Halopiger salifodinae sp. nov., an extremely halophilic archaeon isolated from a salt mine

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A novel extremely halophilic archaeon KCY07-B2^T was isolated from a salt mine in Kuche county, Xinjiang province, China. Colonies were cream-pigmented and cells were pleomorphic rod-shaped. Strain KCY07-B2^T was able to grow at 25–50 °C (optimum 37–45 °C) and pH 6.0–8.0 (optimum 7.0). The strain required at least 1.9 M NaCl for growth. $MgCl_2$ was not required. Cells lysed in distilled water. Polar lipid analysis revealed the presence of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester, derived from both $C_{20}C_{20}$ and $C_{20}C_{25}$ glycerol diethers, together with five glyolipids. The bis-sulfated glycolipid S_2-DGD-1 was present. The DNA G+C content was 62.5 mol%. Analysis of the 16S rRNA gene sequence revealed that strain KCY07-B2^T was closely related to *Halopiger xanaduensis* SH-6^T and *Halopiger aswanensis* 56^T (95.8 % and 95.5 % similarity, respectively). On the basis of its phenotypic, chemotaxonomic and genotypic characteristics, strain KCY07-B2^T is considered to represent a novel species of the genus *Halopiger*, for which the name *Halopiger salifodinae* sp. nov. is proposed. The type strain is KCY07-B2^T (=JCM 18547^T=CGMCC 1.12284^T).

The genus *Halopiger* was originally proposed by Gutiérrez *et al.* (2007). The type strain of the type species, *Halopiger xanaduensis* SH-6^T, was isolated from Shangmatala salt lake, Inner Mongolia, China. After that, the type strain of another novel species, *Halopiger aswanensis* 56^T (Hezayen *et al.*, 2010), was isolated from the surface of hypersaline salt soils close to Aswan, Egypt. The 16S rRNA gene sequence similarity between the two species mentioned above was 99 %. During our surveys on halophilic archaeal diversity of a salt mine in Kuche county, Akesu area in Xinjiang province of China, an extremely halophilic archaeon named KCY07-B2^T was obtained.

NOM-3 medium (Cui *et al.*, 2011) was used for isolation and it contained (l^{-1} distilled water) 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 $MgCl_2 . 6H_2O$, 184.0 g NaCl, 1.0 g 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 (adjusted to pH 7.0 with 1 M NaOH). The medium was solidified with 2.0 % agar. About 3 g salt sample was suspended in 30 ml liquid NOM-3 medium containing 100 μg streptomycin ml^{-1} and 100 μg ampicillin ml^{-1} to enrich halophilic archaea at 37 °C for 2 weeks. The enrichment culture was diluted and spread onto NOM-3 plates which were incubated at 37 °C. After 3 weeks of incubation, a cream-pigmented colony was picked up and purified by repeated restreaking. The isolate was routinely cultured on NOM-3 medium and maintained at −80 °C with 25 % (v/v) glycerol.

Cell morphology and motility were determined by using an optical microscope (BX40; Olympus) and a transmission electron microscope (JEM-1230; JEOL) (Huo *et al.*, 2010). Gram staining was performed by following the method outlined by Dussault (1955). To determine the growth conditions of strain KCY07-B2^T, we used various NaCl concentrations (0.9–5.4 M, at intervals of 0.5 M) or various $MgCl_2 . 6H_2O$ concentrations (0, 0.1, 0.2, 0.5, 0.8, 1, 1.5, 1.8 and 2 M) in NOM-3 medium. The ranges of pH and temperature for growth were determined according to the methods of Zhang *et al.* (2013).

