see Discussion), but three main groups were identified by all three methods and these

results were reasonably well supported by bootstrap analysis data (data not shown). The three groups were (i) all *Photobacterium* species plus *Vibrio damsela*, *V. costicola*, and *V. hollisae*, (ii) *Vibrio logei* plus *Vibrio fisheri*, and (iii) all remaining *Vibrio* species listed in Table 1.

In addition, a number of pairs of taxa were clearly closely related (members of a monophyletic taxon as determined by all three methods). These pairs included *Vibrio cholerae* and *Vibrio mimicus* (100% of bootstrap), *Vibrio ordalii* and *V. anguillarum* (100%), *Vibrio fluvialis* and *Vibrio furnisii* (84%), and finally, but not surprisingly, the two strains of *Vibrio metschnikovii* (73%). *V. costicola* and *V. hollisae* were found to be related by all three methods (the relationship was supported by a moderate bootstrap value, 71%), but their monophyly could have been an artifact caused by the well-known effect of long-branch attraction.

DISCUSSION

Comparisons of nearly complete small-subunit rRNA sequences confirmed that the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* are phylogenetically linked with the *Enterobacteriaceae* and *Pasteurellaceae* to form a monophyletic unit. Our data suggest that this topology is robust since it was obtained independently of the species selected as outgroups and of the phylogenetic method used. This result was also supported by very high bootstrap replication values (Fig. 1). Thus, these taxa form a monophyletic unit within the gamma 3 subgroup of the *Proteobacteria* (43). The genus *Alteromonas* appears to belong to this cluster, as a sister group, but the presence of a single representative sequence for all *Alteromonas* species precludes any definitive conclusion. We examined the relative positions of the families *Vibrionaceae* (restricted to the genera *Vibrio* and *Photobacterium*), *Aeromonadaceae*, *Enterobacteriaceae*, and *Pasteurellaceae*, and our data identified the *Vibrionaceae* as the deepest radiation and a sister group of the three other families (Fig. 2 and 3). The relative positions of the *Aeromonadaceae*, *Enterobacteriaceae*, and *Pasteurellaceae* are more difficult to determine, most probably because of the long internal branches without internodes that characterize the *Aeromonadaceae* and the *Pasteurellaceae* and also because bacteria belonging to the *Pasteurellaceae* (13) seem to have a high rate of mutation. Previous reports based on small-subunit rRNA oligonucleotide cataloging (18), rRNA-DNA hybridization (6, 7, 18), 5S rRNA sequencing (12, 28), and analyses of small-subunit rRNA sequences (23, 29, 30) readily identified the genus *Aeromonas* as a well-defined monophyletic taxon but were contradictory with regard to the phylogenetic position of this taxon. This is not surprising since the genus *Aeromonas* is characterized by a long internal branch and its position can be artifactual in some analyses, making it appear to be a radiation deeper than it should be; we have observed that neighbor-joining methods are sometimes more sensitive to such problems, probably because they work by grouping close neighbors first. Our results that strongly support the hypothesis that the *Aeromonadaceae* is a sister group of the *Enterobacteriaceae* and *Pasteurellaceae* were derived from longer sequences with more constant domains and/or more sequences than the results of previous studies, and the validity of the different topologies was assessed by using several methods and bootstrap analysis. In conclusion, although they are clearly genetically related, the four genera previously placed in the family *Vibrionaceae* can no longer be placed in a single taxon on the basis of genetic data and, more specifically, small-subunit rRNA sequence data (see below).

Our analyses revealed a robust monophyletic unit formed by *Plesiomonas shigelloides* and the *Enterobacteriaceae* (Fig. 3), which is in agreement with 5S rRNA data that also relate *Plesiomonas shigelloides* to the genus *Proteus* (27) and with previous small-subunit rRNA analysis data (29). Our data show that *Plesiomonas shigelloides* is genetically more closely related to *Proteus vulgaris* than to any other bacterium represented in the set of sequences available (Fig. 3). *Plesiomonas shigelloides* branched deeply, as a sister group of all of the other enterobacteria sequenced; more data are necessary to decide whether a new family, the *Plesiomonadaceae*, with one species, *Plesiomonas shigelloides*, should be created or whether this species should be placed in the *Enterobacteriaceae*.

