Halobacillus salsuginis sp. nov., a moderately halophilic bacterium from a subterranean brine

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A moderately halophilic, endospore-forming, Gram-positive, catalase- and oxidase-positive, motile, rod-shaped, aerobic bacterium, designated strain JSM 078133^T, was isolated from a subterranean brine sample collected from a salt mine in Hunan Province, China. Strain JSM 078133^T was able to grow with 1–23 % (w/v) total salts (optimum, 5–10 %) and at pH 6.0–10.0 (optimum, pH 7.5) and 10–45 °C (optimum, 30 °C). *meso*-Diaminopimelic acid was present in the cell-wall peptidoglycan. The predominant respiratory quinone was menaquinone 7 (MK-7) and the major cellular fatty acids were anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{15:0}. The genomic DNA G + C content of strain JSM 078133^T was 42.2 mol%. Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that strain JSM 078133^T belongs to the genus *Halobacillus*, and was related most closely to the type strains of *Halobacillus campisalis* (97.1 % sequence similarity), *Halobacillus alkaliphilus* (97.0 %) and *Halobacillus yeomjeoni* (96.8 %). The combination of phylogenetic analysis, DNA–DNA relatedness data, phenotypic characteristics and chemotaxonomic data supported the view that strain JSM 078133^T represents a novel species of the genus *Halobacillus*, for which the name *Halobacillus salsuginis* sp. nov. is proposed. The type strain is JSM 078133^T (=DSM 21185^T=KCTC 13236^T).

The genus *Halobacillus* was proposed by Spring *et al.* (1996) to accommodate two novel species, *Halobacillus litoralis* and *Halobacillus trueperi*, and the transfer of *Sporosarcina halophila* (Claus *et al.*, 1983) to *Halobacillus* as *Halobacillus halophilus*. At the time of writing, the genus comprises 16 recognized species, the three named above plus *Halobacillus salinus* (Yoon *et al.*, 2003), *H. karajensis* (Amoozegar *et al.*, 2003), *H. localis* (Yoon *et al.*, 2004), *H. aidingensis* and *H. dabanensis* (Liu *et al.*, 2005), *H. yeomjeoni* (Yoon *et al.*, 2005), *H. campisalis* (Yoon *et al.*, 2007), *H. profundus* and *H. kuroshimensis* (Hua *et al.*, 2007), *H. faecis* (An *et al.*, 2007), *H. alkaliphilus* (Romano *et al.*, 2008), *H. mangrovi* (Soto-Ramírez *et al.*, 2008) and *H. seohaensis* (Yoon *et al.*, 2008). In a recent study of the

microbial diversity in the ancient salt deposit of the Xiangli Salt Mine in Hunan Province, China, a moderately halophilic bacterium, designated JSM 078133^T, was isolated from a subterranean brine sample. Data from the present polyphasic taxonomic study indicate that this strain represents a novel species of the genus *Halobacillus*.

Strain JSM 078133^T was isolated from a subterranean brine sample by plating 1:10 serial dilutions of the sample on Difco marine agar 2216 (MA) supplemented with 5 % (w/v) NaCl (hereafter MA5) at 30 °C for 2 weeks. After primary isolation and purification, the isolate was kept either as serial transfers on MA5 slants, or as lyophilized cultures at 4 °C or deep-frozen at –80 °C in Difco marine broth 2216 (MB) supplemented with 5 % (w/v) NaCl and 20 % (v/v) glycerol. Unless indicated otherwise, morphological and physiological studies were performed with cells grown on MA5 at pH 7.5 and 30 °C. Two reference strains, *H. campisalis* KCTC 13144^T and *H. alkaliphilus* DSM

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JSM 078133^T is FJ456889.