All the following biochemical and nutritional tests were carried out on strains KCY07-B2^T and *Halopiger aswanensis* 56^T in triplicate in NOM-3 medium. Oxidase and catalase activities, nitrate and nitrite reduction were tested according to the protocols of Dong & Cai (2001). Indole, methyl red, Voges–Proskauer test, Simmons citrate test and H_2S production as well as hydrolysis of aesculin, casein, gelatin and starch were examined as described by Zhu (2011). Esterase activity was detected as outlined by Gutiérrez & González (1972). The production of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, phosphatase and β-galactosidase were assessed as described by Oren *et al.* (1997). To analyse the use of

Abbreviations: GL, glycolipid; PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester; S_2-DGD-1, bis-sulfated diglycosyl diether.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Halopiger salifodinae* KCY07-B2^T is JX014296.

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

accessory electron acceptors, sodium thiosulfate (20 mM), sodium sulfite (5 mM), sodium sulfate (20 mM), sodium nitrite (5 mM), sodium nitrate (20 mM), L-arginine and DMSO (5 g l^{-1}) were respectively added to sterile NOM-3 medium. Oxygen was removed as described by Grishchenkov *et al.* (2000). The same medium lacking L-cysteine and resazurin was used as an aerobic control. To test the utilization of single carbon sources, fish peptone, sodium formate, sodium acetate, sodium lactate and sodium pyruvate were omitted from NOM-3 medium, and yeast extract was added at concentration of 0.05 g l^{-1}. Organic acids were autoclaved for 20 min at 121 °C. Sugars, alcohols and amino acids were sterilized by filter-sterilization. Each carbon source was added at a concentration of 5 g l^{-1} (Cui *et al.*, 2011). Acid production from carbohydrates was evaluated according to the method of Gerhardt *et al.* (1994). Antibiotic sensitivity tests were performed on NOM-3 agar plates with antibiotic discs containing the following dosages (μg per disc, unless indicated): amoxicillin (10), ampicillin (10), bacitracin (0.04 IU), carbenicillin (100), cefotaxime (30), cefoxitin (30), cephalothin (30), ceftriaxone (30), chloramphenicol (30), ciprofloxacin (5), erythromycin (15), gentamicin (10), kanamycin (30), lincomycin (2), nalidixic acid (30), neomycin (30), nitrofurantoin (300), norfloxacin (10), novobiocin (30), nystatin (100), penicillin G (10 IU), polymyxin (300 IU), rifampicin (5), streptomycin (10), sulfamethoxazole (300), tetracycline (30), tobramycin (10) and vancomycin (30).

Polar lipids were extracted and separated on silica gel 60 F_{254} aluminium-backed thin-layer plates (10 × 10 cm, Merck 5554) and further analysed as Minnikin *et al.* (1984) and Cui *et al.* (2011) described. The solvent system chloroform/methanol/water (65 : 24 : 4, by vol.) was used in the first dimension and chloroform/glacial acetic acid/methanol/water (80 : 15 : 12 : 4, by vol.) was used in the second dimension. The TLC plates were sprayed with sulfuric acid/ethanol (1 : 1, v/v) and heated at 120 °C for 10 min to reveal total lipids.

Genomic DNA was extracted and purified by using the method described by Ng *et al.* (1995). The 16S rRNA gene was amplified by PCR using the universal archaea primers: forward 5′-ATTCCGGTTGATCCTGCCGGA-3′ and reverse 5′-AGGAGGTGATCCAGCCGCAG-3′. PCR products were cloned into vector pMD18-T (TaKaRa) and then sequenced. The sequence was compared with those from closely related species with EzTaxon-e server (Kim *et al.*, 2012). Multiple sequences were aligned with CLUSTAL W1.8 (Thompson *et al.*, 1994). Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987), the maximum-parsimony (Fitch, 1971) and the maximum-likelihood (Felsenstein, 1981) methods with the MEGA 5 program package (Tamura *et al.*, 2011). Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model (Kimura, 1980) for the neighbour-joining method. The DNA G+C content was determined by reversed-phase HPLC as described by Mesbah & Whitman (1989).

