Bacillus solisalsi sp. nov., a halotolerant, alkaliphilic bacterium isolated from soil around a salt lake

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A novel Gram-positive, motile, rod-shaped bacterium isolated from a saline soil in China was characterized by a polyphasic taxonomic approach. The strain, designated YC1^T, was halotolerant [tolerating up to 15 % (w/v) NaCl] and alkaliphilic (growing at a broad pH range of 5–13). 16S rRNA gene sequence analysis revealed that the isolate belonged to the genus *Bacillus*, showing highest similarity to *Bacillus macauensis* JCM 13285^T (98.0 %). However, DNA–DNA hybridization indicated low levels of genomic relatedness with *B. macauensis* JCM 13285^T (8.5 %). The major isoprenoid quinone was MK-7 and the cellular fatty acid profile consisted of significant amounts of iso-C_{15:0} (38.6 %) and anteiso-C_{15:0} (35.9 %). The predominant polar lipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The G + C content of the genomic DNA was 41.8 mol%. On the basis of the polyphasic evidence from this study, strain YC1^T (=KCTC 13181^T=CGMCC 1.6854^T) should be classified as the type strain of a novel species of the genus *Bacillus*, for which the name *Bacillus solisalsi* sp. nov. is proposed.

Many haloalkaliphilic and/or alkalitolerant *Bacillus* species that belong to rRNA group 6 (Ash *et al.*, 1991; Nielsen *et al.*, 1994; Yoon *et al.*, 2004; Nogi *et al.*, 2005; Lim *et al.*, 2006; Borchert *et al.*, 2007) are widely distributed in different saline and alkaline habitats with a high pH and a variable (up to saturation) salt concentration. This group of bacteria has attracted much interest because its members can secrete various potentially useful enzymes (Horikoshi, 2006). Recently, some haloalkaliphilic and alkalitolerant species have been isolated from saline soil samples (Olivera *et al.*, 2005; Nowlan *et al.*, 2006; Lee *et al.*, 2006; Ghosh *et al.*, 2007). This study describes the characterization and identification of a novel halotolerant and alkaliphilic

species of the genus *Bacillus* that was isolated from a soil sample around Yuncheng Salt Lake (34° N 111° E), which is located in the South Shanxi Basin of Shanxi Province, China. Yuncheng Salt Lake is the third largest sodium sulfate inland lake in the world with an area of 132 km² and it was formed in the Quaternary Period of the Cenozoic Era. After the seas dried up and mountains formed, a large amount of saline mineral substances gathered and, after a long period of deposition and evaporation, a natural salt lake was formed.

Strain YC1^T was isolated using the dilution plating technique on Luria–Bertani (LB) medium which contained the following (g l⁻¹): yeast extract, 5; tryptone, 10; and NaCl, 10. The pH was adjusted to 7.0 with 1 M HCl. The organism was incubated at 30 °C and routinely cultivated on LB agar or in LB broth at the same temperature under aerobic conditions.

The primers used for PCR amplification of the 16S rRNA gene were 5'-AGAGTTTGATCCTGGCTCAG-3' (forward) and 5'-AAGGAGGTGATCCAAGCCGCA-3' (reverse), corresponding to positions 8–27 and 1521–1540, respectively, in the 16S rRNA gene sequence of *Escherichia coli* (Brosius *et al.*, 1978). The 1424 bp sequence of strain YC1^T

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Abbreviations: AL, aminolipid; APL, aminophospholipid; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain YC1^T is EU046268.

