Halobacillus seohaensis sp. nov., isolated from a marine solar saltern in Korea

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A Gram-positive or Gram-variable, motile, moderately halophilic, rod-shaped bacterial strain, ISL-50^T, was isolated from a marine solar saltern in Korea and subjected to a polyphasic taxonomic study. Strain ISL-50^T grew optimally at pH 7.0–8.0 and 37 °C in the presence of 8 % (w/v) NaCl. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain ISL-50^T belonged to the genus *Halobacillus*. Strain ISL-50^T exhibited 16S rRNA gene sequence similarity values of 95.3–98.2 % with respect to the type strains of recognized *Halobacillus* species. The cell-wall peptidoglycan was based on meso-diaminopimelic acid, the predominant menaquinone was MK-7 and the major fatty acids were anteiso-C_{15:0} and anteiso-C_{17:0}. The DNA G + C content was 39.3 mol%. DNA–DNA relatedness data and differential phenotypic properties revealed that strain ISL-50^T was distinguishable from the recognized *Halobacillus* species. On the basis of phenotypic, chemotaxonomic, phylogenetic and genetic data, strain ISL-50^T represents a novel species of the genus *Halobacillus*, for which the name *Halobacillus seohaensis* sp. nov. is proposed. The type strain is ISL-50^T (=KCTC 13145^T=CCUG 54358^T).

The genus *Halobacillus* was created by Spring *et al.* (1996) and, at the time of writing, comprises 14 species with validly published names: *Halobacillus halophilus*, *H. litoralis* and *H. trueperi* (Spring *et al.*, 1996), *H. salinus* (Yoon *et al.*, 2003), *H. karajensis* (Amoozegar *et al.*, 2003), *H. locisalis* (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.*, 2007b) and *H. mangrovi* (Soto-Ramírez *et al.*, 2008). The genus *Halobacillus* is differentiated from phylogenetically related genera of the family *Bacillaceae* by having cell-wall peptidoglycan based on L-Orn–D-Asp (Spring *et al.*, 1996; Nunes *et al.*, 2006; An *et al.*, 2007a). However, *H. campisalis*, which was described recently, was found to contain meso-diaminopimelic acid (meso-DAP) instead of L-ornithine as the diamino acid in the cell-wall peptidoglycan (Yoon *et al.*, 2007). In this study, we report on the taxonomic characterization of another *Halobacillus*-like bacterial strain, ISL-50^T, that contains meso-DAP as the diamino acid in the cell-wall peptidoglycan.

Strain ISL-50^T was isolated from a sediment collected from a marine solar saltern at Byunsan, Korea, by means of the dilution-plating technique at 30 °C on marine agar 2216

(MA; Difco) supplemented with 8 % (w/v) NaCl. The type strains of 13 *Halobacillus* species were used as reference strains for DNA–DNA hybridization. *H. halophilus* KCTC 3685^T, *H. litoralis* KCTC 3687^T, *H. trueperi* KCTC 3686^T and *H. faecis* KCTC 13121^T were obtained from the Korean Collection for Type Cultures (Taejeon, Korea), *H. karajensis* DSM 14948^T, *H. profundus* DSM 18394^T and *H. kuroshimensis* DSM 18393^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany), *H. salinus* HSL-3^T, *H. locisalis* MSS-155^T, *H. yeomjeoni* MSS-402^T and *H. campisalis* ASL-17^T were obtained from our previous studies (Yoon *et al.*, 2003, 2004, 2005, 2007) and *H. aidingensis* JCM 12771^T and *H. dabanensis* JCM 12772^T were obtained from the Japan Collection of Microorganisms (Saitama, Japan).

Morphological, physiological and biochemical characteristics of strain ISL-50^T were investigated by using routine cultivation on MA supplemented with 6 % (w/v) NaCl at 37 °C. Cell morphology was examined by using light microscopy (E600; Nikon) and transmission electron microscopy. Flagellation was determined by using a Philips CM-20 transmission electron microscope with cells from exponentially growing cultures. For this purpose, the cells were negatively stained with 1 % (w/v) phosphotungstic acid and the grids were examined after being air-dried. Growth under anaerobic conditions was determined after incubation in a Forma anaerobic chamber on MA supplemented with 6 % (w/v) NaCl and on MA supplemented with 6 % (w/v) NaCl and potassium nitrate (0.1 %,

Abbreviation: meso-DAP, meso-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ISL-50^T is EF612763.

