Phylogenetic Relationships between Some Members of the Genera *Deleya*, *Halomonas*, and *Halovibrio*

S. J. DOBSON,* T. A. McMEEKIN, AND P. D. FRANZMANN

Department of Agricultural Science and the Cooperative Research Centre for the Antarctic and Southern Ocean Environment, University of Tasmania, Box 252C, Hobart, Tasmania 7001, Australia

The genera *Halomonas* and *Deleya*, which constitute the family *Halomonadaceae*, are difficult to differentiate on the basis of phenotypic and chemotaxonomic attributes. DNA-rRNA hybridization studies have indicated that some *Halomonas* spp. have the same level of relationship to the type species of the genus *Deleya* as some *Deleya* spp. A phylogenetic analysis of the 16S rRNA sequences of seven members of the *Halomonadaceae* indicated that the members of the genera *Halomonas* and *Deleya* do not form separate monophyletic subgroups, confirming the lack of any phylogenetic support for retention of these taxa as separate genera. A phylogenetic analysis of the 16S rRNA sequence of *Halovibrio variabilis* confirmed that this species belongs in the *Halomonadaceae*. All of the members of the *Halomonadaceae* examined and *Halovibrio variabilis* possess a cytosine residue at position 486 (*Escherichia coli* numbering), which is an extremely rare attribute among the prokaryotes and has been reported in only one other species, *Listonella anguillarum*. Several other signature characteristics which define this group in the gamma subclass of the *Proteobacteria* are identified. The Jukes-Cantor distances between members of the family *Halomonadaceae*, including *Halovibrio variabilis*, range from 0.086 to 0.000 (the levels of similarity between the 16S rRNA sequences range from 92.6 to 100%). The members of the genera *Halomonas*, *Deleya*, and *Halovibrio* form a monophyletic group and share common chemotaxonomic and phenotypic characteristics. Subgroups containing members of the genera *Halomonas*, *Deleya*, and *Halovibrio* cannot be resolved on the basis of phylogenetic, chemotaxonomic, or phenotypic data. Our data indicate that the members of the genera *Halomonas*, *Deleya*, and *Halovibrio* should be united in a single genus.

The genus *Halomonas* was created to classify a group of moderately halophilic, gram-negative, rod-shaped strains isolated from a saltern. These strains had a 61 mol% G+C in their DNA and could tolerate salt concentrations between 20 and 32% (27). The type species was *Halomonas elongata*. Subsequently, four additional species of the genus *Halomonas* were described to accommodate groups of strains isolated from estuarine, Antarctic lake, and Dead Sea waters (11, 13, 15, 16). The members of the genus *Halomonas* had G+C values in the range from 59 to 64 mol% and were moderately halophilic and/or halotolerant.

The genus *Deleya* was created to accommodate a group of marine species (4). This genus was created on the basis of a distinct lineage observed in DNA-rRNA hybridization studies and on the basis of chemotaxonomic features. The members of this genus were strictly aerobic and motile by means of flagella, accumulated poly- β -hydroxybutyrate, had a requirement for Na⁺, and had DNA G+C contents in the range from 52 to 68 mol%. The type species was “*Deleya aesta*”. *Deleya aquamarina* was later shown to be synonymous with “*D. aesta*” and replaced it as the type species (1). Two additional, moderately halophilic species have been described for strains isolated from saline soils and water bodies (22, 26).

A comparison of 16S rRNA oligonucleotide catalogs grouped *Halomonas elongata* and “*Flavobacterium halmephilum*” as a distinct lineage within the gamma subclass of the purple bacterium phylum, now known as the class *Proteobacteria* (24, 31). “*F. halmephilum*” was transferred to the genus *Halomonas* as *Halomonas halmophila* (13). A comparison of the catalogs of *Halomonas elongata* and

Halomonas halmophila with the catalogs of *Halomonas subglaciescola* and “*D. aesta*” resulted in the description of the family *Halomonadaceae* to accommodate the genera *Halomonas* and *Deleya* (13).

The genera *Halomonas* and *Deleya* are difficult to differentiate on the basis of the phenotypic attributes, fatty acid profiles, polar lipid profiles, and respiratory lipoquinones of their members (5, 12, 16, 23). rRNA-DNA hybridization studies have demonstrated that some *Halomonas* and *Deleya* species have similar melting temperatures [$T_{m(e)}$, the midpoint of thermal melting profiles for strand separation of DNA-DNA or DNA-RNA duplexes] in relation to *D. aquamarina* (18).

In this study the 16S rRNA sequences of six species representing the genera *Halomonas* and *Deleya* were determined to clarify the phylogenetic relationships of these organisms and to determine whether generic distinctions within this group of organisms are warranted.

The genus *Halovibrio* was created to accommodate an obligately halophilic organism with a vibroid morphology isolated from Great Salt Lake (9). The 16S rRNA sequence of *Halovibrio variabilis* was determined as this organism has been observed to have chemotaxonomic features similar to those of members of the genera *Deleya* and *Halomonas* (12).

MATERIALS AND METHODS

Microorganisms. *Halomonas subglaciescola* ACAM 12^T (= DSM 4683^T) (T = type strain) and *Halomonas meridiana* ACAM 246^T (= DSM 5425^T) were obtained from the Australian Collection of Antarctic Microorganisms (ACAM), University of Tasmania, Hobart, Tasmania, Australia. *Halomonas elongata* DSM 2851^T (= ATCC 33173^T), *D. aquamarina* DSM 30161^T (= ATCC 14400^T), *Deleya marina*

* Corresponding author.

DSM 4741^T (= ATCC 25374^T), *Deleya halophila* DSM 4770^T, and *Halovibrio variabilis* DSM 3051^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. The strains were grown at 25°C on marine broth (Difco) supplemented with agar.

DNA isolation, polymerase chain reaction amplification, and sequencing of polymerase chain reaction products. The methods used to extract DNA and directly sequence the isolated 16S ribosomal DNA polymerase chain reaction products have been described previously (6). Sequencing primer 361-341 (*Escherichia coli* numbering) (7) was used instead of primer 129-109.

