Halarchaeum salinum sp. nov., a moderately acidophilic haloarchaeon isolated from commercial sea salt

Yuto Yamauchi, Hiroaki Minegishi, Akinobu Echigo, Yasuhiro Shimane, Hirokazu Shimoshige, Masahiro Kamekura, Takashi Itoh, Noriyuki Doukyu, Akira Inoue and Ron Usami

Correspondence
Yuto Yamauchi
vn1100054@toyo.jp

1Graduate School of Interdisciplinary New Science, Toyo University, 2100 Kujirai, Kawagoe, Saitama 350-8585, Japan
2Bio-Nano Electronics Research Center, Toyo University, 2100 Kujirai, Kawagoe, Saitama 350-8585, Japan
3Japan Agency for Marine-Earth Science and Technology, 2-15 Natsushima, 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

Three halophilic archael strains, MH1-34-1T, MH1-16-1 and MH1-224-5 were isolated from commercial salt samples produced from seawater in Indonesia, the Philippines and Japan, respectively. Cells of the three strains were pleomorphic and stained Gram-negative. Strain MH1-34-1T was orange–red pigmented, while MH1-16-1 and MH1-224-5 were pink-pigmented. Strain MH1-34-1T was able to grow at 12–30 % (w/v) NaCl (with optimum at 18 % NaCl, w/v) at pH 4.5–7.2 (optimum, pH 5.2–5.5) and at 15–45 °C (optimum, 42 °C). Strains MH1-16-1 and MH1-224-5 grew in slightly different ranges. These strains required at least 1 mM Mg^{2+} for growth. The 16S rRNA gene sequences of strains MH1-34-1T, MH1-16-1 and MH1-224-5 were almost identical (99.8–99.9 % similarities), and the closest relative was Halarchaeum acidiphilum MH1-52-1T with 98.4 % similarities. The DNA G + C contents of MH1-34-1T, MH1-16-1 and MH1-224-5 were 59.3, 60.8 and 61.0 mol%, respectively. The level of DNA–DNA relatedness amongst the three strains was 90–91 %, while that between each of the three strains and Halarchaeum acidiphilum MH1-52-1T was 51–55 %. Based on the phenotypic, genotypic and phylogenetic analyses, it is proposed that the isolates should represent a novel species of the genus Halarchaeum, for which the name Halarchaeum salinum sp. nov. is proposed. The type strain is MH1-34-1T (=JCM 16330T=CECT 7574T).

Many haloarchaeal strains grow well at neutral to slightly alkaline pH, and alkaliophilic haloarchaea have also been isolated from various sources. Recently, Minegishi et al. (2008) showed the presence of moderately acidophilic haloarchaeal strains in many solar salt samples. They isolated more than 50 strains capable of growth in a medium adjusted to pH 4.5, from 28 out of 240 commercially available salt samples. Most strains grew at pH range of 4.5–6.0, and none of them showed growth at pH higher than 6.5. As judged from partial (about 500 bp, corresponding to nucleotides 21–520 of Halobacterium salinarum DSM 3754T) 16S rRNA gene sequences, they formed four clusters consisting of four to 25 strains. Strain MH1-52-1, a representative of the most acidophilic strains was classified as Halarchaeum acidiphilum gen. nov., sp. nov. (Minegishi et al., 2010). Unfortunately most of the isolates died during storage for a year, except for strain MH1-34-1T and a few others. In this study we repeated the isolation of acidophilic haloarchaeal strains using the same medium as Minegishi et al. (2008), and obtained 28 strains capable of growth at pH 4.5 from 583 salt samples. Full-length 16S rRNA gene sequences of the 28 strains showed that two isolates, MH1-16-1 and MH1-224-5, were almost identical to strain MH1-34-1T. In the present study, we report on the phenotypic and

The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequences of strains MH1-34-1T, MH1-16-1 and MH1-224-5 are AB372514.2, AB693106 and AB693105, respectively.

Two supplementary figures and a supplementary table are available with the online version of this paper.
phylogenetic characterization of halo-acidophilic strains MH1-34-1T and the two new isolates.

