Phylogenetic Inferences and Taxonomic Consequences of 16S Ribosomal DNA Sequence Comparison of Chromohalobacter marismortui, Volcaniella eurihalina, and Deleya salina and Reclassification of *V. eurihalina* as *Halomonas eurihalina* comb. nov.

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The phylogenetic positions of the moderately halophilic bacteria *Chromohalobacter marismortui*, *Volcaniella eurihalina*, and *Deleya salina* were determined by PCR amplification of rRNA genes and direct sequencing. The resulting data were compared with data for other bacteria obtained from 16S rRNA sequence databases. *C. marismortui*, *V. eurihalina*, and *D. salina* clustered phylogenetically within the gamma subclass of the *Proteobacteria* and are closely related to other species on the *Halomonas-Deleya* branch. *C. marismortui* belongs in the family *Halomonadaceae* and has the characteristic 16S rRNA signatures defined for this family, including the distinctive cytosine residue at position 486 found in all members of the *Halomonadaceae*. *V. eurihalina* is closely related to the type species of the genus *Halomonas*, *Halomonas elongata*, and we formally propose that *V. eurihalina* should be transferred to the genus *Halomonas* as *Halomonas eurihalina* comb. nov. The type strain of this species is strain F9-6 (= ATCC 49336). *D. salina* is not as closely related to other species belonging to the *Halomonas-Deleya* complex, but is more closely related to *Halomonas elongata* than to *Deleya aquamarina*, the type species of the genus *Deleya*. A polyphasic approach will be necessary to determine the natural taxonomic positions of the species belonging to the genera *Halomonas* and *Deleya*, as well as *C. marismortui*, *V. eurihalina*, *Halovibrio variabilis*, and *Paracoccus halodenitrificans*.

Moderately halophilic bacteria grow optimally in media containing between 3 and 15% NaCl (12). Taxonomically, this physiological group of organisms is represented by a limited number of species, including some methanogenic members of the *Archaea*, as well as a wide variety of *Bacteria* (27). In such hypersaline environments, gram-negative rods identified as members of the genera *Halomonas*, *Deleya*, *Pseudomonas*, and *Vibrio* are the most abundant taxonomic groups (21, 22). Very few studies to determine the phylogenetic status of moderately halophilic members of the *Bacteria* have been carried out. Only a few studies of moderately halophilic gram-negative rods have been described previously; these studies were based on 16S rRNA cataloging data (7, 16–18) and, more recently, on 16S rRNA sequence comparisons. The results of the latter studies permitted the authors to place two recently described species, *Flavobacterium gondwanense* and *Flavobacterium saliens*, in the “*Flavobacterium-Bacteroides*” phylum (3). In addition, a new moderately halophilic organism, *Arthromonas aqueoelei*, was recently isolated from petroleum reservoir production fluid (1). A 16S rRNA sequence analysis revealed that *A. aqueoelei* represents a deeply branching lineage in the gamma subclass of the *Proteobacteria* that is specifically related to halophilic species belonging to the genus *Ectothiorhodospira* (1). More recently, Dobson et al. performed a 16S rRNA sequence analysis of several species of the genera *Halomonas*, *Deleya*, and *Halovibrio*, including some moderately halophilic representatives, and determined the phylogenetic relationships of these microorganisms (4). Thus, the members of the genera *Halomonas*, *Deleya*, and *Halovibrio* cluster within the gamma subclass of the *Proteobacteria*, but these organisms do not form separate monophyletic subgroups, suggesting that the species of these three genera might be combined in a single genus (4). In addition, Miller et al. (14) performed an analysis in which they determined the 16S ribosomal (rDNA) sequence of *Paracoccus halodenitrificans* and found that this moderate halophile was inappropriately placed in the genus *Paracoccus* and is in fact closely related to the genera *Deleya* and *Halomonas* in the family *Halomonadaceae*.

