Haloarcula argentinensis sp. nov. and *Haloarcula mukohataei* sp. nov., Two New Extremely Halophilic Archaea Collected in Argentina

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Strains arg-1^T (T = type strain) and arg-2^T, two new strains of extremely halophilic archaea, were isolated from the soils of the Argentine salt flats. The taxonomic features of arg-1^T were similar to, but distinct from, those of the type strain of *Haloarcula vallismortis* and other *Haloarcula* species. On the 16S rRNA phylogenetic tree, strain arg-1^T formed a cluster together with *Haloarcula* species. Strain arg-2^T differed in its glycolipid composition but still was more closely related to the genus *Haloarcula* than to other established genera. We propose that strain arg-1^T be classified as a member of a new species, *Haloarcula argentinensis*, and that strain arg-2^T be classified as a member of *Haloarcula mukohataei* sp. nov., although arg-2^T may belong to a new genus or a subgenus of the genus *Haloarcula*. The type strain of *H. argentinensis* is strain arg-1 (= JCM 9737), and the type strain of *H. mukohataei* is strain arg-2 (= JCM 9738).

Extremely halophilic archaea have been isolated from various hypersaline environments and have been classified into four nonalkaliphilic genera and two alkaliphilic genera on the basis of phenotypic and genetic criteria (4). Recent phylogenetic analysis of 16S rRNA genes revealed a great degree of taxonomic diversity at the genus level within the family *Halobacteriaceae* (9, 17). Seven genera of nonalkaliphilic halobacteria have been described (the genera *Halobacterium*, *Halococcus*, *Haloarcula*, *Haloferax*, *Halorubrum* [*Halorubrobacterium*], *Halobaculum*, and *Natrialba*), and two genera of alkaliphilic halobacteria have been described (the genera *Natronobacterium* and *Natronococcus*) (9, 17, 21).

Strains arg-1^T (T = type strain) and arg-2^T, two new halophiles collected in Argentina, were found to possess the light-driven proton pump retinal proteins cruxrhodopsin-1 and cruxrhodopsin-2, respectively (28, 29). The results of phylogenetic analyses performed with bacteriorhodopsin homologs suggested that the halobacterial strains containing members of the cruxrhodopsin family are distributed in one genus, the genus *Haloarcula* (19), and the light-driven ion pump of the cruxrhodopsin family was identified in the type species of this genus, *Haloarcula vallismortis* (13). We propose that the two new isolates from Argentina should be classified in the genus *Haloarcula* on the basis of the results of a 16S rRNA phylogenetic analysis and a DNA-DNA hybridization analysis.

MATERIALS AND METHODS

Strains and culture conditions. Strain arg-1^T was collected in Salinas Chica on the Valdez Peninsula in Chubut, Argentina, and strain arg-2^T was collected in Salinas Grandes in the Andes highlands in Jujuy, Argentina. Reference strains, including *Haloarcula vallismortis* IFO 14741^T (= ATCC 29715^T), *Halobacterium saccharovororum* IFO 14717^T (= DSM 1137^T), *Halobacterium cutinubrum* IFO 14715^T (= DSM 669^T), *Halobacterium sodomense* IFO 14740^T (= ATCC 33755^T), *Haloferax volcanii* IFO 14742^T (= ATCC 29605^T), and *Haloferax mediterranei* IFO 14739^T (= ATCC 33500^T), were purchased from the Institute for Fermentation, Osaka, Japan. *Haloarcula marismortui* JCM 8966^T (= ATCC 43049^T), *Haloarcula japonica* JCM 7785^T (= T. Takashina TR-1^T), *Haloarcula hispanica* JCM 8911^T (= IAM 13179), and "*Haloarcula sinaiensis*" JCM 8862 were purchased from the Japan Collection of Microorganisms, Institute of Physical and Chemical Research (RIKEN), Saitama, Japan. *Halobacterium cutinubrum* has been transferred to *Halobacterium salinarum* (34). *Halobacterium saccharovororum* and *Halobacterium sodomense* have been transferred to the new genus *Halorubrum* and renamed *Halorubrum saccharovororum* and *Halorubrum sodomense*, respectively (17). (Another new genus, the genus *Halorubrobacte-*

rium, has also been proposed for strains of *Halobacterium saccharovororum* and *Halobacterium sodomense* [9].) The new names are used in this paper.

