Halarchaeum acidiphilum gen. nov., sp. nov., a moderately acidophilic haloarchaeon isolated from commercial solar salt

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A novel halophilic archaeon, strain MH1-52-1^T, was isolated from solar salt imported from Australia. Cells were pleomorphic, non-motile and Gram-negative. Strain MH1-52-1^T required at least 3.0 M NaCl and 1 mM Mg^{2+} for growth. Strain MH1-52-1^T was able to grow at pH 4.0–6.0 (optimum, pH 4.4–4.5) and 15–45 °C (optimum, 37 °C). The diether phospholipids phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester, derived from both $C_{20}C_{20}$ and $C_{20}C_{25}$ archaeol, were present. Four unidentified glycolipids were also detected. The 16S rRNA gene sequence showed the highest similarity to that of *Halobacterium noricense* A1^T (91.7 %); there were lower levels of similarity to other members of the family *Halobacteriaceae*. The G+C content of its DNA was 61.4 mol%. Based on our phenotypic, genotypic and phylogenetic analyses, it is proposed that the isolate should be classified as a representative of a new genus and species, for which the name *Halarchaeum acidiphilum* gen. nov., sp. nov. is proposed. The type strain of *Halarchaeum acidiphilum* is MH1-52-1^T (=JCM 16109^T =DSM 22442^T =CECT 7534^T).

Halophilic archaea are classified within the family *Halobacteriaceae* of the order *Halobacteriales*. The family *Halobacteriaceae* consists of a large group of aerobic microbes that live and grow in hypersaline environments such as salt lakes, salterns, solar salts and subsurface salt formations. Recently, it has been suggested that haloarchaeal strains can grow within saline microniches in non-saline environments, and the novel haloarchaeal species *Haladaptatus paucihalophilus* and *Halosarcina pallida* were isolated from a spring with a low salt concentration (Savage *et al.*, 2007, 2008). Recent studies have reported that some strains can survive for several days in distilled water or 0.5 % salt solution (Savage *et al.*, 2007; Fukushima *et al.*, 2007). These studies have shown that the order *Halobacteriales* is more diverse than previously believed. Currently, haloarchaeal strains are classified in 27 genera that display a wide variety of physiological characteristics including range of pH for growth. Optimal growth occurs either at neutral to slightly alkaline pH or only at alkaline pH. *Halococcus hamelinensis* 100A6^T and *Halococcus qingdaonensis* CM5^T are exceptions, in being able to grow at acidic pH (4.0), but they grow up to pH 9.0, with optimum growth at pH 6.0 (Goh *et al.*, 2006; Wang *et al.*, 2007).

In a previous study, we isolated a few strains of moderately acidophilic haloarchaea able to grow only at pH 4.0–6.0 from solar salt samples commercially available in Japan (Minegishi *et al.*, 2008). In the present study, we report on the phylogenetic and phenotypic characterization of a representative strain, MH1-52-1^T. We propose that the strain represents a novel genus and species.

Strain MH1-52-1^T was isolated from solar salt imported from Australia. A salt sample (1.0 g) was dissolved in 4 ml MH1 medium [containing (l^{-1}) 4.0 g Casamino acids (Difco), 2.0 g yeast extract (Difco), 2.0 g L-glutamic acid, 2.0 g trisodium citrate dihydrate, 5.0 g K_2SO_4, 1.0 g $MgCl_2.6H_2O$ (5 mM), 1.0 g NH_4Cl, 1.0 g KH_2PO_4, 0.004 g $FeSO_4.7H_2O$, 200 g

Abbreviations: ML, maximum-likelihood; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MH1-52-1^T is AB371717.

Growth curves at different pH values and Mg^{2+} concentrations, a TLC plate showing polar lipids and a 16S rRNA gene sequence-based maximum-likelihood tree are available as supplementary material with the online version of this paper.

