Halogranum rubrum gen. nov., sp. nov., a halophilic archaeon isolated from a marine solar saltern

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Two extremely halophilic archaea, strains RO2-11^T and HO2-1, were isolated from two Chinese marine solar salterns, Rudong solar saltern and Haimen solar saltern, respectively. Cells of the two strains were polymorphic and Gram-stain-negative; colonies were red-pigmented. The two strains grew at NaCl concentrations of 2.6–4.3 M (optimum 3.9 M) and required at least 0.1 M Mg²⁺ for growth. They were able to grow over a pH range of 6.0–8.0 and a temperature range of 20–50 °C, with optimal pH of 7.5 and optimal temperature of 37 °C. The major polar lipids of strain RO2-11^T and strain HO2-1 were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and three glycolipids, two of them chromatographically identical to S-DGD-1 and DGD-1, the third unidentified. The 16S rRNA gene sequence similarity of strain RO2-11^T and strain HO2-1 was 99.3% and highest sequence similarity with the closest relative (*Haloferax larsenii*) was 91.4%. Based on the data obtained, the two isolates could not be classified in any recognized genus of the family *Halobacteriaceae*. Strain RO2-11^T and strain HO2-1 are thus considered to represent a novel species of a new genus within the family *Halobacteriaceae*, for which the name *Halogranum rubrum* gen. nov., sp. nov. is proposed. The type strain is RO2-11^T (=CGMCC 1.7738^T =JCM 15772^T).

The extremely halophilic aerobic archaea of the order *Halobacteriales* are mainly found in diverse hypersaline environments such as salt lakes, artificial crystallizer ponds of marine solar salterns, salt soil, salty fermented food and salted hides (Oren, 2006; Zvyagintseva & Tarasov, 1988). Some of them can also be isolated from low-salt habitats such as low-salt, sulfide-rich springs (Savage *et al.*, 2007, 2008), seawater (Rodriguez-Valera *et al.*, 1979) and sediments of seashore marshes (Purdy *et al.*, 2004). At the time of writing, the family *Halobacteriaceae*, the single family described within the order *Halobacteriales*, accommodated 27 recognized genera (Oren *et al.*, 2009). Some of these halophilic archaea were first isolated from

thalassohaline hypersaline environments and the others were obtained from athalassohaline environments, showing that both kinds of saline ecosystems harbour rich communities of halophilic archaea.

Diverse hypersaline environments are commonly found in China (Xu *et al.*, 1999; Xin *et al.*, 2000; Castillo *et al.*, 2006). In addition to many inland salt lakes, soda lakes and salt mines, there are more than 100 artificial marine solar salterns built along the 18 000 km coastline of Eastern China, for the production of salt from seawater. These coastal solar salterns have been a long-term target for the study of microbiota (Shao *et al.*, 2004). In this paper, we report on the isolation and characterization of two novel halophilic archaeal strains that represent a novel species in a novel genus of the family *Halobacteriaceae*.

Rudong solar saltern (32.2699° N 121.3999° E) and Haimen solar saltern (32.0918° N 121.4909° E) are two artificial marine solar salterns located in Jiangsu, China. After more than thirty years flourishing, both of them stopped production in recent years. At the time of sampling, March 28, 2008, Rudong solar saltern had been

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains RO2-11^T and HO2-1 are EU887283 and FJ889134, respectively.

Scanning electron micrographs of strains RO2-11^T and HO2-1, a phase-contrast micrograph of strain RO2-11^T, thin-layer chromatograms and a maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences of strains RO2-11^T, HO2-1 and other haloarchaea are available with the online version of this paper.

