Diversity of Alkaliphilic Halobacteria: Proposals for Transfer of *Natronobacterium vacuolatum*, *Natronobacterium magadii*, and *Natronobacterium pharaonis* to *Halorubrum*, *Natrialba*, and *Natronomonas* gen. nov., Respectively, as *Halorubrum vacuolatum* comb. nov., *Natrialba magadii* comb. nov., and *Natronomonas pharaonis* comb. nov., Respectively

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The 16S rRNA genes of three species of the genus *Natronobacterium* ( *Natronobacterium gregoryi*, *Natronobacterium pharaonis*, and *Natronobacterium vacuolatum*) were sequenced and compared to that of the previously sequenced species *Natronobacterium magadii*. The sequences revealed that *Natronobacterium pharaonis* was phylogenetically distinct from the other members of the genus and also from other recognized genera of the family Halobacteriaceae. However, *Natronobacterium vacuolatum* and *Natronobacterium magadii* were found to be most closely related to the genera *Halorubrum* and *Natrialba*, respectively. An unidentified haloalkaliphile, strain SSL1, was also closely related to *Natronobacterium magadii* and *Natrialba asiatica*. On the basis of phylogenetic tree reconstructions, signature bases specific for individual genera, and sequences of spacer regions between 16 and 23S rRNA genes, we propose the following changes: *Natronobacterium pharaonis* to be transferred to the genus *Natronomonas* gen. nov. as *Natronomonas pharaonis* gen. nov., comb. nov.; *Natronobacterium vacuolatum* to be transferred to the genus *Halorubrum* as *Halorubrum vacuolatum* comb. nov.; and *Natronobacterium magadii* to be transferred to the genus *Natrialba as Natrialba magadii*.

The extremely halophilic, aerobic Archaea are classified within the family Halobacteriaceae, which currently contains nine valid genera: *Halobacterium*, *Halococcus*, *Haloarcula*, *Haloferax*, *Halorubrum*, *Halobaculum*, *Natronobacterium*, and *Natronomonas* (3, 5, 7, 13, 16, 17).

The alkaliphilic members of the *Halobacteriaceae* form a distinct physiological group as they require not only high NaCl concentrations but also high pH (between 8.5 and 11) and low Mg$^{2+}$ concentrations (less than 10 mM) for growth. They have been isolated from a variety of alkaline, hypersaline lakes and soils (3, 15, 18, 20–22). Microscopically, the initial isolates consisted of rods and cocci and were accordingly separated into two genera, *Natronobacterium* and *Natronomonas*, by Tindall et al. (21). *Natronobacterium* currently contains four recognized species, *Natronobacterium gregoryi*, *Natronobacterium magadii*, *Natronobacterium pharaonis*, and *Natronobacterium vacuolatum*, with *Natronobacterium gregoryi* as the type species. The present classification of these isolates is based largely on chemotaxonomic criteria, particularly phenotypic properties and lipid composition. Glycolipid analysis, which has been of great use in the taxonomy of the neutrophilic halobacteria, has had little impact in the classification of the natronobacteria, as these lack major amounts of glycolipids in their membranes (3, 15). The complete sequence of the 16S rRNA gene of only one species, *Natronobacterium magadii*, has been reported, and comparison to other halobacteria has clearly shown that it represents a distinct genus (7, 12). However, the sequences of the other three species of *Natronobacterium* (*Natronobacterium gregoryi*, *Natronobacterium pharaonis*, and *Natronobacterium vacuolatum*) have not been published, so phylogenetic support for their inclusion in this genus is lacking. DNA-DNA hybridization of the four species indicated that they have little sequence homology (31 to 38%) (15), and while this is good evidence for their classification as separate species, it does not preclude their representing different genera.