The basal culture (BC) medium contained (per liter of tap water) 156 g of NaCl, 4 g of KCl, 20 g of MgSO₄ · 7H₂O, 13 g of MgCl₂ · 6H₂O, 1 g of CaCl₂ · 2H₂O, 0.2 g of NaHCO₃, and 0.5 g of KBr (pH 7.5), as described elsewhere (22). Liquid medium was solidified by adding 1.6% agar before autoclaving. Carbohydrate utilization tests were performed with BC medium containing 0.1 g of yeast extract per liter and each sugar at a concentration of 0.1%. Acidification of the culture medium was monitored with a pH meter.

For the following tests, BC medium was enriched with 5 g of yeast extract per liter and 1 g of D-glucose per liter. The growth response to salt was examined by using NaCl concentrations ranging from 60 to 260 g/liter. The MgCl₂ · 6H₂O requirement was examined by using MgCl₂ · 6H₂O concentrations ranging from 0 to 75 g/liter.

Cell growth was monitored by determining the turbidity at 660 nm. Cell motility and cell shape were examined by optical microscopy (Olympus model BH-2 microscope; magnification, ×1,000) without fixation or after acetic acid fixation (2).

Biochemical tests. Antibiotic resistance was tested in the enriched culture medium (liquid medium and solid medium) in which the concentrations of NaCl and MgCl₂ · 6H₂O were 200 and 20 g/liter, respectively. Susceptibility was determined by examining the production of visible colonies in solid medium containing each antibiotic. The class of fructose-bisphosphate aldolase was determined by the method of Dhar and Altekar (1). *dam* methylation of genomic DNA was tested with restriction enzymes *Mbo*I and *Sau*3AI (15). Other bacteriological tests were carried out as described by Smibert and Krieg (27).

Lipid analysis. Lipids were extracted by the method of Kates and Kushwaha (12), dissolved in chloroform-methanol (1:2, vol/vol), and chromatographed on silica gel thin-layer plates (Kieselgel 60 F₂₅₄; Merck) by performing two-dimensional thin-layer chromatography (TLC), as described by Minnikin et al. (18). The glycolipids on the plates were visualized as dark blue spots following a short pyrolysis at 150°C after the plates were sprayed with 0.5% α-naphthol in methanol-water (1:1) and sulfuric acid-ethanol (1:1). All other lipids were visualized as yellow-brown spots after further pyrolysis at about 250°C. The *R_f* values were compared with the *R_f* values of authentic standards (33) from the type strains of halobacteria. Chain lengths of core lipids were analyzed by the thin-layer chromatographic procedure described by Ross et al. (23).

DNA base composition and DNA-DNA reassociation. DNA was isolated and purified as described previously (7). The G+C content was determined by the thermal denaturation method. DNA-DNA reassociation studies were carried out as described by Tindall et al. (32), with minor modifications; DNA fragments were labeled with [α-³²P]dCTP by using the random priming method.

16S rDNA amplification and sequencing. Two sets of primers were designed on the basis of the highly conserved regions of halobacterial 16S ribosomal DNA (rDNA) sequences. One set (primer f1 [5'-ATTCGGTTGATCCTGC3'] and primer r1 [5'-TTTAAGTTTCATCCTTG]) was designed for PCR amplification of the region corresponding to positions 1 to 859 (*Halobacterium halobium* 16S rDNA numbering). The another set (primer f2 [5'-AACCGATTAGATACCC 3'] and primer r2 [5'-GTGATCCAGCCGAGATTC3']) was designed for the region from position 723 to position 1472 (16). The PCR was performed by a routine method (24) for 30 cycles, each of which consisted of denaturation for 30 s at 96°C, annealing for 30 s at 37°C, and polymerization for 3 min at 72°C. The products were separated by electrophoresis, cloned into pUC18 in *Escherichia coli*, and sequenced by the dideoxy chain termination method (26).

