Halobacillus campisalis sp. nov., containing meso-diaminopimelic acid in the cell-wall peptidoglycan, and emended description of the genus *Halobacillus*

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A Gram-positive or variable, motile and coccoid or oval-shaped bacterial strain, ASL-17^T, was isolated from a marine solar saltern of the Yellow Sea in Korea and its exact taxonomic position was investigated by a polyphasic approach. Strain ASL-17^T grew optimally at pH 7.0–8.0 and 37 °C. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain ASL-17^T is most closely affiliated phylogenetically to the genus *Halobacillus*. Strain ASL-17^T exhibited 16S rRNA gene sequence similarity values of 97.7–98.6% to the type strains of recognized *Halobacillus* species. Interestingly, strain ASL-17^T had cell-wall peptidoglycan based on meso-diaminopimelic acid, unlike other *Halobacillus* species. It contained MK-7 as the predominant menaquinone. The major fatty acids were anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. The DNA G + C content was 42.1 mol%. DNA–DNA relatedness data and differential phenotypic properties demonstrated that strain ASL-17^T can be differentiated from recognized *Halobacillus* species. On the basis of phenotypic, chemotaxonomic, phylogenetic and genetic data, strain ASL-17^T represents a novel species of the genus *Halobacillus*, for which the name *Halobacillus campisalis* sp. nov. is proposed. The type strain is ASL-17^T (=KCTC 13144^T =CCUG 54360^T).

The genus *Halobacillus* was created by Spring *et al.* (1996) through the reclassification of *Sporosarcina halophila* as *Halobacillus halophilus* and the description of two novel species, *Halobacillus litoralis* and *Halobacillus trueperi*. Subsequently, six further *Halobacillus* species, *Halobacillus salinus* (Yoon *et al.*, 2003), *Halobacillus karajensis* (Amoozegar *et al.*, 2003), *Halobacillus locisalis* (Yoon *et al.*, 2004), *Halobacillus aidingensis* and *Halobacillus dabanensis* (Liu *et al.*, 2005) and *Halobacillus yeomjeoni* (Yoon *et al.*, 2005), have been described. In this study, we describe a bacterial strain, ASL-17^T, which is related phylogenetically to the genus *Halobacillus*. However, strain ASL-17^T was found to have cell-wall peptidoglycan based on meso-diaminopimelic acid instead of L-ornithine, as found in other members of the genus *Halobacillus*. Accordingly, the aim of the present study was to determine the exact taxonomic position of strain ASL-17^T by a polyphasic characterization that included determination of the phenotypic and chemotaxonomic properties, detailed phylogenetic analysis based on 16S rRNA gene sequences and genetic analysis.

Strain ASL-17^T was isolated from a sediment collected from a marine solar saltern of the Yellow Sea, 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 nine *Halobacillus* species were used as reference strains for DNA–DNA hybridization: *H. halophilus* KCTC 3685^T, *H. litoralis* KCTC 3687^T and *H. trueperi* KCTC 3686^T were obtained from the Korean Collection for Type Cultures (KCTC), Taejeon, Korea; *H. karajensis* DSM 14948^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany; *H. salinus* HSL-3^T, *H. locisalis* MSS-155^T and *H. yeomjeoni* MSS-402^T were obtained in our laboratory (Yoon *et al.*, 2003, 2004, 2005); and *H. aidingensis* JCM 12771^T and *H. dabanensis* JCM 12772^T were obtained from the Japan Collection of Microorganisms (JCM), Saitama, Japan.

The morphological, physiological and biochemical characteristics of strain ASL-17^T were investigated using routine cultivation on MA supplemented with 6% (w/v) NaCl at 37 °C. Cell morphology was examined by light microscopy (Nikon E600) 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