The TLC analysis of the polar lipid composition of the novel strain and *B. macauensis* and a transmission electron micrograph of cells of strain YC1^T are available as supplementary figures with the online version of this paper.

was compared with sequences retrieved from GenBank using the BLAST program (<http://www.ncbi.nlm.nih.gov/blast/>) to determine an approximate phylogenetic affiliation. Phylogenetic analysis was performed using the PHYLIP (Felsenstein, 1993) and MEGA version 3.1 (Kumar *et al.*, 2001) software packages after multiple alignment of the data with CLUSTAL_X (Thompson *et al.*, 1997). Distances (distance options according to the Kimura two-parameter model; Kimura, 1980, 1983) and clustering were based on the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods. Bootstrap analysis (1000 replications) was used to evaluate the topology of the neighbour-joining tree (Felsenstein, 1985). Comparison of the 16S rRNA gene sequences showed that strain YC1^T was clearly affiliated with the genus *Bacillus* and was most closely related to *Bacillus macauensis* JCM 13285^T (98.0% similarity); sequence similarities with type strains of other recognized species of the genus *Bacillus* were less than 96.7%. A phylogenetic tree based on the neighbour-joining method indicated that strain YC1^T formed a monophyletic clade adjacent to *B. macauensis* JCM 13285^T with a high bootstrap resampling value of 99% (Fig. 1).

Chromosomal DNA from strain YC1^T was extracted and purified according to the procedure of Yoon *et al.* (1996). The DNA G+C content, measured according to the method of Cashion *et al.* (1997), was 41.8 mol%. This value is slightly higher than that recorded for *B. macauensis* JCM 13285^T (40.8 mol%). DNA–DNA hybridization between strain YC1^T and *B. macauensis* JCM 13285^T was measured as described by De Ley *et al.* (1970) with the modifications described by Huß *et al.* (1983) and Escara & Hutton (1980). Renaturation rates were computed with the TRANSFER.BAS program described by Jahnke (1992). At the DNA–DNA level, there was 8.5% genetic relatedness between strain YC1^T and *B. macauensis* JCM 13285^T, which provided decisive evidence that the two strains belong to different species when the recommended

threshold value of 70% DNA–DNA relatedness for the delineation of genomic species is considered (Stackebrandt *et al.*, 2002).

Analysis of isoprenoid quinones was performed according to Komagata & Suzuki (1987) on cells harvested from an LB broth culture incubated at 30 °C for 2 days. Peptidoglycan diamino acids were analysed in cell-wall preparations as described by Bousfield *et al.* (1985). The presence of *meso*-diaminopimelic acid as the diagnostic diamino acid of the cell-wall peptidoglycan and MK-7 as the predominant menaquinone were features that were consistent with those of numerous *Bacillus* species, including *B. macauensis* JCM 13285^T (Zhang *et al.*, 2006) and *Bacillus barbaricus* DSM 14730^T (Täubel *et al.*, 2003).

Polar lipids were extracted, examined by two-dimensional TLC and identified by using published procedures (Minnikin *et al.*, 1984). Fatty acid methyl esters were prepared and identified following the instructions of the Microbial Identification system (MIDI), as described by Sasser (1990), after cells were grown on tryptic soy agar (Merck) for 2 days at 30 °C. The type strain of the closely related species *B. macauensis* (JCM 13285^T) was also cultured under the same nutrient and temperature conditions and characterized concurrently with strain YC1^T. The polar lipids present in strain YC1^T and *B. macauensis* were essentially similar, showing only slight quantitative differences; the polar lipid patterns for strain YC1^T and *B. macauensis* JCM 13285^T are available as Supplementary Fig. S1 in IJSEM Online. The major polar lipids present in both strains were diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) and minor components were an unknown aminolipid (AL) and an unknown aminophospholipid (APL); glycolipids were not detected. The polar lipids DPG, PG, PE, AL and APL have also been detected in *B. barbaricus* DSM 14730^T. The absence of glycolipids in strain YC1^T confirmed its close phylogenetic relatedness to *B.*

Fig. 1. Phylogenetic tree obtained by neighbour-joining analysis based on 16S rRNA gene sequences showing the positions of strain YC1^T and some related members of the genus *Bacillus*. Only bootstrap values >50% (expressed as percentages of 1000 resamplings) are shown at nodes. The maximum-likelihood tree showed essentially the same topology (data not shown). Bar, 0.01 substitutions per nucleotide position.

macauensis and *B. barbaricus*, neither of which have glycolipids in their polar lipid profile, and its distant phylogenetic relatedness to *Bacillus subtilis*, which has been reported to contain glycolipids (Brundish *et al.*, 1965; Bishop *et al.*, 1967; Kämpfer *et al.*, 2006).

