Salsuginibacillus halophilus sp. nov., a halophilic bacterium isolated from a soda lake

Shu-Juan Cao, Jian-Hang Qu, Hong-Li Yuan and Bao-Zhen Li

Correspondence

Hong-Li Yuan

hlyuan@cau.edu.cn

State Key Lab for Agrobiotechnology, Key Laboratory of Agro-Microbial Resource and Application, Ministry of Agriculture, College of Biological Sciences, China Agricultural University, Beijing 100193, PR China

A Gram-stain-positive, rod-shaped, endospore-forming, halophilic, alkalitolerant bacterium, designated halo-1^T, was isolated from sediment of Xiarinaoer soda lake, located in the Inner Mongolia Autonomous Region of China. Strain halo-1^T grew in the presence of 9–30 % (w/v) NaCl (optimum 19 %) and at pH 5–10 (optimum pH 9). The cell-wall peptidoglycan contained meso-diaminopimelic acid and the major respiratory isoprenoid quinone was MK-7. The predominant cellular fatty acids of the isolate were anteiso-C_{15:0} (58.35 %), anteiso-C_{17:0} (12.89 %) and C_{16:0} (6.52 %). The polar lipids contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, glycolipid and a phospholipid of unknown structure. The DNA G + C content of the strain was 46.4 mol%. On the basis of 16S rRNA gene sequence similarity, strain halo-1^T showed the highest similarity (93.9 %) to *Salsuginibacillus kocurii* CH9d^T. Strain halo-1^T could be clearly differentiated from its closest phylogenetic relative on the basis of several phenotypic, genotypic and chemotaxonomic features. Therefore, strain halo-1^T represents a novel species, for which the name *Salsuginibacillus halophilus* sp. nov. is proposed, with the type strain halo-1^T (=CGMCC 1.7653^T =NBRC 104934^T).

Soda lakes represent the most alkaline naturally occurring environments on earth, with pH values generally greater than 10, occasionally reaching 12. These lakes are characterized by the presence of large amounts of Na₂CO₃ (usually as Na₂CO₃ · 10H₂O or Na₂CO₃ · NaHCO₃ · 2H₂O) and depletion of Mg²⁺ and Ca²⁺ because of the insolubility of these cations as carbonate minerals under alkaline conditions. Such lakes are also somewhat saline, due to the concomitant increase in Cl[−] (Duckworth *et al.*, 1996).

In order to investigate alkaliphilic and halophilic bacteria, we isolated some strains from sediment of Xiarinaoer soda lake, which is located in Inner Mongolia, China. The sample was collected in March 2006 and the temperature was −1 °C, the pH was 9.91 and the salinity of the water was 84 g NaCl l^{−1} at the time of sampling. The sediment was suspended in liquid base complex medium and vortexed for about 1 min. The suspension was allowed to settle, serially diluted with liquid medium and spread on plates of the base complex medium. The base complex medium for isolation and maintenance contained 7.5 g casein hydrolysate, 10 g yeast extract (Difco), 3 g sodium citrate, 2 g KCl, a trace amount of FeSO₄, 200 g NaCl and

10 g Na₂CO₃ in 1 l distilled water. If required, the medium was solidified by adding agar (18 g l^{−1}) (Pan *et al.*, 2006). The pH was adjusted to 9 with 30 % (w/v) Na₂CO₃ solution and plates were incubated at 37 °C for 1 week. The reference type strain *Salsuginibacillus kocurii* DSM 18087^T was grown using the medium and conditions reported by Carrasco *et al.* (2007).

A 16S rRNA gene fragment from strain halo-1^T was amplified using individual bacterial colony PCR (Güssow & Clackson, 1989). The primers used for PCR were 27F (5'-GAGAGTTTGATCCTGGCTCAG) and 1495R (5'-CTACGGCTACCTTGTACGA) (covering positions 27–1495 in the *Escherichia coli* numbering system; Brosius *et al.*, 1978). PCR products were sequenced using an Applied Biosystems DNA Sequencer 3730 with the software provided by the manufacturer. An almost-complete 16S rRNA gene sequence of strain halo-1^T (1438 bp) was initially compared with reference sequences in the GenBank database using BLAST (Altschul *et al.*, 1997).