Commercial salt samples (0.3 g each) were dissolved in 1 ml sterile 5% NaCl solution, and one drop of each was spread on agar plates of MH1 medium of the following composition (1–1): 4.0 g Casamino acids (Difco), 2.0 g yeast extract (Difco), 2.0 g l-glutamic acid, 2.0 g trisodium citrate. H₂O, 200 g NaCl, 5.0 g K₂SO₄, 50.0 g MgCl₂·6H₂O, 1.0 g NH₄Cl, 1.0 g KH₂PO₄, 4 mg FeSO₄·6H₂O, 2.0 ml trace metal solution, pH adjusted to 4.5 with 40% KOH and 20 g Bacto-agar (Difco) when necessary. The trace metal solution contained (l⁻¹ distilled water): 2.0 g Na₂S₂O₃·5H₂O, 1.0 g CaCl₂·2H₂O, 0.3 g CoCl₂·6H₂O, 0.1 g BaCl₂·2H₂O, 0.1 g MnCl₂·4H₂O, 0.04 g ZnCl₂, 0.1 g Na₂MoO₄·2H₂O, 0.1 g NiCl₂·6H₂O, 0.04 g AlCl₃, 0.02 g Na₂WO₄·2H₂O, 0.02 g H₂BO₃, pH adjusted to 4.0 with HCl. The medium was autoclaved for 20 min at 121 °C. The pH of the medium was not changed by autoclaving. After incubation for 2–4 weeks at 37 °C, colonies were picked up and transferred to new plates. Strains were purified by repeated streaking, and 16S rRNA gene sequences were determined according to the protocol of Minegishi et al. (2012). Three strains selected for further study, MH1-34-1T, MH1-16-1 and MH1-224-5, were isolated from commercial salt samples from seawater from Indonesia (solar salt), Philippines (solar salt) and Japan (salt produced by boiling concentrated sea water), respectively. Phenotypic tests were performed according to the protocols of Minegishi et al. (2010) and Oren et al. (1997). The analyses were conducted using liquid or solidified MH1 medium at 37 °C. Colony morphology was observed on agar medium. Gram-staining was performed according to the protocol of Dussault (1955). Cell morphology and motility were examined using phase-contrast microscopy (BX53F, Olympus). Cells of strains MH1-34-1T, MH1-16-1 and MH1-224-5 were non-motile and pleomorphic, approximately 0.5–1.1 μm in diameter when grown in the MH1 medium. The cells stained Gram-negative. Colonies of MH1-34-1T were orange to red pigmented, while MH1-16-1 and MH1-224-5 were pink pigmented. Strain MH1-34-1T was able to grow at 12–30 % (w/v) NaCl (optimum, 18 %, (w/v)) at pH 4.5–7.2 (optimum, pH 5.2–5.5) and at 15–45 °C (optimum, 42 °C). Final pH after growth was the same as that before inoculation. Strain MH1-16-1 was able to grow at 16–27% (w/v) NaCl (optimum, 24 %, (w/v)) at pH 4.5–6.5 (optimum, pH 5.5) and at 15–50 °C (optimum, 45 °C). Strain MH1-224-5 was able to grow at 15–30% (w/v) NaCl (optimum, 21 %, (w/v)) at pH 4.5–6.5 (optimum, pH 5.0) and 15–45 °C (optimum, 40 °C). The three strains required at least 1 mM Mg²⁺ for growth. Cells of all strains did not lyse when suspended in distilled water, but they died immediately, as judged from the fact that no colonies appeared at all when one drop of the cell suspension was spread on MH1 agar plates and incubated for two weeks. Tests for catalase and oxidase activities and for the hydrolysis of starch, gelatin, Tween 80 and 81 were performed as described by Gonzalez et al. (1978). Reduction of nitrate was detected by using the sulfanilic acid and x-naphthylamine reagent (Smibert & Krieg, 1994). H₂S formation was determined by the black sulfide precipitate in the medium containing 0.5% (w/v) sodium thiosulfate. Indole production from tryptophan and the utilization of sugars and organic acids were assessed as described by Oren et al. (1997). The three strains did not hydrolyse starch, gelatin, Tween 80 and casein. Strain MH1-34-1T did not hydrolyse urea, but MH1-16-1 and MH1-224-5 hydrolysed urea. The strains produced indole from tryptophan but did not produce H₂S from thiosulfate. Utilization of carbon sources for growth was determined in a modified MH1 liquid medium which contained 0.1% Casamino acids (Difco) and 0.5% carbon sources. Yeast extract (Difco), l-glutamic acid and trisodium citrate were omitted. Results are shown in Table S1, available in IJSEM online. Sensitivity to antimicrobial agents was determined by using BD SensiDiscs (Becton Dickinson) or by the methods of Gutiérrez et al. (2008) on MH1 agar plates. Results are shown in the species description, Table 1 and Table. S1. Polar lipids were extracted from the cells with chloroform:methanol and developed on silica gel plates as described previously (Kamekura, 1993). The major polar lipids of strains MH1-34-1T, MH1-16-1 and MH1-224-5 were C₂₀C₂₀ and C₂₀C₂₅ archaeol derivatives of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester. The strains also contained at least two to four glycolipids that have yet to be identified (Fig. S1). Total DNA was extracted by the method of Cline et al. (1989). The 16S rRNA gene sequences were determined as described previously (Minegishi et al., 2012), and relevant sequences retrieved from the DNA Data Bank of Japan (Miyazaki et al., 2003; Pearson & Lipman, 1988) were aligned using CLUSTAL X 2.0.12 (Larkin et al., 2007). The phylogenetic tree was reconstructed by the neighbour-joining (NJ) method (Saitou & Nei, 1987) and evaluated by bootstrap sampling, expressed per 1000 replicates (Felsenstein, 1985). Maximum-likelihood (ML) analysis was performed with RAXML 7.0.4 using the general time reversible (GTR)+Γ model (Stamatakis et al., 2005), and support values for ML tree was obtained by bootstrapping (1000 replicates) using CONSENSE in PHYLIP (Felsenstein, 2002). The 16S rRNA gene sequence similarities amongst strains MH1-34-1T (AB372514 ver.2, 1473 bp), MH1-16-1 (AB693106, 1473 bp) and MH1-224-5 (AB693105, 1474 bp) were 99.8–99.9%, and the most closely related species with a validly published name was Halarchaeum acidiphilum MH1-52-1T (AB371717, 1474 bp) with 98.4% similarities. The phylogenetic position was also confirmed in trees generated using NJ and ML algorithms (Figs 1 and S2). The results suggested that three strains might represent a novel taxon.

