Halogranum gelatinilyticum sp. nov. and *Halogranum amylolyticum* sp. nov., isolated from a marine solar saltern, and emended description of the genus *Halogranum*

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Two extremely halophilic archaeal strains, designated TNN44^T and TNN58^T, were isolated from Tainan marine solar saltern near Lianyungang city, Jiangsu province, China. Cells of the two strains were pleomorphic and Gram-stain-negative; colonies were red-pigmented. Strains TNN44^T and TNN58^T were able to grow at 20–50 °C (optimum 37 °C for both), in the presence of 1.4–5.1 M NaCl (optimum 3.4–3.9 M NaCl) and at pH 5.5–9.0 (optimum pH 6.5–7.0); neither strain required Mg²⁺ for growth. Cells lysed in distilled water. On the basis of 16S rRNA gene sequence analysis, strains TNN44^T and TNN58^T were related closely to *Halogranum rubrum* RO2-11^T (96.2 and 97.2 % similarity, respectively). The polar lipids of the two strains were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate (trace), and one major glycolipid and one minor glycolipid chromatographically identical to sulfated mannosyl glucosyl diether and mannosyl glucosyl diether, respectively; other trace unidentified lipids were also detected. The DNA G + C content of strains TNN44^T and TNN58^T was 64.0 and 62.0 mol%, respectively. The level of DNA–DNA relatedness between strains TNN44^T and TNN58^T was 37.2 %, and these two strains showed a low level of DNA–DNA relatedness with *Halogranum rubrum* RO2-11^T (40.6 and 44.4 %, respectively). Two novel species of the genus *Halogranum* are proposed to accommodate these two strains, *Halogranum gelatinilyticum* sp. nov. (type strain TNN44^T = CGMCC 1.10119^T = JCM 16426^T) and *Halogranum amylolyticum* sp. nov. (type strain TNN58^T = CGMCC 1.10121^T = JCM 16428^T).

Marine solar salterns, man-made shallow ponds for the production of halite from seawater, serve as comfortable habitats for diverse halophilic micro-organisms. Among these halophilic micro-organisms, halophilic archaea are probably the dominant microbes at saltern crystallizer ponds (Oren, 2002). To understand better the biogeographical distribution and diversity of halophilic archaeal communities within different marine solar salterns along the coastline of eastern China (Cui *et al.*, 2010), brine and sediment samples were collected from Tainan marine solar saltern (34° 35′ 38″ N 119° 28′ 56″ E) near Lianyungang city, Jiangsu province, and their halophilic archaeal

diversity was examined based on analysis of a library of PCR-amplified 16S rRNA genes and by cultivation. Of 65 isolates examined, one strain showed 99.9 % 16S rRNA gene sequence similarity to the type strain of *Salinibacter ruber* (Antón *et al.*, 2002); the other 64 strains belonged to the family *Halobacteriaceae*. Two of these halophilic archaeal strains, designated TNN44^T and TNN58^T, were closely related to *Halogranum rubrum*, the single member of the genus *Halogranum*. Based on the data presented in this study, we suggest that strains TNN44^T and TNN58^T represent two novel species of the genus *Halogranum*.

Neutral oligotrophic haloarchaeal medium (NOM) was used for isolation, and comprised (per litre): 0.05 g yeast extract, 0.25 g fish peptone, 1.0 g sodium pyruvate, 5.4 g KCl, 0.3 g K₂HPO₄, 0.25 g CaCl₂, 0.25 g NH₄Cl, 26.8 g MgSO₄ · 7H₂O, 23.0 g MgCl₂ · 6H₂O and 184.0 g NaCl (pH adjusted to 7.0–7.2 with 1 M NaOH solution) (Cui *et al.*, 2010). The medium was solidified with 2.0 % agar. The strains were routinely grown aerobically at 37 °C in NOM-3 medium (NOM series medium) with the following modifications (per litre): 1.0 g yeast extract, 0.25 g fish

Abbreviations: PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester; PGS, phosphatidylglycerol sulfate; (S)-DGD, (sulfated) mannosyl glucosyl diether.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains TNN44^T and TNN58^T are GQ282624 and GQ282623, respectively.

Three supplementary figures are available with the online version of this paper.

peptone, 0.25 g sodium formate, 0.25 g sodium acetate, 0.25 g sodium lactate and 0.25 g sodium pyruvate.