A transmission electron micrograph of a cell of strain ISL-50^T is available with the online version of this paper.

w/v), both of which had been prepared anaerobically using nitrogen. The pH range for growth was determined in marine broth 2216 (MB; Difco) supplemented with 6 % (w/v) NaCl and 1 % (w/v) L-malate adjusted to various pH values (pH 4.5–9.5, using increments of 0.5 pH units) by the addition of HCl, NaHCO₃ or Na₂CO₃. Growth in the absence of NaCl was investigated by using trypticase soy broth, using supplementation with 0.45 % (w/v) MgCl₂·6H₂O and 1 % (w/v) L-malate, prepared according to the Difco formula except that NaCl was excluded. Growth at different NaCl concentrations [2–25 % (w/v), using 1 % increments] was investigated in MB supplemented with 1 % (w/v) L-malate. Growth at various temperatures (4, 10, 20, 25 and 30–40 °C, using 1 °C increments) was measured on MA supplemented with 6 % (w/v) NaCl. Catalase and oxidase activities and hydrolysis of casein, starch and Tweens 20, 40, 60 and 80 were determined as described by Cowan & Steel (1965). Hydrolysis of hypoxanthine, tyrosine and xanthine was tested on MA supplemented with 6 % (w/v) NaCl, using the substrate concentrations described by Cowan & Steel (1965). Hydrolysis of aesculin, gelatin and urea and reduction of nitrate were investigated as described by Lanyi (1987), using artificial seawater supplemented with 6 % (w/v) NaCl. The artificial seawater contained the following [(1 distilled water)⁻¹]: 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O and 1.3 g CaCl₂·2H₂O (Bruns *et al.*, 2001). Susceptibility to antibiotics was determined on MA plates supplemented with 6 % (w/v) NaCl using antibiotic discs (Advantec) with the following concentrations (µg, unless indicated otherwise): polymyxin B, 100 U; streptomycin, 50; penicillin G, 20 U; chloramphenicol, 100; ampicillin, 10; cephalothin, 30; gentamicin, 30; novobiocin, 5; tetracycline, 30; kanamycin, 30; lincomycin, 15; oleandomycin, 15; neomycin, 30; carbenicillin, 100. Acid production from carbohydrates was tested as described by Leifson (1963) using supplementation with 7 % (w/v) NaCl. Utilization of various substrates for growth was determined as described by Baumann & Baumann (1981), using supplementation with 7 % (w/v) NaCl, 2 % (v/v) Hutner's mineral salts solution (Cohen-Bazire *et al.*, 1957) and 1 % (v/v) vitamin solution (Staley, 1968). Enzyme activities were determined by using the API ZYM system (bioMérieux).

Cell biomass for DNA extraction and for the analysis of cell-wall and isoprenoid quinones was obtained from cells grown in MB supplemented with 6 % (w/v) NaCl and 1 % (w/v) L-malate at 37 °C. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), with the exception that RNase T1 was used in combination with RNase A to minimize the contamination of RNA. The 16S rRNA gene was amplified by means of a PCR with two universal primers, as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and the phylogenetic analysis were performed as described by Yoon *et al.* (2003). The isomer type of the diamino acid in the cell-wall peptidoglycan was analysed by

using TLC according to the method described by Komagata & Suzuki (1987). Preparation of whole-cell hydrolysates and determination of the isomer type of the diamino acid were also performed at the DSMZ, using TLC and the solvent system of Rhuland *et al.* (1955). Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987), using reversed-phase HPLC. For analysis of cellular fatty acids, cell mass from strain ISL-50^T was harvested from agar plates after cultivation for 7 days at 37 °C on MA supplemented with 6 % (w/v) NaCl. Fatty acids were extracted and fatty acid methyl esters were prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G+C content was determined by using the method of Tamaoka & Komagata (1984) with the modification that the DNA was hydrolysed by using nuclease P1 (Sigma) and the resultant nucleotides were analysed by reversed-phase HPLC. DNA–DNA hybridization was performed fluorometrically by using the method of Ezaki *et al.* (1989) with photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded, and the means of the remaining three values were quoted as the DNA–DNA relatedness values.

