Halosarcina limi sp. nov., a halophilic archaeon from a marine solar saltern, and emended description of the genus *Halosarcina*

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A halophilic archaeon, strain RO1-6^T, was isolated from a marine solar saltern in eastern China. Cells of strain RO1-6^T were pleomorphic and motile and stained Gram-negative. Strain RO1-6^T grew well on complex medium and colonies were red-pigmented. It was able to grow at 20–50 °C (optimum 37 °C), in 2.1–5.1 M NaCl (optimum 3.9 M NaCl), in 0.05–0.70 M MgCl₂ (optimum 0.30 M MgCl₂) and at pH 6.5–8.0 (optimum pH 7.0). Cells lysed in distilled water and the minimal NaCl concentration to prevent cell lysis was 12 % (w/v). The major polar lipids of strain RO1-6^T were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and two glycolipids that were chromatographically identical to S-DGD-1 and S₂-DGD. The 16S rRNA gene sequence of strain RO1-6^T showed similarities of 96.9 and 96.4 % to those of the type strains of *Halosarcina pallida* and *Halogeometricum borinquense*, respectively, members of the most closely related recognized genera within the family *Halobacteriaceae*. The DNA G+C content of strain RO1-6^T was 61.2 mol%. Phenotypic characterization and phylogenetic analysis revealed that strain RO1-6^T is related to *Halosarcina pallida* and represents a novel species of the genus *Halosarcina*, for which the name *Halosarcina limi* sp. nov. is proposed; the type strain is RO1-6^T (=CGMCC 1.8711^T =JCM 16054^T).

More than 100 marine solar salterns, artificial shallow ponds for the production of sea salt from seawater, are located in tropical and subtropical offshore areas along 18 000 km of coast of marginal sea in eastern China. These solar salterns mainly consist of three kinds of ponds, evaporation ponds, concentration ponds and crystallizer ponds, in which diverse halotolerant and halophilic micro-organisms survive. Halophilic archaea are often distributed in concentration ponds and crystallizer ponds (Cui *et al.*, 2007; Xu *et al.*, 2007). To understand better the halophilic archaeal diversity of marine solar salterns of eastern China, water and sediment samples were collected from several marine solar salterns and low-nutrient medium was used for the isolation of micro-organisms. The taxonomy of strain RO1-6^T, which represents a novel species in the

genus *Halosarcina* of the family *Halobacteriaceae*, was determined in this study.

Strain RO1-6^T was isolated from saline soil of Rudong marine solar saltern (32.2699° N 121.3999° E), Jiangsu Province, China. The neutral oligotrophic haloarchaeal medium (NOM) used for the isolation procedure was modified according to the DBCM2 medium from the online Halohandbook (Dyall-Smith, 2008) and contained the following ingredients (g l⁻¹): yeast extract, 0.05; fish peptone, 0.25; sodium pyruvate, 1.0; KCl, 5.4; K₂HPO₄, 0.3; CaCl₂, 0.25; NH₄Cl, 0.25; MgSO₄·7H₂O, 26.8; MgCl₂·6H₂O, 23.0; and NaCl, 184.0 (pH adjusted to 7.0–7.2 with 1 M NaOH solution). Strains were routinely grown aerobically at 37 °C in CM2 medium containing (g l⁻¹): Casamino acids (Difco), 0.5; yeast extract (Difco), 0.5; sodium pyruvate, 0.5; fish peptone, 0.5; glucose, 5.0; sodium glutamate, 0.5; trisodium citrate, 3.0; KCl, 2.0; K₂HPO₄, 0.3; CaCl₂, 0.5; MgSO₄·7H₂O, 20; and NaCl, 230.0 (pH 7.0–7.2).

Phenotypic tests were performed according to the proposed minimal standards for the description of novel taxa in the order *Halobacteriales* (Oren *et al.*, 1997). The type strains *Haloferax volcanii* CGMCC 1.2150^T, *Haladaptatus*

Abbreviations: PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester.

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain RO1-6^T is EU931577.