Phylogenetic analysis on the basis of 16S rRNA sequences. Sequence data for halobacterial 16S rRNAs were obtained from DNA Data Bank of Japan

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Nucleotide sequence accession numbers. The 16S rDNA sequences of *Haloarcula* sp. strain arg-1^T (*Haloarcula argentinensis*), *Haloarcula* sp. strain arg-2^T (*Haloarcula mukohataei*), and *Haloarcula vallismortis* IFO 14741^T have been deposited in the DDBJ under accession numbers D50849, D50850, and D50851, respectively.

Growth conditions and other features. At 40°C, strain arg-1^T grew optimally in the presence of 2.5 M NaCl, whereas strain arg-2^T grew optimally in the presence of 3.0 to 3.5 M NaCl. No growth was detected at NaCl concentrations below 1.5 M for arg-1^T or below 2.0 M for arg-2^T. Strain arg-1^T grew well in the presence of 4.5 M NaCl, but the growth of strain arg-2^T in the presence of 4.5 M NaCl was rather poor (the growth yield was one-third the growth yield obtained under optimum growth conditions). At the optimum NaCl concentration for each strain, arg-1^T grew optimally in the presence of 100 mM Mg²⁺ and arg-2^T exhibited almost constant growth at Mg²⁺ concentrations from 3 to 300 mM. The minimum Mg²⁺ concentrations for growth were 30 and 3 mM for arg-1^T and arg-2^T, respectively. In actively growing liquid cultures, the cells of strain arg-1^T were mostly triangular disks and the cells of strain arg-2^T were short rods, as determined by phase-contrast microscopic observations. On agar plates, both strains formed large colonies about 2 mm in diameter after 1 week of culture. After prolonged culture, both strains sometimes produced a glutinous extracellular material like the polyhydroxybutyrate produced by *Haloferax mediterranei*. Both strains had flagella and were motile as determined by microscopic observations. Both strains were susceptible (growth was completely inhibited) to novobiocin (5 µg/ml), pravastatin (10 µg/ml), and kanamycin (10 µg/ml) and resistant to penicillin (10 IU/ml), vancomycin (30 µg/ml), and chloramphenicol (30 µg/ml). Both strains were catalase and oxidase positive and were able to use various kinds of sugars (sucrose, maltose, glucose, galactose, mannose, ribose, and glycerol) and to acidify the medium. Strain arg-1^T growth was stimulated by fructose, and neither strain utilized lactose. Fructose-bisphosphate aldolase was not inhibited by EDTA in both strains, which is characteristic of a class I enzyme (1). The genomic DNAs of both strains were hydrolyzed by both *Mbo*I and *Sau*3AI, which suggested that adenosines in the GATC sequences in the genomes were not methylated.

Figure 1 consists of four panels (a, b, c, d) showing immunoblotting results. Panel (a) shows a Western blot of DGD-2 and TGD-2 in whole cell lysates from DGD-2 and TGD-2 strains. Panel (b) shows a Western blot of DGD-2 and TGD-2 in whole cell lysates from DGD-2 and TGD-2 strains. Panel (c) shows a Western blot of DGD-2 and TGD-2 in whole cell lysates from DGD-2 and TGD-2 strains. Panel (d) shows a Western blot of DGD-2 and TGD-2 in whole cell lysates from DGD-2 and TGD-2 strains.

sitions of glycolipids, but cochromatography of lipids from arg-2^T and *Haloarcula marismortui* produced four distinct glycolipid spots (data not shown).

16S rDNA and phylogenetic analysis. PCR amplification of the 16S rDNAs of strains arg-1^T and arg-2^T and *Haloarcula vallismortis* with two sets of primers produced an 859-base fragment from the 5' end of the 16S rDNA and a 750-base fragment from the 3' end, which overlapped by 137 bases. As two overlapping sequences, which included one of the most variable regions of the 16S rRNA gene, were identical for each fragment pair for the three strains, each pair was considered amplified from one 16S rRNA gene, although the results of Southern analysis suggested that at least four and three genes were present in arg-1^T and arg-2^T, respectively. The overall sizes of the 16S rDNAs of strains arg-1^T and arg-2^T and *Haloarcula vallismortis* were 1.472, 1.474, and 1.472 bp, respectively.

