Alkalibacillus flavidus sp. nov., isolated from a marine solar saltern

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A Gram-stain-positive, motile, rod-shaped bacterial strain, ISL-17^T, was isolated from a marine solar saltern of the Yellow Sea, Korea, and its taxonomic position was investigated by means of a polyphasic study. Strain ISL-17^T grew optimally at pH 8.5–9.0, at 37 °C and in the presence of approximately 10 % (w/v) NaCl. It contained *meso*-diaminopimelic acid as the diagnostic diamino acid in the peptidoglycan, MK-7 as the predominant menaquinone and iso-C_{15:0}, anteiso-C_{15:0} and iso-C_{16:0} as the major fatty acids. The DNA G + C content was 48.1 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain ISL-17^T fell within the genus *Alkalibacillus*, clustering with *Alkalibacillus salilacus* BH163^T with a bootstrap resampling value of 100 %. Strain ISL-17^T exhibited 98.2 % 16S rRNA gene sequence similarity to *A. salilacus* BH163^T and 95.8–96.5 % similarity to the type strains of the other *Alkalibacillus* species. The mean DNA–DNA relatedness value between strain ISL-17^T and *A. salilacus* KCTC 3916^T was 19 %. The phenotypic properties of strain ISL-17^T, together with its phylogenetic and genetic distinctiveness, enable this strain to be differentiated from recognized *Alkalibacillus* species. On the basis of phenotypic, phylogenetic and genetic data, strain ISL-17^T represents a novel species within the genus *Alkalibacillus*, for which the name *Alkalibacillus flavidus* sp. nov. is proposed; the type strain is ISL-17^T (=KCTC 13258^T =CCUG 56753^T).

The genus *Alkalibacillus* was proposed by Jeon *et al.* (2005), who reclassified *Bacillus haloalkaliphilus* Fritze 1996 as *Alkalibacillus haloalkaliphilus* and described a novel species, *Alkalibacillus salilacus*. Subsequently, two more species, *Alkalibacillus filiformis* (Romano *et al.*, 2005) and *Alkalibacillus silvisoli* (Usami *et al.*, 2007), have been described. Members of the genus *Alkalibacillus* can be differentiated from those of phylogenetically related genera, i.e. *Filobacillus* and *Tenuibacillus*, on the basis of peptidoglycan type and 16S rRNA gene sequence similarity (Jeon *et al.*, 2005). Members of the genus *Alkalibacillus* have been isolated from alkaline and highly saline mud, a salt lake, water of a mineral pool with gas bubbles and non-saline surface soil (Jeon *et al.*, 2005; Romano *et al.*, 2005; Usami *et al.*, 2007). In this study, the taxonomic characterization is reported of an *Alkalibacillus*-like bacterial strain, ISL-17^T, isolated from a marine solar saltern in Korea.

Strain ISL-17^T was isolated from a marine solar saltern from the Yellow Sea, Korea, by means of the standard dilution plating technique at 30 °C on marine agar 2216 (MA; Difco) supplemented with 6 % (w/v) NaCl (total

NaCl concentration 7.945 %, w/v). *Alkalibacillus salilacus* KCTC 3916^T, which was used as a reference strain for DNA–DNA hybridization and fatty acid analysis, was obtained from the Korean Collection for Type Cultures. The morphological, physiological and biochemical characteristics of strain ISL-17^T were investigated using cells routinely cultivated on MA supplemented with 8 % (w/v) NaCl (8 % NaCl MA) at 37 °C. Cell morphology was examined by light microscopy (Nikon E600) and transmission electron microscopy. Flagellation was determined using a Philips CM-20 transmission electron microscope with cells from exponentially growing cultures; for this purpose, cells were negatively stained with 1 % (w/v) phosphotungstic acid and grids were examined after being air-dried. Growth under anaerobic conditions was determined after incubation in a Forma anaerobic chamber on 8 % NaCl MA and on 8 % NaCl MA supplemented with potassium nitrate (0.1 %, w/v), both of which had been prepared anaerobically under a nitrogen atmosphere. Growth in the absence of NaCl was investigated using trypticase soy broth prepared according to the formula of the Difco medium except that NaCl was omitted. Growth at 2.0–28.0 % (w/v) NaCl (in 1.0 % increments) was investigated in marine broth 2216 (MB; Difco). The pH range for growth was determined in MB supplemented with 8 % (w/v) NaCl (8 % NaCl MB) adjusted to pH 4.5–