Phylogenetic analysis. The 16S rRNA sequences of *D. aquamarina*, *D. halophila*, *D. marina*, *Halomonas elongata*, *Halomonas subglaciescola*, *Halomonas meridiana*, and *Halovibrio variabilis* were aligned manually, taking into account secondary structure considerations, with the 16S rRNA sequence of *Halomonas halmophila* ATCC 19717^T and the 16S rRNA sequences of the following 38 organisms belonging to the gamma subclass of the *Proteobacteria* (culture collection strain designations that are identified in the data base are indicated): *Acinetobacter* sp. strain ATCC 33604, *Aeromonas hydrophila* ATCC 7966^T, *Alteromonas haloplanktis* ATCC 14393, *Arhodomonas oleiferhydrans* ATCC 49307, *Cardiobacterium hominis* ATCC 15826^T, *Coxiella burnetii* str. Q177, *Chromatium tepidum* ATCC 43061^T, *Chromatium vinosum* ATCC 17899^T, *Citrobacter freundii* ATCC 29935, *Ectothiorhodospira halochloris* ATCC 35916^T, *Ectothiorhodospira halophila* str. SL 1, *Ectothiorhodospira shaposhnikovii*, *Erwinia carotovora* ATCC 5713, *Erwinia herbicola*, *Escherichia coli*, "*Flavobacterium lutescens*" ATCC 27951, *Haemophilus influenzae* ATCC 33391^T, *Hafnia alvei* ATCC 13337^T, *Legionella pneumophila* subsp. *pneumophila* ATCC 33152^T, *Marinobacter hydrocarbonoclasticus* ATCC 49840^T, *Marinomonas vaga* ATCC 27119^T, *Methylococcus capsulatus* str. BATH, *Methylobacter methanica* str. 81Z, *Oceanospirillum linum* ATCC 11336^T, *Pasteurella multocida* ATCC 43137^T, *Plesiomonas shigelloides* ATCC 14029^T, *Proteus vulgaris* IFAM 1731, *Pseudomonas aeruginosa* ATCC 25330, *Pseudomonas mendocina* ATCC 25411^T, *Ruminobacter amylophilus* ATCC 29477, *Serratia marcescens* ATCC 13880^T, str. symbiont P of *Acyrtosyphon pisum*, str. symbiont S of *Acyrtosyphon pisum*, *Vibrio parahaemolyticus* ATCC 17802^T, *Wolbachia persica* ATCC VR 331, *Xanthomonas maltophilia* ATCC 13637^T, *Xylella fastidiosa* ATCC 35880, and *Yersinia enterocolitica* ATCC 9610^T. The sequences of most of these species were obtained from the rRNA database project (21); the exceptions were the sequences of *Alteromonas haloplanktis*, *Marinobacter hydrocarbonoclasticus*, and *Marinomonas vaga*, which were obtained from GenBank and then aligned in the rRNA database project format.

Phylogenetic relationships were estimated by distance analysis by using the PHYLIP version 3.4, package. Jukes-Cantor evolutionary distances were calculated by using the DNADIST program, and a phylogenetic tree was derived by using the FITCH program, random order input of sequences, and the global rearrangement option (8). Parsimony analysis was done by using the branch and bound method available in PAUP version 3.0 (25). Both the parsimony and distance trees were unrooted with *Pasteurella multocida* as the outgroup. Bootstrap analysis (2,000 replications) was performed for both the distance and parsimony methods. The parsimony bootstrap analysis was performed by using a heuristic search method because of computing limitations.

TABLE 1. Positions at which the 16S rRNA sequence of *Halomonas elongata* determined in this study differed from the sequence determined by Gauthier et al.

Position(s) (<i>E. coli</i> numbering)	Residue(s) as determined by:	
	This study	Gauthier et al. ^a
184	C	
932	C	
1003-1004	GA	GGA
1009	T	C
1168	C	T
1208	C	
1403	C	A
1461-1467	ATCACCA	AGTCACA

^a Data from reference 14.

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in the GenBank data base under accession numbers M93352 through M93358.

RESULTS

The number of bases determined for the 16S rRNA genes of the seven species ranged from 1,402 to 1,458 (average, 1,432), which represented 96.2% of the sequence in the region from position 29 to position 1521 (*Escherichia coli* numbering). The sequence of *Halomonas elongata* ATCC 33173^T was compared with the sequence obtained by Gauthier et al. (14). There were nine differences between the two sequences (Table 1). A reexamination of the sequencing autoradiographs resolved the differences in favor of the results of this study.

The 16S rRNA sequences of all of the members of the *Halomonadaceae* which we examined had a cytosine residue at 486, which is a highly distinctive signature. An examination of 436 sequences in the rRNA database project (21) did not reveal any organism that possessed a C residue at this site. According to a recent description of the partial 16S rRNA sequence of *Listonella anguillarum*, this organism also contains a C at this position (19). The closest relatives of *Listonella anguillarum*, *Vibrio fischeri* and *Vibrio logei*, do not contain a cytosine residue at this position (19). Most other members of the *Bacteria* possess a uracil residue; the exception is *Planctomyces staleyii*, which has an adenine residue. A deletion occurs in members of the *Archaea*.

A comparison of the sequences of the members of the *Halomonadaceae* with the sequences of 38 other members of the gamma subclass of the *Proteobacteria* revealed several characteristics which formed a distinctive signature for the *Halomonadaceae* within the gamma subclass (Table 2). Other members of the gamma subclass contained a maximum of 5 of the 17 characteristics identified. With one exception (*Listonella anguillarum*), in the gamma subclass the signature at position 486 met the stringent condition of having a constant composition among members of the *Halomonadaceae* and a different and constant composition in all other members of the subclass (29). Similarly, for the signature at position 776 there was only one other known member of the gamma subclass which had the same composition as the members of the *Halomonadaceae*; all other members of the subclass had a different and constant composition.