The G+C contents of the total DNA of strains MH1-34-1T, MH1-16-1 and MH1-224-5, determined by the HPLC

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Table 1. Differential characteristics amongst strains

1, MH1-34-1T; 2, MH1-16-1; 3, MH1-224-5; 4, *Halarchaeum acidiphilum* MH1-52-1T. +, Positive; –, negative; w, weakly positive.

<table>
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<td>Non-pigmented</td>
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<td>16–27 (24)</td>
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![Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strains MH1-34-1T, MH1-16-1 and MH1-224-5 and some other related haloarchaeal strains. Bootstrap values are shown per 1000 replicates. GenBank accession numbers are shown in parentheses. Bar, 0.01 changes per nucleotide position.](image-url)
Halarchaeum salinum sp. nov. is proposed.

Description of Halarchaeum salinum sp. nov.

Halarchaeum salinum (sa.li’nun. L. neut. adj. salinum salted, saline).

Cells are pleomorphic and approximately 0.5–1.1 μm in diameter. Colonies are approximately 0.8–1.0 mm in diameter, circular, smooth and orange–red or pink pigmented. Colonies are hard to pick up with a needle. Able to grow from 12–16 % to 27–30 % (w/v) NaCl, with optimum at 18–24 % NaCl (w/v), from pH 4.5 to pH 6.5–7.2, with optimum at pH 5.0–5.5 and from 15 °C to 45–50 °C, with optimum at 40–45 °C. Requires at least 1 mM Mg²⁺ for growth. Cells are not lysed in distilled water, but die immediately. Catalase- and oxidase-negative. H₂S is not produced from thiosulfate. Indole formation is negative. Does not reduce nitrate under aerobic conditions. Does not hydrolyse starch, gelatin, Tween 80, cellulose, mannann and casein. Capable of using sodium fumarate and sodium hydrolyse starch, gelatin, Tween 80, cellulose, mannan and L-sorbose, trehalose, sodium acetate, sodium citrate, sodium phosphate methyl ester, derived from both C₂₀C₂₀ and C₂₀C₂₅ archaeol. Several unidentified glycolipids are present. The G+C contents of the strains DNA are 59.3–61.0 mol%.

The type strain is MH1-34-1 T (=JCM 16330T=CECT 7574T) isolated from solar salt produced in Sulawesi, Indonesia.

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References


