Halolamina pelagica gen. nov., sp. nov., a new member of the family *Halobacteriaceae*

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Two extremely halophilic archaeal strains, TBN21^T and TBN49, were isolated from the Taibei marine solar saltern near Lianyungang city, Jiangsu province, China. Cells of the two strains were pleomorphic and Gram-negative and colonies were red. Strains TBN21^T and TBN49 were able to grow at 25–50 °C (optimum 37 °C), at 1.4–5.1 M NaCl (optimum 3.4–3.9 M) and at pH 5.5–9.5 (optimum pH 7.0–7.5) and neither strain required Mg^{2+} for growth. Cells lysed in distilled water and the minimal NaCl concentration to prevent cell lysis was 8 % (w/v). The major polar lipids of the two strains were phosphatidic acid, phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate and eight glycolipids; three of these glycolipids (GL3, GL4 and GL5) were chromatographically identical to sulfated mannosyl glucosyl diether (S-DGD-1), galactosyl mannosyl glucosyl diether (TGD-1) and mannosyl glucosyl diether (DGD-1), respectively. Phylogenetic analysis revealed that strains TBN21^T and TBN49 formed a distinct clade with their closest relative, *Halobaculum gomorrense* JCM 9908^T (89.0–89.5 % 16S rRNA gene sequence similarity). The DNA G+C contents of strains TBN21^T and TBN49 were 64.8 and 62.7 mol%, respectively. DNA–DNA hybridization between strains TBN21^T and TBN49 was 90.1 %. The phenotypic, chemotaxonomic and phylogenetic properties suggest that strains TBN21^T and TBN49 represent a novel species in a new genus within the family *Halobacteriaceae*, for which the name *Halolamina pelagica* gen. nov., sp. nov. is proposed. The type strain of *Halolamina pelagica* is TBN21^T (=CGMCC 1.10329^T =JCM 16809^T).

The past 5 years have witnessed the rapid expansion of the family *Halobacteriaceae*, the single family described within the order *Halobacteriales*. As many novel species have been discovered and added to established genera (Oren *et al.*, 2009), more and more isolates representing novel genera have been cultivated and described, such as *Halovivax asiaticus* (Castillo *et al.*, 2006a), *Halostagnicola larsenii* (Castillo *et al.*, 2006b), *Haladaptatus paucihalophilus* (Savage *et al.*, 2007), *Haloquadratum walsbyi* (Burns *et al.*, 2007), *Haloplanus natans* (Bardavid *et al.*, 2007), *Halopiger xanaduensis* (Gutiérrez *et al.*, 2007), *Halosarcina pallida* (Savage *et al.*, 2008), *Halonotius pteroides* (Burns *et al.*, 2010), *Halogranum rubrum* (Cui *et al.*, 2010a), *Halopelagius inordinatus* (Cui *et al.*, 2010c), *Natronoarchaeum mannanilyticum* (Shimane *et al.*, 2010), *Halarchaeum acidiphilum* (Minegishi *et al.*, 2010) and *Halomarina oriensis* (Inoue *et al.*, 2011). This expansion indicates that the family *Halobacteriaceae* is more diverse than was previously recognized. During our surveys of the halophilic archaeal diversity of marine solar salterns of eastern China, we isolated two pleomorphic, thin-slice-shaped strains that were phylogenetically related to *Halobaculum* (*Hbl.*) *gomorrense* Oren *et al.* 1995 (89.0–89.5 % 16S rRNA gene sequence similarity to the type strain). In this study, we characterize these two strains as representing a novel species in a new genus of the family *Halobacteriaceae*.

Strains TBN21^T and TBN49 were isolated from brine sampled from Taibei marine solar saltern (34° 43′ 38″ N 119° 17′ 48″ E) near Lianyungang city of Jiangsu province, China. The neutral oligotrophic haloarchaeal medium (NOM) used for the isolation procedure contained the following (l^{-1}): 0.05 g yeast extract (Oxoid), 0.25 g fish peptone (Sinopharm Chemical Reagent Co., Ltd), 1.0 g sodium pyruvate, 5.4 g KCl, 0.3 g K_2HPO_4, 0.25 g $CaCl_2$, 0.25 g NH_4Cl, 26.8 g $MgSO_4 . 7H_2O$, 23.0 g $MgCl_2 . 6H_2O$ and 184.0 g NaCl (adjusted to pH 7.0–7.2 with 1 M NaOH) (Cui *et al.*, 2010a). 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 (l^{-1}): 1.0 g

Abbreviations: DGD, mannosyl glucosyl diether; S-DGD, sulfated mannosyl glucosyl diether; TGD, galactosyl mannosyl glucosyl diether.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains TBN21^T and TBN49 are GU208826 and GU208827.