Colonies of strain KCY07-B2^T on NOM-3 agar plates after 2 weeks of incubation were 0.1–0.3 mm in diameter, cream-pigmented, circular and elevated. Cells were Gram-stain-negative, non-motile and pleomorphic rod-shaped (Fig. S1 available in IJSEM Online). Strain KCY07-B2^T grew at 25–50 °C, pH 6–8, 1.9–5.4 M NaCl and 0–1.5 M $MgCl_2$ with optimal growth at 37–45 °C, pH 7.0, 2.9–3.4 M NaCl and 0.5–1 M $MgCl_2$. Cells lysed in distilled water. Catalase, oxidase and phosphatase activities, H_2S production and hydrolysis of aesculin were positive. Nitrate reduction, indole, methyl red, Voges–Proskauer and Simmons citrate tests, hydrolysis of starch, casein and gelatin, as well as the production of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease and β-galactosidase were negative. Anaerobic growth did not occur with sodium thiosulfate, sodium sulfite, sodium sulfate, sodium nitrite, sodium nitrate, L-arginine or DMSO. Acid was produced from D-glucose and D-mannose. More detailed characteristics of strain KCY07-B2^T are given in the species description.

The polar lipids of strain KCY07-B2^T were $C_{20}C_{20}$ and $C_{20}C_{25}$ derivatives of phosphatidylglycerol (PG) and phosphatidylglycerol phosphate methyl ester (PGP-Me) together with five glyolipids. One of the glycolipids (GL1) was the bis-sulfated diglycosyl diether (S_2-DGD-1) while phosphatidylglycerosulfate was absent (Fig. S4).

A total of 18 complete 16S rRNA gene sequences were obtained. No heterogeneity was found in the 16S rRNA gene of strain KCY07-B2^T. Based on 16S rRNA gene sequence analysis, strain KCY07-B2^T was affiliated with the family *Halobacteriaceae* and closely related to *Halopiger xanaduensis* SH-6^T (95.8 %) and *Halopiger aswanensis* 56^T (95.5 %), while it shared low sequence similarity (<95 %) with other species of the family *Halobacteriaceae* (*Halobiforma lacisalsi* AJ5^T, 94.8 %; *Haloterrigena thermotolerans* PR5^T, 94.7 %; *Haloterrigena turkmenica* DSM 5511^T, 94.6 %; *Natrinema versiforme* XF10^T, 94.6 %; *Haloterrigena limicola* AX-7^T, 94.5 % and *Halobiforma haloterrestris* 135^T, 94.5 %). The three strains KCY07-B2^T, *Halopiger xanaduensis* SH-6^T (95.8 %) and *Halopiger aswanensis* 56^T formed an independent cluster on the phylogenetic trees (Fig. 1, Fig. S2 and Fig. S3). The genomic G+C content of strain KCY07-B2^T was 62.5 mol%.

Strain KCY07-B2^T was phylogenetically closest to *Halopiger xanaduensis* SH-6^T and *Halopiger aswanensis* 56^T. All three strains shared the same main polar lipids (PG, PGP-Me and S_2-DGD-1). There was little difference in their DNA G+C contents (62.5 mol%, 63.1 mol% and 66.3 mol%, respectively). Therefore, strain KCY07-B2^T should belong to the genus *Halopiger*. However, differences still existed among these three strains. The colonies of strain KCY07-B2^T were cream-pigmented while those of the other two strains were red and pink, respectively. The ranges of pH, temperature and NaCl concentration for growth were also different. Strain KCY07-B2^T shared low 16S rRNA gene

