**Halobaculum magnesiiphilum** sp. nov., a magnesium-dependent haloarchaeon isolated from commercial salt

Hirokazu Shimoshige,1 Tomoaki Yamada,2 Hiroaki Minegishi,1 Akinobu Echigo,1 Yasuhiro Shimane,3 Masahiro Kamekura,4 Takashi Itoh5 and Ron Usami1,6

1Bio-Nano Electronics Research Centre, Toyo University, 2100 Kujirai, Kawagoe, Saitama 350-8585, Japan
2Department of Biological Applied Chemistry, Graduate School of Engineering, Toyo University, 2100 Kujirai, Kawagoe, Saitama 350-8585, Japan
3Japan Agency for Marine-Earth Science and Technology, 2-15 Natsushima-cho, Yokosuka, Kanagawa 237-0061, Japan
4Halophiles Research Institute, 677-1 Shimizu, Noda, Chiba 278-0043, Japan
5Japan Collection of Microorganisms, RIKEN BioResource Center, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
6Graduate School of Interdisciplinary New Science, Toyo University, 2100 Kujirai, Kawagoe, Saitama 350-8585, Japan

Two extremely halophilic archaea, strains MGY-184T and MGY-205, were isolated from sea salt produced in Japan and rock salt imported from Bolivia, respectively. Both strains were pleomorphic, non-motile, Gram-negative and required more than 5 % (w/v) NaCl for growth, with optimum at 9–12 %, in the presence of 2 % (w/v) MgCl₂·6H₂O. In the presence of 18 % (w/v) MgCl₂·6H₂O, however, both strains showed growth even at 1.0 % (w/v) NaCl. Both strains possessed two 16S rRNA genes (**rrnA** and **rrnB**), and they revealed closest similarity to *Halobaculum gomorrense* JCM 9908T, the single species with a validly published name of the genus *Halobaculum*, with similarity of 97.8 %. The **rrnA** and **rrnB** genes of both strains were 100 % similar. The **rrnA** genes were 97.6 % similar to the **rrnB** genes in both strains. DNA G+C contents of strains MGY-184T and MGY-205 were 67.0 and 67.4 mol%, respectively. Polar lipid analysis revealed that the two strains contained phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester derived from C₂₀C₂₀ archaeol. The DNA–DNA hybridization value between the two strains was 70 % and both strains showed low levels of DNA–DNA relatedness (48–50 %) with *Halobaculum gomorrense* JCM 9908T. Physiological and biochemical characteristics allowed differentiation of strains MGY-184T and MGY-205 from *Halobaculum gomorrense* JCM 9908T. Therefore, strains MGY-184T and MGY-205 represent a novel species of the genus *Halobaculum*, for which the name *Halobaculum magnesiiphilum* sp. nov. is proposed; the type strain is MGY-184T (=JCM 17821T=KCTC 4100T).

Members of the family *Halobacteriaceae* (42 genera and 138 species, at the time of writing) require high concentrations of NaCl for growth, with optimum concentrations of 10–30 % (w/v) (Grant et al., 2001), and they display a wide variety of physiological characteristics including ranges of salinity, temperature and pH for growth, etc. Recently, it has been reported that strains of the *Halobacteriaceae* can grow at low salinities (Purdy et al., 2004; Savage et al., 2007, 2008). We have been interested in haloarchaeal strains that exhibited tolerance to high temperature, low pH (Minegishi et al., 2010), high Mg concentration, etc. It has been well known that most of the haloarchaeal strains require a high concentration of magnesium as well for their growth
growth. *Halobacterium salinarum DSM 3754T* needs at least 1.0–2.0 % (w/v) (50–100 mM) MgCl₂·6H₂O for growth (Grant, 2001). *Halobaculum gomorrense* DS2807T (Oren et al., 1995) isolated from the Dead Sea containing 10.5–14.3 % (w/v) MgCl₂·6H₂O (Neev & Emery, 1967) is exceptional in requiring extremely high concentrations of MgCl₂, optimal growth being observed in media containing 0.6–1.0 M MgCl₂·6H₂O in the presence of 2.1 M NaCl. *Halorubrum sodomense* also required high concentrations of MgCl₂·6H₂O, with optimal growth at 0.6–1.2 M in the presence of 2 M NaCl (Oren, 1983). Furthermore, *Halofex volcanii* DS2T isolated from the Dead Sea showed tolerance for high, 1.2–1.4 M, MgCl₂·6H₂O (Mullakhanbai & Larsen, 1975; Cohen et al., 1983; Torreblanca et al., 1986).