The aim of this study was to determine the phylogenetic relationships among the natronobacteria and in particular to ascertain whether the four members of the genus *Natronobacterium* were indeed close relatives. For this purpose, 16S rRNA sequences of the three unsequenced species of *Natronobacterium* and of one unclassified haloalkaliphilic strain, SSL1, were determined, as well as sequences from four other strains of halobacteria. These sequences were incorporated into phylogenetic tree reconstructions of the family *Halobacteriaceae*.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** The following strains of the genus *Natronobacterium* were used in this study: *Natronobacterium gregoryi* NCIMB 2189 T, *Natronobacterium pharaonis* JCM 8858 T, *Natronobacterium vacuolatum* JCM 8858 T (derived from DSM 2160 T via IFO 14720 T), *Natronobacterium vacuolatum* JCM 9060 T (deposited by W. D. Grant), and strain SSL1 (microscopically long rods, originally isolated from an alkaline saline brine from Sambhar Salt Lake, India, and deposited with the American Type Culture Collection as *Natronobacterium* sp. strain ATCC 43988) (22, 23). These strains were grown at 37°C in haloalkaliphilic medium as previously described (20, 23).

*Halobacterium trapanicum* NCIMB 767 was obtained from the National Collection of Industrial and Marine Bacteria in 1994 and deposited as JCM 8979. Three species of the genus *Halococcus* (*Halococcus saccharolyticus* ATCC 49257, *Halococcus maliic*...
Sequencing of 16S rRNA genes and thin-layer chromatographic analysis of polar lipids. Genomic DNAs from the four species of Natronobacterium were extracted as described before (7). Halobacterium trapanicum JCM 8979 and species of Halococcus which do not lyse with N-lauroyl sarcosine were grown on quartz sand, and DNA was recovered by ethanol precipitation. 16S rRNA-encoding genes were amplified by PCR, cloned into plasmid pUC119, and sequenced (7, 8). Final sequences of these genes were unambiguous, as regions which were difficult to read on one strand were easily read by sequencing the complementary strand. Phylogenetic tree reconstructions based on the 16S rRNA sequences were performed as described previously (7).

Membrane lipids were extracted, and thin-layer chromatography was performed as described before (7).

Sequencing of spacer regions. Genomic DNAs from five species of the genus Halorubrum (Halorubrum saccharovorum, Halorubrum sodonense, Halorubrum lacusprofundi, Halorubrum coriense, and Halorubrum distributurn) were extracted (7) and kept at −30°C. Spacer regions between the 16S rRNA and 23S rRNA genes from the three species of the genus Natronobacterium and the five species of the genus Halorubrum were amplified by PCR with a set of specific oligonucleotide primers. The sense primer, 5'TTAAGTCTGAACAGGTAGGCTATG AG, was based on the sequence from nucleotides 2332 to 2356 of the Halobacterium halodurum rRNA operon (accession no. X03407), and the antisense primer, 3'-CTGCACGGTTCGACGCTATTCG, was designed based on nucleotides 2595 to 2596 of the same sequence. Both primers are situated in highly conserved regions of the PCR products which were expected to overlap by 51 bp with the 3'-termini of the 16S rRNA genes and by 71 bp with the 5'-termini of the 23S rRNA genes, were cloned and sequenced as described above. Sequences of spacer regions from the following halobacteria were taken or extracted from operon sequence databases: Halobacterium cutirubrum JCM 9872, Halococcus salifodinae DSM 8989, and Halococcus morrhuae, the type species, branched just before the natronococci, whereas Natronobacterium magadii and strain SSL1 branched before Natrilalba. The exact branching order of Natronobacterium magadii and SSL1 was not clear in parsimony or distance matrix trees, but the bootstrap values indicate a clear separation of Natronobacterium magadii and Natronobacterium gregoryi. Natronobacterium vacuolatum was most closely related to the genus Halorubrum. Natronobacterium pharaonis turned out to be only distantly related to the other genera. Even the sequence most similar to that of Natronobacterium pharaonis, i.e., Haloarcula marismortui A, showed a low similarity value of 89.9%. The lack of bootstrap values at the branch point indicates that parsimony and distance methods could not confidently identify the deepest branching order among the three (bootstrap values, 54 to 55%). However, the maximum likelihood method (the tree shown) gave strongly supported branch lengths in this region. The evidence from sequence similarity and tree reconstructions appears to present a clear case for this isolate representing a new genus.