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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, 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 that was adjusted to various pH values (pH 4.5–9.5 at intervals of 0.5 pH units). Growth in the absence of NaCl was investigated using trypticase soy broth, using supplementation with 0.45 % (w/v) $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, prepared according to the formula of the Difco medium except that NaCl was omitted. Growth at various NaCl concentrations was investigated in MB or trypticase soy broth (Difco). Growth at various temperatures (4–50 °C) 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 nitrate reduction were investigated as described previously (Lanyi, 1987) with the modification that artificial seawater supplemented with 6 % (w/v) NaCl was used for preparation of media. The artificial seawater contained (1^{-1} distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5.94 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.3 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Bruns *et al.*, 2001). H_2S production was tested as described previously (Bruns *et al.*, 2001). Susceptibility to antibiotics was investigated on MA plates supplemented with 6 % (w/v) NaCl by using antibiotic discs with the following amounts: polymyxin B, 100 U; streptomycin, 50 µg; penicillin G, 20 U; chloramphenicol, 100 µg; ampicillin, 10 µg; cephalothin, 30 µg; gentamicin, 30 µg; novobiocin, 5 µg; tetracycline, 30 µg; kanamycin, 30 µg; lincomycin, 15 µg; oleandomycin, 15 µg; neomycin, 30 µg; carbenicillin, 100 µg. 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 analyses of cell-wall and isoprenoid quinones was obtained from cultivation in MB supplemented with 6 % (w/v) NaCl 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 contamination with RNA. The 16S rRNA gene was amplified by PCR using two universal primers as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and 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 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 was also performed at the DSMZ by 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 cellular fatty acid analysis, cell mass of strain ASL-17^T was harvested from agar plates after cultivation for 3 days at 37 °C on MA supplemented with 6 % (w/v) NaCl. The 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 the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed using nuclease P1 (Sigma) and the resultant nucleotides were analysed by reversed-phase HPLC. 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. The highest and lowest values obtained in each sample were excluded, and the means of the remaining three values were quoted as DNA–DNA relatedness values.

Morphological, cultural, physiological and biochemical characteristics of strain ASL-17^T are given in the species description and in Table 1. The almost-complete 16S rRNA gene sequence of strain ASL-17^T determined in this study comprised 1522 nucleotides, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. Comparative 16S rRNA gene sequence analysis revealed that strain ASL-17^T was most closely related phylogenetically to the genus *Halobacillus*. In the phylogenetic tree based on the neighbour-joining algorithm, strain ASL-17^T fell within the radiation of the cluster comprising *Halobacillus* species, joining the type strain of *H. halophilus* with a bootstrap confidence value of 90.3 % (Fig. 1). The relationship between strain ASL-17^T and the type strain of *H. halophilus* was also maintained in trees based on the maximum-likelihood and maximum-parsimony algorithms (Fig. 1). Strain ASL-17^T exhibited 16S rRNA gene sequence similarity values of 97.7–98.6 % to the type strains of *Halobacillus* species and of less than 97.1 % to other species used in the phylogenetic analysis. Strain ASL-17^T had cell-wall peptidoglycan based on *meso*-diaminopimelic acid as the diagnostic diamino acid, unlike previously described *Halobacillus* species, which have cell-wall peptidoglycan based on *L*-Orn–D–Asp (Spring *et al.*, 1996; Amoozegar *et al.*, 2003; Yoon *et al.*, 2003, 2004, 2005; Liu *et al.* 2005). The predominant isoprenoid quinone detected in strain ASL-17^T was menaquinone-7 (MK-7). The fatty acid profile of strain ASL-17^T was composed of the following (each constituting >0.5 % of total fatty acids): branched fatty acids anteiso- $\text{C}_{15:0}$ (50.8 %), anteiso- $\text{C}_{17:0}$ (21.2 %), iso- $\text{C}_{16:0}$ (12.5 %), iso- $\text{C}_{15:0}$ (5.1 %), iso- $\text{C}_{14:0}$ (3.5 %) and

Table 1. Differential phenotypic properties of *Halobacillus* species

Species: 1, *H. campisalis* sp. nov. (strain ASL-17^T); 2, *H. halophilus* (data from Claus *et al.*, 1983; Spring *et al.*, 1996); 3, *H. trueperi* (Spring *et al.*, 1996); 4, *H. litoralis* (Spring *et al.*, 1996); 5, *H. salinus* (Yoon *et al.*, 2003); 6, *H. karajensis* (Amoozegar *et al.*, 2003); 7, *H. locisalis* (Yoon *et al.*, 2004); 8, *H. dabanensis* (Liu *et al.*, 2005); 9, *H. aidingensis* (Liu *et al.*, 2005); 10, *H. yeomjeoni* (Yoon *et al.*, 2005). +, Positive; –, negative; w, weakly positive; v, variable; ND, no data available; DAP, diaminopimelic acid. All species are positive for catalase and oxidase. All species are negative for nitrate reduction (no data for *H. karajensis*), urease, anaerobic growth and hydrolysis of tyrosine (no data for *H. karajensis*).