off production for less than one year, Haimen solar saltern for more than five years. Plenty of salt granules could be found in the saline soil of Rudong marine solar saltern, but not in the soil of Haimen marine solar saltern. To isolate halophilic archaea, soil samples from each of the two solar salterns were collected (using a sterile steel spoon) into sterile 50 ml plastic tubes with screw caps. The tubes were carried back to the laboratory at room temperature. 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 (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. One gram soil was suspended in 9 ml liquid NOM medium, serially diluted with the same medium and then plated onto NOM agar plates. The plates were incubated in the dark at 37 °C for at least 8 weeks in sealed plastic bags. The plate with 50 to 100 colonies was selected for picking the single colony. To ensure purity, a single colony of each strain was restreaked twice onto NOM plates. Strain RO2-11^T was isolated from the saline soil of Rudong marine solar saltern and strain HO2-1 from the soil of Haimen marine solar saltern. The strains were routinely grown aerobically at 37 °C in a modified R2A (Reasoner & Geldreich, 1985) 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; $MgSO_4 \cdot 7H_2O$, 20.0 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 *Haloferax volcanii* CGMCC 1.2150^T, *Haloferax larsenii* CGMCC 1.5347^T, *Halorubrum litoreum* CGMCC 1.5336^T, *Halorubrum lipolyticum* CGMCC 1.5332^T, *Haloarcula marismortui* CGMCC 1.1784^T, *Halosarcina pallida* BZ256^T, *Haladaptatus paucihalophilus* JCM 13897^T and *Halogeometricum borinquense* JCM 10706^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). Minimal salt concentration to prevent cell lysis was tested by suspending washed cells in serial sterile saline solutions containing NaCl ranging from 0 to 15 % (w/v), and the stability of the cells was detected by light microscopic examination.

The Gram stain 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 $NaNO_3$ ($5\text{ g }l^{-1}$) 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 ($5\text{ g }l^{-1}$) were tested in completely filled 10 ml stoppered tubes. Starch hydrolysis was determined on NOM 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 (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 $5\text{ g }l^{-1}$. Sensitivity to antimicrobial agents was performed as described by Gutiérrez *et al.* (2008).

Cells of strains RO2-11^T and HO2-1 were motile and polymorphic, showing different shapes (short rods, irregular polyhedra and ovals, 1–2 μm mean cell dimensions; see Supplementary Fig. S1 available in IJSEM Online) when grown in MR2A liquid medium, Gram-stain-negative and able to grow at a range of NaCl concentrations (2.6–4.3 M, with optimal growth at 3.9 M NaCl). Magnesium was required for growth within the range 0.1–0.7 M (optimum growth at 0.3 M). The growth pH range was 6.0–8.0 (optimum growth at pH 7.5) and the isolates grew at 20–50 °C (optimum growth at 37 °C). The two strains were catalase- and oxidase-positive. They hydrolysed gelatin, casein and Tweens 20, 40, 60 and 80; starch was hydrolysed weakly. They were able to grow in defined and complex media; D-glucose, D-mannose, D-galactose, maltose, sucrose, lactose and glycerol as carbon sources yielded the best growth. Acid was produced when they were grown on carbohydrates. The two strains differed in utilizing single carbon sources for growth; strain RO2-11^T used succinate, L-malate and fumarate as single carbon source supporting growth, but strain HO2-1 did not utilize them. More detailed results of phenotypic tests and nutritional features of the two strains are given in the genus and species descriptions.

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). The amplified products were cloned into the pEASY-T vector (TransGen Biotech) and transformed into cells of *Escherichia coli* Mach1. Twenty clones of strains RO2-11^T and HO2-1 were randomly picked and sequenced at the Sino-GenoMax Company

(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). Phylogenetic trees were reconstructed by 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 replicates) are shown next to the branches. 16S rRNA gene sequence similarity was calculated by comparison with sequences of related halophilic archaea from the online EzTaxon server (Chun *et al.*, 2007).

Sixteen complete 16S rRNA gene sequences (1473 nt each) of strains RO2-11^T and HO2-1 were obtained. Sequence comparisons indicated that both strains have one kind of 16S rRNA gene sequence; they are 99.3% similar to each other. Both strains showed low levels of 16S rRNA gene sequence similarity to other members of the family *Halobacteriaceae*. The most closely related recognized species were representatives of the genera *Haloferax* (*Haloferax larsenii*, 91.4% similarity), *Halorubrum* (*Halorubrum vacuolatum*, 90.5–90.8% similarity) and *Halosarcina* (*Halosarcina pallida*, 90.3–90.7% similarity). Phylogenetic analysis using the neighbour-joining algorithm revealed that the two strains clustered distantly with the *Halorubrum* and *Haloplanus* clade (Fig. 1). The phylogenetic position was also confirmed by a tree generated using the maximum-parsimony algorithm (see Supplementary Fig. S3).

