NOTE

Halomicrobium mukohataei* gen. nov., comb. nov., and emended description of *Halomicrobium mukohataeiAharon Oren,¹ Rahel Elevi,² Satoshi Watanabe,³ Kunio Ihara⁴ and Angela Corcelli⁵Author for correspondence: Aharon Oren. Tel: +972 2 6584951. Fax: +972 2 6528008.
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***Haloarcula mukohataei*, previously isolated from soils of salt flats in Argentina, was originally placed in the genus *Haloarcula* on the basis of 16S rRNA gene sequence comparison. However, its morphology and polar lipid composition differs from that of the other *Haloarcula* species. In addition, its phylogenetic distance from other *Haloarcula* species is rather large, and the 16S rDNA sequence does not contain many of the signature bases characteristic of the genus. Transfer of the species to a new genus was therefore recommended by the International Committee on Systematic Bacteriology Subcommittee on the taxonomy of *Halobacteriaceae*. A full description of the isolate, proposed as a member of a new genus, *Halomicrobium*, as *Halomicrobium mukohataei* comb. nov., is presented. The type strain is strain arg-2^T (= JCM 9738^T = DSM 12286^T = ATCC 700874^T = NCIMB 13541^T).**

Keywords: *Halomicrobium* gen. nov., *Haloarcula mukohataei*, *Halomicrobium mukohataei* comb. nov., halophilic, archaea

Haloarcula mukohataei was isolated by Ihara *et al.* (1997) from soils of salt flats in Argentina. It was placed in the genus *Haloarcula* on the basis of the similarity of its 16S rRNA gene sequence to the 16S rRNA sequences of the other species of the genus. In addition, its retinal proton pump, designated cruxrhodopsin-2, is most similar in amino acid sequence to the proton pumps of *Haloarcula argentinensis* and *Haloarcula vallismortis* (Sugiyama *et al.*, 1994). However, it has been pointed out that there are a number of important properties in which *Haloarcula mukohataei* differs from the other representatives of the genus. It lacks TGD-2, the glycolipid found in all other *Haloarcula* species, but contains a different (unidentified) glycolipid instead. Moreover, the similarity of its 16S rDNA with the 16S rRNA genes of the other *Haloarcula* species is rather low (89.1–90.5% similarity only, compared to $\geq 94.1\%$ among the other *Halo-*

arcula species) and there is a mean similarity of 86.4% between *Haloarcula mukohataei* and members of the other genera of the *Halobacteriaceae*. In addition, the 16S rDNA sequence does not contain many of the signature bases characteristic of the genus (Grant *et al.*, 2001). For these reasons, the International Committee on Systematic Bacteriology Subcommittee on the taxonomy of *Halobacteriaceae* has suggested that transfer of *Haloarcula mukohataei* to a new genus may be justified (Oren & Ventosa, 2000; see also Ventosa, 2001).

Here, we formally propose the transfer of *Haloarcula mukohataei* to a new genus, i.e. *Halomicrobium* gen. nov., as *Halomicrobium mukohataei* comb. nov. We also present an emended description of the species in accordance with the requirements of the minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997).

Haloarcula mukohataei (JCM 9738^T, purchased from the Japan Collection of Microorganisms) was grown in medium (designated medium no. 1) of the following composition (g l⁻¹): NaCl, 200; KCl, 2; MgSO₄ · 7H₂O, 2; trisodium-citrate, 3; Tris, 5; yeast extract, 1; Casamino acids, 5. The pH was adjusted to 7.2 with HCl. For solid media, agar was added to a con-

This paper is dedicated to the memory of Takeshi Tamura (deceased 15 October 1991), who originally described *Haloarcula mukohataei*.

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The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain arg-2^T (= JCM 9738^T = DSM 12286^T = ATCC 700874^T = NCIMB 13541^T) is D50850.

Fig. 1. Phase-contrast micrographs of *H. mukohataei* strain arg-2⁺, grown in medium 1 (top) and in medium 2 (bottom). Bar, 20 µm.

centration of 1.6% (w/v). Where indicated, we used another medium (medium no. 2), routinely employed for growing *Haloarcula* species. Its composition was as follows (g l⁻¹): NaCl, 206; MgSO₄ · 7H₂O, 36; KCl, 0.373; CaCl₂ · 2H₂O, 0.5; MnCl₂, 0.013 mg l⁻¹; and yeast extract, 5 (pH 7). Other halophilic archaea used as reference strains were grown in this medium or in other suitable media, as required. The organism was grown with shaking at 35 °C in 100 or 250 ml Erlenmeyer flasks containing 40 or 100 ml medium, respectively. Growth was assessed by measuring the optical densities of the cultures at 600 nm. The growth media were modified with respect to the content of organic nutrients, buffers, etc., as specified. For growth experiments at different pH values, the buffers PIPES (pH 5.5–6.5), HEPES (pH 7–8) and Tricine (pH 8.5) were added at a concentration of 50 mM.

