Halobiforma lacisalsi sp. nov., isolated from a salt lake in China

Xue-Wei Xu,[1,2] Min Wu,[2] Pei-Jin Zhou[1] and Shuang-Jiang Liu[1]

[1]State Key Laboratory for Microbial Resources at Institute of Microbiology, Chinese Academy of Sciences, ZhongGuanCun, Haidian, Beijing 100080, PR China

[2]College of Life Sciences, Zhejiang University, Hangzhou 310027, PR China

Correspondence
Shuang-Jiang Liu
shuangjiang@hotmail.com

A Gram-negative, motile, neutrophilic and extremely halophilic strain, AJ5^T, was isolated from a salt lake in Xinjiang, China, and subjected to polyphasic taxonomic study. The major polar lipids of the isolate were $C_{20}C_{20}$ and $C_{20}C_{25}$ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and glycolipid. The DNA G+C content was 64·9 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain AJ5^T clustered with members of the genus *Halobiforma*, exhibiting high sequence similarity to the 16S rRNA gene sequences of *Halobiforma nitratireducens* (96·3 %) and *Halobiforma haloterrestris* (99·0 %). Comparative analysis of phenotypic characteristics and DNA–DNA hybridization between strain AJ5^T and *Halobiforma* species supported the conclusion that AJ5^T represents a novel species within this genus, for which the name *Halobiforma lacisalsi* sp. nov. is proposed. The type strain is AJ5^T (=CGMCC 1.3738^T=JCM 12983^T).

The genus *Halobiforma* was proposed by Hezayen *et al.* (2002) and currently contains two species, *Halobiforma haloterrestris* and *Halobiforma nitratireducens* (formerly *Natronobacterium nitratireducens*; Xin *et al.*, 2001). The halophilic archaea of this genus are motile, Gram-negative rods or coccoid in shape (Hezayen *et al.*, 2002). During a study on microbial producers of bacteriorhodopsin from Ayakekum salt lake (37° N 89° E, altitude of 3880 m) located in Xinjiang, China, a red-pigmented, extremely halophilic strain was isolated. On the basis of 16S rRNA gene sequence analysis, strain AJ5^T is closely related to members of the genus *Halobiforma*. The aim of this study was to determine the taxonomic status of this isolate, and it is proposed that strain AJ5^T represents a novel species of the genus *Halobiforma*.

Strain AJ5^T was isolated from a water sample (pH 7·8) of Ayakekum salt lake. After enrichment of the sample in rich medium (Oesterhelt & Stoeckenius, 1974) at 37 °C with shaking for 1–2 weeks, a pure culture was obtained by plating serial dilutions of enrichment culture and restreaking several times. The organism was grown and maintained on S-G medium (Sehgal & Gibbons, 1960).

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AJ5^T is AY277582.

Polar lipid profiles of strain AJ5^T and related species as revealed by one-dimensional TLC are available as supplementary material in IJSEM Online.

Phenotypic tests were performed according to the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). Cell morphology was examined by using optical microscopy (BX40 microscope; Olympus) without fixation and by Gram staining with acetic acid fixation (Dussault, 1955). Flagellation was examined by using a transmission electronic microscope (H-600; Hitachi) at 100 kV after negative staining with 1 % (w/v) phosphotungstic acid solution containing 25 % NaCl (w/v). The salt range for growth was determined in liquid S-G medium modified with NaCl at final concentrations of 5, 8, 10, 12, 15, 18, 20, 22, 25 or 30 % (w/v). The Mg^{2+} range for growth was tested in liquid S-G medium containing $MgCl_2$ at final concentrations of 0, 0·005, 0·01, 0·02, 0·05, 0·1, 0·2, 0·5 and 1·0 M. The pH range for growth (from pH 5·0 to 9·5, with intervals of 0·5 pH units) was examined in S-G medium containing the following buffers (50 mM): MES at pH 5·0–6·0, PIPES at pH 6·5–7·0, Tricine at pH 7·5–8·5 and CHES at pH 9·0–9·5. The determination of growth temperatures for strain AJ5^T in S-G medium (pH 7·5) was carried out using a TN3F temperature-gradient incubator (Advantec). The growth rate was determined by measuring culture turbidity at 600 nm. Anaerobic growth was tested in the presence of nitrate, L-arginine or DMSO (5 g l^{-1} in each case) in filled stoppered tubes. Gelatin hydrolysis was determined as described by Oren *et al.* (2002). The following tests were performed, according to Xin *et al.* (2000), as mentioned previously (Oren *et al.*, 1997): hydrolysis of starch, casein and Tween 80; nitrate and nitrite reduction; production of indole and H_2S; catalase

and oxidase activities; and utilization of sugars, alcohols, amino acids and organic acids.

Total polar lipids were extracted by using the modified method of Kamekura & Kates (1988) and were analysed according to Xin *et al.* (2000) after being separated on silica gel plates (10 × 10 cm) by TLC. Genomic DNA of strain was prepared by using the method of Marmur (1961) and the purity was checked spectrometrically. The G+C content of the DNA was determined by thermal denaturation (T_m) (Marmur & Doty, 1962).