Phenotypic tests were performed according to the proposed minimal standards for description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). *Halogramma rubrum* RO2-11^T, *Haloferax elongans* JCM 14791^T and *Halobacterium salinarum* CGMCC 1.2367 were selected as reference strains in subsequent tests. Cell morphology and motility in exponentially growing liquid cultures were examined by using a Nikon microscope equipped with phase-contrast optics (model E400). Minimal salt concentration to prevent cell lysis was tested by suspending washed cells in serial sterile saline solutions containing NaCl at 0–15% (w/v), and the stability of the cells was detected by light microscopy.

Gram staining was performed by following the method outlined by Dussault (1955). Most miscellaneous biochemical tests and nutritional tests were performed as described and proposed by Oren *et al.* (1997). Briefly, growth and gas formation with nitrate as electron acceptor were tested in 9 ml stoppered tubes, completely filled with liquid NOM medium to which NaNO₃ (5 g l⁻¹) had been added, and containing an inverted Durham tube. The formation of gas from nitrate was detected based on the presence of gas bubbles in Durham tubes, and 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 9 ml stoppered tubes. Starch hydrolysis was determined on NOM agar plates supplemented with soluble starch (2 g l⁻¹) and was detected by flooding the plates with Lugol's iodine solution. Gelatin hydrolysis was performed by growing colonies on NOM agar plates amended with 1% (w/v) gelatin and flooding the plates with Frazier's reagent (McDade & Weaver, 1959) 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 a tube with the 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 the NOM medium and the compound to be tested was added at a concentration of 5 g l⁻¹. Antibiotic susceptibilities were determined according to the method described by Gutiérrez *et al.* (2008) on NOM agar plates with discs containing the following antibiotics (µg per disc, unless otherwise indicated): ampicillin (10), anisomycin (20), aphidicolin (20), bacitracin (0.04 IU per disc), chloramphenicol (30), ciprofloxacin (5), erythromycin (15), gentamicin (10), kanamycin (30), nalidixic acid (30), neomycin (30), norfloxacin (10), novobiocin (30), penicillin G (10 IU per disc), rifampicin (5), streptomycin (10), tetracycline (30) and vancomycin (30).

Polar lipids were extracted by using a chloroform/methanol system and were analysed via one- and two-dimensional TLC, as described by Kates (1986). Merck silica gel 60 F₂₅₄ aluminium-backed thin-layer plates were used in TLC analysis. The plate for detecting phospholipids was subjected to two-dimensional development, with the first solvent of chloroform/methanol/water (65:25:4, by vol.) followed by the second solvent of chloroform/methanol/acetic acid/water (80:12:15:4, by vol.), then sprayed with the phosphate stain reagent (Vaskovsky & Kostetsky, 1968). To determine the phospholipid and glycolipid composition of each strain extract, the two-dimensional development plate was sprayed with sulfuric acid/ethanol (1:2, v/v) followed by heating at 150 °C for 3 min to detect lipids. To compare the polar lipid composition of the two novel strains with the reference strains, the plate was subjected to single development in the above-mentioned second dimensional development solvent, and then sprayed with the same sulfuric acid/ethanol reagent followed by heating at 150 °C for 3 min to detect lipids.

Genomic DNA from halophilic archaeal strains was prepared as described by Ng *et al.* (1995). The 16S rRNA gene was amplified via PCR by 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 denaturing at 95 °C in the first cycle; 1 min denaturing at 95 °C, 1 min annealing at 60 °C and 1.5 min elongation at 72 °C; final extension step at 72 °C for 10 min). The 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 cells of *Escherichia coli* Mach1. Sixteen transformants were randomly picked and sequenced at the Sino-GenoMax Company Limited (Beijing, China), to determine whether the two novel strains possessed multiple distinct 16S rRNA gene sequences. Multiple sequence alignments were performed by using the CLUSTAL W program integrated in MEGA 4 software (Tamura *et al.*, 2007). Phylogenetic trees were reconstructed with the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) algorithms in MEGA 4 software. The percentages of trees in which the associated taxa clustered together in the bootstrap test were determined based on 1000 replicates. Levels of 16S rRNA gene sequence similarity were calculated by comparison with sequences of related halophilic archaea from the online EzTaxon server (Chun *et al.*, 2007). The DNA G+C content was determined by the HPLC method (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).