A halobacterial phylogenetic tree was constructed by the neighbor-joining method on the basis of the 16S rRNA nucleotide sequences (Fig. 2). Strain arg-1^T was included in the *Haloarcula* cluster. Although arg-2^T was not closely related to the other members of the genus *Haloarcula*, it was still closer to the genus *Haloarcula* than to any other genus. A bootstrap confidence value of 99% was obtained when strain arg-2^T was placed on the *Haloarcula* branch.

DNA-DNA reassociations. The DNA-DNA hybridization values obtained for the species of each genus of non-alkaliphilic halobacteria and strains arg-1^T and arg-2^T showed that strains arg-1^T and arg-2^T were members of the genus *Haloarcula* (Table 1). Closer investigation with members of the genus *Haloarcula* showed that arg-2^T was different from previously described *Haloarcula* species, while arg-1^T was very closely related to *Haloarcula hispanica* or *Haloarcula marismortui*. How-

FIG. 2. Phylogenetic tree for the extremely halophilic archaea constructed by the neighbor-joining method. The sequence data used were data for the following strains and were obtained from the DDBJ (accession numbers are given in parentheses): *Halorubrum saccharovororum* NCIMB 2081^T (X82167), *Halorubrum lacusprofundi* ACAM34^T (X82170), *Halorubrum sodomense* ATCC 33755^T (X82169), *Halorubrum trapanicum* NRC 34021^T (X82168), *Halobaculum gomorrense* DSM 9297^T (L37444), *Haloferax volcanii* ATCC 29605^T (K00421), *Haloferax denitrificans* ATCC 35960^T (D14128), *Haloferax gibbonsii* ATCC 33959^T (D13378), *Haloferax mediterranei* ATCC 33500^T (D11107), *Natrilaiba asiatica* JCM 9576^T (D14124), B1^T (= JCM 9577) (D14124), L-11 (D14126), *Halobacterium trapanicum* NCIMB 767^T (D14125), *Natronobacterium magadii* NCIMB 2190^T (X72495), *Natronococcus occultus* NCIMB 2192^T (Z28378), *Natronococcus amylolyticus* Ah-36^T (D43628), *Halococcus morrhuae* ATCC 17082^T (X00662), *Halobacterium salinarum* (*Halobacterium halobium*) DSM 671 (M38280), *Haloarcula mukohataei* JCM 9738^T (D50850), *Haloarcula marismortui* A ATCC 34049^T (X61688), "*Haloarcula sinaiensis*" minor ATCC 33800 (D14129), *Haloarcula vallismortis* #1 ATCC 29715^T (U17593) (5, 6, 9, 11, 14, 16, 17, 20). Bootstrap percentages are indicated at the nodes. The tree was rooted by using the 16S rDNA sequence of *Methanosarcina thermophila* DSM 1825 (accession number M59140) as the outgroup.

ever, a DNA-DNA hybridization value of around 60% is the recommended cutoff point for delineating species (8), and thus arg-1^T was identified as a member of a species independent from previously described *Haloarcula* species.

DISCUSSION

Newly isolated strains arg-1^T and arg-2^T were classified as extremely halophilic aerobic archaea because (i) TLC of whole-organism methanolysates revealed that they contain ether-linked isoprenoid lipids, (ii) they could grow in the presence of antibiotics effective against eubacteria (penicillin, kanamycin, and chloramphenicol) but not in the presence of antibiotics effective against eukaryotes (pravastatin and anisomycin), (iii) they could grow aerobically only in the presence of high concentration of NaCl and lysed at low levels of salinity, and (iv) they possessed bacterial rhodopsins (28, 29) which have been found only in members of the order *Halobacteriales* (4).

Glycolipid analysis is one of the most commonly used conventional methods for determining the genus of bacterial isolates (33). Members of the genus *Halobacterium* contain 1-*O*-[β -D-galactose-(3'-SO₄⁻)-(1'→6')- α -D-mannose-(1'→2')- α -D-glucose]-2,3-di-*O*-phytanyl-*sn*-glycerol and 1-*O*-[β -D-galactose-(3'-SO₄⁻)-(1'→6')- α -D-mannose-(3'←1')- α -D-galactofuranose-(1'→2')- α -D-glucose]-2,3-di-*O*-phytanyl-*sn*-glycerol as their major glycolipids, members of the genus *Haloferax* contain S-DGD, and members of *Haloarcula* contain a triglycosyl diether (TGD-2). Members of the genus *Halorubrum* contain glycolipids that differ slightly in each case analyzed, although they are still based on a glycosyl-mannosyl

