Halocalculus aciditolerans gen. nov., sp. nov., an acid-tolerant haloarchaeon isolated from commercial salt

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Three halophilic archaeal strains, MH2-243-1T, MH2-93-1 and MH2-91-1 were isolated from commercial salt samples from Japan, Australia, and Bolivia. Strain MH2-243-1T was able to grow in the presence of 12–30 % (w/v) NaCl (optimum, 18 % NaCl), at pH 4.5–7.0 (optimum, pH 6.0) and at 20–60 °C (optimum, 40 °C). Strains MH2-91-1 and MH2-93-1 grew in slightly different ranges. The orthologous 16S rRNA gene sequences of the three strains were almost identical (99.8–99.9 % similarities), and the closest relative was Salarchaeum japonicum JCM 16327T with 94.2–94.3 % 16S rRNA gene sequence similarities, followed by strains of members of the closely related genera Halobacterium and Haloarchaeum. The RNA polymerase subunit B’ gene (rpoB') sequence also showed the highest similarity (86.6 %) to that of Salarchaeum japonicum JCM 16327T. The DNA G+C contents of strains MH2-243-1T, MH2-93-1 and MH2-91-1 were 68.5, 68.8 and 68.3 mol%, respectively. DNA–DNA relatedness values amongst the three strains were 97–99 %. The polar lipids of the three strains were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, and at least seven unidentified glycolipids. The polar lipid composition differed from those of Salarchaeum japonicum and species of the genera Halobacterium and Haloarchaeum. Based on the phenotypic and phylogenetic analyses, it is proposed that the isolates represent a novel species of a new genus, for which the name Halocalculus aciditolerans gen. nov., sp. nov. is proposed. The type strain of the type species is MH2-243-1T (=JCM 19596T=KCTC 4149T) isolated from solar salt produced in Japan. MH2-93-1 (=JCM 19595) and MH2-91-1 (=JCM 19594) are additional strains of the type species.

Many haloarchaeal strains grow well in neutral to slightly alkaline media, and alkaliphilic haloarchaea have been isolated from various sources (Oren, 2012). In 2008, we showed the presence of moderately acidophilic haloarchaeal strains capable of growth in the medium MH1 adjusted to pH 4.5. They were isolated from many solar salt samples obtained from Australia, Indonesia, Japan, Mexico, the Philippines, etc. (Minegishi et al., 2008). Most strains grew at a pH range of 4.5–6.0, and none of them showed growth at pH higher than 6.5. A representative
strain, MH1-52-1T, was classified as a new genus and species, *Halarchaeum acidophilum*, the first acidophilic haloarchaeon described (Minegishi et al., 2010a). Subsequently, our group proposed three more species of this genus, *Halarchaeum salinum* (Yamauchi et al., 2013a), *Halarchaeum rubridurum* (Yamauchi et al., 2013b) and *Halarchaeum nitratireducens* (Minegishi et al., 2013), using the medium MH1.

In this study, we modified the medium slightly. The new medium, MH2, differed from MH1 medium we used in previous papers (Minegishi et al., 2008, 2010a) in an increased concentration of MgCl2·6H2O (1.0 to 20.0 g l\(^{-1}\)) and a slightly higher pH (4.5 to 5.0). Full-length 16S rRNA gene sequences of 29 isolated haloarchaeal strains showed that most isolates were members of the genus *Halarchaeum*, but three isolates, MH2-243-1T, MH2-93-1 and MH2-93-1, possessed low level of similarity to members of the family *Halobacteriaceae*. Strains MH2-243-1T, MH2-93-1 and MH2-91-1 were isolated from commercial marine solar salt samples produced in Japan (Okinawa prefecture) and Australia (taken from the Great Barrier Reef), and a rock salt sample from Bolivia (mined from the Andes), respectively. The pH of 25% aqueous solution of these salt samples were 9.7, 8.6 and 6.2, respectively. In the present study, we report the phenotypic and phylogenetic characterization of these halophilic acid-tolerant strains and propose that they represent a novel species of a new genus of the family *Halobacteriaceae*.