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

yeast extract, 0.25 g fish 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). The type strains *Halogranum rubrum* RO2-11^T, *Hbl. gomorrense* JCM 9908^T, *Halorubrum litoreum* Fa-1^T, *Halonotius* (*Hns.*) *pteroides* 1.15.5^T and *Haloferax volcanii* CGMCC 1.2150^T were selected as reference strains. Cell morphology and motility in exponentially growing liquid cultures were examined using a microscope equipped with phase-contrast optics (Nikon model E400). Minimal salt concentrations to prevent cell lysis were tested by suspending washed cells in serial sterile saline solutions containing 0–15 % (w/v) NaCl and the stability of the cells was detected by light microscopic examination.

Gram staining was performed by following the method outlined by Dussault (1955). Most biochemical 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 to which $NaNO_3$ (5 g l^{-1}) had been added and containing an inverted Durham tube. The formation of gas from nitrate was detected by the presence of gas bubbles in the 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 2 g soluble starch l^{-1} and 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 H_2S was tested by growing the isolates and reference strains in a tube with liquid NOM supplemented with 0.5 % (w/v) sodium thiosulfate; 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 NOM and the compound to be tested was added at a concentration of 5 g l^{-1}. Antibiotic susceptibilities were determined by the method of Gutiérrez *et al.* (2008) on NOM agar plates with antibiotic discs containing the following amounts (μg per disc, unless indicated): ampicillin (10), anisomycin (20), aphidicolin (20), bacitracin (0.04 IU), chloramphenicol (30), ciprofloxacin (5), erythromycin (15), gentamicin (10), kanamycin (30), nalidixic acid (30), neomycin (30), norfloxacin (10), novobiocin (30), penicillin G (10 IU), rifampicin (5), streptomycin (10), tetracycline (30) and vancomycin (30).

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 F_{254} aluminium-backed thin-layer plates were used in TLC analysis. In two-dimensional TLC, the first solvent was chloroform/methanol/water (65 : 25 : 4, by vol.) and the second solvent was chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, by vol.), which was also used in one-dimensional TLC. All TLC plates were sprayed with sulfuric acid/ethanol (1 : 2, v/v) followed by heating at 150 °C for 3 min to detect phospholipids and glycolipids.

Genomic DNA from halophilic archaeal strains was prepared as described by Ng *et al.* (1995). The 16S rRNA gene was amplified by 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 as described previously (Cui *et al.*, 2010b). 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. Twenty transformants of each strain were picked randomly and sequenced at the SinoGenoMax Company Limited (Beijing, China) to determine whether the two strains possessed multiple distinct 16S rRNA genes. Multiple sequence alignments were performed using the CLUSTAL W program integrated in MEGA 5 software (http://www.megasoftware.net/) (Kumar *et al.*, 2008). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) algorithms in MEGA 5 software. 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 using the pairwise-distance computing function of MEGA 5 in comparison with sequences of related halophilic archaea. The DNA G+C content was determined by thermal denaturation method (T_m) (Marmur & Doty, 1962). 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 TBN21^T and TBN49 were non-motile and showed pleomorphic thin-slice shapes when grown in liquid NOM-3 (Supplementary Fig. S1, available in IJSEM Online). Cells stained Gram-negative and colonies were red. Strains TBN21^T and TBN49 were able to grow at 25–50 °C (optimum 37 °C), 1.4–5.1 M NaCl (optimum 3.4–3.9 M) and pH 5.5–9.5 (optimum pH 7.0–7.5) and neither strain required Mg^{2+} for growth. Cells lysed in distilled water and the minimal NaCl concentration to prevent cell lysis was 8 % (w/v). Strains TBN21^T and TBN49 hydrolysed starch weakly but did not hydrolyse gelatin, Tween 80 or casein. Both strains produced H_2S from sodium thiosulfate, but did not produce indole from tryptophan. They were able to grow in defined and complex media: D-glucose, D-mannose, D-galactose, acetate, pyruvate and DL-lactate yielded the best growth as single carbon sources.

Strain TBN49 could reduce nitrate to nitrite, but strain TBN21 could not. More detailed results of phenotypic tests and nutritional features of the two strains are given in the species description.