In this paper, we focused on halophilic archaeal strains able to grow in media with very high concentrations of MgCl₂·6H₂O (30 % w/v, 1.48 M), supplemented with very low NaCl concentrations (2.0 % w/v). We isolated two strains that required extremely high concentrations of MgCl₂, strain MGY-184T from sea salt commercially available in Japan and strain MGY-205 from rock salt imported from Bolivia.

Using microspatulas, 328 salt samples purchased in various places in Japan were spread on agar plates of modified JCM medium no. 225 (225M1 medium) containing the following (l⁻¹): 1.0 g Casamino acids (Difco), 1.0 g yeast extract (Difco), 2.0 g soluble starch, 20.0 g NaCl (0.34 M), 300.0 g MgCl₂·6H₂O (1.48 M), 0.13 g CaCl₂·2H₂O and 5.0 g K₂SO₄. The medium was adjusted to pH 6.8 with 25 % (w/v) KOH, solidified with 23.0 g Bacto-agar (Difco). MgCl₂·6H₂O, which is very hygroscopic in a normal atmosphere, was taken from a newly opened 500 g bottle. Media with 30 % (w/v) MgSO₄·7H₂O was unsatisfactory since it formed precipitate after autoclaving. Agar of slightly higher concentration than usual (1.5–2.0 % w/v) was needed to solidify the medium containing high magnesium concentrations. After incubation at 37 °C for at least 4 weeks, 97 colonies appeared from 25 salt samples, and they were picked up, transferred to fresh agar plates, and pure cultures were obtained by plating serial dilutions and repeated transfers on the agar plates. The magnesium-dependent growth of these isolates was determined using 225M1 liquid medium containing 2 % (w/v) NaCl and 0–30 % (w/v) MgCl₂·6H₂O, and two strains were selected on the basis of the highest concentration of MgCl₂·6H₂O for optimal growth. Several strains were also isolated from magnesium-rich commercial bittern brines (mother liquor), but it was not possible to transfer cultures on to agar plates. Strain MGY-184T was isolated from sea salt ‘Salt of Oga Peninsula’ produced by Oga Craft Centre (Akita prefecture, Japan). According to the manufacturer’s information, the salt was produced by concentrating seawater by heating in large iron pans. Strain MGY-205 was isolated from rock salt labelled ‘Sal de Sol’ imported from Bolivia. The salt was harvested from the Andes Mountains in south Bolivia.

Phenotypic tests were performed according to the proposed minimal standards for the descriptions of new taxa in the order *Halobacteriales* (Oren et al., 1997). Physiological and chemotaxonomic analyses were conducted using liquid or solidified 225M1 medium (pH 6.8) supplemented with 1 g sodium pyruvate l⁻¹ at 37 °C. Liquid cultures were routinely incubated at 37 °C statically in an incubator. Growth curves were determined by monitoring the increase in the OD₆₀₀.

NaCl and MgCl₂·6H₂O tolerance was tested at pH 6.8 and 37 °C. As expected from the concentration of NaCl (2.0 % w/v) and MgCl₂·6H₂O (30 % w/v) in the isolation medium, both strains grew in 6–30 % (w/v) MgCl₂·6H₂O in the presence of 2 % (w/v) NaCl, with optimum growth at 15–21 % (w/v) MgCl₂·6H₂O. Both strains showed no growth in the absence of added NaCl, and required at least 1.0 % (w/v) NaCl in the presence of 18 % (w/v) MgCl₂·6H₂O. In the presence of a low concentration, 2 % (w/v), of MgCl₂·6H₂O, however, they grew at 6–30 % (w/v) NaCl with optimum at 9–12 % (w/v) NaCl (see Fig. S1, available in IJSEM Online).

Colonies of strains MGY-184T and MGY-205 were circular, smooth and red after incubation for 4–8 weeks at 37 °C. Morphology of cells grown in liquid medium (after 8 weeks incubation) was observed using a phase-contrast microscope (Axiovert 135, Zeiss). Cells were Gram-negative, non-motile and pleomorphic, mostly with disc morphology and a few short rods, approximately 0.2–0.6 × 0.3–0.9 mm. The morphology of MGY-184T and MGY-205 was obviously different from that of *Halobaculum gomorrense* JCM 9908T (see Fig. S2). Cells lysed in distilled water. The temperature range for growth was 25–55 °C, with optimum growth at 45 °C. Growth in liquid 225M1 medium occurred within the pH range 5.5–9.5, with optimum at pH 6.5.

