Halobacillus salinus sp. nov., isolated from a salt lake on the coast of the East Sea in Korea

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A Gram-positive, rod-shaped, endospore-forming, halophilic bacterium (strain HSL-3^T) was isolated from a salt lake near Hwajinpo beach on the East Sea in Korea and was subjected to a polyphasic taxonomic study. Strain HSL-3^T grew optimally in the presence of 2–10% (w/v) NaCl. Strain HSL-3^T showed poor growth in the absence of NaCl and grew in the presence of less than 23% NaCl. The cell wall peptidoglycan type of strain HSL-3^T was A4 β based on L-Orn–D-Asp. The predominant menaquinone found in strain HSL-3^T was menaquinone-7 (MK-7). Strain HSL-3^T had a cellular fatty acid profile containing large amounts of branched fatty acids; the major fatty acids were anteiso-C_{15:0}, iso-C_{15:0} and iso-C_{16:0}. The DNA G + C content of strain HSL-3^T was 45 mol%. Phylogenetic analysis based on 16S rDNA sequences showed that strain HSL-3^T falls within the radiation of the cluster comprising *Halobacillus* species. Strain HSL-3^T exhibited levels of 16S rDNA similarity of 97.4–98.4% to the type strains of *Halobacillus* species. Levels of DNA–DNA relatedness between strain HSL-3^T and the type strains of all validly named *Halobacillus* species were in the range 7.3–9.2%. On the basis of phenotypic and phylogenetic data and the genomic distinctiveness, strain HSL-3^T (= KCCM 41590^T = JCM 11546^T) should be placed in the genus *Halobacillus* as the type strain of a novel species, for which the name *Halobacillus salinus* sp. nov. is proposed.

Originally, most aerobic or facultatively anaerobic, Gram-positive or Gram-variable, endospore-forming rods were assigned to the genus *Bacillus* (Claus & Berkeley, 1986). Developments in molecular biological methods and chemical analysis revealed the taxonomic heterogeneity of the genus *Bacillus* (Ash *et al.*, 1991; Priest, 1981; Slepecky & Hemphill, 1991; Stackebrandt & Liesack, 1993). In particular, 16S rRNA sequence analyses revealed the presence of several phylogenetically distinct lineages within the genus *Bacillus* (Ash *et al.*, 1991; Nielsen *et al.*, 1994). Subsequently, some phylogenetic groups have been proposed as the new genera *Alicyclobacillus* (Wisotzkey *et al.*, 1992), *Aneurinibacillus*, *Brevibacillus* (Shida *et al.*, 1996), *Marinibacillus* (Yoon *et al.*, 2001), *Paenibacillus* (Ash *et al.*, 1993), *Salibacillus* (Wainø *et al.*, 1999) and *Virgibacillus* (Heyndrickx *et al.*, 1998). In addition, some aerobic or facultatively anaerobic, endospore-forming rods have been described as the new genera *Amphibacillus* (Niimura *et al.*,

1990), *Halobacillus* (Spring *et al.*, 1996), *Gracilibacillus* (Wainø *et al.*, 1999) and *Jeotgalibacillus* (Yoon *et al.*, 2001). Nevertheless, there are few clear-cut differences in phenotypic, and particularly chemotaxonomic, properties among these genera, with some exceptions, including the genera belonging to *Bacillus* rRNA group 2 (Niimura *et al.*, 1990; Shida *et al.*, 1997; Wainø *et al.*, 1999; Yoon *et al.*, 2001). Most of them are characterized chemotaxonomically by having MK-7 as the predominant menaquinone, meso-diaminopimelic acid or L-lysine at position 3 of the peptide subunit of the peptidoglycan and one or more of anteiso-C_{15:0}, iso-C_{15:0}, C_{16:0} and iso-C_{16:0} as the major fatty acids.