NaCl, 2.0 ml trace metal solution] adjusted to pH 4.5 with 40 % KOH. In some experiments, the pH was adjusted to 3.5–6.5 at intervals of 0.2 or 0.5 pH units and the NaCl and $MgCl_2 . 6H_2O$ concentrations were modified as described below. The trace metal solution contained (l^{-1}) 2.0 g $Na_2S_2O_3 . 5H_2O$, 1.0 g $CaCl_2 . 2H_2O$, 0.3 g $CoCl_2 . 6H_2O$, 0.1 g $BaCl_2 . 2H_2O$, 0.1 g $MnCl_2 . 4H_2O$, 0.1 g $ZnCl_2$, 0.1 g $Na_2MoO_4 . 2H_2O$, 0.1 g $NiCl_2 . 6H_2O$, 0.04 g $AlCl_3$, 0.02 g $Na_2WO_4 . 2H_2O$ and 0.02 g H_3BO_3, adjusted to pH 4.0 with 2.0 M HCl. The medium was autoclaved for 20 min at 121 °C. There was no change in the pH of the medium after autoclaving.

After incubation at 37 °C for 1 week without shaking, 0.1 ml of the culture was spread evenly on an MH1 agar plate (pH 4.5), solidified with 20 g Bacto agar (Difco) l^{-1}. After incubation at 37 °C for 2 weeks, colonies were picked up and transferred to fresh agar plates at the same pH and pure cultures were obtained by plating serial dilutions and repeated transfers on agar plates.

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 MH1 medium at 37 °C. Liquid cultures were incubated on a shaking incubator at 100 r.p.m. Growth rates were determined by monitoring the increase in OD_{600} (1 cm light path).

Colonies of strain MH1-52-1^T were circular, smooth and non-pigmented after incubation for 7 days at 37 °C. Pigmentation was not observed even after 3–4 weeks of incubation. Cell morphology was studied using phase-contrast microscopy (Zeiss Axiovert 135). Cells were Gram-negative, non-motile and pleomorphic, with triangular and disc morphology, approximately 1.5–2.0 × 2.0–2.5 μm, and lysed in distilled water. The temperature range for growth was 15–45 °C, with optimum growth at 37 °C. Growth in liquid MH1 medium (5 mM Mg^{2+}) occurred at pH 4.2–4.8, with optimum growth at pH 4.4 (OD_{600} 1.5 after 4 days). NaCl and $MgCl_2 . 6H_2O$ tolerance was tested at pH 4.5 and 37 °C. Strain MH1-52-1^T grew in 18–30 % (w/v) NaCl, with optimum growth at 21–24 % (w/v) NaCl. Growth was observed from 1 mM (OD_{600} 0.6 after 4 days) up to 500 mM (OD_{600} 0.8 after 3 days) Mg^{2+} in medium with 20 % (w/v) NaCl, with an optimum at 50 mM. In medium with 250 mM Mg^{2+}, growth was observed over a slightly broader pH range of 4.0–6.0 with an optimum at pH 4.5 after incubation for 3 days. Cells aggregated in medium containing more than 20 mM Mg^{2+} after incubation for 7 days. The pH of the medium was measured during growth in medium buffered with 100 mM citrate adjusted to 4.4 and 4.6 (Supplementary Fig. S1, available in IJSEM Online). The pH did not change until cell growth reached an OD_{600} of 1.2 and then a gradual increase in pH was observed (Supplementary Fig. S1c, d). According to Johnson (2007), there is no common agreement on the pH boundary that delineates acidophily in micro-organisms, but a useful guide is that extreme acidophiles have an optimum pH for growth of <3.0 and that moderate acidophiles grow optimally at pH 3–5. We believe that strain MH1-52-1^T is the first moderate acidophile reported in the *Halobacteriaceae*.

Antibiotic sensitivity was determined by using BD Sensi-Discs (Becton Dickinson) except for discs containing anisomycin and pravastatin, which were prepared in our laboratory. Strain MH1-52-1^T was sensitive to (per disc) anisomycin (50 μg), bacitracin (10 U), novobiocin (30 μg), pravastatin (50 μg), rifampicin (5 μg) and tetracycline (30 μg) and resistant to ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (120 μg), kanamycin (30 μg), neomycin (30 μg), penicillin G (10 U), streptomycin (300 μg) and vancomycin (30 μg).