Polar lipids were extracted using a chloroform/methanol system and analysed using 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 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.) 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 and double development in the solvent chloroform/methanol/acetic acid/water (85:22.5:10:4, by vol.). Strains RO2-11^T and HO2-1 contained phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and three glycolipids, two of them (GL1 and GL2) chromatographically identical to S-DGD-1 and DGD-1; the third one (GL3), showing faster migration than the above two glycolipids, was still unidentified (Supplementary Fig. S2). The absence of phosphatidylglycerol sulfate (PGS) within this group helps to differentiate the two isolates from neutrophilic members of the genera *Halorubrum*, *Haloplanus* and *Haladaptatus*, which contain PGS (Pesenti *et al.*, 2008; Bardavid *et al.*, 2007; Savage *et al.*, 2007). The glycolipid profile sets strains RO2-11^T and HO2-1 apart from members of the genera *Haloferax* and *Halosarcina*, which possess S-DGD-1 and DGD-1 (Allen *et al.*, 2008; Savage *et al.*, 2008), and the member of the genus *Haloquadratum*, which only contains S-DGD-1 (Burns *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). The DNA G+C contents of strains RO2-11^T and HO2-1 are 55.7 and 57.6 mol%, respectively. These values are lower than those of the genera *Halorubrum* (61.9–71.2 mol%; Pesenti *et al.*, 2008), *Halosarcina* (65.4 mol%; Savage *et al.*, 2008), *Haloplanus* (66.2 mol%; Elevi Bardavid *et al.*, 2007) and *Haloferax* (59.5–65.3 mol%; Allen *et al.*, 2008) and of *Haloquadratum borinquense* (59.1 mol%; Montalvo-Rodriguez *et al.*, 1998), but higher than the value reported for *Haloquadratum walsbyi* (46.9 mol%; Burns *et al.*, 2007). The DNA relatedness between strains RO2-11^T and HO2-1 was 89.2%. The data show that the two strains

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain RO2-11^T, strain HO2-1 and other close relatives within the family *Halobacteriaceae*. Bootstrap values (%) are based on 1000 replicates and are shown for branches with more than 50% bootstrap support. Bar, 0.02 substitutions per nucleotide position.

should be classified in the same species, since the generally accepted threshold value to separate two species is 70 % (Stackebrandt & Goebel, 1994).

In consideration of the morphological and physiological properties of the new isolates, the low levels of 16S rRNA gene sequence similarity with other genera within the family *Halobacteriaceae* and distinctive components of polar lipids, we suggest that strains RO2-11^T and HO2-1 represent a novel species of a new genus within the family *Halobacteriaceae*, for which the name *Halogramum rubrum* gen. nov., sp. nov. is proposed. Characteristics that distinguish strains RO2-11^T and HO2-1 from other genera within the family *Halobacteriaceae* are shown in Table 1.

Description of *Halogramum* gen. nov.

Halogramum (Ha.lo.gra'num. Gr. n. *hals halos* salt; L. neut. n. *granum* granule; N.L. neut. n. *Halogramum* salty granule shape).

Cells are polymorphic under optimal growth conditions and stain Gram-negative. Aerobic heterotrophs. Cells lyse in distilled water. Oxidase and catalase tests are positive. Temperatures between 20 and 50 °C support growth. Sugars are metabolized, in some cases with formation of acids. Cells contain phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester, but phosphatidylglycerol sulfate is absent. Three glycolipids are present, two of them chromatographically identical to S-DGD-1 and DGD-1, the third unidentified. The genomic DNA G+C

content is 55.7–57.6 mol%. The type species is *Halogramum rubrum*. Recommended three-letter abbreviation: *Hgn*.

Description of *Halogramum rubrum* sp. nov.

Halogramum rubrum (ru'brum. L. neut. adj. *rubrum* red).

