Haloplanus aerogenes sp. nov., an extremely halophilic archaeon from a marine solar saltern

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Halophilic archaeal strain TBN37^T was isolated from Taibei marine solar saltern near Lianyungang city of Jiangsu province, China. Cells were pleomorphic, flat and contained gas vesicles. Cells of strain TBN37^T stained Gram-negative and the colonies were pink-pigmented. The strain was able to grow at 25–50 °C (optimum, 37–40 °C), with 1.4–5.1 M NaCl (optimum, 2.1 M NaCl), with 0–1.0 M MgCl₂ (optimum, 0.01 M MgCl₂) and at pH 6.0–9.0 (optimum, pH 7.5). Cells lysed in distilled water and the minimal NaCl concentration to prevent cell lysis was 8 % (w/v). The major polar lipids of strain TBN37^T were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate and one major glycolipid chromatographically identical to sulfated mannosyl glucosyl diether (S-DGD-1). On the basis of 16S rRNA gene sequence analysis, strain TBN37^T was closely related to *Haloplanus natans* and *Haloplanus vescus*, with the same similarity of 97.4 %. The DNA G + C content of strain TBN37^T is 64.1 mol%. DNA–DNA hybridization values between strain TBN37^T and *Haloplanus natans* JCM 14081^T and between strain TBN37^T and *Haloplanus vescus* RO5-8^T were 37.6 % and 42.1 %, respectively. It was concluded that strain TBN37^T represents a novel species of the genus *Haloplanus*, for which the name *Haloplanus aerogenes* sp. nov. is proposed. The type strain is TBN37^T (=CGMCC 1.10124^T =JCM 16430^T).

Halophilic archaea, members of the family *Halobacteriaceae*, have been isolated from various hypersaline environments and are currently classified into more than 27 genera (Oren *et al.*, 2009). The genus *Haloplanus* was first proposed by Bardavid *et al.* to accommodate three flat-shaped strains isolated from an experimental mesocosm filled with a mixture of water from the Dead Sea and the Red Sea, Israel (Bardavid *et al.*, 2007). More recently, *Haloplanus vescus* (Cui *et al.*, 2010), a novel species isolated from Rudong marine solar saltern located in Jiangsu Province of China, was added to the genus *Haloplanus*. The most interesting characteristic of members of this genus is that their cells are pleomorphic and flat, and in static liquid medium always float to the surface, which reveals that they may contain gas vesicles. In 2008, during our surveys on halophilic archaeal diversity of Taibei marine solar saltern, another marine solar saltern near Lianyungang city of Jiangsu province, an extremely halophilic archaeal strain related to the genus

Haloplanus was obtained. In this study, we characterize strain TBN37^T as a novel species of the genus *Haloplanus*.

Strain TBN37^T was isolated from sediment of Taibei marine solar saltern (34° 43' 38" N 119° 17' 48" E), which is an artificial marine solar saltern near Lianyungang city of Jiangsu province, China. The neutral oligotrophic haloarchaeal medium (NOM) used for the isolation procedure 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; NaCl, 184.0 (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 (g l⁻¹): yeast extract, 1.0; fish peptone, 0.25; sodium formate, 0.25; sodium acetate, 0.25; sodium lactate, 0.25; sodium pyruvate, 0.25.

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 *Haloplanus natans* JCM 14081^T, *Haloplanus vescus* RO5-8^T and *Haloferax elongans* JCM 14791^T together with strain *Halobacterium salinarum* CGMCC 1.2367 (=ATCC 33170) were selected as reference strains for positive and negative testing. Cell morphology and motility in exponentially

Abbreviations: PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester; PGS, phosphatidylglycerol sulfate; S-DGD-1, sulfated mannosyl glucosyl diether.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TBN37^T is GQ282625.

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

growing liquid cultures were examined using a Nikon microscope equipped with phase-contrast optics (model E400). The 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 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 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 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 l^{-1} soluble starch 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 the NOM liquid medium 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 the NOM medium and the compound to be tested was added at a concentration of 5 g l^{-1} . Antibiotic susceptibilities were determined by the Gutiérrez (Gutiérrez *et al.*, 2008) method on NOM agar plates with antibiotic discs containing the following concentrations (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 using a chloroform/methanol system and analysed using one- and two-dimensional TLC, as described by 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 is chloroform/methanol/water (65:25:4, by vol.) and the second solvent is chloroform/methanol/acetic acid/water (80:12:15:4, by vol.), which is 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 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 step 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. Ten transformants 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 in MEGA 4 software (Tamura *et al.*, 2007). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) algorithms in MEGA 4 software including 16S rRNA gene sequences representing genera of the family *Halobacteriaceae* with validly published names; phylogenetic trees representing just the most relevant branches are given. 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). 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 strain TBN37^T were motile, pleomorphic and flat, and contained gas vesicles (see Supplementary Fig. S1 available in IJSEM Online). The cells stained Gram-negative and colonies were pink-pigmented. Strain TBN37^T was able to grow at $25\text{--}50^\circ\text{C}$ (optimum, $37\text{--}40^\circ\text{C}$), with 1.4–5.1 M NaCl (optimum, 2.1 M NaCl), with 0–1.0 M MgCl_2 (optimum, 0.01 M MgCl_2) and at pH 6.0–9.0 (optimum pH 7.5). Cells lysed in distilled water and the minimal NaCl concentration to prevent cell lysis was 8 % (w/v). Strain TBN37^T produced indole from tryptophan, but did not hydrolyse gelatin, Tween 80, starch or casein. H_2S was not produced from sodium thiosulfate. The remarkable phenotypic characteristic that distinguished strain TBN37^T from *Haloplanus natans* JCM 14081^T and *Haloplanus vescus* RO5-8^T was that strain TBN37^T could grow under anaerobic conditions with nitrate and produce gas from nitrate, while related members of the same genus could not. More detailed results of phenotypic features of strain TBN37^T are given in the species description.