Alignments of sequences were carried out using CLUSTAL W version 1.8 (Thompson *et al.*, 1994). Similarity was calculated by the method of Jukes & Cantor (1969) in the CLUSTAL W program. Phylogenetic analysis of multiple sequence alignments was performed with software MEGA version 3.1 (Kumar *et al.*, 2004). Phylogenetic tree reconstruction was carried out by the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1993) and maximum-parsimony (Kluge & Farris, 1969) methods.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain halo-1^T is EU581835.

Maximum-parsimony and minimum-evolution 16S rRNA gene sequence-based phylogenetic trees are available as supplementary material with the online version of this paper.

With respect to 16S rRNA gene sequence similarity, strain halo-1^T was most closely related to *Salsuginibacillus kocurii* CH9d^T (93.9 %); similarity to members of other genera was lower than 92 %. These rather low levels of sequence similarity suggest that strain halo-1^T belongs to a novel taxonomic group.

The phylogenetic analysis showed that strain halo-1^T belonged to the *Firmicutes*. It formed a phyletic branch with *S. kocurii* CH9d^T in the neighbour-joining analysis (Fig. 1). Phylogenetic trees constructed using the minimum-evolution and maximum-parsimony methods gave topologies similar to that of the neighbour-joining tree (Supplementary Fig. S1, available in IJSEM Online).

To characterize strain halo-1^T phenotypically, standard phenotypic tests were performed on both strain halo-1^T and its closest neighbour *S. kocurii* DSM 18087^T using the alkaline, saline medium described by Carrasco *et al.* (2007); the concentration of NaCl was adjusted to 19 % for growth of strain halo-1^T. Gram staining was performed using the method described by Dussault (1955). To determine cell morphology, bacterial cultures grown on plates for about 2 days at 37 °C were stained using crystal violet and then examined by microscopy. Motility was tested using the alkaline, saline medium using plates containing 0.15 % agar; the occurrence of diffuse growth along the stab line in the medium was observed. Spore formation was tested using cultures grown for 3 weeks on alkaline, saline medium with CaCl₂ (1 mM), MnCl₂ (50 nM) and MgCl₂ (1 mM) added. Colony morphology was observed after 2 days of incubation on solid alkaline, saline medium. Growth at different concentrations of NaCl was determined in liquid alkaline, saline medium with 0 and 1–30 % (w/v) NaCl (in increments of 2 %). The pH for growth was tested in liquid medium at pH 5.0–13.0 (in increments of

0.5 pH units) adjusted with 30 % (w/v) Na₂CO₃ solution. The temperature range for growth was determined by incubation in the same liquid medium for 1 day to 3 weeks at 4, 18, 28, 37 and 50 °C. Growth was scored as OD₆₀₀. The catalase reaction was performed by placing a small amount of the culture incubated for 24 h on a slide and adding a drop of 3 % H₂O₂ solution. The presence of gas bubbles indicated a positive reaction. Oxidase was examined by daubing cultures incubated for 24 h on filter paper soaked with *N,N*-dimethyl *p*-phenylenediamine solution; the appearance of a red colour within 10 s indicated a positive reaction. Utilization of carbohydrates was assessed using liquid medium containing 2.0 g (NH₄)₂SO₄, 0.5 g NaH₂PO₄, 0.5 g K₂HPO₄, 0.2 g MgSO₄, 0.02 % (w/v) yeast extract and 19 % (w/v) NaCl for halo-1^T or 10 % (w/v) NaCl for *S. kocurii* DSM 18087^T, with the tested carbohydrate at 0.5 % (w/v), in 1 l distilled water; the pH was adjusted to 9.0. Incubation was at 37 °C, and the result was determined spectrophotometrically by measuring the OD₆₀₀ after 3 days. Other tests including aesculin hydrolysis, phenylalanine deaminase, antibiotic sensitivity and other tests listed in Table 1 and the species description were performed according the methods described by Dong & Cai (2001); *S. kocurii* DSM 18087^T was also tested as a control.