Tests for catalase and oxidase activities and for the hydrolysis of starch, gelatin, casein and Tween 80 were performed as described by Gonzalez et al. (1978). Both strains were catalase- and oxidase-negative and did not hydrolyse starch, gelatin, casein and Tween 80. Biochemical tests were performed according to the methods outlined by Gerhardt et al. (1994). Both strains did not show β-galactosidase, phosphatase, urease, lysine and ornithine decarboxylases activities. The two strains did not grow anaerobically in the presence of nitrate, L-arginine or DMSO (5 g l⁻¹) as tested in filled stopped tubes (Oren et al., 1997). Reduction of nitrate was detected by using the sulfanilic acid and naphthylamine reagent (Smibert & Krieg, 1994). Reduction of nitrite was detected. H₂S formation, determined by monitoring the production of a black sulfide precipitate in 225M1 medium containing 0.5 % (w/v) sodium thiosulfate, was negative. Indole production from tryptophan, assessed as described by Oren et al. (1997), was negative. Utilization of sugars and

---

**H. Shimoshige and others**
organic acids and formation of acid from sugars and organic acids was assessed in a modified 225M1 medium in which the yeast extract and soluble starch were omitted. After incubation for 4 weeks, acid formation was determined by using duotest pH indicator paper (Macherey–Nagel). The results are included in the species description. Detailed physiological and biochemical characteristics that distinguish strains MGY-184T and MGY-205 from Halobaculum gomorrense JCM 9908T are shown in Table 1.

Polar lipids were extracted with chloroform:methanol as described previously (Kamekura, 1993). One-dimensional TLC was performed by using HPTLC silica gel 60 plates (20 x 10 cm, Merck) in the solvent system, chloroform:methanol:acetic acid:water (85:22.5:10:4, by vol.). Phospholipids were detected as blue spots by spraying with Dittmer–Lester reagent (Minnikin et al., 1984). Glycolipids were detected as purple spots by spraying with 0.5 % (w/v) a-naphthol in methanol:water (1:1) and then with sulfuric acid:ethanol (1:1), followed by heating at 160°C. Both strains contained phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester as phospholipids, derived from C_{20}C_{20} archaeol, but not phosphatidylglycerol sulfate. Both strains also contained sulfated diglycosyl diether-1 (S-DGD-1) as a glycolipid (see Fig. S3). The overall polar lipids of both strains were similar to those of Halobaculum gomorrense JCM 9908T.

Total DNA was extracted by the method of Cline et al. (1989). The G+C contents of the total DNA of strains MGY-184T and MGY-205, determined by the HPLC method of Tamaoka & Komagata (1984), were 67.0 and 67.4 mol%, respectively. The DNA G+C contents of both strains were lower than that of Halobaculum gomorrense (70 mol%). The 16S rRNA genes were amplified by PCR with the following forward and reverse primers: 5'-ATTCGGGTTGATCTGCCCAGG-3' and 5'-AGGAGGTGATCCAGCGCAG-3' (Fukushima et al., 2007). The amplified DNA was cloned into the pCR2.1 T vector using the TA Cloning kit (Invitrogen) and sequenced using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kits (Applied Biosystems) with the following primers: 1F: 5'-ATTCCGGTTGATCTGCCCAGG-3', 317F: 5'-CCGGGCCCTAGGGGCGCAG-3', 1134F: 5'-GGGCAAC-GTATTGTCAGTAT-3', 351R: 5'-GTAAAGGTTTCGCG-CCTGCT-3', 920R: 5'-GGCGGCCATGCAACCTCCTCT-3' and 1295R: 5'-CTACCGAATCCGCGTTATCCAGCG-3' on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The sequence data showed that strains MGY-184T (1472 bp) and MGY-205 (1472 bp) possessed at least two 16S rRNA genes, rrnA and rrnB (97.6 % similarity) and rrnA and rrnB of the two strains were exactly the same. Similarity search