A phase-contrast light micrograph of cells of strain RO1-6^T, TLC plates showing polar lipids and a maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences are available as supplementary material with the online version of this paper.

paucihalophilus JCM 13897^T, *Haloterrigena saccharovitans* CGMCC 1.3730^T, *Halogeometricum* (*Hgm.*) *borinquense* JCM 10706^T and *Halosarcina* (*Hsn.*) *pallida* BZ256^T were selected as reference strains. Cell morphology and motility in exponentially growing liquid cultures were examined using a Leica microscope equipped with phase-contrast optics (model DM LB2). Minimal salt concentration to prevent cell lysis was tested by suspending washed cells in sterile saline solutions containing NaCl concentrations of 0–15 % (w/v) and cell integrity was detected by light microscopic examination.

Gram staining was performed by following the method outlined by Dussault (1955). Most miscellaneous biochemical tests and nutritional tests were performed as described and cited by Bardavid *et al.* (2007). Briefly, growth and gas formation with nitrate as electron acceptor were tested in 10 ml stoppered tubes completely filled with liquid growth medium to which NaNO₃ (5 g l⁻¹) had been added and containing an inverted Durham tube. The formation of nitrite was monitored colorimetrically. Anaerobic growth in the presence of L-arginine and DMSO (5 g l⁻¹) was tested in completely filled 10 ml stoppered tubes. Starch hydrolysis was determined on NOM agar plates supplemented with 2 g soluble starch l⁻¹ and detected by flooding the plates with Lugol's iodine solution. Gelatin hydrolysis was determined by growing colonies on NOM agar plates amended with 0.5 % (w/v) gelatin and flooding the plates with Frazier reagent after growth was established. Esterase activity was detected as outlined by Gutiérrez & González (1972). Tests for catalase and oxidase activities were performed as described by Gonzalez *et al.* (1978). Production of H₂S was tested by growing the isolates and reference strains in tubes containing NOM liquid medium supplemented with 0.5 % (w/v) sodium thiosulfate; a filter-paper strip impregnated with lead acetate was used for H₂S detection (Cui *et al.*, 2007). To test for growth on single carbon sources, fish peptone and sodium pyruvate were omitted from NOM medium and the compound to be tested was added at a concentration of 5 g l⁻¹. Sensitivity to antimicrobial agents was assessed as described by Gutiérrez *et al.* (2008).

Cells of strain RO1-6^T were motile, pleomorphic (rods and deformed cocci; see Supplementary Fig. S1, available in IJSEM Online) when grown in CM2 liquid medium. Cells stained Gram-negative and colonies were red-pigmented. Strain RO1-6^T was able to grow at 20–50 °C (optimum 37 °C), in 2.1–5.1 M NaCl (optimum 3.9 M NaCl), in 0.05–0.70 M MgCl₂ (optimum 0.30 M MgCl₂) and at pH 6.5–8.0 (optimum pH 7.0). Cells lysed in distilled water and the minimum NaCl concentration to prevent cell lysis was 12 % (w/v). Strain RO1-6^T produced H₂S from sodium thiosulfate, but did not produce indole from tryptophan. Tweens 20, 40, 60 and 80, casein and gelatin were not hydrolysed, but starch was hydrolysed weakly. The isolate was able to grow in defined and complex media. The following substrates were utilized as single carbon and energy sources for growth: D-glucose, D-mannose,

D-galactose, sucrose, lactose, starch, glycerol, acetate, pyruvate, DL-lactate, L-alanine and L-glutamate. Acid was produced during growth on carbohydrates. More detailed results of phenotypic tests and nutritional features of strain RO1-6^T are given in the species description. Based on phenotypic properties, strain RO1-6^T is related to *Hsn. pallida* rather than to *Hgm. borinquense*.

Polar lipids were extracted using a chloroform/methanol system and analysed using one- and two-dimensional TLC, as described previously (Kates, 1986). Merck silica gel 60 F₂₅₄ aluminium-backed thin-layer plates were used in TLC analysis. To detect phospholipids, the plate was subjected to two-dimensional development, with chloroform/methanol/water (65:25:4, by vol.) as the first solvent and chloroform/methanol/acetic acid/water (85:12:15:4, by vol.) as the second solvent, followed by spraying with phosphate stain reagent. To detect glycolipids, the plate was subjected to single development in chloroform/methanol/acetic acid/water (85:22.5:10:4, by vol.) and then sprayed with sulfuric acid/ethanol (1:3, v/v) followed by heating at 150 °C for 3 min to detect phospholipids and glycolipids. Strain RO1-6^T contained phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me) and two glycolipids, the major one being chromatographically identical to S-DGD-1 and the minor one chromatographically identical to S₂-DGD (Supplementary Fig. S2). Based on major polar lipid analysis, strain RO1-6^T is similar to *Halosarcina* except for a minor glycolipid spot. The major glycolipid profile also sets strain RO1-6^T apart from *Hgm. borinquense*, which possesses an unidentified non-sulfate-containing glycolipid (Montalvo-Rodríguez *et al.*, 1998).