18525^T, were obtained from the KCTC (Korean Collection for Type Cultures) and the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen), respectively. Cell morphology was examined by using light microscopy (model BH 2; Olympus). Gram staining was carried out by using the standard Gram reaction combined with the KOH lysis method (Gregersen, 1978). Flagella and endospores were examined according to the methods of Leifson and Schaeffer-Fulton, respectively (Smibert & Krieg, 1994). Growth was tested at various temperatures (5–55 °C, at increments of 5 °C) and different pH (5.0–11.0, at increments of 0.5 pH units) in MB supplemented with 5 % (w/v) NaCl and on MA5 plates. For pH experiments, the buffer solutions described by Chen *et al.* (2007) were used. Growth at different total salt contents [0, 0.1 and 0.5 % (w/v), and 1–30 % (w/v) at increments of 1 %] was determined with both MB and MA prepared according to the formula of Atlas (1993). The concentration of all salts contained in MB or MA was changed (proportions maintained) as an integer in the salt-response experiment. In order to determine the specific ionic requirement of the new isolate, growth at different concentrations (0.1, 0.5, 1 and 3 %, w/v) of NaCl or MgCl₂·6H₂O or the mixture of NaCl plus MgCl₂·6H₂O (1:1) was also tested in MB and on MA with all other trace salts omitted. Methyl red and Voges–Proskauer tests, H₂S production from L-cysteine, hydrolysis of aesculin, indole production, nitrate and nitrite reduction, and phenylalanine deaminase, lysine decarboxylase, ornithine decarboxylase and urease were determined as recommended by Smibert & Krieg (1994). Hydrolysis of casein, cellulose, DNA, gelatin, hypoxanthine, starch, Tweens (20, 40, 60 and 80), tyrosine and xanthine was determined as described by Cowan & Steel (1965). Determination of acid production from carbohydrates, as well as utilization of carbon and nitrogen sources, was performed as recommended by Ventosa *et al.* (1982). Motility, growth under anaerobic conditions, antibiotic susceptibility, and catalase and oxidase activities were determined as described by Chen *et al.* (2007). Other enzymic activities were tested by using API ZYM systems (bioMérieux) according to the manufacturer's instructions with 7.5 % (w/v) NaCl. The morphological, physiological and biochemical characteristics of strain JSM 078133^T are summarized in the species description below and in Table 1.

Genomic DNA was isolated according to Hopwood *et al.* (1985) and the G + C content was determined by using the HPLC method (Mesbah *et al.*, 1989). The 16S rRNA gene was amplified and sequenced as described by Cui *et al.* (2001). The resulting 16S rRNA gene sequence of strain JSM 078133^T was compared with sequences obtained from public databases (GenBank/EMBL/DDBJ) to find the most closely related species. Phylogenetic analyses were performed by using the software package MEGA version 4.1 (Tamura *et al.*, 2007) after multiple alignment of the sequence data with CLUSTAL_X (Thompson *et al.*, 1997). Distances were calculated by using distance options according to Kimura's two-parameter model (Kimura,

1980) and clustering was performed with the neighbour-joining method (Saitou & Nei, 1987). Maximum-parsimony (Kluge & Farris, 1969) and minimum-evolution (Felsenstein, 1997) trees (not shown) were generated by means of methods contained within the software package MEGA version 4.1 (Tamura *et al.*, 2007). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by means of 1000 resamplings (Felsenstein, 1985). DNA–DNA hybridization experiments were carried out by using photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989).

The DNA G + C content of strain JSM 078133^T was 42.2 mol%. The almost-complete 16S rRNA gene sequence (1541 bp) was determined. Phylogenetic analyses based on 16S rRNA gene sequences revealed that strain JSM 078133^T belongs to the genus *Halobacillus*, and was related most closely to the type strains of *H. campisalis* (97.1 % sequence similarity), *H. alkaliphilus* (97.0 %) and *H. yeomjeoni* (96.8 %). Levels of 16S rRNA gene sequence similarity between strain JSM 078133^T and the type strains of other recognized *Halobacillus* species were in the range 96.7 % (*H. dabanensis*) to 94.8 % (*H. kuroshimensis*). In the neighbour-joining phylogenetic tree, however, strain JSM 078133^T and the type strains of *H. campisalis*, *H. alkaliphilus*, *H. halophilus* (96.4 % 16S rRNA gene sequence similarity) and *H. seohaensis* (95.9 %) formed a subcluster separate from other recognized *Halobacillus* species, in which strain JSM 078133^T occupied a distinct clade (Fig. 1).