TABLE 2. Signature characteristics of the 16S rRNA gene for members of the *Halomonadaceae* and frequency of occurrence among other members of the gamma subclass of the *Proteobacteria*

Signature site ^a	Residue(s) in <i>Halomonadaceae</i>	Presence of the same residue(s) in ^b :																								No. of organisms with the same residue ^c	
		<i>Acinetobacter calcoaceticus</i>	<i>Alteromonas haloplanktis</i>	<i>Arthomonas oleiferhydrans</i>	<i>Cardiobacterium hominis</i>	<i>Coxiella burnetii</i> str. Q177	<i>Ectothiorhodospira halochloris</i> str. A	<i>Ectothiorhodospira halophila</i> str. SL1	<i>Ectothiorhodospira shaposhnikovii</i>	<i>Erwinia herbicola</i>	“ <i>Flavobacterium lutescens</i> ”	<i>Haemophilus influenzae</i>	<i>Legionella pneumophila</i>	<i>Marinobacter hydrocarbonoclasticus</i>	<i>Methylococcus capsulatus</i> str. BATH	<i>Methylobacter methanica</i> str. 81Z	<i>Marinomonas vaga</i>	<i>Oceanospirillum linum</i>	<i>Pasteurella multocida</i>	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas mendocina</i>	<i>Ruminobacter amylophilus</i>	str. symbiont P of <i>Acyrtosiphon pisum</i>	<i>Vibrio parahaemolyticus</i>	<i>Wolbachia persica</i>		<i>Xanthomonas maltophilia</i>
76–93	6-pair stem								+				+			+									+		4
484	A														+												1
486	C																										0
640	G												+					+									3
660 ^d	A		+															+									1
668–738	A–U	+			+			+							+										+		5
669–737	A–U			+	+	+																+	+		+		6
745 ^d	U	+						+		+		+						+		+	+				+		7
776	U							+																			1
1124	U																+	+						+			3
1297	U										+				+	+		+							+	+	7
1298	C			+											+	+			+						+	+	4
1423	A					+							+				+					+					4
1424	C			+			+	+	+															+			5
1439	U												+									+					2
1462	A												+									+					2
1464	C					+												+									2

^a *E. coli* numbering.^b Most sequences were obtained from the rRNA database project (21). The exceptions were the *Alteromonas haloplanktis*, *Marinobacter hydrocarbonoclasticus*, and *Marinomonas vaga* sequences, which were obtained from GenBank.^c The number of organisms belonging to the gamma subclass of the *Proteobacteria* out of a sample of 38 available sequences (excluding members of the *Halomonadaceae*) which contained the same 16S rRNA signature characteristic as the members of the *Halomonadaceae*.^d Residues 660 and 745 are paired.

Table 3 shows the evolutionary distances calculated by the Jukes-Cantor equation and the levels of sequence similarity between members of the genera *Deleya*, *Halomonas*, and *Halovibrio* and some representatives of the gamma subclass of the *Proteobacteria*. *D. aquamarina* and *Halomonas meridiana* were the most highly related organisms, having identical sequences over the determined region. The distances between other members of the three genera ranged from 0.026 to 0.086, and the levels of sequence similarity ranged from 92.5 to 97.6%. The closest relative of members of the *Halomonadaceae* was *O. linum*. The evolutionary distances between *O. linum* and members of the *Halomonadaceae* ranged from 0.11 to 0.125, and the levels of sequence similarity ranged from 88.8 to 90.4%.

The phylogenetic tree derived by distance analysis is shown in Fig. 1. Two trees of equal minimal length were found by parsimony analysis (Fig. 2). One of these (Fig. 2b) had a topology similar to that of the tree derived from distance analysis. The topology of the tree in Fig. 2b also was the result obtained from a distance and parsimony analysis of a conserved data set, in which positions at which less than 50% of the taxa had the same nucleotide were eliminated from the data set. The members of the genera *Halomonas* and *Deleya* did not form separate monophyletic

subgroups in the phylogenetic trees. This confirmed that there is no phylogenetic basis to distinguish the two genera and was consistent with results obtained from rRNA-DNA (18), chemotaxonomic (12, 23), and phenotypic (5, 16) studies.

Halovibrio variabilis clustered with the members of the *Halomonadaceae* (Fig. 1 and 2) and was most closely related to *D. aquamarina* and *Halomonas meridiana*, with a level of 16S rRNA sequence similarity of 96.7%. *Halovibrio variabilis* was clearly a member of the *Halomonadaceae* and is treated as such below.

The members of the *Halomonadaceae* were separated into the following three clusters on the distance tree (Fig. 1) and one of the trees derived from parsimony analysis (Fig. 2b): (i) cluster 1, containing *D. aquamarina*, *Halomonas meridiana*, *Halovibrio variabilis*, and *Halomonas subglaciescola*; (ii) cluster 2, containing *Halomonas elongata*, *Halomonas halmophila*, and *D. halophila*; and cluster 3, containing *D. marina*. However, bootstrap analyses in which both the parsimony and distance methods were used indicated that the internal topology of the *Halomonadaceae* group was not stable (Fig. 3). Strong support existed only for a *D. aquamarina*-*Halomonas meridiana*-*Halovibrio variabilis* sub-cluster within cluster 1 described above.

TABLE 3. 16S rRNA evolutionary distances as calculated by the Jukes-Cantor equation and levels of 16S rRNA similarity for members of the genera *Deleya*, *Halomonas*, and *Halovibrio* and some other members of the gamma subclass of the *Proteobacteria*