The genus *Halobacillus* is clearly differentiated from other related genera in the cell-wall peptidoglycan type; members of the genus have peptidoglycan based on L-Orn–D-Asp (Spring *et al.*, 1996), whereas other related genera contain meso-diaminopimelic acid or L-lysine at position 3 of the cell-wall peptidoglycan (Shida *et al.*, 1997; Wainø *et al.*, 1999; Yoon *et al.*, 2001). The genus *Filobacillus*, which has been described recently, contains L-ornithine at position 3 of the cell-wall peptidoglycan but has peptidoglycan based on L-Orn–D-Glu (Schlesner *et al.*, 2001). The genus

Abbreviations: MA, marine agar 2216; MB, marine broth 2216.

The GenBank accession number for the 16S rDNA sequence of strain HSL-3^T is AF500003.

Halobacillus was proposed with two novel species, *Halobacillus litoralis* and *Halobacillus trueperi*, as well as *Sporosarcina halophila*, which was transferred to the genus as *Halobacillus halophilus* (Spring *et al.*, 1996). Since then, no further *Halobacillus* species have been proposed. Recently, a halophilic bacterial strain, HSL-3^T, was isolated from a salt lake on the coast of the East Sea in Korea. This organism was considered to be a member of the genus *Halobacillus* from the results of preliminary study based on partial 16S rDNA sequence comparison. Accordingly, the aim of the present study was to determine the exact taxonomic position of strain HSL-3^T with a combination of phenotypic properties, detailed phylogenetic analysis based on nearly complete 16S rDNA sequence and genomic relatedness. On the basis of the data presented below, strain HSL-3^T should be placed in the genus *Halobacillus* as a novel species, for which we propose the name *Halobacillus salinus* sp. nov.

Strain HSL-3^T was isolated from a salt lake near Hwajinpo beach on the East Sea in Korea by the dilution-plating technique on marine agar 2216 (MA) (Difco). For investigation of morphological and physiological characteristics, strain HSL-3^T was generally cultivated on MA and in marine broth 2216 (MB) (Difco) at 30 °C. Cell biomass of strain HSL-3^T for analysis of the cell wall and menaquinones and for DNA extraction was obtained from MB cultures at 30 °C. Strain HSL-3^T was cultivated on a horizontal shaker at 150 r.p.m. and broth cultures were checked microscopically for purity before being harvested by centrifugation. For fatty acid methyl ester (FAME) analysis, cell mass of strain HSL-3^T and reference strains was obtained from agar plates after 4 days cultivation at 30 °C on MA and MA supplemented with approximately 8.1 % (w/v) NaCl. The reference strains were *H. halophilus* KCTC 3685^T, *H. litoralis* KCTC 3687^T and *H. trueperi* KCTC 3686^T. Cell morphology was examined by light microscopy and transmission electron microscopy (TEM). Flagellation type was examined by TEM using cells from exponentially growing culture. The cells were negatively stained with 1 % (w/v) phosphotungstic acid and after air drying, the grids were examined by using a model CM-20 transmission electron microscope (Philips). Gram reaction was determined using the bioMérieux Gram Stain kit according to the manufacturer's instructions. Catalase activity was determined by bubble production in a 3 % (v/v) hydrogen peroxide solution. Oxidase activity was determined by oxidation of 1 % *p*-aminodimethylaniline oxalate. Urease activity was determined as described by Cowan & Steel (1965) with the modification that 3 % (w/v) NaCl was added. Hydrolysis of casein, starch and Tween 80 was determined as described by Cowan & Steel (1965). Hydrolysis of aesculin and gelatin and nitrate reduction were determined as described by Lanyi (1987) with the modification that 3 % (w/v) NaCl was added. Hydrolysis of hypoxanthine, tyrosine and xanthine was examined on MA with concentrations of substrates described previously (Cowan & Steel, 1965). Acid production from carbohydrates

was determined as described by Leifson (1963). Growth under anaerobic conditions was determined after incubation in an anaerobic chamber with MA that had been prepared anaerobically. Growth at various NaCl concentrations was investigated on MA and in MB. Growth at various temperatures was measured on MA at 4–55 °C.