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10.5 (at intervals of 0.5 pH unit) by using sodium acetate/acetic acid and Na₂CO₃ buffers. Growth at 4, 10, 20, 25, 28, 30, 35, 37, 40, 45, 50, 55 and 60 °C was measured on 8 % NaCl MA. Catalase and oxidase activities were determined as described by Cowan & Steel (1965). Hydrolysis of casein, starch, hypoxanthine, tyrosine and xanthine was tested on 8 % NaCl MA using the substrate concentrations described by Cowan & Steel (1965). Hydrolysis of aesculin, gelatin, Tweens 20, 40, 60 and 80 and urea and nitrate reduction were investigated as described previously (Lányi, 1987) with the modification that artificial seawater supplemented with 8 % (w/v) NaCl was used for preparation of media. The artificial seawater contained (l⁻¹ distilled water) 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). H₂S production was tested as described previously (Bruns *et al.*, 2001). Susceptibility to antibiotics was investigated

on 8 % NaCl MA by using antibiotic discs containing the following: 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) and carbenicillin (100 µg). Acid production from carbohydrates was tested as described by Leifson (1963) with the modification that medium was supplemented with 8 % (w/v) NaCl. Enzyme activities were determined by using the API ZYM system (bioMérieux).

Cell biomass for DNA extraction and for the analysis of the cell wall and isoprenoid quinones was obtained from cultures grown in 8 % NaCl MB at 37 °C. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996) except that RNase T1 was

Table 1. Differential characteristics of strain ISL-17^T and the type strains of other *Alkalibacillus* species

Strains: 1, ISL-17^T; 2, *A. salilacus* BH163^T (data from Jeon *et al.*, 2005; Usami *et al.*, 2007); 3, *A. haloalkaliphilus* DSM 5271^T (Fritze, 1996; Usami *et al.*, 2007); 4, *A. filiformis* 4AG^T (Romano *et al.*, 2005; Usami *et al.*, 2007); 5, *A. silvisoli* BM2^T (Usami *et al.*, 2007). All strains have rod-shaped cells. +, Positive; –, negative; w, weakly positive; ND, no data available.

Characteristic	1	2	3	4	5
Motility	+	+	+	–	+
Gram stain	+	+	+	+	+
Colony colour†	LY	C	CWH	WH–T	C
NaCl concentration for growth (%)					
Range	4.0–26.0	5.0–20.0	>0 to 25.0	0–18.0	5.0–25.0
Optimum	10.0	10.0–12.0	5.0–10.0	10.0	10.0–15.0
Growth pH:					
Range	5.5–9.5	7.0–9.0	>7 to >9.7	7.0–10.0	7.0–10.0
Optimum	8.5–9.0	8.0	9.7	9.0	9.0–9.5
Growth temperature (°C)					
Range	10 to <60	15–40	<50	15–45	20–50
Optimum	37	30	30–37	30	30–37
Catalase	+	+	+	w	+
Oxidase	+	–	+	–	–
Nitrate reduction to nitrite	–	+	–	–	–
Hydrolysis of:					
Aesculin	–	+	+	–	–
Casein	–	–	–/w	–	+
Gelatin	–	–	+	+	+
Starch	–	–	w	–	–
Acid production from					
L-Arabinose	–	+	ND	ND	ND
D-Fructose	–	+	–	–	–
D-Galactose	–	–	–	–	+
Lactose	–	+	ND	ND	ND
Maltose	+	–	–	–	+
Trehalose	–	–	–	–	+
D-Mannitol	–	–	–	–	+
Susceptibility to streptomycin	+	–	–	–	+
DNA G + C content (mol%)	48.1	41.0	37–38	39.5	37.0

*Data from Fritze (1996); reported as Gram-stain-negative by Jeon *et al.* (2005) and Usami *et al.* (2007).

†C, Cream; CWH, creamy white; LY, light yellow; WH–T, white to transparent.

used in combination with RNase A to minimize contamination with RNA. The 16S rRNA gene was amplified by PCR using two universal primers, 5'-GAGTTTGATCCTGGCTCAG-3' and 5'-AGAAAGGAGGTGATCCAGCC-3', 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). Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987) using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. For cellular fatty acid analysis, cell mass of strain ISL-17^T was harvested from 8 % NaCl MA after cultivation for 3 days at both 37 and 30 °C, and cell mass of *A. salilacus* KCTC 3916^T was harvested from 8 % NaCl MA after cultivation for 3 days at 30 °C. 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 are quoted as DNA–DNA relatedness values.