The rather long internal branch and deep radiation that isolate all of the *Aeromonas* species sequenced from all other bacteria (Fig. 3) support the decision of the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Vibrionaceae* (21) to accept the new family *Aeromonadaceae* (as proposed by Colwell et al. [12]) to comprise the single genus *Aeromonas*. This long internal branch suggests that all known (sequenced) species in this genus might be derived from a recent common ancestor. It is difficult (i) to determine a root for the *Aeromonadaceae* (because there is no very closely related outgroup), and (ii) to determine with precision the internal phylogeny of the *Aeromonadaceae* (there are few differences between species). Although these circumstances could in part explain the disagreement observed previously between the results of small-subunit rRNA analysis and the results of chromosomal DNA-DNA pairing experiments (29), recently Sneath (36) also suggested that recombination events between rRNA genes might significantly affect all phylogenetic methods in which small-subunit rRNA sequences are used to determine relationships between *Aeromonas* species.

A restricted family *Vibrionaceae* that includes two genera, *Vibrio* and *Photobacterium*, was supported by the results of all of the methods used (Fig. 2 and 3). Bootstrap values increased when species such as *V. cholerae* or *V. costicola* were removed, a result that we interpret more as an artifact resulting from the high mutation rate in the rRNA sequences of *V. cholerae* or *V. costicola* than an indication that these bacteria do not belong to the *Vibrionaceae*. Thus, most *Vibrio* and *Photobacterium* species form a robust monophyletic unit; the single exception is *V. marinus* (Fig. 2 and 3). The external position of *V. marinus* has also been observed during analyses of 5S rRNA sequences (26, 27) and partial small-subunit rRNA sequences (23). Our *Alteromonas haloplanktis* sequence suggests a close link with *V. marinus*. However, several differences between the corresponding parts of our complete *V. marinus* sequence and the partial sequences published previously (23) are difficult to explain by a difference in strains. It is likely that one of these sequences is not "true" *V. marinus* sequence, and the exact phylogenetic position of *V. marinus* remains to be determined. Within the restricted family *Vibrionaceae*, our only robust result was a division of all species into three subgroups. Other relationships proved to be extremely difficult to establish with certainty, probably for two reasons: (i) the members of some branches exhibited particularly high rates of mutation (typically *V. costicola* and *V. hollisae*), while the members of other branches exhibited low rates of mutation (for example, *V. proteolyticus* and *V. logei*) and (ii) many species exhibited low numbers of differences in their rRNA sequences (Table 2 shows distances between sequences). While some of these problems could possibly be tackled by using a more sophisticated approach than the methods that we employed, the low numbers of differences between many pairs of sequences and