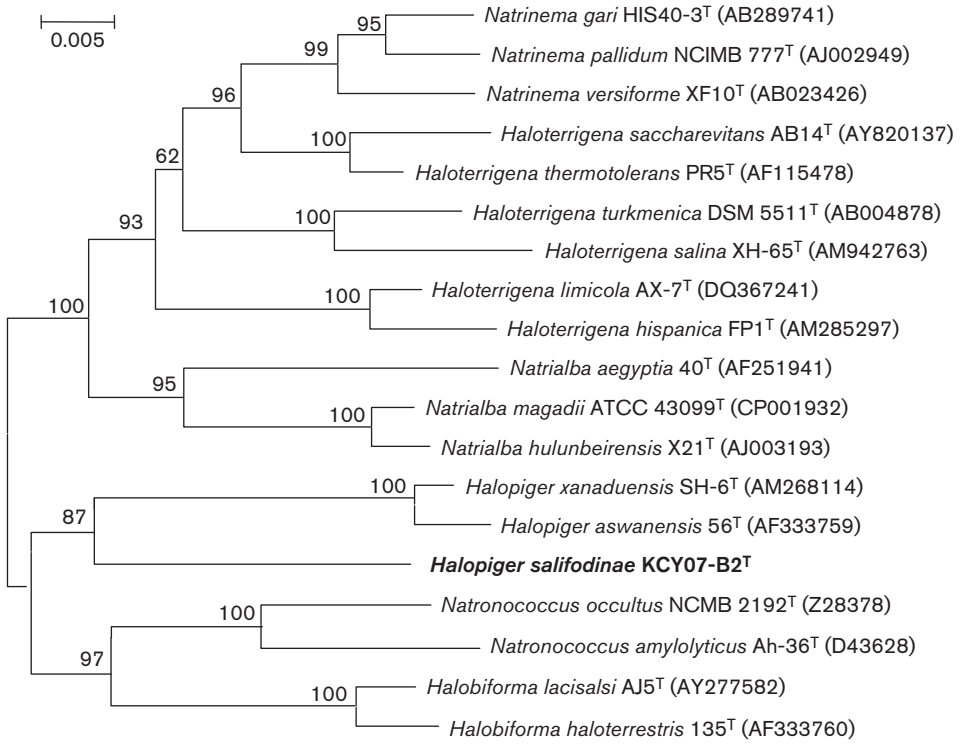

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships of strain KCY07-B2^T and related species. Bootstrap values based on 1000 replications are shown as percentages at branching points. Bar, 0.005 substitutions per nucleotide position.

sequence similarity with the other two strains (<96 %). *Halopiger aswanensis* 56^T contained more polar lipids than strain KCY07-B2^T (Fig. S4). The detailed differences in physiological and biochemical characterization among three strains are summarized in Table 1.

Based on the phenotypic, chemotaxonomic and genotypic characteristics described above, strain KCY07-B2^T was identified as a novel species within the genus *Halopiger*, for which the name *Halopiger salifodinae* sp. nov. is proposed.

Description of *Halopiger salifodinae* sp. nov.

Halopiger salifodinae (sa.li.fo.di′nae. L. gen. n. *salifodinae* of a saltpit, salt mine).

Cells are non-motile, strictly aerobic, pleomorphic rod-shaped and Gram-stain-negative. Colonies are cream-pigmented. Extremely halophilic. Growth occurs at 25–50 °C (optimum 37–45 °C), 1.9–5.4 M NaCl (optimum 2.9–3.4 M), 0–1.5 M $MgCl_2$ (optimum 0.5–1 M) and pH 6.0–8.0 (optimum 7.0). Cells lysed in distilled water. Catalase, oxidase and phosphatase activities, H_2S production and hydrolysis of aesculin are positive. Nitrate and nitrite reduction, indole, methyl red, Voges–Proskauer and Simmons citrate tests, hydrolysis of casein, gelatin, starch, Tween 20, Tween 40, Tween 60 and Tween 80, as well as the production of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease and β-galactosidase are negative. Able to use the following substrates as sole carbon sources: acetate, L-asparagine, citrate, fumarate, D-glucose, L-glutamate, glycine, isoleucine, L-lysine, L-malate, D-mannose, L-serine, D-sorbitol, starch, succinate and L-threonine. No growth occurs on D-arabinose, D-fructose, D-galactose, glycerol, lactose, maltose, D-mannitol, propionate, D-raffinose, D-ribose, D-trehalose or D-xylose. Acid is produced from D-glucose and D-mannose. Sensitive to the following antibiotics (μg per disc, unless indicated): ciprofloxacin (5), norfloxacin (10), novobiocin (30), rifampicin (5), nitrofurantoin (300) and nystatin (100). Resistant to amoxicillin (10), ampicillin (10), bacitracin (0.04 IU), carbenicillin (100), cefotaxime (30), cefoxitin (30), ceftriaxone (30), cephalothin (30), chloramphenicol (30), ciprofloxacin (5), erythromycin (15), gentamicin (10), kanamycin (30), lincomycin (2), nalidixic acid (30), neomycin (30), penicillin G (10 IU), polymyxin (300 IU), rocephin (30), streptomycin (10), sulfamethoxazole (300), tetracycline (30), tobramycin (10) and vancomycin (30). The polar lipids are $C_{20}C_{20}$ and $C_{20}C_{25}$ derivatives of PG and PGP-Me together with five glycolipids. S_2-DGD-1 is present.