Unless specified otherwise, tests for phenotypic properties were carried out as indicated in the Proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). Most tests were performed as outlined by Holding & Collee (1971). Appropriate positive and negative controls were included in all tests. Growth and gas formation with nitrate as electron acceptor were tested in 150 ml stoppered bottles, completely filled with growth medium to which NaNO₃ (5 g l⁻¹) had been added, and

containing an inverted test tube. The formation of nitrite was monitored colorimetrically. Anaerobic growth in the presence of 5 g l-arginine · HCl l⁻¹ was tested in completely filled 15 ml stoppered tubes. Controls without arginine were included, and incubations were performed in the dark; *Halobacterium salinarum* NRC 817 served as the positive control. Hydrolysis of starch was examined on agar plates supplemented with 2 g soluble starch l⁻¹. Starch hydrolysis was detected by flooding the plates with iodine solution. Gelatin hydrolysis was determined by growing colonies on agar plates amended with 0.4% gelatin and flooding the plates with a solution of 15% (w/v) HgCl₂ in 20% (w/v) HCl after growth was obtained. Hydrolysis of Tween 20 and Tween 80 was tested as outlined by Gutiérrez & González (1972): each was added at a concentration of 1 ml l⁻¹ to autoclaved medium no. 1 supplemented with 1 g CaCl₂ · 2H₂O l⁻¹. Indole production was detected with Kovacs' reagent after having grown the cells in media supplemented with 0.1 L-tryptophan g l⁻¹. To test for growth on single carbon sources, yeast extract and Casamino acids were omitted from the media (medium no. 1 or medium no. 2 supplemented with 50 mM PIPES buffer, pH 7), and the compound to be tested was added at a concentration of 5 g l⁻¹, together with 1 g NH₄Cl l⁻¹, 1.36 g KH₂PO₄ l⁻¹ and 0.01 g FeSO₄ · 7H₂O l⁻¹. The outcome was considered to be positive when growth was obtained in at least two successive transfers in this medium.

Cell morphology was examined using a Zeiss Axiovert model 135TV microscope equipped with phase-contrast optics. For photography, drops of culture were mixed on a microscope slide with an equal volume of melted 1% agarose containing 20% (w/v) NaCl, then covered with a cover-slip. For electron microscopic examination, drops of culture were applied on glow-discharged, carbon-coated Formvar grids for 2 min. The drops were then washed off with a solution of 1% uranyl acetate, pH 4.5. After air-drying, the grids were viewed in a Philips TECNAI 211 electron microscope operating at 100 kV.

Lipids were extracted using the Bligh–Dyer method (Bligh & Dyer, 1959) as modified for extreme halophiles (Kates, 1986). Dried lipid extracts were dissolved in chloroform/methanol 1:1. Electrospray ionization/mass spectra were obtained with an API 165 mass spectrometer (Perkin-Elmer Sciex) equipped with an ion-spray interface. The samples were continuously introduced into the mass spectrometer (at a flow rate of 10 µl min⁻¹) by a Harvard model 11 syringe pump. The instrumental conditions were as follows: nebulizer gas flow (air), 1.2 l min⁻¹; curtain gas flow (nitrogen), 1.2 l min⁻¹; needle voltage, 5600 V; orifice voltage, –150 V; ring voltage, –200 V; mass range, 50–2000 a.m.u. in steps of 0.1 Da; dwell time, 0.2 ms. With the orifice voltage used, collision-induced dissociation/MS spectra were obtained showing [M–H]⁺ and [M–2H]²⁺ parent ions as well as some fragmentation ions.

Fig. 2. Uranyl acetate-stained cells of *H. mukohataei* strain arg-2^T, showing flagella. Bar, 0.5 μm.

Most properties relevant to the taxonomic description of *Haloarcula mukohataei* have already been presented by Ihara *et al.* (1997). To complete the description, we have determined a number of additional phenotypic characteristics.

We can confirm that the cells are short rods ($1\text{--}3 \times 0.5\text{ }\mu\text{m}$) when grown in medium no. 1 (Fig. 1, top). However, cell length is variable and depends, to a large extent, on the growth medium used: exponentially growing cells in medium no. 2 were significantly longer ($3\text{--}8 \times 0.5\text{ }\mu\text{m}$; Fig. 1, bottom). The typical flat pleomorphic shapes so characteristic of most *Haloarcula* species were never observed. When suspended in solutions containing less than 10% (w/v) NaCl, cells lost their native rod shape and became spherical. Electron microscopic examination after staining with uranyl acetate showed multiple flagella inserted peritrichously, but, occasionally, tufts of flagella originating from one site were observed (Fig. 2). It should be noted that because of exposure to the staining solution (which was low in salt), cells lost their native rod shape during sample preparation.

Optimal growth with the normal rod-shaped morphology was obtained at about 45 °C. Above this temperature, cells grew rapidly, but assumed a spherical morphology. No growth was obtained above 52 °C. The pH range for growth was 6.2–8.0; no growth was observed below pH 6.0 or above pH 8.2.