During the last decade, our studies of hypersaline environments have led to the description of several new organisms (26, 27). On the basis of phenotypic data, the moderately halophilic gram-negative species *Chromohalobacter marismortui* (28) and *Volcaniella eurihalina* (20) could be closely related to the genus *Halomonas*, the genus *Deleya*, the genus *Halovibrio*, or the recently described species *A. aqueoelei*. Furthermore, in 1991 a new *Deleya* species, *Deleya salina*, was described (25); this species and the two moderate halophiles mentioned above have not been studied previously from a phylogenetic point of view.

In this study, the 16S rDNA sequences of several representatives of the moderately halophilic species *C. marismortui*, *Volcaniella eurihalina*, and *D. salina* were determined in order to clarify the phylogenetic relationships of these organisms.

**MATERIALS AND METHODS**

**Strains and culture conditions.** The six moderately halophilic strains used in this study are listed in Table 1. All of the strains were grown in a medium containing 0.5% (wt/vol) yeast extract (Difco) and a salt mixture having a final concentration of 10% (29). The pH was adjusted to 7.5, and the cultures were incubated at 37°C in an orbital shaker at 200 strokes per min. When necessary, solid media were prepared by adding 2% (wt/vol) Bacto Agar (Difco).

**Isolation of genomic DNA and 16S rDNA sequence analysis.** Cells were harvested at approximately the late exponential phase by centrifugation, washed,
and resuspended in 560 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). The cells were lysed by adding 25 µl of 10% sodium dodecyl sulfate (Sigma) containing 0.1 mg of proteinase K (Sigma) and incubating the preparations for 1 h at 37°C. DNA was extracted and precipitated by the CTAB miniprep protocol for bacterial genomic DNA preparations (31).

PCR amplification of the 16s rRNA gene by using the forward primer 16F27: and the reverse primer 16R1488: and direct sequence determinations of the PCR-amplified DNAs were carried out by using automated (Applied Biosystems, Inc.) DNA cycle sequencing (10). Sequence data were aligned with known 16s rRNA and rDNA sequences by using evolutionarily conserved primary sequences and secondary structures as references (8, 32). Evolutionary distances, including a correction factor for reverse mutations (9), were calculated for sequence pairs by using a “mask” (13) for nonhomologous or uncertain nucleotide positions. Dendrograms were generated by using a pairwise, weighted, least-squares distance method (15).

Nucleotide sequence accession numbers. The EMBL accession numbers for the 16s rDNA nucleotide sequences of the strains used in this study are shown in Table 1.

RESULTS AND DISCUSSION

The 16s rDNA primary sequences of strains (Table 1) belonging to the moderately halophilic species C. marismortui, Volcaniella euhalina, and D. salina comprised 1,460 nucleotides (corresponding to nucleotide positions 28 to 1,487; Escherichia coli numbering system), which represented more than 95% of the estimated total primary structures.

The 16s rDNA gene sequences of the gram-negative moderate halophiles C. marismortui, Volcaniella euhalina, and D. salina were compared with the 16s rRNA sequences of a wide variety of strains (Table 2) available in previously published studies and with sequences obtained from the EMBL RNA database. The genera Chromohalobacter, Volcaniella, and Deleya clustered within the gamma subclass of the Proteobacteria, and the sequences of members of these genera exhibited high levels of homology (99.5 to 90.3%) with the sequences of other moderately halophilic bacteria belonging to the Halomonas-Deleya complex (Table 2). In contrast, these organisms are not closely related to other moderate halophiles or marine bacteria, such as Vibrio costicola (levels of 16s rDNA sequence similarity, 82.2 to 83.4%), A. aquaeolei (levels of similarity, 85.0 to 86.1%), and Marinobacter hydrocarbonoclasticus (levels of similarity, 86.6 to 89.2%). A dendrogram constructed on the basis of the results of our analysis of the sequences of the moderately halophilic species C. marismortui, Volcaniella euhalina, and D. salina and other members of the gamma subclass of the Proteobacteria is shown in Fig. 1.