Morphological, cultural, physiological and biochemical characteristics of strain ISL-50^T are given in the species description or in Table 1. A transmission electron micrograph of a cell of strain ISL-50^T is available as Supplementary Fig. S1 in IJSEM Online. The almost-complete 16S rRNA gene sequence of strain ISL-50^T determined in this study comprised 1522 nt, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. Comparative 16S rRNA gene sequence analysis revealed that strain ISL-50^T has the closest phylogenetic affiliation with the genus *Halobacillus*. In a phylogenetic tree constructed by using the neighbour-joining algorithm, strain ISL-50^T fell within the radiation of the cluster comprising *Halobacillus* species, forming a cluster with the type strains of *H. campisalis* and *H. halophilus* (Fig. 1). The relationships among strain ISL-50^T, *H. campisalis* and *H. halophilus* were also maintained in trees based on the maximum-likelihood and maximum-parsimony algorithms (Fig. 1). Strain ISL-50^T exhibited 16S rRNA gene sequence similarity values of 95.3–98.2 % with respect to the type strains of *Halobacillus* species and values <95.6 % with respect to other species used in the phylogenetic analysis.

Strain ISL-50^T possessed cell-wall peptidoglycan containing *meso*-DAP as the diagnostic diamino acid. The predominant isoprenoid quinone detected in strain ISL-50^T was MK-7; minor amounts (approx. 3 %) of MK-6 and MK-8 were also present. The fatty acid profile of strain ISL-50^T was composed of the following (each constituting >0.5 % of total fatty acids): branched fatty acids anteiso-C_{15:0} (69.2 %), anteiso-C_{17:0} (13.5 %), iso-C_{16:0} (5.6 %), iso-C_{15:0} (4.6 %), iso-C_{14:0} (2.5 %), iso-C_{17:0} (0.8 %) and anteiso-C_{17:1} (0.5 %) and straight-chain fatty acids C_{15:0}