Cells of strains TNN44^T and TNN58^T were pleomorphic short rods, irregular cocci and ovals when grown in NOM liquid medium (see Supplementary Fig. S1 available in IJSEM Online). Cells of strain TNN44^T were motile, while those of strain TNN58^T were not. Cells stained Gram-negative and colonies were red-pigmented. Strains TNN44^T

and TNN58^T were able to grow at 20–50 °C (optimum 37 °C for both), in the presence of 1.4–5.1 M NaCl (optimum 3.4–3.9 M NaCl) and at pH 5.5–9.0 (optimum pH 6.5–7.0); neither strain required Mg²⁺ for growth. Cells lysed in distilled water and the minimal NaCl concentrations to prevent cell lysis were 10 % (w/v) for strain TNN44^T and 8 % (w/v) for strain TNN58^T. The two strains produced indole from tryptophan, but did not produce H₂S from sodium thiosulfate. Strain TNN44^T hydrolysed gelatin and starch (the latter only weakly); by contrast, strain TNN58^T did not hydrolyse gelatin but hydrolysed starch strongly. The two strains did not hydrolyse Tween 80 or casein. They were able to grow in defined and complex media; D-glucose, D-mannose, D-galactose, sucrose, lactose, glycerol, acetate, pyruvate and DL-lactate as single carbon sources yielded the best growth. Acid was produced during growth on some carbohydrates. Detailed results of phenotypic tests and nutritional characteristics of the two strains are given in the species descriptions below.

The polar lipids of the two strains were phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), trace phosphatidylglycerol sulfate (PGS), one major glycolipid (GL1) and a minor glycolipid (GL2) chromatographically identical to sulfated mannosyl glucosyl diether (S-DGD-1) and mannosyl glucosyl diether (DGD-1), respectively; other trace unidentified glycolipids (GL3, GL4, GL5 and GL6) were also detected (Supplementary Fig. S2). The polar lipid profiles of the two strains were largely chromatographically identical to those of *Halogramum rubrum* RO2-11^T (Supplementary Fig. S2). The polar lipid composition thus supports the classification of strains TNN44^T and TNN58^T in the genus *Halogramum*.

Sixteen complete 16S rRNA gene sequences of strains TNN44^T and TNN58^T were obtained. Comparisons indicated that the two strains each have one type of 16S rRNA gene sequence, and that they share 96.4 % similarity. The two strains were related closely to *Halogramum rubrum* RO2-11^T (96.2 and 97.2 % 16S rRNA gene sequence similarity, respectively). Phylogenetic analysis using the neighbour-joining algorithm revealed that strains TNN44^T and TNN58^T clustered tightly with *Halogramum rubrum* RO2-11^T (Fig. 1). This phylogenetic position was also confirmed in the tree generated with the maximum-parsimony

algorithm (Supplementary Fig. S3). Phylogenetic analysis based on 16S rRNA gene sequences thus also supported the placement of strains TNN44^T and TNN58^T in the genus *Halogramum*.

The DNA G+C content of strains TNN44^T and TNN58^T were 64.0 and 62.0 mol%, respectively. These values are higher than those reported for *Halogramum rubrum* (55.7–57.6 mol%) (Cui *et al.*, 2010). The level of DNA–DNA relatedness between strains TNN44^T and TNN58^T was 37.2 %, showing that the two strains should be classified as representing two different species, as the generally accepted threshold value to separate two species is 70 % (Stackebrandt & Goebel, 1994). Strains TNN44^T and TNN58^T showed low levels of DNA–DNA relatedness to *Halogramum rubrum* RO2-11^T (40.6 and 44.4 %, respectively), which indicated that the two strains are related more closely to *Halogramum rubrum* RO2-11^T than to each other at the DNA–DNA hybridization level and represent two distinct species of the genus *Halogramum*.

This polyphasic taxonomic study provides evidence that strains TNN44^T and TNN58^T represent two novel species of the genus *Halogramum*, for which the names *Halogramum gelatinilyticum* sp. nov. (type strain TNN44^T = CGMCC 1.10119^T = JCM 16426^T) and *Halogramum amylolyticum* sp. nov. (type strain TNN58^T = CGMCC 1.10121^T = JCM 16428^T) are proposed. Characteristics that distinguish strains TNN44^T and TNN58^T from *Halogramum rubrum* RO2-11^T are shown in Table 1.