**Table 1.** Characteristics that distinguish strains MGY-184T and MGY-205 from Halobaculum gomorrense JCM 9908T

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>1</th>
<th>2</th>
<th>3*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size (μm)</td>
<td>0.2–0.6 x 0.3–0.9</td>
<td>0.2–0.6 x 0.3–0.9</td>
<td>0.5–1 x 5–10</td>
</tr>
<tr>
<td>NaCl optimum (% w/v)</td>
<td>9–12§</td>
<td>9–12§</td>
<td>9–15§</td>
</tr>
<tr>
<td>MgCl₂, 6H₂O optimum (% w/v)</td>
<td>15–21§</td>
<td>15–21§</td>
<td>12–20∥</td>
</tr>
<tr>
<td>Temperature optimum (°C)</td>
<td>45</td>
<td>45</td>
<td>40</td>
</tr>
<tr>
<td>Hydrolysis of starch</td>
<td>–</td>
<td>–</td>
<td>+ ( + )</td>
</tr>
<tr>
<td>Acid produced from:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-Fructose</td>
<td>–</td>
<td>–</td>
<td>– ( + )</td>
</tr>
<tr>
<td>d-Galactose</td>
<td>–</td>
<td>–</td>
<td>+ ( + )</td>
</tr>
<tr>
<td>Lactose</td>
<td>–</td>
<td>–</td>
<td>– ( + )</td>
</tr>
<tr>
<td>Maltose</td>
<td>–</td>
<td>–</td>
<td>+ ( + )</td>
</tr>
<tr>
<td>d-Mannitol</td>
<td>+</td>
<td>+</td>
<td>– ( - )</td>
</tr>
<tr>
<td>d-Xylose</td>
<td>–</td>
<td>–</td>
<td>+ ( + )</td>
</tr>
<tr>
<td>Citrate</td>
<td>–</td>
<td>–</td>
<td>– ( + )</td>
</tr>
<tr>
<td>Fumarate</td>
<td>–</td>
<td>–</td>
<td>+ ( + )</td>
</tr>
<tr>
<td>Sensitivity to:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anisomycin (50 μg ml⁻¹)</td>
<td>–</td>
<td>–</td>
<td>+ ( + )</td>
</tr>
<tr>
<td>Aphidicolin (25 μg ml⁻¹)</td>
<td>–</td>
<td>–</td>
<td>+ ( + )</td>
</tr>
<tr>
<td>Pravastatin (50 μg ml⁻¹)</td>
<td>–</td>
<td>–</td>
<td>+ ( + )</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>67.0</td>
<td>67.4</td>
<td>70</td>
</tr>
</tbody>
</table>

*Data in parentheses from this study.
†Determined in the presence of 2.0 % MgCl₂, 6H₂O.
‡Determined in the presence of 8 % MgCl₂, 6H₂O.
§Determined in the presence of 2.0 % NaCl.
∥Determined in the presence of 12 % NaCl.
performed in NCBI BLAST tools (http://www.ncbi.nlm.nih.gov/BLAST) showed that these sequences were most closely related to AB477982 (97.8 %) and AB477983 (96.2 %) of Halobaculum gomorrense JCM 9908T. Lower similarities (<90.5 %) were found with 16S rRNA gene sequences from other species of the family Halobacteriaceae. The 16S rRNA gene sequences of related strains retrieved from the DNA Data Bank of Japan (Miyazaki et al., 2003; Pearson & Lipman, 1988; Lipman & Pearson, 1985) were aligned using CLUSTAL_X 2.0.10 (Larkin et al., 2007). A phylogenetic tree was reconstructed by using the neighbour-joining (NJ) method (Saitou & Nei, 1987) and evaluated by bootstrap sampling (Felsenstein, 1985). Maximum-likelihood (ML) analyses were performed using PAUP* 4.0b10 (Swofford, 2002). Bootstrap values per 1000 replicates are shown. GenBank accession numbers are given in parentheses. Bar, 0.01 changes per nucleotide position.

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strains MGY-184T, MGY-205 and some other related haloarchaeal strains. Bootstrap values per 1000 replicates are shown. GenBank accession numbers are shown in parentheses. Bar, 0.01 changes per nucleotide position.

International Journal of Systematic and Evolutionary Microbiology 63

H. Shimoshige and others

Descriptive data for strains MGY-184T and MGY-205 are shown in Table 1. These strains were most closely related to AB477982 (97.8 %) and AB477983 (96.2 %) of Halobaculum gomorrense JCM 9908T, respectively. These data clearly support the conclusion that the two strains are members of the same species, but different from Halobaculum gomorrense.