Signature bases of 16S rRNA genes. By using the aligned sequences, a number of signature sequences (24) which were specific for each genus were revealed. These are as follows: Halobacterium (Halobacterium cutirubrum, Halobacterium halodurum, and strain Y12), 77C, 106G, 128T, 213C, 222T, 407A, and 1383T; Haloarcula (Haloarcula marismortui, Haloarcula salinaensis, Haloarcula vallismortis, and Haloarcula adriensis), 235T, 255A, 665A, 670G, 675C, 885C, 937T, 968C, 972T, 1173A, 1200T, 1202T, 1243T (− means a gap), 1250A, and 1458A; Haloferax (the four recognized species), 1109A, 1218T, 1220T, and 1227A; Halococcus (two strains of Halococcus morrhuae, Halococcus saccharolyticus, Halococcus salifodinae, and Halobacterium trapanicum JCM 8979, but not Halococcus turkmenicus), 25T, 46C, 90C, 200C, 211C, 215A, 495A, 499A, 1246C, 1271T, and 1280A; Natrilalba (Natrilalba asiatica), 385G, 502G, and 1108A; Halobaculum (Halobaculum gomoneurense), 106T, 213A, 246T, 521T, 603A, 690T, 702C, 706A, 777C, and 943T; Natronobacterium gregoryi and two Natronococcus spp. (Natronococcus occultus and Natronococcus amylolyticus),
FIG. 1. Phylogenetic tree reconstruction of the *Halobacteriaceae* with complete 16S rRNA sequences. The representative tree shown here was derived by using maximum likelihood (fDNAm1). Branches with poorly supported lengths have been collapsed. Bootstrap values (100 replicates) shown at the nodes were obtained by parsimony (above) and distance matrix (below) methods and added to the maximum likelihood tree. Only bootstrap values above 70% are shown. The scale bar represents 0.02 expected changes per site.
sequence. If sequences from members of the other genera were introduced, the alignment was severely disrupted. This was also reflected by the tRNA\textsuperscript{A\textsubscript{1}}\textsuperscript{genes (72 bp), which were largely conserved among the five \textit{Halorubrum} spp. and \textit{Natronobacterium vacuolatum}, except for a one-base difference in \textit{Halorubrum lacusprofundi} (\textsuperscript{52}C\textsuperscript{T}). Alignment with the other seven tRNA\textsuperscript{A\textsubscript{1}}\textsuperscript{genes revealed differences in nine bases. Three of these (bases 5, 17, and 68) are conserved among the five recognized species of the \textit{Halorubrum-Natronobacterium vacuolatum} group but differ from the other sequences, at bases 5 and 68, which form a hydrogen bond, T and A in the \textit{Halorubrum-Natronobacterium vacuolatum} group but C and G in the rest; and at base 17, which is in the D arm of the cloverleaf secondary structure, C in the \textit{Halorubrum-Natronobacterium vacuolatum} group but T in the rest.