The type strain KCY07-B2^T (=JCM 18547^T=CGMCC 1.12284^T) was isolated from a salt mine in Kuche county, Xinjiang province, China. The DNA G+C content of the type strain is 62.5 mol% (HPLC).

Table 1. Differential characteristics among strains KCY07-B2^T, *Halopiger xanaduensis* SH-6^T and *Halopiger aswanensis* 56^T

Taxa: 1, strain KCY07-B2^T; 2, *Halopiger xanaduensis* SH-6^T; 3, *Halopiger aswanensis* 56^T. Data for strains KCY07-B2^T and *Halopiger aswanensis* 56^T were taken from this study. The data for *Halopiger xanaduensis* SH-6^T was taken from Gutiérrez *et al.* (2007). All strains were positive for catalase and oxidase activities; all strains were negative for hydrolysis of casein, indole, methyl red test, nitrate reduction and anaerobic growth. The following substrates could be used by all strains as the sole carbon sources: acetate, L-asparagine, L-glutamate and L-serine. No growth occurred with glycerol, D-mannitol, propionate, D-raffinose, D-ribose or D-trehalose. Acid was produced from D-glucose by all three strains. All strains were sensitive to novobiocin and resistant to ampicillin, cephalothin, chloramphenicol, erythromycin, gentamicin, kanamycin, nalidixic acid, neomycin, penicillin G, polymyxin, streptomycin, tetracycline and vancomycin. −, Negative; +, positive; R, resistant; S, susceptible.

Characteristic	1	2	3
Cell morphology	Pleomorphic rods	Pleomorphic and mostly long rods	Pleomorphic rods
Pigmentation	Cream	Red	Pink
Maximum growth temperature (°C)	50	45	55
Temperature optimum (°C)	37–45	37	45
pH range	6.0–8.0	6.0–11.0	6–9.2
pH optimum	7.0	7.5–8.0	7.5
NaCl range (M)	1.9–5.4	2.5–5.0	1.7–5.2
NaCl optimum (M)	2.9–3.4	4.3	3.8–4.3
H_2S production	+	−	+
Indole production	−	−	+
Urease	−	+	−
Nitrite from nitrate	−	+	+
Hydrolysis of starch	−	−	+
Hydrolysis of gelatin	−	+	−
Hydrolysis of Tween 80	−	+	+
Acid production from:			
Arabinose	−	+	+
Fructose	−	−	+
L-Malate	−	−	+
Maltose	−	−	+
D-Mannose	+	−	−
Starch	−	−	+
Xylose	−	+	+
Antibiotic tolerance			
Rifampicin (5 μg)	S	R	S
Bacitracin (0.04 IU)	R	S	S
Sulfamethoxazole (300 μg)	R	S	S
DNA G+C content (mol%)	62.5*	63.1	66.3*
Polar lipids	PG, PGP-Me, GL1 (S_2-DGD-1), GL2, GL3, GL4, GL6	PG, PGP-Me, S2-DGD-1	PG, PGP-Me, GL1 (S_2-DGD-1), GL2, GL3, GL5, GL6, GL7, L1, L2

*The DNA G+C contents of strains KCY07-B2^T and *Halopiger aswanensis* 56^T were determined by HPLC under identical conditions.

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