Preparation of cell wall and determination of peptidoglycan structure were carried out by the methods described by Schleifer & Kandler (1972) with the modification that TLC on cellulose sheets was used instead of paper chromatography. Menaquinones were analysed as described previously (Komagata & Suzuki, 1987) using reverse-phase HPLC. For quantitative analysis of cellular fatty acid compositions, a loop of cell mass was harvested and FAMES were prepared and identified following the instructions of the Microbial Identification System (MIDI). Chromosomal DNA was isolated and purified according to the method described previously (Yoon *et al.*, 1996), with the exception that RNase T1 was used together with RNase A. The G+C content was determined by the method of Tamaoka & Komagata (1984). DNA was hydrolysed and the resultant nucleotides were analysed by reverse-phase HPLC.

16S rDNA was amplified by PCR using two universal primers as described previously (Yoon *et al.*, 1998). The PCR product was purified with a QIAquick PCR purification kit (Qiagen). Sequencing of the purified 16S rDNA was performed using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems) as recommended by the manufacturer. The purified sequencing reaction mixtures were electrophoresed automatically using an Applied Biosystems model 377 automatic DNA sequencer. Alignment of sequences was carried out with CLUSTAL W software (Thompson *et al.*, 1994). Gaps at the 5' and 3' ends of the alignment were omitted from further analysis. Phylogenetic trees were inferred by using three tree-making algorithms, the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) methods in the PHYLIP package (Felsenstein, 1993). Evolutionary distance matrices for the neighbour-joining method were calculated with the algorithm of Jukes & Cantor (1969) with the DNADIST program. The stability of relationships was assessed by a bootstrap analysis based on 1000 resampling of the neighbour-joining dataset by using the programs SEQBOOT and CONSENSE of the PHYLIP package. DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. Of the values obtained, the highest and lowest values in each sample were excluded and the remaining three values were used for calculation of similarity values. DNA relatedness values are the means of three values.

Strain HSL-3^T was Gram-positive but changed to Gram-variable as cultures aged. Cells of strain HSL-3^T were rods, measuring approximately 0.7–1.1 µm wide by 1.5–4.0 µm

Fig. 1. Light micrograph of cells of strain HSL-3^T from exponentially growing culture (3 days cultivation on MA). Bar, 5 μm.

long in 3 day cultures at 30 °C on MA (Fig. 1). The cells showed no difference in their morphology on MA or MA containing approximately 10% NaCl. Cells of strain HSL-3^T were motile by means of peritrichous flagella. Ellipsoidal endospores were observed at central or sub-terminal positions in swollen sporangia. Colonies of strain HSL-3^T were smooth, circular to slightly irregular, slightly raised, pale orange-yellow in colour and 2–3 mm in diameter after 3 days on MA. Strain HSL-3^T grew optimally at 30–37 °C; it grew at 10 and 45 °C but not at 4 °C or temperatures above 46 °C. Strain HSL-3^T grew well in the pH range 6.0–8.0, with an optimum of approximately 7.0, and growth was observed at pH 5.0 but not at pH 4.5. Strain HSL-3^T grew optimally in the presence of 2–10% (w/v) NaCl, and showed poor growth in the absence of NaCl. It grew in the presence of 23% NaCl but not in the presence of more than 24% NaCl. Strain HSL-3^T did not grow under anaerobic conditions on MA. Strain HSL-3^T showed catalase and oxidase activities but no urease activity. Aesculin, casein and Tween 80 were hydrolysed and no hydrolysis of hypoxanthine, starch, tyrosine or xanthine was observed. Nitrate was not reduced to nitrite. Acid was produced from D-cellobiose, D-fructose, D-glucose, maltose, D-mannitol, D-mannose, sucrose and D-trehalose and produced weakly from D-galactose, D-melezitose and melibiose. Phenotypic properties of strain HSL-3^T and *Halobacillus* species are summarized in Table 1.