Table 1. Differential phenotypic properties of *Halobacillus* species

Taxa: 1, strain ISL-50^T; 2, *H. halophilus*, data from Claus *et al.* (1983) and Spring *et al.* (1996); 3, *H. campisalis*, data from Yoon *et al.* (2007); 4, *H. trueperi*, data from Spring *et al.* (1996); 5, *H. litoralis*, data from Spring *et al.* (1996); 6, *H. salinus*, data from Yoon *et al.* (2003); 7, *H. karajensis*, data from Amoozegar *et al.* (2003); 8, *H. locisalis*, data from Yoon *et al.* (2004); 9, *H. dabanensis*, data from Liu *et al.* (2005); 10, *H. aidingensis*, data from Liu *et al.* (2005); 11, *H. yeomjeoni*, data from Yoon *et al.* (2005); 12, *H. profundus*, data from Hua *et al.* (2007); 13, *H. kuroshimensis*, data from Hua *et al.* (2007); 14, *H. faecis*, data from An *et al.* (2007b). All were positive for catalase and oxidase and negative for nitrate reduction (not determined for *H. karajensis*), urease, anaerobic growth and hydrolysis of tyrosine (not determined for *H. karajensis*). +, Positive reaction; –, negative reaction; w, weakly positive reaction; v, variable reaction; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Cell morphology	Rods	Cocci or oval-shaped	Cocci or oval-shaped	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods or long filamentous rods	Rods	Rods	Rods
Flagellation	Single	Single or peritrichous	Peritrichous	Peritrichous	Peritrichous	Peritrichous	Absent	Single	Peritrichous	Peritrichous	Single	Absent	Absent	Absent
Gram staining	+(v)	+	+(v)	+	+	+(v)	+	+(v)	+	+	+(v)	+	+	+
Spore shape	Ellipsoidal	Spherical	Spherical	Ellipsoidal or spherical	Ellipsoidal or spherical	Ellipsoidal	Ellipsoidal or spherical	Ellipsoidal	Ellipsoidal	Ellipsoidal	Ellipsoidal	Ellipsoidal or spherical	Ellipsoidal or spherical	Ellipsoidal or spherical
Spore position	Central or subterminal	Central or lateral	Central	Central or subterminal	Central or subterminal	Central or subterminal	Central or subterminal	Central or subterminal	Central or subterminal	Central or subterminal	Central or subterminal	Central	Terminal	Central
Colony colour	Yellowish-white	Orange	Light yellow	Orange	Orange	Pale orange–yellow	Cream or white	Light orange–yellow	Cream to orange	Orange	Light yellow	Pale yellow	Yellow–orange	Orange
Maximum temp. for growth (°C)	38	40	41	44	43	45	49	42	50	40	48	47	48	45
Growth at:														
4 °C	+	–	+	–	–	–	–	–	–	–	–	–	–	–
pH 5.0	–	–	–	–	–	+	–	+	+	–	–	–	–	–
pH 5.5	–	–	+	–	–	+	–	+	+	–	–	+	+	+
0.5 % NaCl	–	–	+	+	+	+	–	–	+	+	+	+	+	+
25 % NaCl	–	–	–	+	+	–	–	–	+	–	–	+	+	–
Hydrolysis of:														
Aesculin	–	–	+	–	–	+	+	+	–	–	–	+	+	ND
Casein	+	+	+	–	–	+	+	–	+	+	+	+	+	ND
Gelatin	–	+	–	+	+	+	+	–	–	+	+	–	+	+
Starch	+	+	+	–	–	–	+	+	+	+	–	+	+	ND
Tween 80	–	–	–	–	–	+	–	+	–	–	+	+	+	ND
Acid production from:														
D-Fructose	–	–	+	+	+	+	+	+	+	+	–	+	+	+
D-Galactose	–	–	+	+	–	w	–	–	–	–	–	–	–	+
Maltose	–	–	–	+	+	+	+	–	+	+	+	+	+	+
Sucrose	+	–	+	+	+	+	–	+	+	+	+	+	+	+
D-Xylose	+	–	–	–	+	–	–	–	+	–	–	+	–	–

Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
D-Glucose	–	–	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannitol	+	–	–	–	+	+	+	–	+	+	+	+	–	+
Trehalose	+	–	+	+	+	+	ND	+	+	+	+	+	–	+
Cell-wall type	meso-DAP	L-Orn-D-Asp	meso-DAP	L-Orn-D-Asp	L-Orn-D-Asp	L-Orn-D-Asp	L-Orn-D-Asp	L-Orn-D-Asp	L-Orn-D-Asp	L-Orn-D-Asp	L-Orn-D-Asp	L-Orn-D-Asp	L-Orn-D-Asp	L-Orn-D-Asp
DNA G+C content (mol%)	39.3	40.1–40.9	42.1	43	42	45	41.3	44.0	41.4	42.2	42.9	43.3	42.1	46.5

*Positive when Mg^{2+} ion is added.

(1.8 %) and $C_{16:0}$ (1.4 %). This fatty acid profile is similar to those of *Halobacillus* species, although there are differences in the proportions of some fatty acids, probably because of differences in cultivation conditions and extraction procedures (Liu *et al.*, 2005; Yoon *et al.*, 2005, 2007). The DNA G+C content of strain ISL-50^T was 39.3 mol%. These chemotaxonomic properties are sufficient to support the result of the phylogenetic classification, i.e. that strain ISL-50^T could be a member of the genus *Halobacillus*, although there is a difference in the type of diagnostic diamino acid between strain ISL-50^T and other *Halobacillus* species (except for *H. campisalis*). Differences in the peptidoglycan types within the same genus have been shown in some genera (Schleifer & Kandler, 1972; Takeuchi & Hatano, 1998; Rheims *et al.*, 1999; Abd El-Rahman *et al.*, 2002).