Organism	Evolutionary distance or level of 16S rRNA similarity (%) ^a														
	<i>Deleya aquamarina</i>	<i>Halomonas meridiana</i>	<i>Halovibrio variabilis</i>	<i>Halomonas subglaciescola</i>	<i>Halomonas halmophila</i>	<i>Halomonas elongata</i>	<i>Deleya halophila</i>	<i>Deleya marina</i>	<i>Oceanospirillum linum</i>	<i>Marinobacter hydrocarbonoclasticus</i>	<i>Pseudomonas aeruginosa</i>	<i>Marinomonas vaga</i>	<i>Ruminobacter amylophilus</i>	<i>Wolbachia persica</i>	<i>Pasteurella multocida</i>
<i>Deleya aquamarina</i>		100	96.7	95.3	94.0	94.0	94.0	93.6	89.5	88.4	88.2	87.5	85.3	84.9	82.8
<i>Halomonas meridiana</i>	0.000		96.7	95.0	93.7	93.8	93.9	93.6	89.1	88.2	88.0	87.2	85.0	84.6	82.7
<i>Halovibrio variabilis</i>	0.036	0.035		94.5	92.5	93.1	94.0	92.6	88.8	88.1	87.5	86.9	84.4	83.1	82.6
<i>Halomonas subglaciescola</i>	0.051	0.053	0.059		94.8	94.8	94.5	93.2	89.2	88.1	87.4	88.1	83.8	83.2	82.0
<i>Halomonas halmophila</i>	0.065	0.068	0.082	0.055		97.6	95.7	93.5	89.0	86.8	87.3	87.3	83.3	83.0	81.9
<i>Halomonas elongata</i>	0.066	0.068	0.075	0.056	0.025		96.7	94.1	89.4	87.6	87.7	87.7	83.5	83.5	82.1
<i>Deleya halophila</i>	0.068	0.069	0.068	0.061	0.047	0.036		95.2	90.2	88.7	87.8	88.5	84.0	83.7	82.7
<i>Deleya marina</i>	0.073	0.073	0.085	0.077	0.073	0.067	0.056		90.4	89.0	87.9	88.2	84.7	84.1	82.8
<i>Oceanospirillum linum</i>	0.118	0.121	0.125	0.120	0.120	0.118	0.111	0.110		89.7	88.4	89.4	84.2	84.2	83.8
<i>Marinobacter hydrocarbonoclasticus</i>	0.132	0.133	0.134	0.134	0.148	0.140	0.132	0.128	0.113		88.1	88.3	84.1	83.6	82.2
<i>Pseudomonas aeruginosa</i>	0.133	0.134	0.141	0.142	0.140	0.137	0.142	0.140	0.127	0.133		88.2	86.0	84.3	82.6
<i>Marinomonas vaga</i>	0.142	0.145	0.149	0.132	0.142	0.138	0.133	0.137	0.115	0.130	0.130		84.3	85.3	83.8
<i>Ruminobacter amylophilus</i>	0.170	0.171	0.180	0.186	0.191	0.190	0.192	0.183	0.178	0.182	0.156	0.178		82.8	85.3
<i>Wolbachia persica</i>	0.176	0.179	0.199	0.196	0.197	0.192	0.198	0.193	0.180	0.191	0.179	0.167	0.198		81.7
<i>Pasteurella multocida</i>	0.203	0.202	0.204	0.211	0.210	0.210	0.211	0.210	0.185	0.208	0.201	0.185	0.164	0.214	

^a The values on the lower left are 16S rRNA evolutionary distances as calculated by the Jukes-Cantor equation, and the values on the upper right are levels of 16S rRNA similarity, expressed as percentages.

An examination of the sequence similarity values also suggested that clusters 1 through 3 did not represent unreservedly distinct subgroups within the *Halomonadaceae* (Table 3). The differences in the distances of *Halomonas subglaciescola* and *D. halophila* from members of different subgroups were marginal. *Halomonas subglaciescola* was related to the other members of the *D. aquamarina* subgroup at similarity levels of 94.5 to 95.3% and to *Halomonas elongata* and *Halomonas halmophila* at a similarity level of 94.8%. *D. halophila* was related to the other members of the *Halomonas elongata* subgroup at similarity levels of 95.7 to 96.7% and to *D. marina* at a similarity level of 95.2%. Therefore, *Halomonas subglaciescola* and *D. halophila* were not clearly aligned with one subgroup but were intermediate between subgroups.

The inclusion of *Halomonas subglaciescola* in cluster 1 also was not supported by an examination of the character data. There were more positions at which *Halomonas subglaciescola* was monophyletic with regard to *Halomonas elongata*, *Halomonas halmophila*, and *D. halophila* than at which it was monophyletic with regard to the other members of cluster 1. Similarly, the uncertainty about the placement of *D. halophila* was evident from the character data. The number of sequence positions at which the identity of the base in *D. halophila* was the same as that in *H. halmophila* and *H. elongata* and different from the identities in all other members of the *Halomonadaceae* was five, compared with the seven positions at which the identity of the base in *D. halophila* was the same as that in *D. marina* and different from the identities in all other members of the *Halomonadaceae*.

FIG. 1. Phylogenetic tree derived by distance analysis of 16S rRNA sequences, showing the relationships among some members of the genera *Deleya*, *Halomonas*, and *Halovibrio* and some other members of the gamma subclass of the *Proteobacteria*. *Pasteurella multocida* is the outgroup organism. Bar = 0.03 evolutionary distance unit as calculated by the Jukes-Cantor equation.

FIG. 2. Equally most parsimonious trees derived from an analysis of the 16S rRNA sequences of some members of the genera *Deleya*, *Halomonas*, and *Halovibrio* and some other members of the gamma subclass of the *Proteobacteria*. *Pasteurella multocida* is the outgroup organism. Bar (length of horizontal branches) = 50 nucleotide differences.

DISCUSSION

Six oligonucleotides from the catalogs of *Halomonas elongata* and *Halomonas halmophila* were identified previously as unique to these organisms within the gamma subclass of the *Proteobacteria* (31); however, within the wider set of organisms examined in this study, only one of these signatures, occurring at positions 1297 and 1298 (Table 2), had value as a signature for the *Halomonadaceae*. An additional 16 characteristics present in the 16S rRNA sequences of all members of the genera *Deleya*, *Halomonas*, and *Halovibrio* examined and sparsely distributed among the other members of the gamma subclass of the *Proteobacteria* provide a defining signature for this family (Table 2). One of these signature characteristics, a cytosine residue at position 486, was extremely rare among the prokaryotes and has been reported for only one other species, *Listonella anguillarum*.

The identical 16S rRNA sequences of *D. aquamarina* and *Halomonas meridiana* suggested that these two names may

be synonyms. The 16S rRNA data were consistent with the similar fatty acid profiles of the two organisms (Table 4). *Halomonas meridiana* and "*D. aesta*" (a synonym of *D. aquamarina*) also have identical polar lipid patterns (12). The current, widely accepted definition of a bacterial species is organisms that exhibit levels of DNA-DNA hybridization of approximately 70% or more and $\Delta T_{m(e)}$ values of $\leq 5^\circ\text{C}$ (28).