Our sequence analysis revealed that C. marismortui is genetically distinct from the nonhalophilic bacteria for which sequence data are available (Fig. 1). This phylogenetic relationship is consistent with the results of previous DNA-RNA hybridization studies (11), which showed that C. marismortui should be included in the family Halomonadaceae, a family that Franzmann et al. (7) proposed in order to bring together the species of the genera Halomonas and Deleya. The taxonomic features of the genus Chromohalobacter (28) are consistent with those defined by Franzmann et al. (7) for the family Halomonadaceae, including the G+C content of the genomic DNA. In addition, the 16s rRNA gene sequences of C. marismortui, Volcaniella euhalina, and D. salina contain the 17 signature nucleotides which define the family Halomonadaceae, including the cytosine residue at position 486, which is a distinctive signature of all members of the Halomonadaceae (4).

Dobson et al. (4) proposed that all Halomonas, Deleya, and Halovibrio species should be united in a single genus, since these organisms constitute a coherent phylogenetic group. However, we believe that such a single genus would be too heterogeneous if all species belonging to these genera were included. This is reflected by the genomic DNA G+C content range for the single genus (52 to 68 mol%) (11), a range that is clearly larger than the ranges that are currently accepted for genera (19). However, although several subgroups may be detected in the Halomonas-Deleya complex, it remains to be seen to what extent these subgroups are indicative of subunits in a single genus or of several genera. It is clear from this study and previous studies that C. marismortui should be considered a member of a separate genus. There is phenotypic evidence that C. marismortui is different enough from the Halomonas-Deleya complex to warrant classification in a separate genus (28). However, additional chemotaxonomic data will be necessary to confirm the taxonomic status of these organisms.

We sequenced the 16s rRNA of the type strain of C. marismortui, ATCC 17056, as well as the 16s rDNAs of three other isolates, A-65, A-100, and A-492. The sequences of the latter three strains differed at only one position (position 488; E. coli numbering). However, when these sequences were compared with the C. marismortui ATCC 17056 (T = type strain) sequence, we found 39 positions at which the sequences differed (level of sequence difference, 2.7%). These data reflect not only the phenotypic and genotypic differences reported previously (28, 29), but also the different sources of isolation of these organisms. C. marismortui ATCC 17056 was originally isolated from the Dead Sea (5), an athalassohaline environment, while the other three strains were obtained from marine salterns located near the Mediterranean Sea (28, 29). Previous DNA homology experiments demonstrated that the isolates obtained from marine salterns formed a single DNA hybridization group (with levels of homology between 84 and 100%), while the levels of DNA homology between these isolates and C. marismortui ATCC 17056 ranged from 66 to 91% (28). It is now thought that organisms belonging to the same species should exhibit levels of DNA relatedness of approximately 70% or more and that they should be coherent phenotypically (30). As recently pointed out, this species concept corresponds to an estimated level of 16s rRNA sequence identity of 97% or more (3% or approximately 45 nucleotide differences) (24). Our 16s rDNA sequence data support the close relationships determined previously for the strains obtained from marine salterns and what could be considered the limit of the species.
| Strain or species | Chromohalobacter marismortui ATCC 17056 | Chromohalobacter marismortui A-65 | Volcanicella eurihalina | Deleya salina | Halomonas elongata | Halomonas halophila | Halomonas meridiana | Halomonas subglaciescola | Deleya marina | Deleya aquamarina | Deleya halophila | Halobaterio variabilis | Vibrio algolyticus | Vibrio costicola | Ectothiorhodospina halochloris | Aeromorphae aquaeolei | Alteromonas haloplanktis | Marinomonas vaga | Marinobacter hydrocarbono-clasticus | Oceanospirillum linum | Photobacterium phosphorum | Escherichia coli | Pseudomonas aeruginosa | Paracoccus halodenitrificans |
|------------------|----------------------------------------|-------------------------------|----------------------|-------------|------------------|------------------|------------------|------------------|--------------|----------------|----------------|----------------|------------------|----------------|------------------------|------------------|-----------------------------|--------------------|--------------------------|------------------|--------------------------|------------------|
|                  | 97.0                                   | 93.6                          | 94.4                | 93.8        | 93.3             | 93.2             | 92.8             | 91.9             | 93.3         | 93.1           | 92.3           | 82.9             | 82.3             | 84.1             | 84.8             | 85.4             | 82.6          | 85.1           | 86.9           | 88.4           | 84.2           | 83.6           | 86.3           | 92.8           |