Strain ISL-50^T exhibited DNA–DNA relatedness values of 9–22 % with respect to the type strains of 13 recognized *Halobacillus* species: *H. campisalis* ASL-17^T (19 %), *H. halophilus* KCTC 3685^T (22 %), *H. litoralis* KCTC 3687^T (13 %), *H. trueperi* KCTC 3686^T (15 %), *H. karajensis* DSM 14948^T (9 %), *H. salinus* HSL-3^T (17 %), *H. locisalis* MSS-155^T (15 %), *H. yeomjeoni* MSS-402^T (20 %), *H. aidingensis* JCM 12771^T (11 %), *H. dabanensis* JCM 12772^T (18 %), *H. profundus* DSM 18394^T (14 %), *H. kuroshimensis* DSM 18393^T (9 %) and *H. faecis* KCTC 13121^T (20 %). These DNA–DNA relatedness values indicate that strain ISL-50^T represents a genomic species that is different from the recognized *Halobacillus* species (Wayne *et al.*, 1987). Strain ISL-50^T was differentiated from the recognized *Halobacillus* species through differences in several phenotypic characteristics, including flagellum type, hydrolysis of substrates, acid production from substrates and the diamino acid type of the cell-wall peptidoglycan (Table 1). The phylogenetic and genetic distinctiveness and differential phenotypic properties are sufficient to categorize strain ISL-50^T within a species that is distinct from the recognized *Halobacillus* species (Wayne *et al.*, 1987; Table 1). Therefore, on the basis of the data presented, strain ISL-50^T should be placed within the genus *Halobacillus* as a novel species, for which the name *Halobacillus seohaensis* sp. nov. is proposed.

Description of *Halobacillus seohaensis* sp. nov.

Halobacillus seohaensis (seo.ha.en'sis. N.L. masc. adj. *seohaensis* of Seohae, the Korean name for the Yellow Sea in Korea, from which the type strain was isolated).

Cells are rods (0.5–0.9 × 1.0–4.0 µm) on MA supplemented with 6 % (w/v) NaCl at 37 °C. Gram-positive, but Gram-variable in old cultures. Motile by means of a single polar flagellum. Colonies are circular, raised, smooth, glistening, yellowish-white in colour and 1.5–2.0 mm after 7 days incubation at 37 °C on MA supplemented with 6 % (w/v) NaCl. Optimal growth temperature is 37 °C; growth occurs at 4 and 38 °C, but not at 39 °C. Optimal pH for growth is 7.0–8.0; growth occurs at pH 6.0, but not at pH 5.5. Optimal growth occurs in the presence of approximately

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the positions of strain ISL-50^T, other *Halobacillus* species and some other related taxa. Bootstrap values (expressed as percentages of 1000 replications) >40 % are shown at branch points. ● indicates that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Alicyclobacillus acidocaldarius* DSM 446^T (GenBank accession no. X60742) was used as an outgroup (not shown). Bar, 0.01 substitutions per nucleotide position.

8 % (w/v) NaCl; growth occurs in the presence of 1–21 % (w/v) NaCl. Tweens 20, 40 and 60, hypoxanthine and xanthine are not hydrolysed. In assays with the API ZYM system, esterase (C4), esterase lipase (C8) and acid phosphatase are present, but alkaline phosphatase, lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase are absent. L-Malate is utilized, but L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, maltose, D-mannose, sucrose, trehalose, D-xylose, acetate, citrate, succinate, benzoate, pyruvate, salicin, formate and L-glutamate are not. Acid is produced from D-cellobiose, D-ribose and D-sorbitol, but not from L-arabinose, lactose, D-mannose, melezitose, melibiose, raffinose, L-rhamnose or *myo*-inositol. Susceptible to ampicillin, carbenicillin, cephalothin, chloramphenicol, gentamicin, kanamycin, lincomycin, novobiocin, oleandomycin, penicillin G and tetracycline, but not to neomycin, polymyxin B or streptomycin. The cell-wall peptidoglycan contains *meso*-DAP as the diamino acid. The predominant menaquinone is MK-7. The major fatty acids (>10 % of total fatty acids) are anteiso-C_{15:0} and anteiso-C_{17:0}. The DNA G + C content is 39.3 mol %.

The type strain, ISL-50^T (=KCTC 13145^T=CCUG 54358^T), was isolated from a marine solar saltern of the Yellow Sea at Byunsan, Korea.

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