Amann et al. (2) found that a DNA-DNA hybridization value of 50% corresponded to a level of 16S rRNA sequence similarity of approximately 99% among strains belonging to the genus *Fibrobacter* and suggested that genomic sequence conservation varies among groups of strains having similar evolutionary depth as estimated from 16S rRNA sequence similarity data. Fox et al. (10) reported a level of 16S rRNA sequence similarity of 99.8% between two *Bacillus* sp. which exhibited DNA-DNA hybridization values of 23 to 50% and concluded that 16S rRNA sequence identity within the limits

FIG. 3. Bootstrap consensus trees derived from parsimony analysis (a) and distance analysis (2,000 replications) (b), showing the frequency of occurrence of groups where the frequency was more than 50%.

TABLE 4. Levels of major fatty acids in members of the *Halomonadaceae*^a

Species or strain ^b	% (wt/wt)		
	16:0	16:1 + cyc17:0	18:1 + cyc19:0
<i>Halomonas meridiana</i>	15.5	7.7	73.4
" <i>Deleya aesta</i> "	17.0	9.1	71.2
<i>Deleya aquamarina</i>	17.4	8.4	70.7
<i>Deleya venusta</i>	15.9	10.7	68.6
<i>Halovibrio variabilis</i>	17.5	13.8	66.0
<i>Halomonas halmophila</i>	20.2	11.3	64.6
<i>Deleya halophila</i>	23.3	10.6	64.6
<i>Halomonas elongata</i>	26.0	9.0	62.8
<i>Deleya cupida</i>	22.6	17.5	56.1
<i>Deleya pacifica</i>	26.2	15.6	55.7
<i>Halomonas subglaciescola</i>	25.4	19.7	52.3
<i>Halomonas subglaciescola</i> ACAM 21	32.0	28.9	38.2
<i>Halomonas halodurans</i>	27.3	41.7	26.7
<i>Deleya marina</i>	30.3	43.1	23.5

^a Data from reference 12.^b Data for the type strain unless indicated otherwise.

of experimental error is not sufficient to guarantee species identity, where species are defined on the basis of DNA-DNA hybridization values of 70% or more. A numerical taxonomic analysis showed that *Halomonas meridiana* and "*D. aesta*" were phenotypically distinct (16). Therefore, DNA-DNA hybridization data will be required to clarify whether *D. aquamarina* and *Halomonas meridiana* are synonyms. Fox et al. (10) state, "In the absence of definitive DNA-DNA data, strains with effectively identical 16S rRNA sequences are best regarded as belonging to the same rRNA species complex or rRNA superspecies. Such strains can be expected to exhibit considerable levels of DNA-DNA homology (typically at least 30%), which need not exceed 70%." According to this definition *D. aquamarina* and *Halomonas meridiana* belong to the same rRNA species complex.

A comparison of DNA-rRNA hybridization values (18) with 16S rRNA similarity values showed that of the species common to both sets of data, *Halomonas subglaciescola* was the closest to *D. aquamarina* (Table 5). There were

TABLE 5. 16S rRNA similarity values, DNA-rRNA $T_{m(e)}$ values, and DNA-DNA homology values for comparisons between *D. aquamarina* DSM 30161^T and other members of the genera *Halomonas* and *Deleya*

Organism	% 16S rRNA similarity	DNA-rRNA $T_{m(e)}$ (°C)	% DNA-DNA homology
<i>Halomonas meridiana</i>	100	— ^b	—
<i>Halovibrio variabilis</i>	96.7	—	—
<i>Deleya venusta</i>	—	79.0	41
<i>Deleya cupida</i>	—	76.4	16
<i>Deleya pacifica</i>	—	76.6	12
<i>Halomonas subglaciescola</i>	95.3	76.2	—
<i>Halomonas halmophila</i>	94.0	76.2	—
<i>Halomonas elongata</i>	94.0	75.6	—
<i>Deleya halophila</i>	94.0	75.2	—
<i>Deleya marina</i>	93.6	75.6	12

^a DNA-rRNA $T_{m(e)}$ values were obtained from reference 18. DNA-DNA homology values were obtained from reference 1. All values are values for type strains. Values were not available for *Halomonas halodurans* and *Deleya salina*.

^b —, data not available.

some differences in the arrangement of the other species common to both sets of data with respect to their similarity to *D. aquamarina*. *Halomonas halmophila*, *Halomonas elongata*, and *D. halophila* had the same level of 16S rRNA sequence similarity with respect to *D. aquamarina*, whereas there was a difference of 1°C in the $T_{m(e)}$ values of the hybrids. The $T_{m(e)}$ values for *Halomonas subglaciescola* and *Halomonas halmophila* were identical, whereas there was a 1.3% difference in the levels of 16S rRNA sequence similarity between these species compared with *D. aquamarina*. In addition, *D. marina* had a higher $T_{m(e)}$ value than *D. halophila* by 0.4°C, but had a lower level of 16S rRNA sequence similarity by 0.4%. These differences were marginal and possibly reflect the use of 23S rRNA for the hybridization studies and the small sensitivities of hybridization to the distribution and identity of mismatched bases. The possibility that these differences might have arisen from the presence of undetermined bases in the 16S rRNA sequences was considered. However, the arrangement of 16S rRNA sequence similarity values was identical when the values were calculated by using a data set from which all positions where there was an undetermined base in any of the sequences were eliminated (data not shown).

Data for DNA-rRNA hybridization between members of the *Halomonadaceae* and *D. aquamarina* revealed the presence of two subgroups (18). The species most closely related to *D. aquamarina* [$\Delta T_{m(e)}$, 3°C] were its synonyms "*D. aesta*" and *Alcaligenes faecalis* subsp. *homari*, *Deleya venusta*, and the misnamed taxa "*Achromobacter viscosum*," "*Pseudomonas bathycetes*," "*Agarobacterium algini-cum*," and "*Achromobacter turbidus*." Related to *D. aquamarina* in the second subgroup [$\Delta T_{m(e)}$, 5 to 7°C] were *Deleya pacifica*, *Deleya cupida*, *Halomonas halmophila*, *Halomonas subglaciescola*, *Halomonas elongata*, *D. marina*, *D. halophila*, and the misnamed taxa "*Achromobacter halophilus*" and "*Chromobacterium marismortui*." The 16S rRNA sequence comparisons done in this study provided information on the relatedness of all combinations of pairs of organisms and not just information on relatedness with respect to a single reference organism. While it appeared from the phylogenetic trees that the members of the *Halomonadaceae* clustered into three subgroups, bootstrap analysis and an examination of distance and character data showed that these clusters were not well supported. On the basis of this phylogenetic analysis, division of the members of the *Halomonadaceae* into three genera in accord with the composition of the three clusters could not be justified.