Cells are motile and polymorphic, showing different shapes (short rods, irregular polyhedra and ovals, 1–2 µm mean cell dimensions) when grown in MR2A liquid medium and stain Gram-negative. Minimal NaCl concentration to prevent cell lysis is 12 % (w/v). Colonies on agar plates containing 3.9 M NaCl are red, elevated and round. Chemo-organotrophic and aerobic. Growth occurs at NaCl concentrations of 2.6–4.3 M, at Mg²⁺ concentrations of 0.1–0.7 M, at pH values in the range 6.0–8.0, and at temperatures between 20 and 50 °C. Optimal NaCl concentration, Mg²⁺ concentration, pH and temperature for growth are 3.9 M, 0.3 M, 7.5 and 37 °C, respectively. Catalase- and oxidase-positive. Does not grow under anaerobic conditions with nitrate, arginine or DMSO. Nitrate reduction to nitrite is observed; no gas formation from nitrate. Nitrite is also reduced. H₂S is produced from Na₂S₂O₃. Negative for indole formation. Tweens 20, 40, 60 and 80, casein and gelatin are hydrolysed; starch is hydrolysed weakly. The following substrates are utilized as carbon sources for growth: D-glucose, D-mannose, D-galactose, maltose, sucrose, lactose, starch, glycerol, D-sorbitol, acetate, pyruvate, DL-lactate, succinate, L-malate, fumarate, L-alanine, L-glutamate and L-ornithine.

Table 1. Differential characteristics between strain RO2-11^T, strain HO2-1 and closely related genera within the order *Halobacteriales*

Taxa: 1, Strains RO2-11^T and HO2-1; 2, *Halorubrum* (Pesenti *et al.*, 2008); 3, *Haloferax* (Allen *et al.*, 2008); 4, *Haloplanus* (Bardavid *et al.*, 2007); 5, *Halogeometricum* (Montalvo-Rodriguez *et al.*, 1998); 6, *Halosarcina* (Savage *et al.*, 2008); 7, *Haloquadratum* (Burns *et al.*, 2007). +, Positive; –, negative; w, weak; +/–, positive or negative; NR, not reported; PGP, phosphatidylglycerol sulfate; UG, unidentified glycolipid.

Characteristic	1	2	3	4	5	6	7
Cell shape	Pleomorphic	Pleomorphic	Pleomorphic	Pleomorphic	Pleomorphic	Coccus	Squares
Motility	+	+/–	+/–	–	+	–	–
Colony colour	Red	Orange–red	Red–pink	Pink	Pink	Pink	Red–pink
Optimum NaCl (M)	3.9	2.5–4.5	1.7–4.3	3.0	3.1–4.3	3.1	3.1
Optimum temperature (°C)	37	37–50	32–50	40	40	30	45
Optimum pH	7.5	7.0–7.5/9.0–10.0	6.4–7.5	7.0	7.0	6.5	7.0
Anaerobic growth with nitrate	–	+/–	+/–	–	+	–	–
Catalase activity	+	+	+	+	+	+	–
Oxidase activity	+	+	+	+	+	+	–
Indole formation	–	+/–	+/–	+	+	+	–
Starch hydrolysis	w	+/–	+/–	–	–	–	–
Casein hydrolysis	+	+/–	+/–	–	+	–	–
Gelatin hydrolysis	+	+/–	+/–	–	+	–	NR
Utilization of glycerol	+	+	+	–	+	+	–
DNA G+C content (mol%)	55.7–57.6	61.9–71.2	59.5–65.3	66.2	59.1	65.4	46.9
Presence of PGS	–	+/–	+	+	–	–	–
Types of glycolipids present	S-DGD-1, DGD-1,UG	S-DGD-3	S-DGD-1, DGD-1	S-DGD-1	UG	S-DGD-1, DGD-1	S-DGD-1

D-Fructose, L-sorbose, D-ribose, D-xylose, D-mannitol, citrate, glycine, L-arginine, L-aspartate and L-lysine are not utilized as carbon sources. Produces acid when grown on D-glucose, D-galactose, maltose, sucrose and glycerol. Sensitive to the following antibiotics (μg or IU per disc): 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 phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, S-DGD-1, DGD-1 and one unidentified glycolipid. The DNA G+C content of the type strain is 55.7 mol% (as determined by HPLC).

The type strain is RO2-11^T (=CGMCC 1.7738^T =JCM 15772^T), and was isolated from a soil sample of Rudong solar saltern in Jiangsu province, China.

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