Cell mass for quinone, cellular fatty acid and cell-wall peptidoglycan analyses was obtained from a culture grown in base complex medium at 37 °C, pH 9.0 and the appropriate optimal NaCl concentration. For analysis of isoprenoid quinones, cells grown aerobically were freeze-dried and subjected to extraction of the lipid fraction as described by Collins (1985). For quantitative analysis of cellular fatty acids, fatty acid methyl ester mixtures were prepared and identified following the manufacturer's instructions for the Microbial Identification System (MIDI Inc.); fatty acids were identified and quantified by the Sherlock MIS software (version 4.5). The type of cell-wall peptidoglycan was determined by TLC (Hasegawa *et al.*, 1983). For polar lipid analyses, cell biomass of strain halo-1^T and *S. kocurii* DSM 18087^T was obtained from cultures grown in alkaline, saline medium at 37 °C for 24 h. Lipids were extracted according to the method of Bligh & Dyer (1959). Polar lipids were analysed by two-dimensional TLC on silica gel plates (Merck) (Minnikin *et al.*, 1977).

Genomic DNA was prepared according to the method of Marmur (1961). The G+C content was analysed by the thermal denaturation method using a BIO-20 UV spectrophotometer according to De Ley *et al.* (1970). For the calculation of DNA G+C content, the equation of De Ley *et al.* (1970) was used and corrected using genomic DNA of *Escherichia coli* K-12 as a reference.

Cells of strain halo-1^T were Gram-stain-positive, aerobic, non-motile straight rods. The oxidase reaction was negative and the catalase reaction was positive. Colonies grown on alkaline, saline agar medium were round,

Fig. 1. Neighbour-joining tree showing the phylogenetic position of strain halo-1^T, *S. kocurii* CH9d^T and other reference strains based on 16S rRNA gene sequences. The sequence of *Paenibacillus massiliensis* 2301065^T was used as an outgroup. Bootstrap values expressed as percentages of 1000 replications are shown at branch points when greater than 50 %. Bar, 0.01 substitutions per nucleotide position.

Table 1. Characteristics that distinguish strain halo-1^T from *S. kocurii* CH9d^T

Data for *S. kocurii* CH9d^T were taken from Carrasco *et al.* (2007) unless indicated.

Characteristic	Strain halo-1 ^T	<i>S. kocurii</i> CH9d ^T
Colony pigmentation	Yellow	Cream
NaCl concentration for growth (% w/v)		
Range	9–30	3–20
Optimum	19	10
pH for growth		
Range	5.0–10.0	5.8–10.0
Optimum	9.0	8.5
Urease	+	–*
H ₂ S production	+	–
Hydrolysis of:		
Gelatin	+	–
Aesculin	–	+†
DNA G + C content (mol%)	46.4	44.7
Utilization of:		
Lactose, maltose, D-salicin, trehalose, glycerol,	+	–
D-sorbitol, inositol		
D-Ribose	–	+
Sensitivity to:		
Tetracycline	–	+
Kanamycin	+	–

*Data from this study.

†Data from this study; reported as negative by Carrasco *et al.* (2007).

convex, entire, yellow and semi-transparent, 1–2 mm in diameter after 2 days of incubation at 37 °C. Spores were present in a central position. Strain halo-1^T was extremely halophilic and alkalitolerant. It grew in media containing 9–30 % (w/v) NaCl and optimally at 19 % (w/v) NaCl. The strain could not grow without NaCl. The pH range for growth was 5.0–10.0 (optimum pH 9.0). For *S. kocurii* DSM 18087^T, the range of NaCl concentration for growth was 3–20 % (w/v) NaCl and optimal growth was observed at 10 % (w/v). H₂S was produced by strain halo-1^T, but not by *S. kocurii* DSM 18087^T. Hydrolysis of gelatin was positive for strain halo-1^T but negative for *S. kocurii* DSM 18087^T. *S. kocurii* DSM 18087^T could hydrolyse aesculin but strain halo-1^T could not. Detailed morphological and physiological characteristics are given in the species description and Table 1.

The major fatty acids of strain halo-1^T were anteiso-C_{15:0} (58.35 %) and anteiso-C_{17:0} (12.89 %), similar to *S. kocurii* DSM 18087^T. The whole-cell fatty acid profile is shown in Table 2. It is possible that the differences between the strains in the proportions of some fatty acids were the result of the different media used for growth. However, the major components are clear. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid, and predominant menaquinone was MK-7, as for phylogenetically related taxa. The polar lipids of strain halo-1^T contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidyletha-

nolamine, glycolipid and a phospholipid of unknown structure. The G + C content of the genomic DNA of strain halo-1^T was 46.4 mol%, a little higher than its closest neighbour *S. kocurii* CH9d^T (44.7 mol%). On the basis of the phylogenetic, morphological and physiological characteristics described above, isolate halo-1^T belongs to the genus *Salsuginibacillus*; however, the low 16S rRNA gene sequence similarity and other differences between halo-1^T and *S. kocurii* DSM 18087^T indicate that strain halo-1^T represents a novel species, for which the name *Salsuginibacillus halophilus* sp. nov. is proposed.