The only group of species in the phylogenetic trees that was well supported by the bootstrap analysis was composed of *D. aquamarina*, *Halomonas meridiana* and *Halovibrio variabilis*, which were related at a 16S rRNA sequence similarity level of 96.7% (Jukes-Cantor values, 0.035 to 0.036). If this similarity level were considered appropriate for the formation of a genus, then *Halomonas subglaciescola* and *D. marina* would be assigned to two separate genera, *Halomonas halmophila* and *Halomonas elongata* would be assigned to another separate genus not well supported by the results of the parsimony bootstrap analysis (Fig. 2b), and the position of *D. halophila* would remain ambiguous. *D. halophila* exhibited levels of sequence similarity to the *Halomonas halmophila*-*Halomonas elongata* group which ranged from 95.7 to 96.7% and a level of sequence similarity to *D. marina* of 95.2%, and the clustering of *D. halophila* with either the *Halomonas halmophila*-*Halomonas elongata* group or *D. marina* was not supported by the results of the bootstrap analyses. Notwithstanding the

uncertainties which would arise from the use of a similarity level of 96 to 96.7% to define genera within the *Halomonadaceae*, the *D. aquamarina*-*Halomonas meridiana*-*Halovibrio variabilis* group might be considered a separate genus if its members were phenotypically or chemotaxonomically distinct from the other members of the *Halomonadaceae*.

In a numerical phenotypic comparison which included *Halomonas meridiana*, "*D. aesta*" (a synonym of *D. aquamarina*), *Halomonas elongata*, *Halomonas halmophila*, and *D. halophila*, *Halomonas meridiana* was more distant from "*D. aesta*" than it was from the other species of the *Halomonadaceae* (16). This indicates that for members of the *Halomonadaceae* relationships derived from numerical phenotypic analyses are not in accord with phylogenetic relationships derived from 16S rRNA data. In a study of aerobic marine bacteria, Baumann et al. (3) similarly observed that numerical phenotypic analyses were not necessarily useful for identifying associations above the species level. Therefore, significant phenotypic characteristics can be identified only in light of known phylogenetic relationships. A phenotypic comparison of all members of the *Halomonadaceae* has not been done; however, an indication of whether phenotypic characteristics might be useful for differentiating the *D. aquamarina*-*Halomonas meridiana*-*Halovibrio variabilis* subgroup from other members of the *Halomonadaceae* is given by the phenotypic data of James et al. (16), who studied *Halomonas meridiana*, "*D. aesta*," and the type strains of four other members of the *Halomonadaceae*. These data were examined to identify characteristics which were identical in *Halomonas meridiana* and "*D. aesta*" and which could differentiate this pair of organisms from the other members of the *Halomonadaceae* examined. The only attributes identified that might be useful for this purpose were utilization of some sole carbon sources. However, it seems unlikely that carbon source utilization patterns would be useful for defining the *D. aquamarina*-*Halomonas meridiana*-*Halovibrio variabilis* group, as one of the characteristic features of *Halovibrio variabilis* is its inability to grow on a wide range of sole carbon sources (10). For example, in so far as the data of James et al. (16) and Fendrich (9) can be compared, the growth of both "*D. aesta*" and *Halomonas meridiana* was stimulated by D-glucose, maltose, mannose, and succinate, whereas the growth of *Halovibrio variabilis* was not.

All members of the *Halomonadaceae* contained the same major fatty acids (12). Thus, subgroups within the *Halomonadaceae* could not be characterized by signature fatty acids. The major fatty acids, 16:0, 16:1, 17:0cyc, 18:1, and 19:0cyc, represented 95.2 to 99.1% of the total fatty acids. The conversion of monounsaturated fatty acids to cyclopropane acids is a one-step synthesis which is sensitive to salt concentration, oxygen tension, growth rate, and temperature (30). The sum of the levels of the monounsaturated fatty acid and its derived cyclopropane fatty acid is a more stable characteristic than the level of the cyclopropane fatty acid alone (17). The relative levels of the fatty acids in the profiles of the members of the *Halomonadaceae* are shown in Table 4 in decreasing order of the level of 18:1 plus 19:0cyc. In this arrangement the members of the *D. aquamarina*-*Halomonas meridiana*-*Halovibrio variabilis* group have higher levels of 18:1 plus 19:0cyc and lower levels of 16:0 than the other members of the *Halomonadaceae*; however, the differences are marginal. The differences in the levels of 16:0, 16:1 plus 17:0cyc, and 18:1 plus 19:0cyc in *Halovibrio variabilis* and *Halomonas halmophila* range from 1 to 3%. A determination of whether this level of difference is significant would require

an estimate of error from repeat determinations of these profiles. If evidence of this difference were repeatable, larger than experimental error, and larger than intraspecies variation, the identification of an unknown strain would still require a concomitant determination of the fatty acid profiles of several reference species of the *Halomonadaceae* to account for the effect of small differences in culture conditions with each experiment. The creation of a separate genus on the basis of such a small difference in fatty acid profiles is not justified in our opinion. The majority of the minor fatty acid components were present at levels of less than 1%. Utilization of these components to define new genera would similarly be unreliable.

Although the *D. aquamarina*-*Halomonas meridiana*-*Halovibrio variabilis* cluster was well supported phylogenetically, phenotypic characteristics which defined this group, could not be identified, polar lipid patterns and the identities of respiratory lipoquinones did not discriminate the group from the other members of the *Halomonadaceae*, and the differences in the proportions of fatty acids were marginal and could not practicably be employed to define the group. The Ad Hoc Committee on Approaches to Taxonomy within the *Proteobacteria* expressed the opinion that "The first step in the identification of bacteria is the assignment of organisms to genera. Therefore, the greatest clarity in circumscription and utility in the choice of characteristics must be accorded to the level of genera. It is completely impracticable to define genera solely on the basis of phylogenetic data" (20). In accord with the opinion of the Ad Hoc Committee on Approaches to Taxonomy within the *Proteobacteria*, we concluded that the *D. aquamarina*-*Halomonas meridiana*-*Halovibrio variabilis* group cannot be described as a separate genus.