Characteristic	1	2	3	4	5	6	7	8	9	10
Cell morphology	Cocci or oval	Cocci or oval	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods or long filamentous rods
Flagellation	Peritrichous	Single or peritrichous	Peritrichous	Peritrichous	Peritrichous	Absent	Single	Peritrichous	Peritrichous	Single
Gram staining	+	+	+	+	+	+	+	+	+	+
Spore shape	Spherical	Spherical	Ellipsoidal or spherical	Ellipsoidal or spherical	Ellipsoidal	Ellipsoidal or spherical	Ellipsoidal	Ellipsoidal	Ellipsoidal	Ellipsoidal
Spore position	Central	Central or lateral	Central or subterminal	Central or subterminal	Central or subterminal	Central or subterminal	Central or subterminal	Central or subterminal	Central or subterminal	Central or subterminal
Colony colour	Light yellow	Orange	Orange	Orange	Pale orange–yellow	Cream or white	Light orange–yellow	Cream to orange	Orange	Light yellow
Maximum temperature for growth (°C)	41	37	44	43	45	49	42	50	40	48
Growth at 4 °C	+	–	–	–	–	–	–	–	–	–
Growth at:										
pH 5.0	–	–	–	–	+	–	+	+	–	–
pH 5.5	+	–	–	–	+	–	+	+	–	–
Growth at:										
0.5 % NaCl	+	–	+	+	+	–	–	+	+	+
25 % NaCl	–	–	+	+	–	–	–	+	–	–
Hydrolysis of:										
Aesculin	+	–	–	–	+	+	+	–	–	–
Casein	+	+	–	–	+	+	–	+	+	+
Gelatin	–	+	+	+	+	+	–	–	+	+
Starch	+	+	–	–	–	+	+	+	+	–
Tween 80	–	–	–	–	+	–	+	–	–	+
Acid production from:										
D-Fructose	+	–	+	+	+	+	+	+	+	–
D-Galactose	+	–	+	–	w	–	–	–	–	–
Maltose	–	–	+	+	+	+	–	+	+	+
Sucrose	+	–	+	+	+	–	+	+	+	+
D-Xylose	–	–	–	+	–	–	–	+	–	–
D-Glucose	+	–	+	+	+	+	+	+	+	+
D-Mannitol	–	–	–	+	+	+	–	+	+	+
Trehalose	+	–	+	+	+	ND	+	+	+	+
Cell-wall type	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
DNA G + C content (mol%)	42.1	40.1–40.9	43	42	45	41.3	44.0	41.4	42.2	42.9

*Positive when Mg²⁺ ions are added.

iso-C_{17:0} (2.5 %); unsaturated fatty acid C_{16:1ω7c} alcohol (1.6 %); straight-chain fatty acids C_{16:0} (1.2 %) and C_{15:0} (0.6 %); and summed feature 4 (iso-C_{17:1} and/or

anteiso-C_{17:1}, 0.6 %). This fatty acid profile was similar as those of *Halobacillus* species, although there are differences in the proportions of some fatty acids, probably because of

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain ASL-17^T (*Halobacillus campisalis* sp. nov.), other *Halobacillus* species and some other related taxa. Bootstrap values (expressed as percentages of 1000 replications) >50% are shown at branch points. Dots indicate 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; not shown) was used as an out-group. Bar, 0.01 substitutions per nucleotide position.

differences in cultivation conditions and extraction procedures (Liu *et al.*, 2005; Yoon *et al.*, 2005). The DNA G+C content of strain ASL-17^T was 42.1 mol%. Strain ASL-17^T is clearly distinguishable from *Halobacillus* species by the difference in the cell-wall peptidoglycan type (Table 1). However, the phylogenetic similarities between strain ASL-17^T and *Halobacillus* species may be too great to place strain ASL-17^T in a new genus separate from the genus *Halobacillus*. Moreover, strain ASL-17^T does not show noteworthy differences from members of the genus *Halobacillus* in isoprenoid quinone or fatty acid profiles. Accordingly, it appears to be appropriate that strain ASL-17^T be classified in the genus *Halobacillus*.