Genomic DNA from the halophilic archaeal strains was prepared as described by Ng *et al.* (1995). 16S rRNA genes were amplified by PCR using primers 0018F and 1518R (Cui *et al.*, 2009). PCR was performed in a thermal cycler (MJ Research PTC-150) for 30 cycles (5 min denaturation step at 95 °C in the first cycle; 1 min denaturation at 95 °C, 1 min annealing at 60 °C and 1.5 min elongation at 72 °C, with a final extension step at 72 °C for 10 min). PCR products were examined on a 1.0 % (w/v) agarose gel and then cloned into the pEASY-T vector (TransGen Biotech) and transformed into *Escherichia coli* Mach1. Ten transformants were picked randomly and sequenced at the SinoGenoMax Company, Beijing, China, to determine whether the strain possessed multiple distinct 16S rRNA gene sequences. Multiple sequence alignments were performed using the CLUSTAL W program integrated in the MEGA4 software (Tamura *et al.*, 2007). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods in MEGA4. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replications) are shown next to the branches. 16S rRNA gene sequence similarities were calculated by comparison with those of related halophilic archaea from the online EzTaxon server (Chun *et al.*, 2007).

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain RO1-6^T and other close relatives within the family *Halobacteriaceae*. Bootstrap values (>50%) are given at nodes. Bar, 0.02 substitutions per site.

Ten complete 16S rRNA gene sequences (1472 nt each) of strain RO1-6^T were obtained. Sequence comparisons indicated that strain RO1-6^T has a single 16S rRNA gene sequence. The 16S rRNA gene sequence of strain RO1-6^T showed similarities of 96.9 and 96.4% to the type strains of *Hsn. pallida* and *Hgm. borinquense*, members of the closest recognized genera within the family *Halobacteriaceae*. Phylogenetic analysis using the neighbour-joining algorithm revealed that strain RO1-6^T clustered with the *Halogeometricum* and *Halosarcina* clades (Fig. 1). The phylogenetic position was also confirmed in a tree generated using the maximum-parsimony algorithm (Supplementary Fig. S3). The phylogenetic relationship between strain RO1-6^T and members of the genera *Halogeometricum* and *Halosarcina* reveals that they form a paraphyletic group. Based on 16S rRNA gene sequence similarities and its

phylogenetic position, strain RO1-6^T is related phylogenetically to members of the genus *Halosarcina* rather than to those of the genus *Halogeometricum*.

The DNA G+C content was determined by the HPLC method (Mesbah *et al.*, 1989). The DNA G+C content of strain RO1-6^T was 61.2 mol%, which is lower than that of *Hsn. pallida* (65.4 mol%; Savage *et al.*, 2008), but higher than the values reported for *Haloquadratum walsbyi* (46.9 mol%; Burns *et al.*, 2007) and *Hgm. borinquense* (59.1 mol%; Montalvo-Rodríguez *et al.*, 1998).

Based on data from this polyphasic taxonomic study, it is concluded that strain RO1-6^T represents a novel species of the genus *Halosarcina* within the family *Halobacteriaceae*, for which the name *Halosarcina limi* sp. nov. is proposed. Characteristics that distinguish strain RO1-6^T from *Hsn. pallida* BZ256^T and *Hgm. borinquense* JCM 10706^T are shown in Table 1.