Given the possibility that in the future phenotypic or chemotaxonomic characteristics might be identified which could define phylogenetic groups at the genus level among the set of organisms currently assigned to the genera *Deleya*, *Halomonas*, and *Halovibrio*, the identification of well-resolved phylogenetic groups within this set of organisms will remain problematic. The position of *D. halophila* is ambiguous at a similarity level of 96%. At a 16S rRNA sequence similarity level of 97% *Halomonas subglaciescola*, *D. halophila*, and *D. marina* would separate into individual groups; *Halomonas elongata* and *Halomonas halmophila* would form a cluster (sequence similarity level, 97.6%) that was not well supported by the results of bootstrap analysis when the parsimony method was used (Fig. 3a); and the *D. aquamarina*-*Halomonas meridiana*-*Halovibrio variabilis* group, which was well supported phylogenetically, would break down, as *Halovibrio variabilis*, which had a level of 16S rRNA sequence similarity to the other two species of 96.7%, would form a separate group.

An examination of the fatty acid profiles of member species of the *Halomonadaceae* not included in the 16S rRNA sequence analysis suggests that the addition of these species to the 16S rRNA phylogenetic tree would result in even less resolution of groups within the tree. The species are arranged in Table 4 in order of decreasing amount of 18:1 plus 19:0cyc. The concomitant trends with respect to the other major fatty acids are increasing amounts of 16:0 and increasing amounts of 16:1 plus 17:0cyc, although these characteristics are not entirely consistent. The levels of 16:1 plus 17:0cyc and 18:1 plus 19:0cyc in ACAM 21 are intermediate in the range of the levels present in *D. marina* and a group of species which includes *D. halophila*, while the level of 16:0 is similar to that in *D. marina*. Given the similarities

that exist between the arrangement of species in Table 4 and in the phylogenetic trees, *Halomonas subglaciescola* ACAM 21 may be predicted to be phylogenetically intermediate between *D. halophila* and *D. marina*. The fatty acid profiles of *D. cupida* and *D. pacifica* were most similar to the profile of *Halomonas subglaciescola* ACAM 12^T, and the levels of 16:1 plus 17:0cyc and 18:1 plus 19:0cyc in these organisms were intermediate in the range for these fatty acids in *Halomonas subglaciescola* and *Halomonas elongata*. The exact phylogenetic position of *D. cupida* and *D. pacifica* is not predictable. The profiles of these organisms fall within a group that contains *Halomonas halmophila*, *Halomonas elongata*, *D. halophila*, and *Halomonas subglaciescola* ACAM 12^T. The differences in the levels of fatty acid types are not large in this group and show no consistent trends.

The analysis of the 16S rRNA data indicates that the set of organisms assigned to the genera *Deleya*, *Halomonas*, and *Halovibrio* are resolved into coherent phylogenetic groups only at very high levels of 16S rRNA sequence similarity (>97%). The question then is whether the phenotypic and chemotaxonomic discrimination between organisms within this group at that level is sufficient to warrant their description as several separate genera rather than separate species. The available phenotypic and chemotaxonomic data suggest that separation of this group of organisms into several different genera is not warranted. The species for which 16S rRNA sequence data were obtained represent the diversity in this group in terms of phenotypes, habitats, fatty acid profiles, and G+C values. An exception may be *D. venusta*, which has the lowest G+C value; however, this species is closely related to *D. aquamarina*, as indicated by rRNA-DNA hybridization data and confirmed by a DNA-DNA hybridization value of 41% (Table 5).

The members of the genera *Deleya*, *Halomonas*, and *Halovibrio* form a coherent phylogenetic group which is homogeneous with respect to chemotaxonomic features (fatty acids and respiratory lipoquinones), and traditional phenotypic features appear not to be useful for delineating subgroups within this set of organisms. It is our opinion that there is sufficient phylogenetic, chemotaxonomic, and phenotypic data to indicate that the members of these three genera should be united in a single genus. Unification of the genera *Deleya*, *Halomonas*, and *Halovibrio* would stabilize the taxonomy of this group. Until the current unsatisfactory taxonomy is clarified, any newly described species belonging to this group will remain in a state of limbo.

ACKNOWLEDGMENTS

We thank Harry Burton of the Australian Antarctic Division for his interest in and support for this project.

This study received funding from grants from the Australian Research Council and the Antarctic Science Advisory Committee. S. J. Dobson was supported by an Australian Postgraduate Research Award. P. D. Franzmann was supported by an Australian Research Council Fellowship.