Strain MH1-52-1^T was catalase- and oxidase-negative and did not hydrolyse starch, gelatin, casein or Tween 80. Reduction of nitrate was not detected by using the sulfanilic acid and α-naphthylamine reagent (Smibert & Krieg, 1994). Detailed physiological and biochemical characteristics of strain MH1-52-1^T are listed in the species description and in Table 1.

Polar lipids were extracted with chloroform/methanol as described previously (Kamekura, 1993). TLC was performed by using HPTLC silica gel 60 plates (20 × 10 cm; Merck) in the solvent system chloroform/methanol/acetic acid/water (85 : 22.5 : 10 : 4, by vol.). Glycolipids were detected as purple spots by spraying with 0.5 % (w/v) α-naphthol in methanol/water (1 : 1) and then with sulfuric acid/ethanol (1 : 1), followed by heating at 160 °C. Strain MH1-52-1^T contained phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester as phospholipids, derived from both $C_{20}C_{20}$ and $C_{20}C_{25}$ archaeol (double spots), but not phosphatidylglycerol sulfate. Strain MH1-52-1^T contained at least four glycolipids that have yet to be identified (see Supplementary Fig. S2), although the two overlapping spots might represent $C_{20}C_{20}$ and $C_{20}C_{25}$ moieties of a glycolipid. Strain MH1-52-1^T may be distinguished from all other known members of the family *Halobacteriaceae* on the basis of its polar lipids.

Total DNA was extracted by the method of Cline *et al.* (1989). The G + C content of the total DNA of strain MH1-52-1^T, determined by the HPLC method of Tamaoka & Komagata (1984), was 61.4 mol%. The 16S rRNA gene was amplified by PCR with forward and reverse primers 5′-ATTCCGGTTGATCCTGCCGG-3′ and 5′-AGGAGGTGATCCAGCCGCAG-3′. Amplified DNA was cloned by using the TA Cloning kit (Invitrogen) and sequenced using the ABI PRISM BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems) with the following primers: 611F (5′-GGTACGTCCGGGGTAGGAGT-3′) and 860R (5′-CCTTTAAGTTTCATCCTTGC-3′) (primers designed in this work) and −20 (5′-GGAAACAGCTATGACCATG-3′) and Rev (5′-GTAAAACGACGGCCAGT-3′) (vector-side primers) on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The sequence of strain MH1-52-1^T (1430 bp) was closest to that of *Halobacterium noricense* A1^T, with 91.7 % similarity. Sequences of related

Table 1. Differential characteristics between strain MH1-52-1^T and closely related genera within the order *Halobacteriales*

Taxa: 1, strain MH1-52-1^T; 2, *Halobacterium*; 3, *Halococcus*; 4, *Haloferax*; 5, *Halorubrum*. Data for reference genera were taken from Savage *et al.* (2008), Yachai *et al.* (2008) and Oren *et al.* (2009). +, Positive; −, negative; +/−, variable depending on the species.

Characteristic	1	2	3	4	5
Morphology	Pleomorphic	Rod	Coccus	Pleomorphic	Pleomorphic/rod
Motility	−	+	−	+/−	+/−
NaCl optimum (%)	21–24	15–26	16–26	10–26	15–27
Temperature optimum (°C)	37	37–45	30–40	32–50	37–50
pH optimum	4.4	7.0–7.5	6.8–9.5	6.4–7.5	7.0–7.5/9.0–10.0
Lysis in distilled water	+	+	−	+	+
DNA G+C content (mol%)	61.4	54.3–71.2	59.5–66.0	59.1–64.5	62.7–71.2

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 with RAxML 7.0.4 using the GTR+Γ model (Stamatakis *et al.*, 2005). Support values for the ML tree were obtained by bootstrapping (1000 replicates) using CONSENSE in PHYLIP (Felsenstein, 2002). The NJ tree (Fig. 1) and ML tree (Supplementary Fig. S3) showed that strain MH1-52-1^T was a member of the family *Halobacteriaceae* and was most closely related to the species of the genus *Halobacterium*. However, strain MH1-52-1^T was distinctly differentiated from the *Halobacterium* species (Oren *et al.*, 2009) in motility, morphology, pigmentation, catalase activity, growth pH range, presence/absence of the $C_{20}C_{25}$ isoprenoid moiety and the glycolipid profile.