Emended description of the genus *Halosarcina* Savage *et al.* 2008

Cells are cocci (sarcina-like clusters) or pleomorphic (rods and deformed cocci) under optimal growth conditions and stain Gram-negative. Cells are motile or non-motile. Aerobic heterotrophs. Colonies are red or pink. Cells lyse in distilled water. Oxidase and catalase tests are positive. Sugars are metabolized, in some cases with formation of acids. Cells contain PG and PGP-Me, but phosphatidylglycerol sulfate is absent. The major glycolipid is chromatographically identical to S-DGD-1. DGD-1 or another glycolipid may be present in some species as a minor component. The type species is *Halosarcina pallida*.

Table 1. Differential characteristics of strain RO1-6^T, *Hsn. pallida* BZ256^T and *Hgm. borinquense* JCM 10706^T

Strains: 1, RO1-6^T; 2, *Hsn. pallida* BZ256^T (data taken from Savage *et al.*, 2008); 3, *Hgm. borinquense* JCM 10706^T (Montalvo-Rodríguez *et al.*, 1998). All three strains are positive for catalase and oxidase.

Characteristic	1	2	3
Cell shape	Pleomorphic	Coccus	Pleomorphic
Motility	+	—	+
Colony colour	Red	Pink	Pink
Optimum NaCl (M)	3.9	3.1	3.1–4.3
Optimum growth temperature (°C)	37	30	40
Optimum pH	7.0	6.5	7.0
Anaerobic growth with nitrate	—	—	+
Gas formation from nitrate	—	—	+
Indole formation	—	+	+
Casein hydrolysis	—	—	+
Gelatin liquefaction	—	—	+
Utilization of fructose	—	+	+
Utilization of xylose	—	—	+
DNA G+C content (mol%)	61.2	65.4	59.1
Glycolipid(s)*	S-DGD-1, S ₂ -DGD	S-DGD-1, DGD-1	UG

*UG, Unidentified glycolipid.

Description of *Halosarcina limi* sp. nov.

Halosarcina limi (li'mi. L. gen. n. *limi* of/from mud).

Displays the following properties in addition to those given in the emended genus description. Cells are motile and pleomorphic (rods and deformed cocci) when grown in CM2 liquid medium. Colonies on agar plates containing 3.9 M NaCl are red, elevated and round. Chemo-organotrophic. Growth occurs at 20–50 °C (optimum 37 °C), in 2.1–5.1 M NaCl (optimum 3.9 M NaCl), in 0.05–0.70 M MgCl₂ (optimum 0.30 M MgCl₂) and at pH 6.5–8.0 (optimum pH 7.0). Minimal NaCl concentration to prevent cell lysis in distilled water is 12 % (w/v). Does not grow under anaerobic conditions with nitrate, arginine or DMSO. Nitrate reduction to nitrite is observed and nitrite is reduced. H₂S is produced from sodium thiosulfate. Negative for indole formation. Tweens 20, 40, 60 and 80, casein and gelatin are not hydrolysed; starch is hydrolysed weakly. Able to grow in defined and complex media. The following substrates are utilized as single carbon and energy sources for growth: D-glucose, D-mannose, D-galactose, sucrose, lactose, starch, glycerol, acetate, pyruvate, DL-lactate, L-alanine and L-glutamate. D-Fructose, L-sorbose, D-ribose, D-xylose, maltose, D-mannitol, D-sorbitol, succinate, L-malate, fumarate, citrate, glycine, L-arginine, L-aspartate, L-lysine and L-ornithine are not utilized as carbon sources. Acid is produced during growth on carbohydrates. Sensitive to the following antibiotics (µg per disc, unless otherwise indicated): rifampicin (5), novobiocin (30), bacitracin (0.04 IU), anisomycin (20) and aphidicolin (20). Resistant to the following antibiotics: erythromycin (15), neomycin (30), chloramphenicol (30), ampicillin (10), penicillin G (10 IU), norfloxacin (10), ciprofloxacin (5), streptomycin (10), kanamycin (30), tetracycline (30), vancomycin (30), gentamicin (10) and nalidixic acid (30). The major polar lipids are PG, PGP-Me and two major glycolipids that are chromatographically identical to S-DGD-1 and S₂-DGD.

The type strain is RO1-6^T (=CGMCC 1.8711^T =JCM 16054^T), isolated from Rudong solar saltern, Jiangsu Province, China. The DNA G+C content of the type strain is 61.2 mol% (as determined by HPLC).

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