The 16S rRNA gene sequence was amplified under the conditions described by Feng *et al.* (2005) with the following primers: primer 1 (5′-ATTCCGGTTGATCCTGC-3′, *Escherichia coli* positions 6–22) and primer 2 (5′-AGGAGGTGATCCAGCCGCAG-3′, *E. coli* positions 1540–1521). The sequence was compared with closely related sequences of reference organisms from the FASTA network service (http://www.ebi.ac.uk/fasta33). Sequence data were aligned with CLUSTAL W, version 1.8 (Thompson *et al.*, 1994). Phylogenetic trees were constructed by the neighbour-joining methods within the MEGA 3 program package (Kumar *et al.*, 2004). DNA–DNA hybridizations were carried out in 2 × SSC at 79 °C, by following the procedure of De Ley *et al.* (1970), as modified by Huß *et al.* (1983), with a Beckman Coulter DU800 spectrophotometer equipped with a high-performance temperature controller.

The cells of strain AJ5^T are Gram-negative and motile by means of several flagella (Fig. 1). The cells are pleomorphic: they mainly occur as long rods (0·5–0·8 × 3·0–5·0 μm) in liquid medium during exponential growth, but occasionally they occur as short rods (0·5–0·8 × 1·2–3·0 μm) or coccoid forms (0·5–1·5 μm in diameter) on solid medium. Colonies grown on S-G agar plates are reddish, smooth, circular, slightly elevated and 0·5–1 mm in diameter after incubation for 6 days at 37 °C. Other phenotypic characteristics of strain AJ5^T are summarized in Table 1 and the species description.

The almost-complete 16S rRNA gene sequence of strain AJ5^T (1471 nt; nucleotide positions 6–1540 according to *E. coli* numbering) showed 99·0 % similarity to the rRNA gene sequences of *H. haloterrestris* and of the halophilic archaeon strain AB1, which was isolated from the same origin but lost during subsequent cultivation, 96·3 % similarity to that of *H. nitratireducens* and less than 95 % similarity to the sequences of other genera. On the basis of 16S rRNA gene sequences, strain AJ5^T clustered with

Table 1. Characteristics that differentiate strain AJ5^T from other *Halobiforma* species

Strains: 1, strain AJ5^T (data from this study); 2, *H. haloterrestris* JCM 11627^T (Hezayen *et al.*, 2002); 3, *H. nitratireducens* AS 1.1980^T (Xin *et al.*, 2001). Symbols: +, positive; −, negative; W, weak; NR, not reported. All *Halobiforma* strains are motile, reduce nitrate, hydrolyse gelatin and Tween 80, are catalase- and oxidase-positive and are Gram-negative.

Characteristic	1	2	3
NaCl range (M)	>1·7	>2·2	2·5–5·2
pH range	6·5–9·0	6·0–9·2	8·0–10·5
pH optimum	7·5	7·5	8·9
Anaerobic growth with DMSO	−	NR	+
Anaerobic growth with nitrate	+	−	+
Indole formation	−	+	−
Hydrolysis of starch	−	−	+
Hydrolysis of casein	−	+	−
Utilization of:			
Fructose	+	−	−
Arabinose	−	+	−
Mannitol	−	NR	+
Maltose	W	+	−
Sucrose	−	+	−
D-Xylose	−	+	−
Acid production from:			
Arabinose	−	+	NR
Maltose	−	+	NR
Sucrose	−	+	NR
D-Xylose	−	+	NR
Sensitivity to tetracycline	W	−	NR
DNA G+C content (mol%)	64·9	66·9	63·8

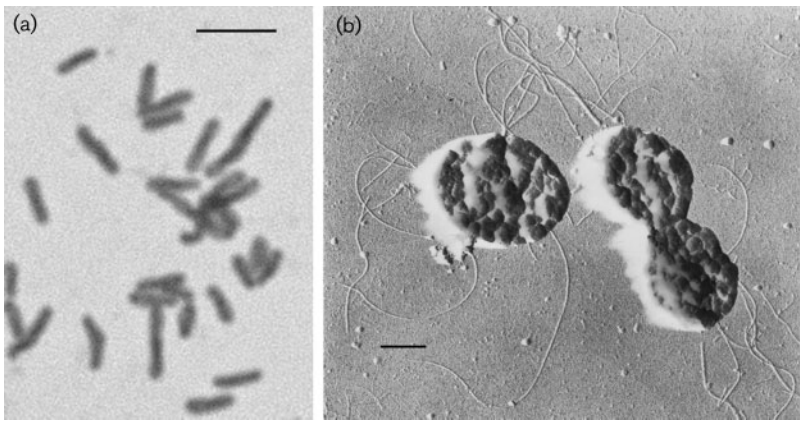

Fig. 1. (a) Phase-contrast photograph of Gram-stained *H. lacisalsi* strain AJ5^T cells grown in S-G broth. (b) Transmission electron micrograph of *H. lacisalsi* strain AJ5^T cells grown on S-G agar plates and stained with 1 % (w/v) phosphotungstic acid solution containing 25 % (w/v) NaCl. Bars: 10 μm (a) and 0·5 μm (b).