Amino acids of whole-cell hydrolysates were analysed as described by Hasegawa *et al.* (1983). Isoprenoid quinones were analysed by HPLC as described by Groth *et al.* (1996). Fatty acids were determined according to Sasser (1990) by using the Microbial Identification System (MIDI; Microbial ID) with cells grown in MB supplemented with 5 % (w/v) NaCl in flasks on a rotary shaker (about 180 r.p.m.) at 30 °C for 3 days. Chemotaxonomic data for strain JSM 078133^T were consistent with its assignment to the genus *Halobacillus*. Strain JSM 078133^T possessed a cell-wall type based on *meso*-diaminopimelic acid and contained menaquinone 7 (MK-7; 98.5 %) as the predominant respiratory quinone with MK-8 (1.5 %) present in trace amounts. The major fatty acids of strain JSM 078133^T were anteiso-C_{15:0} (50.5 % of the total), anteiso-C_{17:0} (18.6 %) and iso-C_{15:0} (10.1 %); iso-C_{14:0} (1.3 %), iso-C_{16:0} (6.7 %), C_{15:0} (1.2 %), C_{16:0} (4.4 %), C_{18:0} (1.3 %) and summed feature 4 (iso-C_{17:1} I and/or anteiso-C_{17:1} B; 1.2 %) were present in minor amounts, and C_{16:1}ω7c alcohol, C_{17:1}ω10c and summed feature 5 (anteiso-C_{18:0} and/or C_{18:2}ω6,9c) were detected at a level <1 %. This fatty acid profile was similar to those of recognized *Halobacillus* species, although there were differences in the proportions of some components, probably because of differences in cultivation conditions and extraction procedures (Liu *et al.*, 2005; Yoon *et al.*, 2005, 2007, 2008).

The results of phylogenetic analysis, and morphological and chemotaxonomic investigations supported the view

Table 1. Characteristics used to distinguish strain JSM 078133^T from phylogenetically related *Halobacillus* species

Taxa: 1, strain JSM 078133^T (*H. salsuginis* sp. nov.); 2, *H. campisalis* (data from Yoon *et al.*, 2007); 3, *H. alkaliphilus* (Romano *et al.*, 2008); 4, *H. yeomjeoni* (Yoon *et al.*, 2005); 5, *H. halophilus* (Claus *et al.*, 1983; Spring *et al.*, 1996; Romano *et al.*, 2008); 6, *H. seohaensis* (Yoon *et al.*, 2008). +, Positive; –, negative; v, variable; ND, no data available. All taxa are positive for catalase and oxidase activity but negative for anaerobiosis and urease.

Characteristic	1	2	3	4	5	6
Colony colour	Cream	Light yellow	Pale orange–yellow	Light yellow	Orange	Yellowish white
Gram staining	+	+	+	+	+	+
Nitrate reduction	+	–	–	–	–	–
Cell morphology	Rods	Cocci/ovoid	Cocci	Rods/long filamentous rods	Cocci/ovoid	Rods
Flagellation	Peritrichous	Peritrichous	ND	Single	Single/peritrichous	Single
Spore shape*	E	S	S	E	S	E
Spore position†	ST	C	ND	C/ST	C/L	C/ST
Temperature range (°C)	10–45	4–41	25–50	15–48	15–40	4–38
pH optimum	7.5	7.0–8.0	9.0	7–8	7.2	7.0–8.0
NaCl range (% w/v)	1–23	0–22	5–25	0.5–21	2–20	1–12
NaCl optimum (% w/v)	5–10	8	10	3–5	10	8
Hydrolysis of:						
Aesculin	+	+	ND	–	–	–
Casein	–	+	–	+	+	+
Gelatin	+	–	–	+	+	–
Starch	+	+	–	–	+	+
Tween 80	–	–	ND	+	–	–
Tyrosine	–	–	+	–	–	–
Acid production from:						
D-Fructose	–	+	+	–	–	–
D-Glucose	+	+	+	+	–	–
D-Galactose	–	+	–	–	–	–
D-Mannitol	–	–	ND	+	–	+
Maltose	+	–	+	+	–	–
Sucrose	+	+	+	+	–	+
Trehalose	+	+	+	+	–	+
D-Xylose	–	–	+	–	–	+
Cell-wall type‡	meso-DAP	meso-DAP	L-Orn–D-Asp	L-Orn–D-Asp	L-Orn–D-Asp	meso-DAP
DNA G + C content (mol%)	42.2	42.1	43.5	42.9	40.1–40.9	39.3

*E, Ellipsoidal; S, spherical.

†C, Central; L, lateral; T, terminal; ST, subterminal.

‡D-Asp, D-asparagine; meso-DAP, meso-diaminopimelic acid; L-Orn, L-ornithine.

that strain JSM 078133^T should be assigned to the genus *Halobacillus*. However, it could be distinguished from its closest phylogenetic relatives in the genus on the basis of several phenotypic characteristics (Table 1). To establish the precise taxonomic position of strain JSM 078133^T, DNA–DNA hybridization experiments were performed between the novel isolate and the type strains of *H. campisalis* and *H. alkaliphilus*. Levels of DNA–DNA relatedness between strain JSM 078133^T and the type strains of these two species were 13.4 and 10.2 %, respectively, values that are well below the threshold value of 70 % recommended by Wayne *et al.* (1987) for assigning strains to the same species. Therefore, on the basis of the data from the present polyphasic taxonomic study, strain

JSM 078133^T should be placed in the genus *Halobacillus* as a member of a novel species, for which we propose the name *Halobacillus salsuginis* sp. nov.