The polar lipids of strain TBN37^T were phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS), and one

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain TBN37^T, members of the genus *Haloplanus*, and other related genera of the family *Halobacteriaceae*. Bootstrap values (>70 %) are given at nodes. Bar, 0.02 substitutions per site.

major glycolipid chromatographically identical to sulfated mannosyl glucosyl diether (S-DGD-1), in a pattern chromatographically identical to the polar lipid profile of *Haloplanus natans* JCM 14081^T (see Supplementary Fig. S2). The polar lipid composition supports classification of strain TBN37^T in the genus *Haloplanus*.

Ten complete 16S rRNA gene sequences of strain TBN37^T were obtained. Sequence comparisons indicated that strain TBN37^T has one kind of 16S rRNA gene sequence. It was closely related to both *Haloplanus natans* and *Haloplanus vescus*, with the same similarity of 97.4 %. Phylogenetic analysis using the neighbour-joining algorithm revealed that strain TBN37^T clustered tightly with *Haloplanus natans* and *Haloplanus vescus*, 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 16S rRNA gene-based phylogenetic analysis results supported the placement of strain TBN37^T in the genus *Haloplanus*.

The DNA G + C content of strain TBN37^T is 64.1 mol%, lower than those of strains representing *Haloplanus natans* (66.1–66.4 mol%) (Bardavid *et al.*, 2007) but higher than that of *Haloplanus vescus* RO5-8^T (62.1 mol%). The DNA–DNA hybridization values between strain TBN37^T and *Haloplanus natans* JCM 14081^T and between strain TBN37^T and *Haloplanus vescus* RO5-8^T were 37.6 % and 42.1 %, respectively, which indicated that strain TBN37^T represents a distinct genospecies of *Haloplanus* since the generally accepted threshold value to separate two species is less than 70 % (Stackebrandt & Goebel, 1994).

This polyphasic taxonomic study provides evidence that strain TBN37^T represents a novel species of the genus *Haloplanus*, for which the name *Haloplanus aerogenes* sp. nov. is proposed. Characteristics that distinguish strain TBN37^T from *Haloplanus natans* JCM 14081^T and *Haloplanus vescus* RO5-8^T are shown in Table 1.

Description of *Haloplanus aerogenes* sp. nov.

Haloplanus aerogenes (ae.ro'ge.nes. Gr. n. *aer* *aeros*, air; N.L. suff. *-genes* (from Gr. v. *gennaō* to produce) producing; N.L. adj. *aerogenes* gas-producing).

Cells are pleomorphic and flat (1.0–2.0 µm) under optimal growth conditions and stain Gram-negative. Cells contain gas vesicles. Colonies on agar plates containing 2.1 M NaCl

are pink, elevated and round. Chemo-organotrophic and aerobic. Growth occurs at 25–50 °C (optimum, 37–40 °C), with 1.4–5.1 M NaCl (optimum, 2.1 M NaCl), with 0–1.0 M MgCl₂ (optimum, 0.01 M MgCl₂) and at pH 6.0–9.0 (optimum, pH 7.5). Cells lyse in distilled water and the minimal NaCl concentration to prevent cell lysis is 8 % (w/v). Catalase- and oxidase-positive. Grows anaerobically in the presence of nitrate and produces gas. Does not grow under anaerobic conditions with arginine or DMSO. Nitrate reduction to nitrite is observed. H₂S is not produced from sodium thiosulfate. Positive for indole formation. Tween 80, casein, starch and gelatin are not hydrolysed. The following substrates are utilized as single carbon and energy sources for growth: D-glucose, D-mannose, D-galactose, sucrose, acetate, pyruvate, DL-lactate, L-alanine, L-arginine and L-glutamate. No growth on D-fructose, L-sorbose, D-ribose, D-xylose, maltose, lactose, starch, glycerol, D-mannitol, D-sorbitol, succinate, L-malate, fumarate, citrate, glycine, L-aspartate, L-lysine or L-ornithine. No acid is produced from sugars. 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

Table 1. Characteristics that distinguish strain TBN37^T from *Haloplanus natans* JCM 14081^T and *Haloplanus vescus* RO5-8^T

Strains: 1, TBN37^T; 2, *Haloplanus natans* JCM 14081^T (Bardavid *et al.*, 2007); 3, *Haloplanus vescus* RO5-8^T (Cui *et al.*, 2010). +, Positive; –, negative. All data from this study.

Characteristic	1	2	3
Optimum NaCl (M)	2.1	3.0	3.1
Optimum pH	7.5	7.0	6.0–6.5
Anaerobic growth with nitrate	+	–	–
Gas formation from nitrate	+	–	–
Utilization of:			
D-Galactose	+	–	–
D-Ribose	–	+	–
Maltose	–	–	+
Sucrose	+	–	–
L-Glutamate	+	–	–
H ₂ S formation	–	+	–
G + C content (mol%)	64.1	66.2	62.1

(10), chloramphenicol (30), neomycin (30), 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, PGS and one major glycolipid chromatographically identical to S-DGD-1. The DNA G+C content of the type strain is 64.1 mol% (as determined by HPLC).

The type strain is TBN37^T (=CGMCC 1.10124^T =JCM 16430^T), isolated from Taibei marine solar saltern in Jiangsu province, China.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (no. 30970006).

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