core-sulfated diglycosyl diether (17). It has been proposed that members of the genus *Halorubrobacterium*, which is a junior objective synonym of the genus *Halorubrum*, contain 1-*O*-[α -D-mannose-(2'-SO₄⁻)-(1'→4')- α -D-glucose]-2,3-di-*O*-phytanyl-*sn*-glycerol (9). The major glycolipids of arg-1^T were TGD-2 and DGD-2 (Fig. 1), which suggested that this strain belongs to the genus *Haloarcula*. The glycolipids of strain arg-2^T were similar to DGD-1 and S-DGD as determined by the TLC analysis. On the basis of the results of the glycolipid analysis alone, arg-2^T might be a member of the genus *Haloferax*. However, arg-2^T contained the phospholipid phosphatidylglycosulfate, which is absent in the genus *Haloferax*. Since mutation of the gene(s) encoding a certain enzyme(s) for glycolipid modification may have occurred without affecting the general phenotype, classification on the basis of glycolipids can only be considered circumstantial evidence. Thus, strain arg-2^T cannot be excluded from the genus *Haloarcula* by a difference in glycolipids.

Analysis of the 16S rRNAs of 26 halobacterial strains (Fig. 2) revealed seven large clusters, six of which corresponded to the genera *Haloarcula*, *Haloferax*, *Halobacterium*, *Halococcus*, *Halorubrum* (*Halorubrobacterium*), and *Halobaculum*. The last cluster included three genera (the genera *Natronobacterium*, *Natronococcus*, and *Natrilaiba*) and some as-yet-unidentified strains. *Halobacterium trapanicum* NCIMB 767^T and NRC 34021^T were originally obtained from the same strain, but the phylogenetic positions of these strains were apparently different (Fig. 2). Moreover, the currently available strain NCIMB 767^T seems to be a member of the genus *Halococcus* as determined by cell shape and resistance to low levels of salinity (6a, 31). It is necessary to reconsider the validity of *Halobacterium trapanicum* ATCC 43102^T, NCIMB 767^T, and NRC 34021^T. Strain arg-1^T was found to fall in the *Haloarcula* cluster. The species status of arg-1^T is further supported by the results of DNA-DNA hybridization experiments (Table 1). On the 16S rRNA tree (Fig. 2), strain arg-2^T branched off before the main branch of the genus *Haloarcula*. On the phylogenetic tree constructed by using bacteriorhodopsins, strain arg-2^T similarly branched before other *Haloarcula* strains (details will be discussed elsewhere). Both of these features suggest that arg-2^T may be only remotely related to the genus *Haloarcula*. However, multiple heterologous 16S rRNAs have been reported for *Haloarcula marismortui* and "*Haloarcula sinaiensis*" (10, 20) (Fig. 2). This suggests that the 16S rRNA cloned from the strain arg-2^T genome may be the most remote 16S rRNA of the heterologous 16S rRNA species in the strain, since at least three 16S rRNA genes were detected in the genome of arg-2^T.

TABLE 1. Levels of DNA-DNA hybridization between the two new strains and various halobacterial strains

Strain	% DNA-DNA hybridization with:	
	arg-1 ^T	arg-2 ^T
<i>Haloferax volcanii</i> IFO 14742 ^T	8	15
<i>Halobacterium salinarum</i> IFO 14716 ^T	1	2
<i>Halococcus morrhuae</i> JCM 8876 ^T	2	1
<i>Halorubrum saccharovororum</i> IFO 14717 ^T	7	19
<i>Haloarcula vallismortis</i> IFO 14741 ^T	43	26
<i>Haloarcula marismortui</i> JCM 8966 ^T	56	28
<i>Haloarcula hispanica</i> JCM 8911 ^T	61	27
<i>Haloarcula japonica</i> JCM 7785 ^T	25	23
" <i>Haloarcula sinaiensis</i> " JCM 8862	41	24
arg-1 ^T (= JCM 9737 ^T)	100	27
arg-2 ^T (= JCM 9738 ^T)	17	100

Therefore, until all three 16S rRNAs are cloned and sequenced, *arg-2^T* cannot be excluded from the genus *Haloarcula*.