Six hundred commercial salt samples (0.3 g each) were dissolved in 1.0 ml sterile 5% NaCl solution, and 10 μl of each were spotted at regular intervals on MH2 medium agar plates (16 spots per plate). MH2 medium comprised (g l\(^{-1}\)); 4.0 Casamino acids (Difco), 2.0 yeast extract (Difco), 2.0 l-glutamic acid, 2.0 Na2S2O3·5H2O, 2H2O, 200 NaCl, 5.0 K2SO4, 20.0 MgCl2·6H2O, 1.0 NH4Cl, 1.0 KH2PO4, 0.004 FeSO4·6H2O, 2.0 ml trace metal solution, pH adjusted to 5.0 with 40% KOH and 20 g Bacto-agar (Difco) added when necessary. The trace metal solution contained (g l\(^{-1}\)); 2.0 Na2S2O3·5H2O, 1.0 CaCl2·2H2O, 0.3 CoCl2·6H2O, 0.1 BaCl2·2H2O, 0.1 MnCl2·4H2O, 0.1 ZnCl2, 0.1 Na2MoO4·2H2O, 0.1 NiCl2·6H2O, 0.04 AlCl3, 0.02 Na2WO4, 2H2O, 0.02 H3BO3, pH adjusted to 4.0 with HCl. The medium was autoclaved for 20 min at 121 °C. After incubation at 37 °C for 2–4 weeks, growth was detected on 17 spots. An aliquot (100 μl) of freshly prepared solutions of each of the 17 salt samples was spread on MH2 agar medium. After incubation for 2–4 weeks at 37 °C, colonies were transferred to new plates and purified by repeated dilution and streaking.

Total DNA was extracted by the method of Cline et al. (1989). The orthologous 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 the CLUSTAL X 2.1 software package (Larkin et al., 2007). A phylogenetic tree was reconstructed by the neighbour-joining (NJ) method (Saitou & Nei, 1987) and was evaluated by bootstrap sampling, expressed as percentages of 1000 replicates (Felsenstein, 1985). Maximum-likelihood (ML) analysis and support values were performed with raxmlGUI version 1.31 (Silvestro & Michalak, 2012; Stamatakis et al., 2005). The 16S rRNA gene sequence similarities amongst strains MH2-243-1T (GenBank accession no. AB844670, 1473 bp), MH2-93-1 (AB844667, 1473 bp) and MH2-91-1 (AB844666, 1473 bp) were 99.9–99.8%, and the most closely related recognized species was *Salarchaeum japonicum* JCM 16327T (AB663360, 1473 bp) with 94.3–94.2% similarity, followed by strains of members of the genera *Halobacterium* and *Halarchaeum*, with similarities of 94.0–92.8% and 92.6–91.3%, respectively. The phylogenetic position was also confirmed in trees generated using NJ (Fig. 1a) and ML algorithms (Fig. S1, available in the online Supplementary Material). The three strains formed a clad clearly separated from *Salarchaeum japonicum* JCM 16327T, and species of the genera *Halobacterium* and *Halarchaeum*. The results suggested that three strains might represent a novel genus. The determination and analysis of DNA-dependent RNA polymerase B gene (rpoB) sequences were performed according to Minegishi et al. (2010b). The rpoB gene sequences of strains MH2-243-1T (GenBank accession no. AB917096, 1827 bp), MH2-93-1 (AB917095, 1827 bp) and MH2-91-1 (AB917094, 1827 bp) were the same, and most closely related to that of *Salarchaeum japonicum* JCM 16327T (86.6% rpoB gene sequence similarity). In the NJ tree reconstructed using rpoB sequences (Fig. 1b), strains MH2-243-1T, MH2-93-1 and MH2-91-1 clustered tightly with each other, while those of species of the genera *Halobacterium*, *Halarchaeum* and *Salarchaeum* formed distinctly separated clades. These results supported the placement of strains MH2-243-1T, MH2-93-1 and MH2-91-1 in a novel genus.

DNA–DNA relatedness amongst the three strains was assessed by using the fluorometric method of Ezaki et al. (1989). The threshold value of 70% DNA–DNA relatedness is generally accepted for differentiation of species (Wayne et al., 1987). The DNA–DNA hybridization values were 99 and 99% (reciprocally), 97 and 99% (reciprocally) and 98 and 99% (reciprocally) between MH2-243-1T and MH2-93-1, between MH2-243-1T and MH2-91-1, and between MH2-93-1 and MH2-91-1, respectively, suggesting that these three strains should be classified within the same species.

The G+C contents of the total DNA of strains MH2-243-1T, MH2-93-1 and MH2-91-1 determined by the HPLC method of Tamaoka & Komagata (1984) were 68.5, 68.8 and 68.3 mol%, respectively. In contrast, DNA G+C contents of the type strains of species of the genera *Halobacterium*, *Salarchaeum* and *Halarchaeum* are slightly lower [54.5–66.4, 64, 59.3–66.4 mol%, respectively (Gutiérrez et al., 1989; Gruber et al., 2004; Yang et al., 2006; Shimane et al., 2011; Minegishi et al., 2010a, 2013; Yamauchi et al., 2013a, b; Saralov et al., 2012)]. These data again suggested the three strains represent a novel taxon.