Strain ASL-17^T exhibited DNA–DNA relatedness values of 11–25% to the type strains of nine recognized *Halobacillus* species: *H. halophilus* KCTC 3685^T (18%), *H. litoralis* KCTC 3687^T (25%), *H. trueperi* KCTC 3686^T (12%), *H. karajensis* DSM 14948^T (12%), *H. salinus* HSL-3^T (19%), *H. locisalis* MSS-155^T (17%), *H. yeomjeoni* MSS-402^T (22%), *H. aidingensis* JCM 12771^T (11%) and *H. dabanensis* JCM 12772^T (18%). These values indicate that strain ASL-17^T represents a genomic species that is different from recognized *Halobacillus* species (Wayne *et al.*, 1987). Strain ASL-17^T can be differentiated from recognized *Halobacillus* species by differences in several phenotypic characteristics and in cell-wall type (Table 1). The phylogenetic and genetic distinctiveness and differential phenotypic properties are sufficient to allocate strain ASL-17^T to a species that is separate from recognized *Halobacillus* species (Wayne *et al.*, 1987; Table 1). Therefore, on the basis of the data presented, strain ASL-17^T should be placed within a novel species of the genus *Halobacillus*, for which the name *Halobacillus campisalis* sp. nov. is proposed.

Description of *Halobacillus campisalis* sp. nov.

Halobacillus campisalis (cam.pi.sa'lis. L. n. *campus* field; L. gen. n. *salis* of salt; N.L. gen. n. *campisalis* of a field of salt).

Cells are cocci or oval (0.7–1.4 × 1.0–1.6 µm) on MA supplemented with 6% (w/v) NaCl at 37 °C. Gram-positive, but Gram-variable in old cultures. Colonies are circular, slightly convex, smooth, glistening, light-yellow in colour and 1.5–2.0 mm after 3 days incubation at 37 °C on MA supplemented with 6% (w/v) NaCl. Mg²⁺ ions are required for growth. Optimal growth temperature is 37 °C; growth occurs at 4 and 41 °C, but not at 42 °C. Optimal pH for growth is 7.0–8.0; growth occurs at pH 5.5 but not at pH 5.0. Optimal growth occurs in the presence of approximately 8% (w/v) NaCl; growth occurs without NaCl and in the presence of 22% (w/v) NaCl, but not in the presence of >23% (w/v) NaCl. Tweens 20, 40 and 60, hypoxanthine and xanthine are not hydrolysed. H₂S is not produced. In assays with the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8) and β-galactosidase are present, but lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. D-Glucose, D-fructose, D-galactose, D-cellobiose, D-mannose, trehalose, sucrose and maltose are utilized, but L-arabinose, D-xylose, acetate, citrate, succinate, benzoate, L-malate, pyruvate, salicin, formate and L-glutamate are not. Acid is produced from D-cellobiose, lactose, D-mannose, D-melezitose, melibiose, D-raffinose and L-rhamnose, but not from L-arabinose, myo-inositol, D-ribose or D-sorbitol. Susceptible to ampicillin, carbenicillin, cephalothin, chloramphenicol, gentamicin, kanamycin, lincomycin, neomycin, novobiocin, oleandomycin, penicillin G, streptomycin and tetracycline, but not to polymyxin B. The cell-wall peptidoglycan contains meso-diaminopimelic acid as the diamino acid. The predominant menaquinone is MK-7. The major fatty acids (>10% of total fatty acids) are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. The DNA G+C content of the type strain is 42.1 mol%.

The type strain, ASL-17^T (=KCTC 13144^T =CCUG 54360^T), was isolated from a marine solar saltern of the Yellow Sea in Korea.

Emended description of the genus *Halobacillus* Spring *et al.* 1996

The description of the genus *Halobacillus* is as given by Spring *et al.* (1996), Yoon *et al.* (2003, 2004, 2005), Amoozegar *et al.* (2003) and Liu *et al.* (2005) with the following amendment. The cell-wall peptidoglycan is based on L-Orn-D-Asp or meso-diaminopimelic acid.

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