The major cellular fatty acids of strain YC1^T were iso-C_{15:0} and anteiso-C_{15:0}, comprising about 38.6 and 35.9%, respectively, of the total fatty acid content. As shown by Kämpfer (1994), the predominance of 14- to 17-carbon iso- and anteiso-branched fatty acids is typical of members of the genus *Bacillus*. The fatty acid profile of strain YC1^T was similar to those of its close relatives, *B. macauensis* JCM 13285^T, *B. barbaricus* DSM 14730^T and *Bacillus gelatini* LMG 21880^T, but the fatty acid compositions were somewhat different (Table 1).

Cell morphology and motility were examined using light microscopy (model BH2; Olympus) and transmission electron microscopy (model H-7650; Hitachi) after 48 h growth on LB agar at 30 °C. For electron microscopic observation, cells were air-dried and negatively stained with 1% (w/v) phosphotungstic acid (see Supplementary Fig. S2 available in IJSEM Online). Gram-staining and analysis of endospore-forming features were performed according to the method described by Smibert & Krieg (1994). Growth at different temperatures and pH was investigated as described by Xu *et al.* (2005) using LB as the basal medium. NaCl tolerance was tested at concentrations between 0 and 20% (w/v) in combination with LB medium. Growth was tested on nutrient agar, Simmons' citrate agar, cetrimide agar and MacConkey agar. Oxidase, catalase and phenylalanine deaminase activities and hydrolysis of casein, gelatin, Tweens 80 and 20, carboxymethylcellulose, aesculin, tyrosine, DNA and starch were investigated as described by Smibert & Krieg (1994). Acid production from carbohydrates was determined using the medium and method described by Yamaguchi & Yokoe (2000). Single carbon source utilization studies were carried out as described by Liu *et al.* (2008). Sensitivity

Table 1. Fatty acid content (%) of strain YC1^T and related species of the genus *Bacillus*

Strains: 1, YC1^T (data from present study); 2, *B. macauensis* JCM 13285^T (present study); 3, *B. barbaricus* DSM 14730^T (Täubel *et al.*, 2003); 4, *B. gelatini* LMG 21880^T (De Clerck *et al.*, 2004).

Fatty acid	1	2	3	4
iso-C _{14:0}	0.7	2.3	9.4	0
C _{14:0}	0.7	1.4	0.9	0
iso-C _{15:0}	38.6	25.5	19.0	60
anteiso-C _{15:0}	35.9	62.3	42.8	9
iso-C _{16:0}	2.5	2.8	6.0	4
C _{16:0}	3.4	0	2.0	2
iso-C _{17:0}	7.7	5.2	0.0	13
anteiso-C _{17:0}	10.0	0.2	2.6	10.0

to antibiotics was tested using the diffusion method as described by Park *et al.* (2007) on LB agar at 30 °C with antibiotic filter-paper discs (8 mm diameter; Sanofi Pasteur). All tests were performed at 30 °C and properties were recorded after 2 days. Additional biochemical features were tested using API 20E kits (bioMérieux) according to the instructions of the manufacturer. Phenotypic characteristics of strain YC1^T are shown in the species description and in Table 2. As shown in Table 2, the novel isolate can be readily differentiated from its closest phylogenetic relatives in the genus *Bacillus* by several phenotypic properties.

On the basis of the above evidence, it is proposed that strain YC1^T should be classified in the genus *Bacillus* as the type strain of a novel species, for which the name *Bacillus solisalsi* sp. nov. is proposed.

Description of *Bacillus solisalsi* sp. nov.

Bacillus solisalsi (so.li.sal'si. L. neut. n. *solum* -i soil; L. adj. *salsus* -a -um salty; N.L. gen. n. *solisalsi* of a salty soil).