The phylogenetic and phenotypic characteristics outlined above indicate that strain MH1-52-1^T represents a novel genus and species, for which the name *Halarchaeum acidiphilum* gen. nov., sp. nov. is proposed.

Description of *Halarchaeum* gen. nov.

Halarchaeum (Hal′ar.chae′um. Gr. n. *hals*, *halos* salt, salt water; N.L. n. *archaeum* ancient one, archaeon, from Gr. adj. *archaios* ancient; N.L. neut. n. *Halarchaeum* a saline archaeon).

Cells are non-motile, Gram-negative and pleomorphic, with triangular and disc morphology. Cells contain phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester, derived from both $C_{20}C_{20}$ and $C_{20}C_{25}$ archaeol, and four unidentified glycolipids. Cells lyse in distilled water. Chemo-organotrophic, growing on a wide range of substrates, including single and complex carbon sources. The type species is *Halarchaeum acidiphilum*. Recommended three-letter abbreviation: *Hla*.

Description of *Halarchaeum acidiphilum* sp. nov.

Halarchaeum acidiphilum (a.ci′di.phi′lum. N.L. neut. n. *acidum* acid; Gr. adj. *philos* loving; N.L. neut. adj. *acidiphilum* acid-loving).

Exhibits the following properties in addition to those given in the genus description. Cells are approximately 2.0 μm in diameter. Colonies are approximately 1.0 mm in diameter, circular, smooth and non-pigmented. Grows in 18–30 % (w/v) NaCl, with optimum growth at 21–24 % (w/v) NaCl. A minimum of 1 mM Mg^{2+} is required for growth. Optimal temperature for growth is 37 °C (range, 15–45 °C). Moderately acidophilic; grows at initial pH 4.0–6.0, with an optimum at pH 4.4–4.5. Does not grow anaerobically with nitrate or DMSO. Does not ferment arginine.

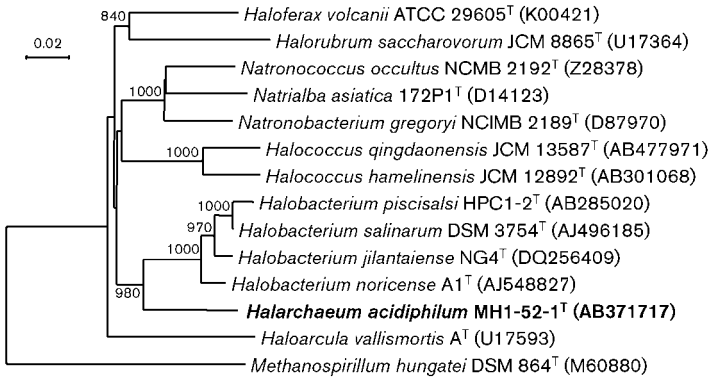

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain MH1-52-1^T and related haloarchaeal strains. Bootstrap values are shown as percentages of 1000 replicates. GenBank accession numbers are shown in parentheses. Bar, 0.02 changes per nucleotide position.

Capable of using the following single carbon substrates: arabinose, cellobiose, fructose, galactose, glucose, glycerol, ribitol, raffinose, sucrose and xylose. Lactose, maltose, mannitol, mannose, ribose, sorbitol, trehalose, citrate and glutamate are not utilized as carbon sources. Able to utilize complex carbon sources such as yeast extract. Sensitive to anisomycin, bacitracin, novobiocin, pravastatin, rifampicin and tetracycline. Resistant to ampicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, neomycin, penicillin, streptomycin and vancomycin. Catalase- and oxidase-negative. Gelatin, starch, casein and Tween 80 are not hydrolysed. Indole is not produced from tryptophan. H_2S is not produced from thiosulfate. Does not reduce nitrate under aerobic conditions. The DNA G+C content of the type strain is 61.4 mol%.

The type strain is MH1-52-1^T (=JCM 16109^T =DSM 22442^T =CECT 7534^T), isolated from solar salt imported into Japan from Australia.

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