The isolate grew in mineral medium (both in medium no. 1 and in medium no. 2) on glucose, galactose, sucrose, maltose or glycerol as single carbon and energy source. No growth was obtained on Na-acetate, Na-succinate, L-glutamate or ribose. Anaerobic growth was observed with nitrate as electron acceptor, with the formation of nitrite and gas. Starch hydrolysis was weakly positive. Gelatin and casein were not degraded. Tween 80 was not hydrolysed; Tween 20 at the concentration employed inhibited growth. No indole was produced from tryptophan. Anaerobic growth on L-arginine was not observed.

Comparison of the electrospray ionization-mass spectra of strain arg-2^T and *Haloarcula vallismortis* ATCC 29715^T (Fig. 3) revealed significant differences. *Haloarcula vallismortis* contains TGD-2 as its major glycolipid ($m/z = 1138$; showing a small peak only, as this lipid is difficult to ionize). No such peak was found in lipid extracts of strain arg-2^T, which instead had a major peak at $m/z = 1056$, a molecular mass corresponding with sulfated diglycosyl diether lipids. Peaks showing the presence of the phytanyl diether derivatives of phosphatidylglycerol ($m/z = 806$), phosphatidylglycerosulfate ($m/z = 885$) and the methyl ester of phosphatidylglycerophosphate ($m/z = 900$) were found in both strains.

The additional information gained on the properties of strain arg-2^T enable the description of the isolate as a

Fig. 3. Electrospray ionization-mass spectra profiles (negative ion) of total lipid extracts of *H. mukohataei* strain arg-2^T (a) and *Haloarcula vallismortis* ATCC 29715^T (b).

member of a new genus, for which we propose the name *Halomicrobium* gen. nov. (recommended abbreviation '*Hmc.*'; Oren & Ventosa, 2000).

Description of *Halomicrobium* gen. nov.

Halomicrobium (Ha.lo.mi.cro'bi.um. Gr. n. *hals*, *halos* salt; Gr. adj. *micros* small; Gr. n. *bios* life; N.L. neutr. n. *Halomicrobium* small, salt-life form).

Rod-shaped bacteria, phylogenetically loosely affiliated with the genus *Haloarcula*. Gram-negative. Aerobic or facultatively anaerobic in the presence of nitrate. Heterotrophic. Pigmented red by bacterioruberin derivatives. May contain retinal proteins. Oxi-

dase- and catalase-positive. Extremely halophilic. The genomic DNA G+C content of the type species is 65 mol%. The type species is *Halomicrobium mukohataei*.

Emended description of *Halomicrobium mukohataei* comb. nov.

Halomicrobium mukohataei comb. nov. (mu.ko. ha'tae.i. N.L. gen. n. *mukohataei* of Yasuo Mukohata, Japanese biochemist and biophysicist).

Gram-negative. Motile by means of flagella inserted peritrichously and/or in tufts. Short or longer rods in actively growing cultures (1–3 or 3–8 × 0.5 µm, depending on the composition of the medium and on growth conditions); pleomorphic in stationary cultures. Colonies are about 2 mm in diameter after one week incubation at 40 °C on 1.5% agar plates containing 20% salt; they are translucent, entire, smooth and red-orange. Chemo-organotrophic. Grows aerobically or anaerobically in the presence of nitrate, with the formation of nitrite and gas. Does not grow anaerobically in the presence of L-arginine. Catalase- and oxidase-positive. Extremely halophilic, requiring between 2.5 and 4.5 M NaCl for growth and at least 2 M NaCl for structural stability. Grows optimally in the presence of 3.0–3.5 M NaCl at 40–45 °C. The pH range for growth is 6.2–8.0. The minimum Mg²⁺ concentration required is 3 mM in solid medium; no concentration dependence for growth was observed at Mg²⁺ concentrations between 3 and 300 mM. Acids are produced from glucose, galactose, mannose, ribose, sucrose, maltose and glycerol. Glucose, galactose, sucrose, maltose and glycerol support growth as single carbon and energy sources. Starch is hydrolysed. Does not hydrolyse gelatin or casein. Tween 80 is not hydrolysed. Does not produce indole from tryptophan. Polar lipids include phosphatidylglycerol, Meposphatidylglycerophosphate, phosphatidylglycerol-sulfate and an unidentified monosulfated diglycosyl diether lipid. Contains a retinal proton pump. The fructose-1,6-bisphosphate aldolase is class I (EDTA-insensitive). Resistant to chloramphenicol (30 µg ml⁻¹), kanamycin (30 µg ml⁻¹) and penicillin (10 IU ml⁻¹). Susceptible to anisomycin (10 µg ml⁻¹), novobiocin (5 µg ml⁻¹) and pravastatin (10 µg ml⁻¹). The genomic DNA G+C content is 65 mol% (as determined by the thermal denaturation method). The type strain, isolated from soil collected from a salt flat in Argentina, is strain arg-2^T (= JCM 9738^T = DSM 12286^T = ATCC 700874^T = NCIMB 13541^T). The GenBank accession number of the 16S rRNA gene of the type strain is D50850.

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