TABLE 2. Genetic distances between *Vibrio* and *Photobacterium* species

Taxon	Genetic distance (%) from ^a :																																	
<i>P. phosphoreum</i>	2.94	1.71	3.02	5.18	5.51	4.02	3.80	4.64	4.64	4.64	4.57	4.41	4.56	6.27	5.98	6.03	5.49	5.89	5.50	5.49	4.89	4.49	4.42	4.58	4.19	5.89	5.27	5.82	5.50	5.42	5.35	4.44	5.24	
<i>P. angustum</i>	2.09	4.95	4.10	3.88	4.64	5.10	5.33	5.33	5.18	5.10	5.26	5.18	6.04	5.75	5.80	5.26	6.03	5.49	5.19	5.56	5.19	4.96	5.42	6.11	5.50	5.27	5.80	5.49	5.58	5.44	5.58	5.44	5.24	
<i>V. damsela</i>	4.27	4.58	4.21	3.34	3.50	4.03	3.96	3.88	3.88	3.96	3.88	3.96	3.88	5.44	5.14	5.28	4.73	5.28	4.74	4.73	4.28	4.42	3.89	3.81	5.13	4.74	5.21	5.05	5.12	4.59	4.33	4.21	4.24	
<i>V. fisheri</i>	6.03	6.13	5.26	4.72	5.56	5.33	5.65	5.72	5.80	5.80	6.89	6.52	6.65	6.11	5.73	5.80	6.11	5.66	5.87	6.12	5.89	5.19	6.73	6.50	6.43	6.34	5.80	5.81	5.93	5.96	5.93	5.93	5.93	
<i>V. logei</i>	2.40	4.48	5.03	5.26	4.87	4.72	4.72	4.87	4.79	5.42	5.12	5.95	5.49	6.42	5.49	6.06	4.81	5.59	5.21	5.59	5.37	5.21	5.05	5.82	5.05	4.05	5.82	4.89	5.44	5.51	5.51	5.51	5.51	
<i>V. proteolyticus</i>	4.50	4.74	4.96	4.58	4.74	4.74	4.89	4.81	4.74	4.52	4.97	4.50	6.06	4.81	5.59	5.21	5.59	5.37	5.21	5.05	5.82	5.05	4.05	5.82	4.89	5.44	5.51	5.51	5.51	5.51	5.51	5.51	5.51	
<i>V. carchariae</i>	1.16	1.31	1.39	1.24	1.16	1.16	1.16	1.08	4.26	3.88	3.94	3.17	3.94	2.78	2.47	2.48	2.32	1.71	1.47	1.86	4.56	3.17	4.26	3.40	3.09	2.71	3.20	3.20	3.20	3.20	3.20	3.20	3.20	3.20
<i>V. harveyi</i>	0.77	0.93	1.24	1.16	1.16	1.16	1.16	1.16	4.18	3.81	4.18	3.63	4.03	3.25	3.33	2.87	3.09	2.64	2.25	2.02	4.57	3.10	4.03	3.72	3.56	3.02	3.10	3.10	3.10	3.10	3.10	3.10	3.10	3.10
<i>V. campbellii</i>	0.54	0.85	0.77	0.77	0.77	0.77	0.70	3.72	3.41	3.71	3.79	4.80	3.02	2.78	2.02	2.47	2.40	1.71	2.79	5.03	2.32	3.26	2.94	2.63	2.56	2.55	2.55	2.55	2.55	2.55	2.55	2.55	2.55	2.55
<i>V. parahaemolyticus</i>	0.31	0.39	0.54	0.46	3.48	3.11	3.48	3.71	4.80	3.71	4.80	3.02	2.78	2.02	2.47	2.40	1.71	2.79	5.03	2.32	3.26	2.94	2.63	2.56	2.55	2.55	2.55	2.55	2.55	2.55	2.55	2.55	2.55	2.55
<i>V. alginolyticus</i>	0.08	0.23	0.15	3.18	2.80	3.33	3.56	4.64	2.79	2.47	1.71	2.24	2.09	1.63	2.79	4.88	2.09	3.18	2.79	2.48	2.25	2.24	2.25	2.24	2.25	2.24	2.25	2.24	2.25	2.24	2.25	2.24	2.25	2.24
<i>V. pelagius</i>	0.08	0.23	0.15	3.18	2.80	3.33	3.56	4.64	2.79	2.47	1.71	2.24	2.09	1.63	2.79	4.88	2.09	3.18	2.79	2.48	2.25	2.24	2.25	2.24	2.25	2.24	2.25	2.24	2.25	2.24	2.25	2.24	2.25	2.24
<i>V. natriegens</i>																																		
<i>V. anguillarum</i>																																		
<i>V. ordalii</i>																																		
<i>V. metschnikovii</i> strain 1																																		
<i>V. metschnikovii</i> strain 2																																		
<i>V. cincinnatiensis</i>																																		
<i>V. diazotrophicus</i>																																		
<i>V. vulnificus</i> strain 1																																		
<i>V. vulnificus</i> strain 2																																		
<i>V. furnisii</i>																																		
<i>V. nereis</i>																																		
<i>V. tubiashi</i>																																		
<i>V. nigripulchritudo</i>																																		
<i>V. gazogenes</i>																																		
<i>V. aestuarianus</i>																																		
<i>V. splendidus</i>																																		
<i>V. mediterranei</i>																																		
<i>V. orientalis</i>																																		
<i>V. navarrensis</i>																																		

^a Distances were calculated as percentages of dissimilarity by using parts of small-subunit rRNA sequences of *Vibrio* and *Photobacterium* strains which were available and which could be aligned without any ambiguity (see Materials and Methods).

the possible existence of crossing-over (as observed for *Aeromonas* small-subunit sequences [36]) suggest that sequences from a different part of the genome will probably be required to help answer these questions.

