Haladaptatus litoreus sp. nov., an extremely halophilic archaeon from a marine solar saltern, and emended description of the genus *Haladaptatus*

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Two extremely halophilic archaea, strains RO1-28^T and RO1-22, were isolated from a marine solar saltern in Jiangsu, China. Both strains required at least 0.05 M Mg²⁺ and 1.7 M NaCl for growth. They were able to grow over a pH range of 6.0–8.5 and a temperature range of 25–55 °C, with optimal pH of 7.0 and optimal temperature of 37–40 °C. Based on 16S rRNA gene sequence analysis, strains RO1-28^T and RO1-22 were closely related to *Haladaptatus paucihalophilus*, the single species of the genus *Haladaptatus*, with similarities of 94.0–95.2%. The major polar lipids of the two strains were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate and three glycolipids chromatographically identical to the glycolipids of *Haladaptatus paucihalophilus* JCM 13897^T. Both strains RO1-28^T and RO1-22 had a DNA G + C content of 54.0 mol% (HPLC). The DNA–DNA hybridization value between the two strains was more than 70% (92%) and both strains showed low levels of DNA–DNA relatedness (32% and 33%) with *Haladaptatus paucihalophilus* JCM 13897^T. It was concluded that strains RO1-28^T and RO1-22 represent a novel species of the genus *Haladaptatus*, for which the name *Haladaptatus litoreus* sp. nov. is proposed. The type strain is RO1-28^T (=CGMCC 1.7737^T =JCM 15771^T).

Extremely halophilic aerobic archaea, members of the order *Halobacteriales*, have been mainly isolated from diverse hypersaline environments such as salt lakes, artificial crystallizer ponds of marine solar salterns, salty fermented food and salted hides (Oren, 2006). A few of them can also be found in low-salt habitats such as low-salt, sulfide-rich springs and seashore marshes (Purdy *et al.*, 2004; Savage *et al.*, 2007, 2008). The genus *Haladaptatus* was first proposed by Savage *et al.* to accommodate the species *Haladaptatus paucihalophilus*, which is coccus- or coccobacillus-shaped and was isolated from Zodletone

Spring, a sulfide- and sulfur-rich but low NaCl concentration spring in south-western Oklahoma, USA (Savage *et al.*, 2007). Strains of *Haladaptatus paucihalophilus* can grow in a wide range of salt concentrations (0.8–5.1 M) and their cells remain viable in distilled water after prolonged incubation, providing evidence that they are adapted to relatively low-salt systems or fluctuating salt concentrations. Each strain of *Haladaptatus paucihalophilus* possesses at least two heterogeneous 16S rRNA gene sequences with 2.7–4.1% divergence. During our surveys on halophilic archaeal diversity of marine solar salterns of Eastern China, two halophilic archaeal isolates related to *Haladaptatus paucihalophilus* were obtained. In this study, we characterize the two strains, RO1-28^T and RO1-22, as a new species of the genus.

Strains RO1-28^T and RO1-22 were isolated from sediment of the Rudong solar saltern (32.2699° N 121.3999° E) in Jiangsu province, China. The neutral oligotrophic haloarchaeal medium (NOM) used for the isolation pro-

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

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains RO1-28^T and RO1-22 are EU887285 and FJ773394, respectively.

Scanning electron micrographs and thin-layer chromatograms are available with the online version of this paper.