Strain HSL-3^T did not contain any diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. From the result of the cell-wall analysis, strain HSL-3^T had peptidoglycan type A4β, based on L-Orn–D-Asp, as described by Schleifer & Kandler (1972). The predominant menaquinone found in strain HSL-3^T was unsaturated menaquinone with seven isoprene units (MK-7). The

cellular fatty acid profile of strain HSL-3^T is represented in Table 2, together with those of the type strains of the three *Halobacillus* species. Strain HSL-3^T and the type strains of the three *Halobacillus* species were found to have similar fatty acid profiles when they were cultivated on either MA or MA containing approximately 10% NaCl (Table 2). Strain HSL-3^T had a cellular fatty acid profile containing large amounts of branched fatty acids, with anteiso-C_{15:0}, iso-C_{15:0} and iso-C_{16:0} as the major fatty acids (Table 2). The DNA G + C content of the strain was 45 mol%.

An almost complete 16S rDNA sequence of strain HSL-3^T was determined directly following PCR amplification. The 16S rDNA sequence of strain HSL-3^T determined in this study comprised 1522 nucleotides, representing approximately 96% of the *Escherichia coli* 16S rRNA sequence. Phylogenetic trees based on 16S rDNA sequences showed that strain HSL-3^T falls within the radiation of the cluster comprising *Halobacillus* species (Fig. 2). In the tree based on the neighbour-joining algorithm, strain HSL-3^T clustered with the clade comprising *H. litoralis* and *H. trueperi*, and the relationship between this cluster and *H. halophilus* was supported by bootstrap analysis at a confidence level of 99.0% (Fig. 2). Strain HSL-3^T exhibited levels of 16S rDNA similarity of 97.4, 98.4 and 98.2%, respectively, to the type strains of *H. halophilus*, *H. litoralis* and *H. trueperi*. Levels of 16S rDNA similarity between strain HSL-3^T and other species used in the phylogenetic analysis were less than 94.6% (Fig. 2). DNA–DNA hybridization was performed to determine the genomic relatedness between strain HSL-3^T and the type strains of the three *Halobacillus* species. Strain HSL-3^T exhibited mean levels of DNA–DNA relatedness of 7.3, 8.5 and 9.2%, respectively, with *H. halophilus* KCTC 3685^T, *H. litoralis* KCTC 3687^T and *H. trueperi* KCTC 3686^T when the DNAs of the four strains were used separately as labelled DNA probes for cross-hybridization.

The morphological and physiological properties indicate the strong possibility of assigning strain HSL-3^T to one of the genera of Gram-positive or -variable, aerobic, endospore-forming rods. The results of 16S rDNA sequence analysis reveal that strain HSL-3^T has the closest phylogenetic affiliation to the genus *Halobacillus*. The results obtained in the chemotaxonomic analyses are consistent with the results of 16S rDNA sequence analysis and phylogenetic inference. The chemotaxonomic data obtained from strain HSL-3^T are most similar to the chemotaxonomic properties characteristic of the genus *Halobacillus* (Spring *et al.*, 1996), although there are a few differences in their cellular fatty acid compositions (Table 2). In particular, the cell-wall peptidoglycan type, based on L-Orn–D-Asp, is a key marker that differentiates strain HSL-3^T and the genus *Halobacillus* from other genera of aerobic or facultative anaerobic, endospore-forming rods. Therefore, in view of the combined morphological, chemotaxonomic

Table 1. Differential phenotypic properties of strain HSL-3^T and *Halobacillus* species

Data for reference species were taken from Claus *et al.* (1983) (*H. halophilus*) and Spring *et al.* (1996) (*H. halophilus*, *H. litoralis*, *H. trueperi*). +, Positive; –, negative; w, weakly positive; v, variable; ND, not determined. All species are peritrichously flagellated and positive for catalase, oxidase and hydrolysis of gelatin. All species are negative for nitrate reduction, urease, anaerobic growth and hydrolysis of tyrosine.