Within the restricted family *Vibrionaceae*, it would certainly be interesting to compare the results obtained from a sequence analysis with data derived from numerical taxonomy. However, this task proved to be very difficult, because relatively few classical studies have included all species, and because some strains have sometimes been wrongly recognized as species (see below). Importantly, in none of the classical numerical taxonomy studies did the authors examine their proposed taxonomic schemes for robustness, and it is very difficult to assess which part of an interpretation relied on extremely solid data and which part was more an extrapolation. The results of a recent thorough study (19) in which the authors used numerical taxonomy (61 different phenotypic tests) to examine 236 strains belonging to the *Vibrionaceae* (extended definition) also suggest that there may be an additional problem. An ordination analysis did not provide a clear separation of vibrios, enteric bacteria, and *Aeromonas* species, in contrast to what is now generally admitted and, in particular, to what had been found by workers in the same laboratory on the basis of an analysis of 5S rRNA sequences (28). Grimes et al. suggested that the phenotypes of species freshly isolated from natural environments could be different from the phenotypes of the same species after some period of laboratory culture. As part of their phenotypes, bacteria certainly contain the products of many regulated genes that are expressed according to the environment in which they grew. Thus, environmentally-linked phenotypes could be reflections of the ecosystems from which the bacteria were obtained, and they could occur for more than several cell cycles (a phenomenon analogous to magnetic remanence). If this phenomenon does exist, as suggested by a comparison of *V. parahaemolyticus* strains isolated from human and marine sources (3), analysis of small-subunit rRNA (or some other structural gene) is certainly a better phylogenetic index and a more efficient taxonomic tool, but phenotypic analysis is the only method that can be used to determine the actual place of a particular microorganism in a natural food web. However, because possible errors in determining sequences could lead to misidentification of species, and because it is not yet known how to relate exactly classical strain identification to small-subunit rRNA sequences, we concluded that it would be wise to reinvestigate previously published molecular data and/or strain identification, at least, when molecular phylogenies (often derived from only a single strain) are in strong disagreement with the results of classical taxonomy derived from repeated biochemical studies. The vibrios are a particularly difficult problem, because a few isolates have probably been misidentified in some studies and because of the phenotypic variations mentioned above (for an example of discrepancy, see references 3 and 11).

For phylogeny and perhaps for sound taxonomy, comparison of sequences is an appropriate approach only if there has been no lateral transfer of genes and if most (or all) bacterial species follow a vertical scheme of evolution. Baumann et al. have analyzed the evolution of rRNA (6), alkaline phosphatase, glutamine synthetase, and superoxide dismutase (4, 5) in *Vibrio* and *Photobacterium* species (8). The high correlation coefficients for the evolutionary pathways of these different molecules led these authors to assume that there has been no major lateral transfer of genes. Under these conditions, the phylogenetic reconstruction obtained from one molecule (a gene tree) represents the evolutionary history of the species (a species tree). Since rRNA sequences are now obtained more easily

than the sequence of any other gene, we rely on such sequences for reconstructing phylogenies from sequences and for estimating tree robustness. However, small-subunit rRNA analysis has some limitations: (i) small-subunit rRNA sequences do not contain highly variable domains that are probably required to properly analyze relationships of closely related organisms (similar species or strains within species), (ii) crossing over between genes of a multigene family (such as rRNA) could affect the reconstruction of phylogenies for closely related species (36), and (iii) bacterial classification should not be restricted to the evolutionary history of a single gene, since the definition of a species is related at least as much to its role in an ecosystem as to its ribosomal history. It is now important to try to correlate the information derived from rRNA analyses with the considerable amount of phenotypic analysis and numerical taxonomy data available (10, 24, 39), bearing in mind that some or many of the phenotypic traits usually analyzed might be short-term adaptative characteristics expressed by a "species" to survive in a particular environment.

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