Phenotypic tests were performed according to Minegishi et al. (2010a) and Oren et al. (1997). The analyses were conducted using liquid or solidified MH2 medium at 37 °C. Colony morphology was observed on agar media.
Halorubrum saccharovorum JCM 8865T (AB663419)
Haloferax volcanii JCM 8879T (AB663383)
Halovivax asiaticus JCM 14624T (AB663452)
Haloterrigena turkmenica JCM 9101T (AB663450)
Halococcus morrhuae JCM 8876T (AB663368)
Haloarcula vallismortis JCM 8877T (AB663358)
Salarchaeum japonicum JCM 16327T (AB663478)

**Halocalculus aciditolerans** MH2-243-1T (AB844670)
**Halocalculus aciditolerans** MH2-93-1 (AB844667)
**Halocalculus aciditolerans** MH2-91-1 (AB844666)

Halobacterium salinarum JCM 8978T (AB663362)
Halobacterium jilantaiense JCM 13558T (AB663359)
Halobacterium noricense JCM 15102T (AB663360)
Halarchaeum acidiphilum MH1-52-1T (AB371717)
Halarchaeum salinum MH1-34-1T (AB372514)
Halarchaeum nitratireducens MH1-136-2T (AB372515)
Halarchaeum rubridurum MH1-16-3T (AB372513)
Methanospirillum hungatei JF-1T (CP000254)

Halorubrum saccharovorum JCM 8865T (AB477200)
Halovivax asiaticus JCM 14624T (AB477198)
Haloterrigena turkmenica JCM 9101T (AB477198)
Halococcus morrhuae JCM 8876T (AB477157)
Haloarcula vallismortis JCM 8877T (AB477148)

**Halocalculus aciditolerans** MH2-243-1T (AB917096)
**Halocalculus aciditolerans** MH2-93-1 (AB917095)
**Halocalculus aciditolerans** MH2-91-1 (AB917094)

Halobacterium salinarum JCM 8978T (AB477200)
Halobacterium jilantaiense JCM 13558T (AB477200)
Salarchaeum japonicum JCM 16327T (AB550269)

**Halocalculus aciditolerans** MH2-243-1T (AB917096)
**Halocalculus aciditolerans** MH2-93-1 (AB917095)
**Halocalculus aciditolerans** MH2-91-1 (AB917094)

Halarchaeum acidiphilum MH1-52-1T (AB550268)
Halarchaeum salinum MH1-34-1T (AB917093)
Halarchaeum nitratireducens MH1-136-2T (AB917091)
Halarchaeum rubridurum MH1-16-3T (AB917092)
Halobacterium salinarum JCM 8978T (AB477151)
Halobacterium noricense JCM 15102T (AB550267)
Methanospirillum hungatei JF-1T (CP000254)
Described by Oren et al.

Utilization of sugars and organic acids were assessed as subsulfite. Indole production from tryptophan and the precipitate in medium containing 0.5 % (w/v) sodium glutamic acid and alpha-naphthylamine reagent (Smibert & Krieg, 1978). Reduction of nitrate was detected by using the sulfanilic acid/water (85 : 22.5 : 10 : 4, by vol.) as described previously (Kamekura, 1993). The major polar lipids are archaeol derivatives of phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), derived from both C20C20 and C20C25 archaeol. The strains also contained at least seven glycolipids that have yet to be identified. Those glycolipids were easily distinguishable from those of Salarchaeum japonicum JCM 16327T in their Rf values (Fig. S2). Double spots (C20C20 and C20C25) overlapping with PGP-Me of Salarchaeum japonicum JCM 16327T were identified as sulfated diglycosyl diether-1 (S-DGD-1) (Shimane et al., 2011). Halarchaeum acidiphilum JCM 16109T also possessed glycolipids (C20C20 and C20C25) with Rf values different from those of the three novel strains. Halobacterium salinarum JCM 8978T was unique in possessing sulfated triglycosyl diether (S-TGD) and sulfated tetraglycosyl diether-1 (S-TeGD) of C20C20 archaeol derivatives. Overall, the polar lipid profiles of strains MH2-243-1T, MH2-93-1 and MH2-91-1 were definitely different from those of species of the three nearest described genera.

The above phenotypic and phylogenetic characteristics indicated that strains MH2-243-1T, MH2-93-1 and MH2-91-1 represent a novel species in a new genus within the family Halobacteriaceae, for which the name Halocalculus aciditolerans gen. nov., sp. nov. is proposed.

**Description of Halocalculus gen. nov.**

*Halocalculus* [Ha.lo.cal.'cu.lus. Gr. n. *hals*, *halos* the sea, salt; L. masc. n. *calculus* pebble, gravel; N.L. masc. n. *Halocalculus* salt (-requiring) pebble-shaped archaeon].