cedure was modified according to the DBCM2 medium from the online Halohandbook (Dyall-Smith, 2008) and contained the following ingredients (l^{-1}): yeast extract, 0.05 g; fish peptone, 0.25 g; sodium pyruvate, 1.0 g; KCl, 5.4 g; K_2HPO_4 , 0.3 g; $CaCl_2$, 0.25 g; NH_4Cl , 0.25 g; $MgSO_4 \cdot 7H_2O$, 26.8 g; $MgCl_2 \cdot 6H_2O$, 23.0 g; NaCl, 184.0 g (pH adjusted to 7.0–7.2 with 1 M NaOH solution). The medium was solidified with 2.0% agar. Strains were routinely grown aerobically at 37 °C in a modified R2A medium (MR2A) containing the following ingredients (l^{-1}): Casamino acids (Difco), 0.5 g; yeast extract (Difco), 0.5 g; sodium pyruvate, 0.5 g; fish peptone, 0.5 g; glucose, 0.5 g; sodium glutamate, 0.5 g; trisodium citrate, 3.0 g; KCl, 2.0 g; K_2HPO_4 , 0.3 g; $CaCl_2$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 20 g; NaCl, 200.0 g (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 *Haladaptatus paucihalophilus* JCM 13897^T, *Haloferax volcanii* CGMCC 1.2150^T and *Haloplanus natans* JCM 14081^T were selected as reference strains in positive and negative testing. Cell morphology and motility in exponentially growing liquid cultures were examined using a Leica microscope equipped with phase-contrast optics (model DM LB2). For scanning electron microscopy examination, 0.5 ml samples were fixed overnight at 4 °C by adding glutaraldehyde to a final concentration of 5.0%. A 10 µl sample was smeared on a polylysine-coated coverslip, and air-dried. The coverslip was then serially dehydrated in 40, 70, 90 and 100% ethanol solutions (10 min at each stage), critical-point dried, mounted on a specimen stub, sputter-coated with gold and viewed in a Hitachi S-4800 scanning electron microscope.

The Gram stain was performed by following the method outlined by Dussault (1955). Growth and gas formation with nitrate as electron acceptor were tested in 10 ml stoppered tubes, completely filled with liquid growth medium to which $NaNO_3$ (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 MR2A agar plates supplemented with 2 g soluble starch per litre and detected by flooding the plates with Lugol's iodine solution. Gelatin hydrolysis was performed by growing colonies on MR2A 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 H_2S was tested by growing the isolates and reference strains in a tube with the MR2A liquid medium supplemented with 0.5% (w/v) $Na_2S_2O_3$; a filter-paper strip impregnated with lead acetate was used for H_2S detection. The other miscellaneous biochemical tests and nutrition tests were performed as described and cited by

Bardavid *et al.* (2007). Sensitivity to antimicrobial agents was performed as described by Gutiérrez *et al.* (2008).

Cells of strains RO1-28^T and RO1-22 were non-motile, coccus-shaped (1.0–1.5 µm) (Supplementary Fig. S1, available in IJSEM Online), Gram-stain-negative and were able to grow in a wide range of salinities (1.7–5.1 M NaCl; optimal growth at 3.4 M and 4.3 M, respectively). Cells did not lyse in distilled water and remained viable under these conditions for up to 2 weeks. Colonies on MR2A agar were pink-pigmented. Both strains utilized D-glucose, D-mannose, maltose, D-galactose and sucrose but not D-fructose or lactose as carbon sources for growth. The novel strains were not able to grow with DMSO, nitrate, nitrite or arginine under anaerobic conditions. More detailed results of phenotypic tests and nutritional features of strains RO1-28^T and RO1-22 are given in the species description. Characteristics that distinguish strains RO1-28^T and RO1-22 from the two described strains of *Haladaptatus paucihalophilus* are shown in Table 1.

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. The plate dotted with sample for detecting phospholipids was subjected to two-dimensional development, with the first solvent of chloroform/methanol/water (65:25:4, by vol.) and followed by the second solvent of chloroform/methanol/acetic acid/water (85:12:15:4, by vol.). The plate for detecting glycolipids was subjected to single development in the solvent chloroform/methanol/acetic acid/water (85:22.5:10:4, by vol.). Strains RO1-28^T and RO1-22 contained phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS) and three glycolipids, in a pattern chromatographically identical to the polar lipid profile of *Haladaptatus paucihalophilus* JCM 13897^T (see Supplementary Fig. S2). Among the three glycolipids, one of them (GL2) was chromatographically identical to S-DGD-1 of *Haloferax volcanii* CGMCC 1.2150^T and *Haloplanus natans* JCM 14081^T, the remaining two (GL1 & GL3) were still unidentified (see Supplementary Fig. S2b, c). The polar lipid composition supports classification of strains RO1-28^T and RO1-22 in the genus *Haladaptatus*.