The polar lipids of strains TBN21^T and TBN49 were phosphatidic acid, phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate and eight glycolipids; three of these glycolipids (GL3, GL4 and GL5) were chromatographically identical to S-DGD-1 (sulfated mannosyl glucosyl diether 1), TGD-1 (galactosyl mannosyl glucosyl diether 1) and DGD-1 (mannosyl glucosyl diether 1), respectively (Supplementary Fig. S2). The glycolipid profile sets strains TBN21^T and TBN49 apart from *Hbl. gomorrense*, which contains S-DGD-1 and DGD-1 (Oren *et al.*, 1995), *Halogranum rubrum*, which contains S-DGD-1, DGD-1 and trace unidentified glycolipids that run faster chromatographically than DGD-1 (Cui *et al.*, 2010a), *Hns. pteroides*, which contains S-DGD-1 (Burns *et al.*, 2010), and *Halorubrum* species, which contain S-DGD-3 (Oren *et al.*, 2009).

Sixteen complete 16S rRNA gene sequences of strain TBN21^T and 19 complete 16S rRNA gene sequences of strain TBN49 were obtained. Sequence comparisons indicated that strains TBN21^T and TBN49 each contain a single 16S rRNA gene and their sequences are 99.9 % similar. Both strains showed relatively low levels of 16S rRNA gene sequence similarity to other members of the family *Halobacteriaceae* and the closest related recognized species were *Hbl. gomorrense* (89.0–89.5 % similarity to the type strain), *Halogranum rubrum* (89.7–89.8 % similarity) and *Halosarcina pallida* (89.9 % similarity). Phylogenetic analysis using the neighbour-joining algorithm revealed that strains TBN21^T and TBN49 formed a distinct clade with *Hbl. gomorrense* (Fig. 1). The phylogenetic position was confirmed in trees generated using the maximum-parsimony and maximum-likelihood algorithms (Supplementary Fig. S3). Phylogenetic analysis of the 16S rRNA gene revealed that the two strains represent a novel taxon.

The DNA G+C contents of strains TBN21^T and TBN49 were 64.8 and 62.7 mol%, respectively. These values are within the range of values reported for *Halogranum* (Cui *et al.*, 2010a) and *Halorubrum* (Oren *et al.*, 2009; Mancinelli *et al.*, 2009) and higher than *Hns. pteroides* (58 mol%) (Burns *et al.*, 2010), but lower than the value reported for *Hbl. gomorrense* (Oren *et al.*, 1995). DNA–DNA hybridization between strains TBN21^T and TBN49 was 90.1 %, showing that the two strains should be classified in the same species, since the generally accepted threshold value to separate two species is 70 % (Stackebrandt & Goebel, 1994).

This polyphasic taxonomic study provides evidence that strains TBN21^T and TBN49 represent a novel species of a new genus within the family *Halobacteriaceae*, for which the name *Halolamina pelagica* gen. nov., sp. nov. is proposed. Characteristics that distinguish strains TBN21^T and TBN49 from members of other genera within the *Halobacteriaceae* are shown in Table 1.

Description of *Halolamina* gen. nov.

Halolamina [Ha.lo.la′mi.na. Gr. n. *hals*, *halos* salt; L. fem. n. *lamina* a thin slice; N.L. fem. n. *Halolamina* thin-slice-shaped salt (organism)].

Cells are pleomorphic and thin-slice-shaped under optimal growth conditions and stain Gram-negative. Aerobic heterotrophs. Cells lyse in distilled water. Oxidase and catalase tests are positive. Extremely halophilic, with growth occurring in media containing 1.4–5.1 M NaCl; known strains grow best at 3.4–3.9 M NaCl. The optimum magnesium concentration varies between 0.01 and 0.05 M. Temperatures of 25–50 °C and pH 5.5–9.5 support growth. Sugars are metabolized, in some cases with the formation of acids. The polar lipids are phosphatidic acid, phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate and eight glycolipids; three of these glycolipids are chromatographically identical to S-DGD-1, TGD-1 and DGD-1. The genomic DNA G+C content is 62.7–64.8 mol%. Known strains have been isolated from marine solar salterns. The type species is *Halolamina pelagica*. Recommended three-letter abbreviation: *Hlm*.

Description of *Halolamina pelagica* sp. nov.

Halolamina pelagica (pe.la′gi.ca. L. fem. adj. *pelagica* of or belonging to the sea).