Emended description of the genus *Halogramum* Cui *et al.* 2010

Cells are pleomorphic under optimal growth conditions and stain Gram-negative. Aerobic heterotrophs. Cells lyse in distilled water. Oxidase- and catalase-positive. Extremely halophilic, with growth occurring in media containing 1.4–5.1 M NaCl; most strains grow best at 3.4–3.9 M NaCl. The optimum magnesium concentration varies between 0.05 and 0.3 M. Growth is observed at 20–50 °C and at pH 5.5–9.0. Sugars are metabolized, in some cases with the formation of acids. The polar lipids are PG, PGP-Me, trace PGS, and one major glycolipid and one minor glycolipid chromatographically identical to S-DGD-1 and DGD-1, respectively; other trace unidentified glycolipids may be present. The genomic DNA G+C content is between 55.7

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strains TNN44^T and TNN58^T and other close relatives within the family Halobacteriaceae. Bootstrap values (percentages) are based on 1000 replicates and are shown for branches with more than 50 % support. Bar, 0.02 substitutions per nucleotide position.

Table 1. Characteristics that distinguish strains TNN44^T and TNN58^T from *Halogranum rubrum* RO2-11^T

Strains: 1, TNN44^T; 2, TNN58^T; 3, *Halogranum rubrum* RO2-11^T (data from Cui *et al.*, 2010). +, Positive; –, negative; w, weakly positive.

Characteristic	1	2	3
Motility	+	–	+
Minimal salt concentration to prevent cell lysis (%)	10	8	12
Optimum NaCl (M)	3.4–3.9	3.4	3.9
Mg ²⁺ required	–	–	+
Optimum pH	7.0	6.5	7.5
Reduction of nitrate to nitrite	–	+	+
Utilization of:			
Maltose	–	+	+
Lactose	w	+	+
D-Mannitol	–	+	–
D-Sorbitol	w	+	+
Succinate	–	+	+
L-Malate	–	+	+
Fumarate	–	+	+
Glycine	–	+	–
L-Alanine	+	+	w
L-Ornithine	+	+	w
Indole formation	+	+	–
Starch hydrolysis	w	+	w
Gelatin hydrolysis	+	–	+
Tween 80 hydrolysis	–	–	+
H ₂ S formation	–	–	+
DNA G + C content (mol%)	64.0	62.0	55.7

and 64.0 mol%. Isolated from marine solar salterns. The type species is *Halogranum rubrum*.

Description of *Halogranum gelatinilyticum* sp. nov.

Halogranum gelatinilyticum [ge.la.ti.ni.ly'ti.cum. N.L. neut. n. *gelatinum* gelatin; N.L. neut. adj. *lyticum* (from Gr. neut. adj. *lutikon*) able to loosen, able to dissolve; N.L. neut. adj. *gelatinilyticum* gelatin-dissolving, referring to the property of being able to hydrolyse gelatin].

Cells are motile, pleomorphic – showing different shapes (short rods, irregular cocci and ovals, mean cell dimensions 1–2 µm) under optimal growth conditions – and stain Gram-negative. Colonies on agar plates containing 3.4–3.9 M NaCl are red, elevated and round. Chemo-organotrophic and aerobic. Growth occurs at 20–50 °C (optimum 37 °C), in the presence of 1.7–5.1 M NaCl (optimum 3.4–3.9 M NaCl) and 0–1.0 M MgCl₂ (optimum 0.1 M MgCl₂), and at pH 5.5–9.0 (optimum pH 7.0). Cells lyse in distilled water and the minimal NaCl concentration to prevent cell lysis is 10% (w/v). Catalase- and oxidase-positive. Does not grow under anaerobic conditions with nitrate, arginine or DMSO.