Genomic DNA from halophilic archaeal strains was prepared as described by Ng *et al.* (1995). The 16S rRNA gene was amplified by PCR, using primers 0018F and 1518R (Cui *et al.*, 2009). The amplified products were cloned into the pEASY-T vector (TransGen Biotech) and transformed into *Escherichia coli* Mach1. Thirty clones of each novel strain were randomly picked and sequenced at the Sino-GenoMax Company Limited (Beijing, China), to determine whether the two strains possessed multiple distinct 16S rRNA gene sequences. Multiple sequence alignments were performed using the CLUSTAL W program integrated into the MEGA4 software (Tamura *et al.*, 2007).

Table 1. Characteristics that distinguish strains RO1-28^T and RO1-22 from *Haladaptatus paucihalophilus* JCM 13897^T and GY252

Strains: 1, Strain RO1-28^T; 2, Strain RO1-22; 3, *Haladaptatus paucihalophilus* DX253^T = JCM 13897^T; 4, *Haladaptatus paucihalophilus* GY252. +, Positive; –, negative; NR, not reported; w, weakly positive. The table is based on direct comparison of the phenotypic properties of the type strains, as well as on published data for the two strains of *Haladaptatus paucihalophilus* (Savage *et al.*, 2007).

Characteristic	1	2	3	4
Optimum NaCl (M)	3.4	4.3	3.1	2.6
NaCl range (M)	1.7–5.1	1.7–5.1	0.8–5.1	0.8–5.1
Optimum growth temperature (°C)	37–40	37–40	30	25
Optimum pH	7.0	7.0	6.5	6.0
pH range	6.0–8.5	6.0–8.5	5.0–7.5	5.0–7.5
Nitrate reduction	+	+	–	–
Utilization of:				
Fructose	–	–	+	+
Xylose	–	–	+	+
Mannitol	+	–	+	+
L-Malate	–	–	+	+
Citrate	–	–	+	+
L-Alanine	+	w	–	–
L-Arginine	+	–	–	–
L-Aspartate	+	+	–	–
DNA G + C content (mol%)	54.0	54.0	60.5 (59.2*)	NR

*Determined in this study.

Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods in the MEGA4 software. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were calculated. 16S rRNA gene sequence similarity was calculated by comparison with sequences of related halophilic archaea from the online EzTaxon server (Chun *et al.*, 2007).

Thirty complete 16S rRNA gene sequences (1472 nt each) of strains RO1-28^T and RO1-22 were obtained. Sequence comparisons indicated that both strains had only one kind of 16S rRNA gene sequence; they were 99.7 % similar to each other. Both strains were closely related to *Haladaptatus paucihalophilus*, the single species of the genus *Haladaptatus*, with similarities of 94.0–95.2 %. Phylogenetic analysis using the neighbour-joining algorithm revealed that strain RO1-28^T and strain RO1-22 clustered with *Haladaptatus paucihalophilus*, forming a branch in the cluster with a bootstrap value of 100 % (Fig. 1). The phylogenetic position was also confirmed in a tree generated using the maximum-parsimony algorithm (see Supplementary Fig. S3).

The DNA G + C content was determined by using the HPLC method of Mesbah *et al.*, (1989). DNA–DNA hybridization analyses were performed according to the thermal denaturation and renaturation method of De Ley *et al.* (1970) as modified by Huß *et al.* (1983). Both strains RO1-28^T and RO1-22 had a DNA G + C content of

54.0 mol%, a relatively low value when compared with *Haladaptatus paucihalophilus* (60.5 mol% reported in the literature; 59.2 mol% determined in this study). The DNA relatedness between strains RO1-28^T and RO1-22 was 92 %. The data show that the two strains should be classified as members of the same species, since the generally accepted threshold value to separate two species is 70 % (Stackebrandt & Goebel, 1994). The DNA relatedness between strains RO1-28^T and RO1-22, and *Haladaptatus paucihalophilus* JCM 13897^T was 32 % and 33 %, respectively.