Cells are non-motile and pleomorphic under optimal growth conditions and stain Gram-negative. Extremely halophilic, acid-tolerant (able to grow from pH 4.5 to 7.5 with optimum growth at pH 6.0) and mesophilic. Requires at least 1 mM Mg2+ for growth. Cells lyse in distilled water. Chemo-organotrophic, growing on a wide range of substrates, including single carbon sources. The polar lipids are archaeol derivatives of PGP-Me, derived from both C20C20 and C20C25 archaeol. The DNA G+C content is between 68.3 and 68.8 mol%.

The type species is *Halocalculus aciditolerans*.

Recommended three-letter abbreviation: Hcl.

**Description of Halocalculus aciditolerans sp. nov.**

Exhibits the following characteristics in addition to those given in the genus description. Cells are pleomorphic, approximately 0.6–1.0 μm. On agar plates, under optimal conditions pale pink micro-colonies are formed (0.2 mm in diameter). Able to grow from 12–15% to 27–30% (w/v) NaCl (optimum 15–18% NaCl), from pH 4.5 to pH 7.0–7.5 (optimum at pH 6.0) and from 18–20°C to 58–60°C (optimum at 40–45°C). Catalase-positive and oxidase-negative. H2S is not produced from thiosulfate. Indole formation is negative. Reduces nitrate under aerobic conditions. Anaerobic growth with nitrate, arginine and DMSO is observed. Does not hydrolyse starch, gelatin, Tween 80, cellulose, mannose, mannan or casein. Capable of using L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, glyceral, lactose, maltose, D-mannitol, D-mannose, raffinose, α-L-rhamnose, ribose, D-sorbitol, sucrose, trehalose, D-xylose, sodium citrate, sodium pyruvate and sodium succinate as single carbon substrate. Sodium DL-lactate is not utilized as a carbon source. Characteristics that vary amongst strains of the species are shown in Table 1. The major polar lipids are archaeol derivatives of PGP-Me, derived from both C20C20 and C20C25 archaeol; seven unidentified glycolipids are also present.

The type strain is MH2-243-1T (=JCM 19596T =KCTC 4149T) isolated from solar salt produced in Japan. The DNA G+C content of the type strain is 68.5 mol%. MH2-93-1 (=JCM 19595) and MH2-91-1 (=JCM 19594) isolated from commercial salt samples, are additional strains of the species.

Acknowledgements
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References

Table 1. Differential characteristics among strains MH2-243-1T, MH2-93-1 and MH2-91-1 and type species of closely related genera within the family Halobacteriaceae

<table>
<thead>
<tr>
<th>Strains: 1, MH2-243-1T (data from this study); 2, MH2-93-1 (this study); 3, MH2-91-1 (this study); 4, Halarchaeum acidiphilum MH1-52-1T (Minegishi et al., 2010a, 2013); 5, Halobacterium salinarum JCM 8978T (Grant, 2001; Gruber et al., 2004; Gutierrez et al., 1989; Oren et al., 2009; Yachai et al., 2008; Yang et al., 2006); 6, Salarchaeum japonicum YSM-79T (Shimane et al., 2011). +, Positive; –, negative. All genera were positive for cell lysis in distilled water and the presence of phosphatidylglycerol and PGP-Me.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<th>6</th>
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<tbody>
<tr>
<td>Cell shape</td>
<td>Pleomorphic</td>
<td>Pleomorphic</td>
<td>Pleomorphic</td>
<td>Pleomorphic</td>
<td>Rod</td>
<td>Short rod</td>
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<td>Motility</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Pigmentation</td>
<td>Pale pink</td>
<td>Pale pink</td>
<td>Pale pink</td>
<td>Non-pigmented</td>
<td>Red</td>
<td>Clear red</td>
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<tr>
<td>Growth range: NaCl (% w/v)</td>
<td>12–30</td>
<td>12–27</td>
<td>15–27</td>
<td>18–30</td>
<td>&gt;17.5</td>
<td>&gt;9</td>
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<td>Temperature (°C)</td>
<td>20–58</td>
<td>20–58</td>
<td>18–58</td>
<td>15–45</td>
<td>20–60</td>
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<td>pH</td>
<td>4.5–7.0</td>
<td>4.5–7.0</td>
<td>4.5–7.5</td>
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<td>5.5–8.5</td>
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<td>Growth optima: NaCl (% w/v)</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>21–24</td>
<td>20–25</td>
<td>15–18</td>
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<td>–</td>
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<td>68.8</td>
<td>68.3</td>
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<td>61.4</td>
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