a The values on the lower left are 16S rDNA evolutionary distances as calculated by the Jukes-Cantor equation, and the values on the upper right are levels of 16S rDNA similarity, expressed as percentages.
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The 16S rDNA sequence data also show that *Volcaniella eurihalina* ATCC 49336<sup>T</sup> is phylogenetically very close to *Halomonas elongata* (Fig. 1). The same 16S rDNA nucleotide sequence was obtained when hypervariable regions of the rDNA of *Volcaniella eurihalina* 41a were studied (data not shown). *Volcaniella eurihalina* and *Halomonas elongata* differ in many phenotypic characteristics (20), have different G+C contents (65.7 mol% for *Volcaniella eurihalina* and 60.5 mol% for *Halomonas elongata*), exhibit low levels of DNA-DNA relatedness (7%), and thus should be considered two separate species (20). The 16S rDNA sequence data indicate that these organisms are very closely related phylogenetically and thus should be placed in the same genus. In addition, the phenotypic characteristics of *Volcaniella eurihalina* are similar to those of the genus *Halomonas* (20). Therefore, we propose that *Volcaniella eurihalina* should be transferred to the genus *Halomonas* as *Halomonas eurihalina* comb. nov. A description of this species is given below. Features that distinguish *Volcaniella eurihalina* from related moderate halophiles have been described elsewhere (20, 25).

Finally, the 16S rDNA sequence of *D. salina* ATCC 49509<sup>T</sup> indicated that this species is a member of the *Halomonas-Deleya* branch (Fig. 1), but is distinct from the other species that we analyzed. However, as in the case of *Deleya halophila*, *D. salina* is more closely related to *Halomonas elongata* than to *Deleya aquamarina* (the type species of the genus *Deleya*). On the basis of the currently available taxonomic data for these microorganisms, it is difficult to establish whether *D. salina* should be included in the genus *Halomonas* or should be placed in a different genus.

In conclusion, our data show that the moderately halophilic species *C. marismortui*, *Volcaniella eurihalina*, and *D. salina* cluster in the gamma subclass of the *Proteobacteria* and belong to the *Halomonas-Deleya* complex. As pointed out previously (4, 6, 11), the taxonomic position of all species included in the genera *Halomonas* and *Deleya* is not clear, and in fact, phenotypic and chemotaxonomic data have so far failed to distinguish species of the genera *Halomonas* and *Deleya* (2, 6, 23, 33). In addition, *Halovibrio variabilis*, *Paracoccus halodenitrificans* (14), and *Volcaniella eurihalina* (formally transferred to the genus *Halomonas* below) are also included on this phylogenetic branch. Since this group of halophiles is widely distributed in different ecological niches, a more detailed study of other strains, as well as new isolates, will clearly be needed to define properly the taxonomic structure of the existing species, and conclusions should be based not only on the currently available data but also on new phenotypic, chemotaxonomic, and molecular data. As in the case of some other groups in the *Bacteria* and *Archaea*, new taxonomic markers are needed to differentiate these microorganisms.

**Transfer of Volcaniella eurihalina to the genus Halomonas as Halomonas eurihalina comb. nov.** The species *Volcaniella euri-
Volcanic euris, eurihalina Ruiz-Berraquero, and Ramos-Cormenzana 1990) comb. nov. has been published previously (20). The G+C content of the microorganism was determined to be 716 MELLADO ET AL. INT. J. SYST. BACTERIOL.

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