On the basis of their phenotypic and chemotaxonomic characteristics, including low levels of DNA–DNA relatedness to *Haladaptatus paucihalophilus* JCM 13897^T, strains RO1-28^T and RO1-22 are considered to represent a novel species of the genus *Haladaptatus*, for which the name *Haladaptatus litoreus* sp. nov. is proposed.

Emended description of the genus *Haladaptatus* Savage *et al.* 2007

Gram-negative cocci or coccobacilli occurring singly or in pairs. Cells did not lyse in distilled water. Colonies are pink-pigmented. Some species possess at least two heterogeneous 16S rRNA gene sequences. Cells contain PG, PGP-Me and PGS. Three glycolipids are present; one of them is chromatographically identical to S-DGD-1, the others are unidentified. Chemo-organotrophic, growing on a wide range of substrates, including single and complex carbon sources. Produce acid from carbohydrates.

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between the members of the genus *Haladaptatus* and related genera within the family *Halobacteriaceae*. Bootstrap values (%) are based on 1000 replicates and are shown for branches with more than 70% bootstrap support. Bar, 0.02 substitutions per nucleotide position.

Hydrolyse casein, starch, gelatin and Tween 80. Grow at a wide range of NaCl concentrations. Sensitive to novobiocin, bacitracin and rifampicin. Resistant to erythromycin, penicillin, ampicillin, chloramphenicol, neomycin, nalidixic acid and gentamicin. The DNA G+C content is between 54.0 and 60.5 mol%. Isolated from low-salt, sulfide- and sulfur-rich spring and saltern crystallizer ponds. The type species is *Haladaptatus paucihalophilus*.

Description of *Haladaptatus litoreus* sp. nov.

Haladaptatus litoreus (li.to're.us. L. masc. adj. *litoreus* living near the sea, of or belonging to the seashore).

Cells are non-motile, coccus-shaped (1.0–1.5 µm) and Gram-stain-negative. Colonies on MR2A agar plates containing 3.4 M NaCl are pink, elevated and round. Chemo-organotrophic and aerobic. Growth occurs at NaCl concentrations of 1.7–5.1 M, at Mg²⁺ concentrations of 0.05–0.7 M, at pH values in the range 6.0–8.5, and within the temperature range 25–55 °C. Optimal NaCl concentration, pH and temperature for growth are 3.4 M, 7.0 and 37–40 °C, respectively. Catalase- and oxidase-positive. Does not grow under anaerobic conditions with nitrate, arginine and DMSO. Nitrate reduction to nitrite is observed. H₂S is not produced from Na₂S₂O₃. Positive for indole formation. Tweens 20, 40, 60 and 80 are hydrolysed. Positive for caseinase, amylase and gelatinase. The following substrates are utilized as carbon sources for growth: D-glucose, D-mannose, D-galactose, maltose, sucrose, starch, glycerol, D-mannitol, acetate, pyruvate, DL-lactate, fumarate, L-alanine, L-arginine, L-aspartate, L-glutamate and L-ornithine. D-Fructose, L-sorbose, D-ribose, D-xylose, lactose, D-sorbitol, succinate, L-malate, citrate, glycine and L-lysine are not utilized as carbon sources. Produces acid when grown on D-glucose, D-galactose, sucrose, starch, maltose and glycerol. Sensitive to the following antibiotics (µg or IU per disc): rifampicin (5), novobiocin (30), bacitracin (0.04 IU) and norfloxacin (10). Resistant to the following antibiotics: erythromycin (15), neomycin (30), chloramphenicol (30), ampicillin (10), penicillin G (10 IU), ciprofloxacin (5), streptomycin (10), kanamycin (30), tetracycline (30), vancomycin (30), gentamicin (10) and nalidixic acid

(30). The major polar lipids are PG, PGP-Me, PGS and three glycolipids, similar to the polar lipid profile of *Haladaptatus paucihalophilus* JCM 13897^T. The DNA G+C content of the type strain is 54.0% (HPLC).

The type strain is RO1-28^T (=CGMCC 1.7737^T =JCM 15771^T), and was isolated from Rudong solar saltern in Jiangsu province, China.

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