REFERENCES

1. Akagawa, M., and K. Yamasato. 1989. Synonymy of *Alcaligenes aquamarinus*, *Alcaligenes faecalis* subsp. *homari*, and *Deleya aesta*: *Deleya aquamarinus* comb. nov. as the type species of the genus *Deleya*. Int. J. Syst. Bacteriol. 39:462-466.
2. Amann, R. L., C. Lin, R. Key, L. Montgomery, and D. Stahl. 1992. Diversity among *Fibrobacter* isolates: towards a phylogenetic classification. Syst. Appl. Microbiol. 15:23-31.
3. Baumann, L., P. Baumann, M. Mandel, and R. D. Allen. 1972. Taxonomy of aerobic marine eubacteria. J. Bacteriol. 110:402-429.
4. Baumann, L., R. D. Bowditch, and P. Baumann. 1983. Description of *Deleya* gen. nov. created to accommodate the marine species *Alcaligenes aestus*, *A. pacificus*, *A. cupidus*, *A. venustus*, and *Pseudomonas marina*. Int. J. Syst. Bacteriol. 33:793-802.
5. Dobson, S. J. 1988. B.Sc. thesis, University of Tasmania, Hobart, Tasmania, Australia.
6. Dobson, S. J., R. R. Colwell, T. A. McMeekin, and P. D. Franzmann. 1993. Direct sequencing of the polymerase chain reaction-amplified 16S rRNA gene of *Flavobacterium gondwanense* sp. nov. and *Flavobacterium salegens* sp. nov., two new species from a hypersaline Antarctic lake. Int. J. Syst. Bacteriol. 43:77-83.
7. Edwards, U., T. Rogall, H. Blöcker, M. Emde, and E. C. Böttger. 1989. Isolation and direct complete nucleotide determination of entire genes. Characterisation of a gene coding for 16S ribosomal genes. Nucleic Acids Res. 17:7843-7853.
8. Felsenstein, J. 1989. PHYLIP—phylogeny inference package (version 3.2). Cladistics 5:164-166.
9. Fendrich, C. 1988. *Halovibrio variabilis* gen. nov. sp. nov., *Pseudomonas halophila* sp. nov. and a new halophilic coccoid eubacterium from Great Salt Lake, Utah, USA. Syst. Appl. Microbiol. 11:36-43.
10. Fox, G. E., J. D. Wisotzkey, and P. Jurtshuk, Jr. 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int. J. Syst. Bacteriol. 42:166-170.
11. Franzmann, P. D., H. R. Burton, and T. A. McMeekin. 1987. *Halomonas subglaciescola*, a new species of halotolerant bacteria isolated from Antarctica. Int. J. Syst. Bacteriol. 37:27-34.
12. Franzmann, P. D., and B. J. Tindall. 1990. A chemotaxonomic study of members of the family *Halomonadaceae*. Syst. Appl. Microbiol. 13:142-147.
13. Franzmann, P. D., U. Wehmeyer, and E. Stackebrandt. 1988. *Halomonadaceae* fam. nov., a new family of the class *Proteobacteria* to accommodate the genera *Halomonas* and *Deleya*. Syst. Appl. Microbiol. 11:16-19.
14. Gauthier, M. J., B. Lafay, R. Christen, L. Fernandez, M. Acquaviva, P. Bonin, and J.-C. Bertrand. 1992. *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new extremely halotolerant, hydrocarbon-degrading marine bacterium. Int. J. Syst. Bacteriol. 42:568-576.
15. Hebert, A. M., and R. H. Vreeland. 1987. Phenotypic comparison of halotolerant bacteria: *Halomonas halodurans* sp. nov., nom. rev., comb. nov. Int. J. Syst. Bacteriol. 37:347-350.
16. James, S. R., S. J. Dobson, P. D. Franzmann, and T. A. McMeekin. 1990. *Halomonas meridiana*, a new species of extremely halotolerant bacteria isolated from Antarctic saline lakes. Syst. Appl. Microbiol. 13:270-278.
17. Jantzen, E., and K. Bryn. 1985. Whole-cell and lipopolysaccharide fatty acids and sugars of Gram-negative bacteria, p. 145-172. In M. Goodfellow and D. E. Minnikin (ed.), Chemical methods in bacterial systematics. Academic Press, Inc., London.
18. Kersters, K. 1992. The genus *Deleya*, p. 3189-3197. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications, vol. 4, 2nd ed. Springer-Verlag, New York.
19. Kita-Tsakamoto, K., H. Oyaizu, K. Nanba, and U. Simudu. 1993. Phylogenetic relationships of marine bacteria, mainly members of the family *Vibrionaceae*, determined on the basis of 16S rRNA sequences. Int. J. Syst. Bacteriol. 43:8-19.
20. Murray, R. G. E., D. J. Brenner, R. R. Colwell, P. De Vos, M. Goodfellow, P. A. D. Grimont, N. Pfennig, E. Stackebrandt, and G. A. Zavarin. 1990. Report of the Ad Hoc Committee on Approaches to Taxonomy Within the *Proteobacteria*. Int. J. Syst. Bacteriol. 40:213-215.
21. Olsen, G. J., N. Larsen, and C. R. Woese. 1991. The ribosomal RNA database project. Nucleic Acids Res. 19:2017-2021.
22. Quesada, E., A. Ventosa, F. Ruiz-Berraquero, and A. Ramos-Cormenzana. 1984. *Deleya halophila*, a new species of moderately halophilic bacteria. Int. J. Syst. Bacteriol. 34:287-292.
23. Skerratt, J. H., P. D. Nichols, C. A. Mancuso, S. R. James, S. J.

- Dobson, T. A. McMeekin, and H. Burton. 1991. The phospholipid ester-linked fatty acid composition of members of the family *Halomonadaceae* and genus *Flavobacterium*. A chemotaxonomic guide. *Syst. Appl. Microbiol.* **14**:8–13.
24. Stackebrandt, E., R. G. E. Murray, and H. G. Trüper. 1988. *Proteobacteria* classis nov., a name for the phylogenetic taxon that includes the "purple bacteria and their relatives." *Int. J. Syst. Bacteriol.* **38**:321–325.
25. Swofford, D. L. 1990. PAUP: phylogenetic analysis using parsimony, version 3.0. Illinois Natural History Survey, Champaign.
26. Valderrama, M. J., E. Quesada, V. Bejar, A. Ventosa, M. C. Guitierrez, F. Ruiz-Berraquero, and A. Ramos-Cormenzana. 1991. *Deleya salina* sp. nov., a moderately halophilic gram-negative bacterium. *Int. J. Syst. Bacteriol.* **41**:377–384.
27. Vreeland, R. H., C. D. Litchfield, E. L. Martin, and E. Elliot. 1980. *Halomonas elongata*, a new genus and species of extremely salt-tolerant bacteria. *Int. J. Syst. Bacteriol.* **30**:485–495.
28. Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichesky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Trüper. 1987. Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. *Int. J. Syst. Bacteriol.* **37**:463–464.
29. Weisburg, W. G., Y. Oyaizu, H. Oyaizu, and C. R. Woese. 1985. Natural relationship between bacteroides and flavobacteria. *J. Bacteriol.* **164**:230–236.
30. Wilkinson, S. G. 1988. Fatty acid composition in members of the *Pseudomonadaceae*, p. 334–337. In C. Ratledge, and S. G. Wilkinson (ed.), *Microbial lipids*, vol. 1. Academic Press, Inc., London.
31. Woese, C. R., W. G. Weisburg, C. M. Hahn, B. J. Paster, L. B. Zablen, B. J. Lewis, T. J. Macke, W. Ludwig, and E. Stackebrandt. 1985. The phylogeny of purple bacteria: the gamma subdivision. *Syst. Appl. Microbiol.* **6**:25–33.