Nitrate is not reduced to nitrite. H₂S is not produced from sodium thiosulfate. Indole formation is positive. Hydrolyses gelatin and starch (the latter only weakly) but not casein or Tween 80. The following substrates are utilized as single carbon and energy sources for growth: D-glucose, D-mannose, D-galactose, sucrose, lactose, starch, glycerol, D-sorbitol, acetate, pyruvate, DL-lactate, L-alanine, L-glutamate and L-ornithine. No growth occurs on D-fructose, L-sorbose, D-ribose, D-xylose, maltose, D-mannitol, succinate, L-malate, fumarate, citrate, glycine, L-arginine, L-aspartate or L-lysine. Acid is produced from D-glucose, D-mannose, D-galactose, sucrose and lactose. Sensitive to the following antibiotics (µg per disc, unless otherwise indicated): novobiocin (30), bacitracin (0.04 IU per disc), anisomycin (20), aphidicolin (20) and rifampicin (5). Resistant to the following antibiotics: erythromycin (15), penicillin G (10 IU per disc), ampicillin (10), chloramphenicol (30), neomycin (30), norfloxacin (10), ciprofloxacin (5), streptomycin (10), kanamycin (30), tetracycline (30), vancomycin (30), gentamicin (10) and nalidixic acid (30). The polar lipids are PG, PGP-Me, trace PGS, and one major glycolipid and one minor glycolipid chromatographically identical to S-DGD-1 and DGD-1, respectively; three trace unidentified glycolipids may be present. The DNA G + C content of the type strain is 64.0 mol% (as determined by HPLC).

The type strain, TNN44^T (=CGMCC 1.10119^T =JCM 16426^T), was isolated from Tainan marine solar saltern near Lianyungang city, Jiangsu province, China.

Description of *Halogranum amylolyticum* sp. nov.

Halogranum amylolyticum [a.my.lo.ly'ti.cum. Gr. n. *amylon* starch; N.L. neut. adj. *lyticum* (from Gr. neut. adj. *lutikon*) able to loosen, able to dissolve; N.L. neut. adj. *amylolyticum* starch-dissolving, referring to the property of being able to hydrolyse starch].

Cells are non-motile, pleomorphic – showing different shapes (irregular cocci and ovals, mean cell dimensions 1–2 µm) under optimal growth conditions – and stain Gram-negative. Colonies on agar plates containing 3.4 M NaCl are red, elevated and round. Chemo-organotrophic and aerobic. Growth occurs at 20–50 °C (optimum 37 °C), in the presence of 1.4–5.1 M NaCl (optimum 3.4 M NaCl) and 0–1.0 M MgCl₂ (optimum 0.05 M MgCl₂), and at pH 5.5–9.0 (optimum pH 6.5). Cells lyse in distilled water and the minimal NaCl concentration to prevent cell lysis is 8% (w/v). Catalase- and oxidase-positive. Does not grow under anaerobic conditions with nitrate, arginine or DMSO. Reduces nitrate to nitrite. H₂S is not produced from sodium thiosulfate. Indole formation is positive. Hydrolyses starch but not gelatin, casein or Tween 80. The following substrates are utilized as single carbon and energy sources for growth: D-glucose, D-mannose, D-galactose, maltose, sucrose, lactose, starch, glycerol, D-mannitol, D-sorbitol, acetate, pyruvate, DL-lactate, succinate, L-malate, fumarate, glycine, L-alanine, L-glutamate and L-ornithine.

No growth occurs on D-fructose, L-sorbose, D-ribose, D-xylose, citrate, L-arginine, L-aspartate or L-lysine. Acid is produced from D-glucose, D-mannose, D-galactose, maltose, sucrose and lactose. Sensitive to the following antibiotics (μg per disc, unless otherwise indicated): novobiocin (30), bacitracin (0.04 IU per disc), anisomycin (20), aphidicolin (20) and rifampicin (5). Resistant to the following antibiotics: erythromycin (15), penicillin G (10 IU per disc), ampicillin (10), chloramphenicol (30), neomycin (30), norfloxacin (10), ciprofloxacin (5), streptomycin (10), kanamycin (30), tetracycline (30), vancomycin (30), gentamicin (10) and nalidixic acid (30). The polar lipids are PG, PGP-Me, trace PGS, and one major glycolipid and one minor glycolipid chromatographically identical to S-DGD-1 and DGD-1, respectively; two trace unidentified glycolipids may be present. The DNA G + C content of the type strain is 62.0 mol% (as determined by HPLC).

The type strain, TNN58^T (=CGMCC 1.10121^T =JCM 16428^T), was isolated from Tainan marine solar saltern near Lianyungang city, Jiangsu province, China.

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