Characteristic	Strain HSL-3 ^T	<i>H. halophilus</i>	<i>H. litoralis</i>	<i>H. trueperi</i>
Cell morphology	Rods	Cocci or oval	Rods	Rods
Gram staining	+	+	+	+
Spore morphology:*				
Shape	E	S	ES	ES
Position	CS	CL	CS	CS
Colony colour	Pale orange-yellow	Orange	Orange	Orange
Growth at/in:				
45 °C	+	–	–	–
pH 5.0	+	–	–	–
pH 5.5	+	–	–	–
0.5 % NaCl	+	–	+	+
25 % NaCl	–	–	+	+
Hydrolysis of:				
Aesculin	+	–	–	–
Casein	+	+	–	–
Starch	–	+	–	–
Tween 80	+	–	–	–
Acid production from:				
D-Fructose	+	–	+	+
D-Galactose	w	–	–	+
Maltose	+	–	+	+
Sucrose	+	–	+	+
D-Xylose	–	–	+	–
D-Glucose	+	–	+	+
D-Mannitol	+	–	+	–
D-Trehalose	+	–	+	+
DNA G + C content (mol%)	45	40–41	42	43

*Spore shape: E, ellipsoidal; S, spherical. Spore position: C, central, L, lateral, S, subterminal.

and phylogenetic data, it is evident that strain HSL-3^T belongs to the genus *Halobacillus*.

Strain HSL-3^T is similar to *Halobacillus* species in its morphological and most of its physiological characteristics (Table 1). However, there are some minor differences between strain HSL-3^T and *Halobacillus* species, including tolerance of NaCl, temperature and pH for growth, the ability to hydrolyse some substrates and acid production from carbohydrates (Table 1). Strain HSL-3^T shows growth in the presence of NaCl up to 23 %, whereas *H. halophilus*, *H. litoralis* and *H. trueperi* respectively show growth in the presence of NaCl up to 15, 25 and 30 % (Claus *et al.*, 1983; Spring *et al.*, 1996). While strain HSL-3^T can grow at temperatures up to 45 °C, the other *Halobacillus* species cannot grow at 45 °C (Claus *et al.*, 1983; Spring *et al.*, 1996). Strain HSL-3^T and the other *Halobacillus* species show differences in growth at pH 5.5 and 5.0. It is noteworthy that the fatty acid profile of strain HSL-3^T also differs from those of the type strains of the other *Halobacillus* species

in the proportions of some fatty acids (Table 2). These fatty acid profiles did not show conspicuous differences on MA containing 2 or 10 % NaCl. Levels of DNA–DNA relatedness are low enough to categorize strain HSL-3^T as a distinct species within the genus *Halobacillus* (Wayne *et al.*, 1987). Therefore, differences in some phenotypic characteristics and genetic distinctiveness together indicate that strain HSL-3^T is a member of a species separate from previously described *Halobacillus* species.

On the basis of the data presented above, strain HSL-3^T should be placed in the genus *Halobacillus* as a novel species, for which we propose the name *Halobacillus salinus* sp. nov.

Description of *Halobacillus salinus* sp. nov.

Halobacillus salinus (sa'li.nus. N.L. adj. *salinus* salted, salty).

Cells are rods, 0.7–1.1 µm wide by 1.5–4.0 µm long in 3 day cultures at 30 °C on MA. Gram-positive but

Table 2. Cellular fatty acid profiles of strain HSL-3^T and other *Halobacillus* species

Values are percentages of total fatty acids. Strain HSL-3^T, *H. halophilus* KCTC 3685^T, *H. trueperi* KCTC 3686^T and *H. litoralis* KCTC 3687^T were cultivated on MA (which contains approx. 1.9 % NaCl) and on MA supplemented with approximately 8.1 % NaCl [to give MA (10 %)]. ND, Not detected.