**DISCUSSION**

In this study, phylogenetic tree reconstructions have clearly shown the genus \textit{Natronobacterium} as being polyphyletic, i.e., it contains a diverse group of organisms that show closer relationships to widely differing groups within the family \textit{Halobacteriaceae}. Each of the four current species probably belongs to a separate genus. Unlike the other halobacterial genera, which possess a variety of membrane glycolipids, the halalkaliphiles contain only trace amounts or no glycolipids. Though phosphatidylglycerol and phosphatidylglycerophosphate-methyl ether are the main membrane phospholipids of the alkaliphiles as in the other genera, minor amounts of additional phospholipids (PL1 to PL4; only the structure of PL2 has been determined previously [11]) have been detected by thin-layer chromatography among the haloalkaliphiles (14, 15). \textit{Natronobacterium gregoryi} possesses phospholipids PL1 and PL3, \textit{Natronobacterium pharaonis} has PL1, \textit{Natronobacterium magadii} has PL3, and \textit{Natronobacterium vacuolatum} contains PL3 and PL4. On the other hand, \textit{Natronococcus occultus} has PL1 and PL2, while \textit{Natronococcus amylyticus} does not contain PL2 (9).

Since the phylogenetic data presented in this study indicate that each of the four species of the genus \textit{Natronobacterium} probably belongs to a separate genus, the known differences in minor phospholipids may be considered as additional taxonomic markers.

We believe that the four species of the genus \textit{Natronobacterium} belong to different genera as discussed below.

\textit{Natronobacterium gregoryi}, which clustered tightly with the two species of the genus \textit{Natronococcus}, should remain the type species of the genus \textit{Natronobacterium}. This is supported by the four signature bases described above.

\textit{Natronobacterium pharaonis} was only distantly related to the other taxa and would appear to represent a novel genus. Sequence comparisons with other 16S rRNA genes, including that of the recently proposed species \textit{Haloarcula mukohataei} (4), showed similarities of less than 89.9%. Ten signature bases were also observed. We propose to transfer \textit{Natronobacterium pharaonis} to \textit{Natronomonas} gen. nov.

\textit{Natronobacterium vacuolatum} showed a 16S rRNA sequence similarity of 95.6% to \textit{Halorubrum saccharovorans}, the type species, indicating that they may belong to the same genus. In addition, a close examination of the 37 aligned sequences, used in reconstructing the tree, has revealed many sequence signatures. As described in Results, sequences of the six species of \textit{Halorubrum} possess 19 genus-specific signature bases (i.e., not found in the other 31 sequences). The \textit{Natronobacterium vacuolatum} sequence shares 16 of these 19 bases (the three bases which are confined to the six recognized species of \textit{Halorubrum} are underlined). On the other hand, there are two positions (781 and 1406) where five of the six species and \textit{Natronobacterium vacuolatum} have the same specific signature bases and two positions (1264 and 1265) where \textit{Halorubrum coriense} (only) has different bases from the other 35 sequences. Furthermore, six bases, 785A, 793T, 1234G, 1242A, 1267T, and 1284A, were confined to the six recognized species of the genus \textit{Halorubrum, Natronobacterium vacuolatum}, and \textit{Halobaculum gomorense}. Altogether, these data seem to support the view that \textit{Natronobacterium vacuolatum} is related to members of the genus \textit{Halorubrum}. No glycolipids have been detected in \textit{Natronobacterium vacuolatum} (15), whereas most of the species of the genus \textit{Halorubrum} are characterized by the presence of glycolipid sulfated diglycosydiethester-3 (S-DGD-3) (7), but an exception has been reported, i.e., sulfated diglycosydiethester-5 (DGD-5) for \textit{Halorubrum trapanicum} NRC 34021 (13). We cannot exclude the possibility that a trace amount of glycolipid is present in \textit{Natronobacterium vacuolatum}.

Given the evidence from 16S rRNA gene sequences and the additional data from spacer region sequences, we believe there is a strong case to support the transfer of \textit{Natronobacterium vacuolatum} to the genus \textit{Halorubrum} and for the strain to be called \textit{Halorubrum vacuolatum} (comb. nov.).