Description of *Salsuginibacillus halophilus* sp. nov.

Salsuginibacillus halophilus (ha.lo.phi'lus. Gr. n. *hals* salt; Gr. adj. *philos* loving; N.L. masc. adj. *halophilus* salt-loving).

Cells are Gram-stain-positive, spore-forming, non-motile rods, 0.5–0.8 × 2.5–4.5 µm. After 48 h, colonies on agar medium are 1–2 mm in diameter, round, convex, entire, yellow and semi-transparent. Growth occurs at 18–50 °C (optimum 37 °C). Halophilic; grows at 9–30 % (w/v) NaCl. Can not grow without NaCl; optimal growth at 19 % (w/v) NaCl. The pH range for growth is 5.0–10.0 (optimum pH 9.0). Catalase-positive and oxidase-negative. Nitrate is reduced to nitrite. Casein, DNA, Tween 80, starch and aesculin are not hydrolysed; gelatin is clearly hydrolysed.

Table 2. Cellular fatty acid profiles of strain halo-1^T and *S. kocurii* CH9d^T

Data for *S. kocurii* CH9d^T were taken from Carrasco *et al.* (2007). —, Not detected/not reported. Strain halo-1^T was grown on base agar medium (pH 9) prepared following the manufacturer's instructions for the Microbial Identification System (MIDI).

Fatty acid	Strain halo-1 ^T	<i>S. kocurii</i> CH9d ^T
C _{10:0}	0.05	—
C _{12:0}	0.15	—
iso-C _{13:0}	0.04	—
anteiso-C _{13:0}	0.21	—
iso-C _{14:0}	5.13	2.0
C _{14:0}	1.27	0.3
Summed feature 1*	0.34	—
anteiso-C _{15:1} A	0.20	—
iso-C _{15:0}	5.28	6.9
anteiso-C _{15:0}	58.35	49.7
C _{15:0}	0.95	—
iso-C _{16:0}	4.40	5.7
Summed feature 3*	0.39	—
C _{16:0}	6.52	—
iso-C _{17:0}	0.54	8.0
anteiso-C _{17:0}	12.89	16.4
C _{17:0} cyclo	0.11	—
iso-C _{17:1} ω5c	—	1.8
C _{17:0}	0.24	—
anteiso-C _{17:1}	—	1.6
C _{18:1} ω9c	0.68	—
C _{18:1} ω7c	0.57	—
C _{18:1} ω5c	0.31	—
C _{18:0}	1.27	—
iso-C _{18:0}	—	0.23
C _{19:0} cyclo ω8c	0.11	—

*Summed features are combinations of fatty acids that cannot be separated by the MIDI system. Summed feature 1 comprises iso-C_{15:1} H and/or C_{13:0} 3-OH; summed feature 3 comprises iso-C_{15:0} 2-OH and/or C_{16:1}ω7c.

H₂S is produced. Indole is not detected. Urease test is positive. Phenylalanine deaminase-negative. The following carbohydrates can be used as sole carbon sources: D-glucose, cellobiose, lactose, maltose, sucrose, D-salicin, trehalose, D-mannitol, glycerol, L-rhamnose, D-sorbitol and inositol. D-Ribose, D-fructose, D-galactose, D-mannose, raffinose, starch, sodium gluconate, D- and L-arabinose, D-xylose, melezitose and inulin are not fermented. Resistant to chloramphenicol (30 µg per disc), ampicillin (10 µg), tetracycline (30 µg) and carbenicillin (100 µg) and sensitive to streptomycin (10 µg) and kanamycin (30 µg). Major cellular fatty acids are anteiso-C_{15:0} and anteiso-C_{17:0}. Cell-wall peptidoglycan contains meso-diaminopimelic acid. Polar lipids include diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, glycolipid and a phospholipid of unknown structure.

Predominant respiratory quinone is MK-7. The DNA G + C content of the type strain is 46.4 mol%.

The type strain, halo-1^T (=CGMCC 1.7653^T =NBRC 104934^T), was isolated from sediment of Xiarinaoer soda lake in Inner Mongolia, China.

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