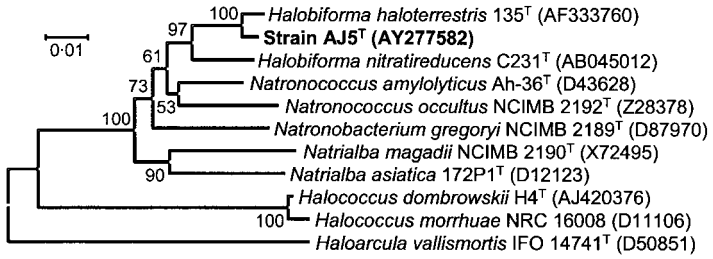

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences from strain AJ5^T and other related organisms. The tree was constructed using the neighbour-joining method, with bootstrap values calculated from 100 resamplings. The number at each branch point represents the percentage bootstrap support. Bar, 0·01 substitutions per nucleotide position.

previously described *Halobiforma* species in the phylogenetic tree, with high bootstrap values (>95 %) (Fig. 2). The polar lipid profile of strain AJ5^T was compared with those of *H. haloterrestris* and *H. nitratireducens* by one-dimensional TLC (see the supplementary figure available in IJSEM Online) and two-dimensional TLC. The results showed that they had very similar polar lipid profiles: all possess $C_{20}C_{20}$ and $C_{20}C_{25}$ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and glycolipid, but lack phosphatidylglycerol sulfate (Hezayen *et al.*, 2002).

Strain AJ5^T is phylogenetically closest to *H. haloterrestris*. However, they differ in that the latter uses arabinose, maltose, sucrose and D-xylose, but not fructose (Table 1). In addition, *H. haloterrestris* grows at pH 6·0, is insensitive to tetracycline and has a higher DNA G+C content (66·9 mol%). They also showed differences in other important phenotypic traits such as anaerobic growth with nitrate, indole formation and casein hydrolysis (Table 1). Strain AJ5^T could be distinguished from *H. nitratireducens* by phenotypic characteristics, especially the pH range for growth (Table 1). Strain AJ5^T is neutrophilic (pH 6·5–9·0 for growth, with an optimum at pH 7·5) while *H. nitratireducens* is alkaliphilic (pH 8·0–10·5 for growth, with an optimum at pH 8·9) (Xin *et al.*, 2001). Furthermore, the DNA–DNA hybridization rates for strain AJ5^T with respect to *H. haloterrestris* and *H. nitratireducens* were 58·4 % and 27·4 %, respectively. Therefore, we suggest that strain AJ5^T represents a novel species of the genus *Halobiforma*, for which the name *Halobiforma lacisalsi* sp. nov. is proposed.

Description of *Halobiforma lacisalsi* sp. nov.

Halobiforma lacisalsi (la.ci.sal′si. L. masc. n. *lacus* lake; L. adj. *salsus -a -um* salted, salt; N.L. gen. n. *lacisalsi* of a salt lake).

Cells are motile, Gram-negative and pleomorphic (rods and coccoid forms). Rods lyse in distilled water. Colonies are reddish, smooth, circular and elevated. Growth requires at least 1·7 M NaCl, optimally 2·6–4·3 M NaCl. Growth occurs at 0–0·5 M $MgCl_2$, optimally at around 0–0·2 M $MgCl_2$. The pH range for growth is 6·5–9·0, with an optimum at pH 7·5. The temperature range for growth is between 24 and 57 °C, with an optimum at 42–45 °C. Chemo-organotrophic. Anaerobic growth occurs in the presence of nitrate. Oxidase- and catalase-positive. Nitrate is reduced and gas is produced. Nitrite is not reduced. Negative for indole formation. Gelatin and Tween 80 are hydrolysed, whereas starch and casein are not. H_2S is produced from thiosulfate. The following substrates are utilized for growth: fructose, glucose, glycerol, glutamate, arginine, lysine, ornithine, acetate, pyruvate and lactate. Mannose, starch, arabinose, lactose, mannitol, rhamnose, sorbitol, maltose, galactose, D-ribose, sucrose, D-xylose, aspartate, glycine, alanine, fumarate, malate, propionate, succinate and citrate are not utilized for growth. Acid is produced from glucose and glycerol. Sensitive to tetracycline and vancomycin, but not to norfloxacin, ciprofloxacin, streptomycin, kanamycin or ampicillin. The major polar lipids are $C_{20}C_{20}$ and $C_{20}C_{25}$ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and glycolipid. The G+C content of the DNA is 64·9 mol% (T_m).

The type strain, strain AJ5^T (=CGMCC 1.3738^T=JCM 12983^T), was isolated from a salt lake in Altun Mountain in China.

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