In contrast, when the amino acid sequences of bacteriorhodopsins from various halobacteria were aligned, a specific insertion of six amino acid residues (L-S/P-A-G-A-E) was commonly found only in those sequences obtained from the *Haloarcula* strains (strains *arg-1^T* and *arg-2^T* and *Haloarcula vallismortis*). This suggests that the insertion occurred after the divergence of the genera (details will be discussed elsewhere).

In conclusion, chemotaxonomic and genetic data indicate that both *arg-1^T* and *arg-2^T* are members of the genus *Haloarcula* in the order *Halobacteriales*. The distinction of both strains from other validly described species in the genus *Haloarcula* is supported by the uniqueness of the glycolipids, the low DNA-DNA hybridization values, and the low levels of sequence similarity of the 16S rDNAs.

Description of *Haloarcula argentinensis* sp. nov. *Haloarcula argentinensis* (ar.gen.ti.nen'sis. M.L. adj. *argentinensis* pertaining to Argentina). Gram negative. Motile by means of polar flagella. Cells are triangular flat disks in the actively growing stage that are ca. 1.0 by 0.3 μ m. Colonies are about 3 mm in diameter after 1 week of incubation at 40°C on 1.5% agar plates containing 20% salt. The colonies are translucent, entire, smooth, orange-red, and mucous on old plates.

Chemoorganotrophic. Growth is strictly aerobic. Growth occurs in the presence of 2.0 to 4.5 M NaCl. Optimum growth occurs in the presence of 2.5 to 3 M NaCl at 40°C. The Mg^{2+} concentration required for growth is 30 mM in solid medium, but the optimum Mg^{2+} concentration is around 100 mM (in medium containing 2.5 M NaCl). Acids are produced from various sugars (sucrose, maltose, glucose, galactose, mannose, ribose, glycerol, and fructose). Resistant to chloramphenicol (30 μ g/ml), kanamycin (30 μ g/ml), and penicillin (10 IU/ml). Susceptible to anisomycin (10 μ g/ml), novobiocin (5 μ g/ml), and pravastatin (10 μ g/ml).

The G+C content of the DNA is 62 mol% (as determined by the thermal denaturation method).

Catalase and oxidase positive. The fructose-bisphosphate aldolase is class I (EDTA insensitive).

Nonsaponifiable ether-linked phosphoglyceride lipids. The major glycolipid is TGD-2.

Contains retinal proteins.

The type strain is strain *arg-1* (= JCM 9737).

Description of *Haloarcula mukohataei* sp. nov. *Haloarcula mukohataei* (mu.ko.ha.tae'i. M.L. adj. *mukohataei*, of Yasuo Mukohata, Japanese biochemist and biophysicist).

Gram-negative. Motile by means of polar flagella. Short rods (ca. 2.0 by 0.5 μ m) in actively growing cultures and pleomorphic in stationary cultures. Colonies are about 2 mm in diameter after 1 week of incubation at 40°C on 1.5% agar plates containing 20% salt. They are translucent, entire, smooth, and red-orange.

Chemoorganotrophic. Growth is strictly aerobic. Growth occurs in the presence of 2.5 to 4.5 M NaCl; optimum growth occurs in the presence of 3.0 to 3.5 M NaCl at 40°C. The minimum Mg^{2+} concentration required for growth is 3 mM in solid medium, but concentration dependence for growth is not observed at Mg^{2+} concentrations between 3 and 300 mM. Acids are produced from many sugars (sucrose, maltose, glucose, galactose, mannose, ribose, and glycerol). Resistant to chloramphenicol (30 μ g/ml), kanamycin (30 μ g/ml), and penicillin (10 IU/ml). Susceptible to anisomycin (10 μ g/ml), novobiocin (5 μ g/ml), and pravastatin (10 μ g/ml).

The G+C content of the DNA is 65 mol% (as determined by the thermal denaturation method).

Catalase and oxidase positive. The fructose-bisphosphate aldolase is class I (EDTA insensitive).

Nonsaponifiable ether-linked phosphoglyceride lipid. The major glycolipid is unknown.

Contains retinal protein.

The type strain is strain *arg-2* (= JCM 9738).

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