Description of *Halobacillus salsuginis* sp. nov.

Halobacillus salsuginis (sal.su'gi.nis. L. gen. fem. n. *salsuginis* of/from brine).

Cells are Gram-positive, moderately halophilic, catalase- and oxidase-positive, aerobic rods, approximately 0.5–0.9 µm wide and 1.5–3.0 µm long, occurring singly, in pairs or in short chains. Motile by means of peritrichous flagella. Cells bear ellipsoidal endospores that lie in subterminal swollen sporangia. Colonies are cream-

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence analysis and constructed by using the neighbour-joining method showing the position of strain JSM 078133^T among related taxa. 'm' indicates a branch that was also found with the minimum-evolution (Felsenstein, 1997) method; asterisks indicate branches that were also recovered using the minimum-evolution and maximum-parsimony (Kluge & Farris, 1969) algorithms. Numbers at nodes indicate bootstrap values (>50%) based on a neighbour-joining analysis of 1000 resampled datasets. Bar, 1 substitution per 100 nt.

coloured, of low convexity and semi-translucent with circular/slightly irregular margins, and are 1–2 mm in diameter after incubation for 3–5 days at 30 °C on MA5. Growth occurs with 1–23% (w/v) total salts (optimum, 5–10%) and at pH 6.0–10.0 (optimum, pH 7.5) and 10–45 °C (optimum, 30 °C). Na⁺ and Mg²⁺ ions are required for growth. Positive for nitrate reduction and phenylalanine deaminase, but negative for methyl red and Voges–Proskauer tests, nitrite reduction, H₂S and indole production, and lysine decarboxylase, ornithine decarboxylase and urease. Aesculin, DNA, gelatin, starch and Tween 40 are hydrolysed, but casein, cellulose, hypoxanthine, Tweens 20, 60 and 80, tyrosine and xanthine are not. Acids are produced from D-arabitol, D-glucose, maltose, sucrose and trehalose, but not from adonitol, L-arabinose, cellobiose, dulcitol, D-fructose, D-galactose, glycerol, *myo*-inositol, D-lactose, D-mannitol, D-mannose, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, D-salicin, D-sorbitol or D-xylose. The following compounds are utilized as sole source of carbon and energy or sole source of carbon, nitrogen and energy: L-arabinose, dextrin, D-fructose, D-glucose, maltose, raffinose, sucrose, adonitol, D-sorbitol, L-alanine, L-asparagine and L-serine. The following are not utilized: cellobiose, D-galactose, D-lactose, D-mannose, melezitose, melibiose, L-rhamnose, D-ribose, D-salicin, trehalose, D-xylose, D-arabitol, glycerol, *myo*-inositol, D-mannitol, acetate, butyrate, citrate, gluconate, propionate, succinate, L-arginine, L-glutamic acid, D-glycine, L-histidine, hydroxy-L-proline, L-isoleucine, L-leucine, L-methionine, L-proline

and L-valine. Constitutive enzymes expressed are alkaline phosphatase, esterase (C4) and esterase lipase (C8), but not acid phosphatase, α -chymotrypsin, cystine arylamidase, α -fucosidase, α -galactosidase, β -galactosidase, *N*-acetyl- β -glucosaminidase, α -glucosidase, β -glucosidase, β -glucuronidase, leucine arylamidase, lipase (C14), α -mannosidase, naphthol-AS-BI-phosphohydrolase, trypsin or valine arylamidase. Susceptible to (μ g per disc): ampicillin (30), carbenicillin (30), chloramphenicol (30), gentamicin (10), kanamycin (30), lincomycin (2), rifampicin (5) and streptomycin (10), but not to nalidixic acid (20), nitrofurantoin (30), novobiocin (30), polymyxin B (30), tetracycline (30) or tobramycin (10). *meso*-Diaminopimelic acid is present in the cell-wall peptidoglycan as the diagnostic diamino acid. Possesses MK-7 as the predominant menaquinone. Major fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{15:0}. The DNA G + C content of the type strain is 42.2 mol%.

The type strain, JSM 078133^T (=DSM 21185^T=KCTC 13236^T), was isolated from a subterranean brine sample collected from the Xiangli Salt Mine in Hunan Province, China.

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