The 16S rRNA gene of \textit{Natronobacterium magadii} showed a high sequence similarity to that of strain SSL1 (95.9%) and to those of \textit{Natratiba asiatica} 172P\textsuperscript{T} (type strain) and \textit{Natratiba asiatica} B1T (93.3 and 93.7%, respectively). The similarity between SSL1 and \textit{Natratiba asiatica} B1T were 95.9 and 96.6%, respectively. These figures (pairwise comparisons) seem to suggest that \textit{Natronobacterium magadii}, strain SSL1, and \textit{Natratiba asiatica} may be members of the same genus, \textit{Natratiba}. On the other hand, the lipid analyses do not support the close 16S rRNA sequence similarities. No glycolipid has been detected in \textit{Natronobacterium magadii}, but strain SSL1 has a minor amount of glycolipid DGD-4 (glucose 1→6-glycosyl glycerol diether [10, 23]). \textit{Natratiba asiatica} has a glycolipid, S2-DGD (mannose-2,6-disulfate 1→2-glycosyl glycerol diether [7]). The structures of these lipids suggest that the enzymes involved in the biosynthesis are different between the two. However, one of the two strains of \textit{Natratiba asiatica}, B1T, is able to grow at alkaline pH (from 5 to 10) (7). Furthermore, \textit{Natronobacterium magadii} and strain SSL1 do share one of the three signature bases of the genus \textit{Natratiba}, 502C, and strain SSL1 shares 1108A. Moreover, 568A is confined to \textit{Natratiba asiatica} B1T, \textit{Natronobacterium magadii}, and strain SSL1. Altogether, these data (high similarities in 16S rRNA genes, signature bases, and growth at alkaline pH) seem to support the view that \textit{Natronobacterium magadii} and strain SSL1 are members of the genus \textit{Natratiba}, and we propose to transfer \textit{Natronobacterium magadii} to the genus \textit{Natratiba} as \textit{Natratiba magadii} and to call strain SSL1 \textit{Natratiba} sp. strain SSL1.

The inferred tree (Fig. 1) also includes three species of the genus \textit{Halococcus} (\textit{Halococcus saccharolyticus}, \textit{Halococcus salifodiniae}, and \textit{Halococcus turkmenicus}) and an unvalidated species of the genus \textit{Halorubrum, Haloarcula aidinensis} (25). Validation of the latter species is currently in progress (26). Further taxonomic investigation of the three species of \textit{Halococcus} will be described in a separate paper.

least 2 M NaCl. Alkaliphilic, growing between pH 7 and 10 with optimum at 8.5. Molars percent G+C content of DNA is 64.3 (Tm), 61.2 to 62.1 (buoyant density). Polar lipids are glycerol diether analogs of phosphatidyglycerol, methyl ester of phosphatidyglycerophosphate, phosphatidic acid, and an unknown phospholipid, PL1. Both diphytanyl moieties (C20 and C20) and phytanylstearterpanyl moieties (C20 and C23) detected.

Type species is Natronomonas pharaonis (Soliman and Trüper 1982) comb. nov. (basonym, Natronobacterium pharaonis). The first alkaliphilic extreme halophile described. Pleomorphic motile rods, originally isolated from the alkaline brines of eutrophic desert lakes of Wadi Natrun, Egypt. Detailed characteristics are described elsewhere by Soliman and Trüper (18).

Type strain is Gabara, deposited with the Deutsche Sammlung von Mikroorganismen as DSM 2160T = ATCC 35678 = NCIMB 2260T.

Strain SP1, which showed 96% homology with DSM 2160T in DNA-DNA hybridization, was isolated from a Kenyan soda lake, Lake Magadi, and deposited as NCIMB 2191 (21). NCIMB 2191 contains a small amount of disesterterpanyl moiety (C25 and C25), whereas DSM 2160T does not (19).

Description of Halorubrum vacuolatum (Mwatha and Grant 1993) comb. nov. (basonym, Natronobacterium vacuolatum). The properties of the type strain M24 (NCIMB 13189) were described previously (15).

Description of Natrithalas magadaii (Tindall et al. 1984) comb. nov. (basonym, Natronobacterium magadii). The properties of the type strain MS3 were described previously (21).

REFERENCES