Table 2. Differential characteristics of strain YC1^T and related species of the genus *Bacillus*

Strains: 1, YC1^T (data from present study); 2, *B. macauensis* JCM 13285^T (Zhang *et al.*, 2006); 3, *B. barbaricus* DSM 14730^T (Täubel *et al.*, 2003); 4, *B. gelatini* LMG 21880^T (De Clerck *et al.*, 2004). +, Positive; -, negative; v, results vary between references; ND, no data available.

Characteristic	1	2	3	4
Growth at 47 °C	+	-	-	+
Growth in NaCl:				
5 %	+	-	-	+
10 %	+	-	-	+
Gelatin hydrolysis	-	+	ND	+
Utilization of:				
Cellobiose	-	+	-	v
D-Fructose	+	v	+	+
D-Galactose	-	+	-	+
D-Glucose	-	+	+	+
Inositol	-	+	-	+
Maltose	-	+	+	+
D-Mannitol	+	+	-	+
N-Acetyl-D-glucosamine	-	v	+	+
Pyruvate	-	+	+	ND
L-Rhamnose	-	+	-	ND
D-Ribose	-	+	+	+
Salicin	+	+	-	-
D-Sorbitol	-	v	-	ND
Sucrose	-	+	-	ND
Trehalose	+	-	+	+
D-Xylose	+	v	-	+
DNA G + C content (mol%)	41.8	40.8	ND	41.5

Cells are Gram-positive, endospore-forming, facultatively anaerobic, motile rods bearing a single lateral flagellum. Cells are 0.9 µm wide and 2.5–3.0 µm long. Endospores are ellipsoidal and form subterminally in swollen sporangia. After 2 days incubation on LB agar, colonies are 0.5–1.0 mm in diameter, cream-coloured, opaque, circular, smooth and convex. Grows at 15–53 °C (optimum, 35–42 °C) and pH 5–13 (optimum, pH 7–10). Tolerates up to 15 % NaCl. Growth occurs on nutrient agar, but not on Simmons' citrate agar, cetrimide agar or MacConkey agar. Catalase- and oxidase-positive. Phenylalanine deaminase-negative. In API 20E tests, positive for β-galactosidase, lysine decarboxylase and Voges-Proskauer reaction. Negative for arginine dihydrolase, arginine decarboxylase, ornithine decarboxylase, indole and H₂S production, urease and nitrate reduction. Starch, DNA, tyrosine, Tween 20, aesculin and casein are hydrolysed, but Tween 80, gelatin and carboxymethylcellulose are not. Acid is produced from trehalose, salicin and D-fructose. Utilizes D-xylose, D-fructose, D-mannose, trehalose, D-mannitol, L-arabinose and salicin, but not D-lactose, sucrose, D-galactose, D-glucose, maltose, melibiose, turanose, cellobiose, D-ribose, melezitose, raffinose, L-rhamnose, L-sorbose, adonitol, L-arabitol, *i*-erythritol, xylitol, D-sorbitol, inositol, dextrin, glycerol, acetate, gluconate, inulin, amygdalin, *N*-acetyl-D-glucosamine, pyruvate or methyl α-glucoside. Sensitive to streptomycin (10 µg), penicillin G (10 IU), ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), tetracycline (30 µg), ofloxacin (5 µg), amoxicillin (10 µg), cefamezin (30 µg), rifampicin (5 µg), gentamicin (10 µg), ceftazidime (30 µg), vancomycin (30 µg), kanamycin (30 µg), carbenicillin (100 µg) and polymyxin B (30 µg). The diagnostic diamino acid of the cell-wall peptidoglycan is *meso*-diaminopimelic acid and MK-7 is the predominant quinone. The cellular fatty acid profile consists of significant amounts of C₁₅ branched-chain acids, iso-C_{15:0} and anteiso-C_{15:0}. Major polar lipids are DPG, PG and PE; APL and AL are minor components.

The type strain, YC1^T (=KCTC 13181^T=CGMCC 1.6854^T), was isolated from a soil sample taken from the Yuncheng Salt Lake in the Shanxi Province, PR China. The DNA G+C content of the type strain is 41.8 mol%.

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