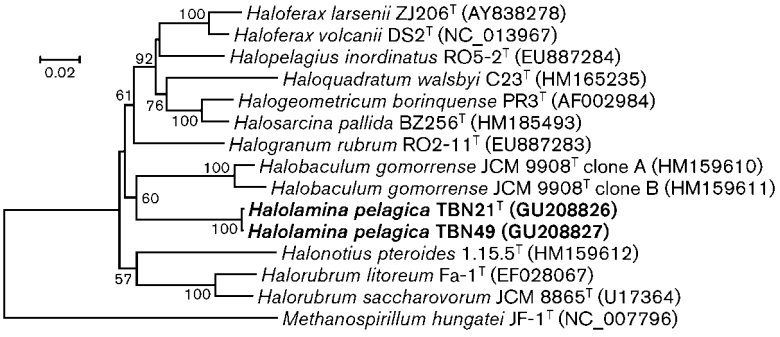

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between strains TBN21^T and TBN49 and close relatives within the family *Halobacteriaceae*. Bootstrap percentages based on 1000 replicates are shown for branches with more than 50 % support. Bar, 0.02 substitutions per nucleotide position.

Table 1. Differential characteristics between strains TBN21^T and TBN49 and closely related genera within the order *Halobacteriales*

Taxa: 1, strains TBN21^T and TBN49 (for variable data, given as TBN21^T/TBN49); 2, *Halobaculum* (data from Oren *et al.*, 1995); 3, *Halogranum* (Cui *et al.*, 2010b); 4, *Halorubrum* (Oren *et al.*, 2009; Mancinelli *et al.*, 2009); 5, *Halonotius* (Burns *et al.*, 2010). +, Positive; −, negative; V, positive or negative, depending on the species; W, weak; PGS, phosphatidylglycerol sulfate.

Characteristic	1	2	3	4	5
Cell morphology	Pleomorphic	Rod	Pleomorphic	Pleomorphic	Flat rod
Motility	−	+	V	V	+
Mg^{2+} required	−	+	V	V	−
Reduction of nitrate to nitrite	−/+	+	V	V	−
Utilization of:					
D-Mannose	+	−	+	V	−
D-Xylose	−	+	−	V	−
Maltose	−	+	V	V	−
Sucrose	−	+	+	V	−
Lactose	−	−	+/W	V	−
Acetate	+	−	+	V	−
Pyruvate	+	−	+	V	+
Presence of PGS	+	−	V	V	−
Glycolipid(s)*	S-DGD-1, DGD-1, TGD-1, 4 UG	S-DGD-1	S-DGD-1, DGD-1, 2–3 UG	S-DGD-3	S-DGD-1
DNA G+C content (mol%)	64.8/62.7	70	55.7–64.0	60.2–71.2	58

*UG, Unidentified glycolipids.

Displays the following properties in addition to those described for the genus. Colonies on agar plates containing 3.4–3.9 M NaCl are red, elevated and round. Chemo-organotrophic. Growth occurs at 25–50 °C (optimum 37 °C), 1.4–5.1 M NaCl (optimum 3.4–3.9 M), 0–0.7 M $MgCl_2$ (optimum 0.01–0.05 M) and pH 5.5–9.5 (optimum pH 7.0–7.5). Minimal NaCl concentration to prevent cell lysis is 8 % (w/v). Does not grow under anaerobic conditions with nitrate, arginine or DMSO. Nitrate reduction to nitrite is observed in some strains. H_2S is not produced from sodium thiosulfate. Indole formation is negative. Hydrolyses starch weakly but does not hydrolyse gelatin, casein or Tween 80. The following substrates are utilized as single carbon and energy sources for growth: D-glucose, D-mannose, D-galactose, starch, acetate, pyruvate and DL-lactate. The following substrates are utilized as single carbon, nitrogen or energy sources for growth: L-alanine, L-arginine, L-glutamate and L-ornithine. No growth occurs on D-fructose, L-sorbose, D-ribose, D-xylose, maltose, sucrose, lactose, glycerol, D-mannitol, D-sorbitol, succinate, L-malate, fumarate, citrate, glycine, L-aspartate or L-lysine. Acid is produced from D-glucose, D-mannose and D-galactose. Sensitive to the following antibiotics (μg per disc, unless indicated): novobiocin (30), bacitracin (0.04 IU), anisomycin (20), aphidicolin (20) and rifampicin (5). Resistant to erythromycin (15), penicillin G (10 IU), 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 DNA G+C content of the type strain is 64.8 mol% (T_m).

The type strain, TBN21^T (=CGMCC 1.10329^T =JCM 16809^T), and reference strain TBN49 (=CGMCC 1.10330 =JCM 16810) were isolated from Taibei marine solar saltern near Lianyungang city, Jiangsu province, China.

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