Antimicrobial compound sensitivity was determined by BD Sensi-Disc (Becton Dickinson), except for disks of anisomycin, and pravastatin, which were prepared in our laboratory. Sensi-Disc (Becton Dickinson), except for disks of anisomyacin (50 μg ml−1), aphidicolin (25 μg ml−1), bacitracin (10 U ml−1), lineozid (30 μg ml−1), novobiocin (30 μg ml−1), rifampicin (5 μg ml−1) and simrastatin (50 μg ml−1), but resistant to ampicillin (10 μg ml−1), chloramphenicol (30 μg ml−1), erythromycin (15 μg ml−1), gentamicin (120 μg ml−1), kanamycin (30 μg ml−1), neomycin (30 μg ml−1), penicillin G (10 U ml−1), streptomycin (300 μg ml−1), tetracycline (30 μg ml−1) and vancomycin (30 μg ml−1). Antimicrobial compounds sensitivity tests on Halobaculum gomorrense JCM 9908T were performed by using 3 ml of the liquid medium used by Oren et al. (1995), because growth on solidified medium was never observed in our laboratory. Halobaculum gomorrense JCM 9908T was sensitive to anisomycin (50 μg ml−1), aphidicolin (25 μg ml−1), bacitracin (10 U ml−1), lineozid (30 μg ml−1), novobiocin (30 μg ml−1), pravastatin (50 μg ml−1), rifampicin (5 μg ml−1) and simrastatin (50 μg ml−1), but resistant to ampicillin (10 μg ml−1), chloramphenicol (30 μg ml−1), erythromycin (15 μg ml−1), gentamicin (120 μg ml−1), kanamycin (30 μg ml−1), neomycin (30 μg ml−1), penicillin G (10 U ml−1), streptomycin (300 μg ml−1), tetracycline (30 μg ml−1) and vancomycin (30 μg ml−1).

On the basis of tolerance to MgCl2 and requirement of NaCl for growth, morphological properties, other phenotypic characteristics (Table 1) and levels of DNA–DNA relatedness, strains MGY-184T and MGY-205 are considered to represent a novel species of the genus Halobaculum, for which the name Halobaculum magnesiophilum sp. nov. is proposed.

Description of Halobaculum magnesiophilum sp. nov.

Halobaculum magnesiophilum [mag.ne.si.i’phi.lum. N.L. n. magnesium; N.L. neut. adj. philum (from Gr. neut. adj. philon) friend, loving; N.L. neut. adj. magnesi-philum magnesium-loving].

Cells are non-motile, Gram-negative and pleomorphic, mostly with disc morphology and a few short rods, approximately 0.2–0.6 × 0.3–0.9 mm. Growth occurs at 6–30 % (w/v) NaCl, with optimum growth at 9–12 % (w/v) NaCl and 6–30 % (w/v) MgCl2, with optimum growth at 15–21 % (w/v) MgCl2. Optimal temperature for growth is 45 °C (range, 25–55 °C). Growth at pH 5.5–9.5, with optimum at pH 6.5. H2S is not produced from sodium thiosulfate. Indole is produced from tryptophan. Nitrate

![Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strains MGY-184T, MGY-205 and some other related haloarchaeal strains. Bootstrap values per 1000 replicates are shown. GenBank accession numbers are shown in parentheses. Bar, 0.01 changes per nucleotide position.](image-url)
reduction to nitrite is observed. Nitrite was reduced. Catalase- and oxidase-negative. Gelatin, starch, casein and Tween 80 are not hydrolysed. β-Galactosidase-, phosphatase-, urease-, lysine and ornithine decarboxylases-negative. Does not grow anaerobically with nitrate or DMSO. Does not ferment arginine. Utilizes glucose, glycerol, mannitol, sucrose, sorbitol, trehalose, acetate and pyruvate as single carbon substrates. Arabinose, fructose, galactose, lactose, maltose, mannose, ribose, xylose, citrate, fumarate, lactate, malate, succinate, alanine, arginine, aspartate, glutamate, glycine and lysine are not utilized as carbon sources. Acid is produced from glucose, glycerol, mannitol, sucrose and trehalose. Able to utilize complex carbon sources such as neoipeptone. Sensitive to bacitracin linezolid, nalidixic acid, novobiocin, rifampicin and simramast. Resistant to ampi-

The type strain is 67.0 mol%.

The type strain is MGY-184T (=JCM 17821=KCTC 4100T), isolated from a sea salt sample, commercially available in Japan. The DNA G+C content of the type strain is 67.0 mol%.

References


Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid–deoxyribonucleic acid hybridization in micro-
dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int J Syst Bacteriol 39, 224–229.


Fukushima, T., Usami, R. & Kamekura, M. (2007). A traditional Japanese-style salt field is a niche for haloarchaeal strains that can survive in 0.5% salt solution. saline Syst 3, 2.