Fatty acid	HSL-3 ^T		KCTC 3685 ^T		KCTC 3686 ^T		KCTC 3687 ^T	
	MA	MA (10 %)	MA	MA (10 %)	MA	MA (10 %)	MA	MA (10 %)
Straight-chain fatty acids								
C _{15:0}	1.5	1.6	0.9	1.5	ND	ND	0.4	1.1
C _{16:0}	1.6	1.0	1.2	0.9	1.2	0.9	1.0	0.6
Branched fatty acids								
anteiso-C _{13:0}	ND	ND	0.2	0.4	ND	ND	ND	0.4
iso-C _{14:0}	8.0	9.4	9.7	12.2	26.4	21.7	1.6	6.4
iso-C _{15:0}	25.1	26.3	5.4	7.5	6.7	7.7	11.5	15.8
anteiso-C _{15:0}	30.7	31.7	44.2	47.3	15.6	25.3	51.1	57.0
iso-C _{16:0}	16.9	15.7	18.1	15.2	35.4	31.5	4.7	5.4
iso-C _{17:0}	5.0	4.2	1.5	1.2	3.1	2.1	3.9	1.5
anteiso-C _{17:0}	6.3	6.2	15.4	11.9	4.3	6.5	15.8	8.2
iso-C _{17:1} ω10c	ND	ND	ND	ND	ND	ND	1.3	0.2
Unsaturated fatty acids								
C _{16:1} ω7c alcohol	4.9	4.0	2.9	1.8	7.5	3.8	3.5	2.0
C _{16:1} ω11c	ND	ND	ND	ND	ND	ND	0.6	ND
Summed features*								
Summed feature 4	ND	ND	0.3	ND	ND	0.5	4.8	1.5
Summed feature 5	ND	ND	0.5	ND	ND	ND	ND	ND

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 4 contained iso-C_{17:1} I and/or anteiso-C_{17:1} B. Summed feature 5 contained anteiso-C_{18:0} and/or C_{18:2}ω6,9c.

Gram-variable in old cultures. Motile by means of peritrichous flagella. Central or subterminal ellipsoidal endospores are observed in swollen sporangia. Colonies are smooth, circular to slightly irregular, slightly raised, pale orange-yellow

Fig. 2. Neighbour-joining tree showing the phylogenetic positions of strain HSL-3^T, *Halobacillus* species and representatives of related taxa based on 16S rDNA sequences. Scale bar represents 0.01 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are shown at branch-points.

in colour and 2–3 mm in diameter after 3 days on MA. The optimal temperature for growth is 30–37 °C. Growth occurs at 10 and 45 °C but not at 4 °C or temperatures above 46 °C. The optimal pH for growth is around pH 7.0 and no growth is observed at pH 4.5. Grows optimally in the presence of 2–10 % (w/v) NaCl and growth is poor in the absence of NaCl. Growth occurs in the presence of 23 % NaCl but not 24 % NaCl. Growth does not occur under anaerobic conditions on MA. Catalase- and oxidase-positive. Urease-negative. Aesculin, casein, gelatin and Tween 80 are hydrolysed. Hypoxanthine, starch, tyrosine and xanthine are not hydrolysed. Nitrate is not reduced to nitrite. Acid is produced from D-cellobiose, D-fructose, D-glucose, maltose, D-mannitol, D-mannose, sucrose and D-trehalose and produced weakly from D-galactose, D-melezitose and melibiose. Acid is not produced from adonitol, L-arabinose, lactose, myo-inositol, D-raffinose, L-rhamnose, D-ribose, D-sorbitol, stachyose or D-xylose. The cell wall contains peptidoglycan based on L-Orn–D-Asp. The predominant menaquinone is MK-7. The major fatty acids are anteiso-C_{15:0}, iso-C_{15:0} and iso-C_{16:0}. The G + C content is 45 mol% (determined by HPLC).

The type strain, strain HSL-3^T (=KCCM 41590^T=JCM 11546^T), was isolated from a salt lake near Hwajinpo beach on the East Sea in Korea.

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