Morphological, cultural, physiological and biochemical characteristics of strain ISL-17^T are given in the species description and in Table 1. The almost-complete 16S rRNA gene sequence of strain ISL-17^T determined in this study comprised 1520 nt, representing approximately 96 % of the

Escherichia coli 16S rRNA gene sequence. In the phylogenetic tree based on the neighbour-joining algorithm, strain ISL-17^T fell within the clade comprising *Alkalibacillus* species, particularly clustering with the type strain of *A. salilacus* at a bootstrap resampling value of 100 % (Fig. 1). The same topology was also found in trees constructed using the maximum-likelihood and maximum-parsimony algorithms (Fig. 1). Strain ISL-17^T exhibited 16S rRNA gene sequence similarity values of 98.2 % to *A. salilacus* BH163^T, 95.8–96.5 % to type strains of the other *Alkalibacillus* species and less than 94.5 % to other species used in the phylogenetic analysis.

Strain ISL-17^T had *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. The predominant menaquinone detected in strain ISL-17^T was MK-7 (at a peak area ratio of approximately 93 %). The cellular fatty acid profiles of strain ISL-17^T cultivated at two different temperatures are shown in Table 2, together with that of *A. salilacus* KCTC 3916^T, determined in this study. Strain ISL-17^T had large amounts of branched and straight-chain fatty acids; the major components (>10 % of total fatty acids) were iso-C_{15:0}, anteiso-C_{15:0} and iso-C_{16:0}. The fatty acid profiles of strain ISL-17^T and *A. salilacus* KCTC 3916^T were similar in that iso-C_{15:0} and anteiso-C_{15:0} are the major fatty acids, although there were differences in the proportions of some fatty acids, particularly iso-C_{16:0} and anteiso-C_{17:0}, between the two strains (Table 1; Jeon *et al.*, 2005). The fatty acid profiles of strain ISL-17^T and the other *Alkalibacillus* species were basically similar, although anteiso-C_{17:0} is one of major components in *A. filiformis* or *A. silvisoli*, but not in strain ISL-17^T, and iso-C_{16:0} is one of major components in strain ISL-17^T, but not in *A. haloalkaliphilus*, *A. filiformis* or *A. silvisoli* (Table 1; Jeon *et al.*, 2005; Romano *et al.*, 2005; Usami *et al.*, 2007). The DNA G + C content of strain ISL-17^T was 48.1 mol%, which is higher than those of other *Alkalibacillus* species (Table 1). Data obtained from these chemotaxonomic analyses sup-

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain ISL-17^T, *Alkalibacillus* species and some other related taxa. Bootstrap values (expressed as percentages of 1000 replications) of >50 % are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms. The sequence of *Alicyclobacillus acidocaldarius* DSM 446^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Table 2. Cellular fatty acid compositions of strain ISL-17^T and *A. salilacus* KCTC 3916^T

Data from this study. Values are percentages of total fatty acids; fatty acids that represent <0.5 % in both strains have been omitted. —, Not detected.

Fatty acid	Strain ISL-17 ^T		<i>A. salilacus</i> KCTC 3916 ^T
	37 °C	30 °C	30 °C
Straight-chain			
C _{15:0}	4.0	2.4	0.4
C _{16:0}	0.6	0.6	1.1
Unsaturated			
C _{16:1} <i>ω</i> 7c alcohol	0.3	1.5	1.2
Branched			
iso-C _{14:0}	13.1	8.1	2.7
iso-C _{15:0}	25.1	27.2	26.8
anteiso-C _{15:0}	24.3	29.9	38.6
iso-C _{16:0}	25.2	19.8	9.9
iso-C _{17:0}	1.0	1.3	1.9
anteiso-C _{17:0}	5.4	7.9	15.6
Unknown			
ECL 14.263	—	0.4	1.0

port the result of the phylogenetic analysis, i.e. that strain ISL-17^T belongs to the genus *Alkalibacillus*.

Strain ISL-17^T exhibited a mean DNA–DNA relatedness value of 19 % with respect to *A. salilacus* KCTC 3916^T, which had an 16S rRNA gene sequence similarity value of greater than 97 % to strain ISL-17^T. Strain ISL-17^T was distinguishable from recognized *Alkalibacillus* species by differences in several phenotypic characteristics as listed in Table 1. The phylogenetic and genetic distinctiveness and differential phenotypic properties of strain ISL-17^T are sufficient to categorize it as a member of a species that is distinct from the four currently recognized *Alkalibacillus* species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). On the basis of the data presented, strain ISL-17^T is considered to represent a novel species of the genus *Alkalibacillus*, for which the name *Alkalibacillus flavidus* sp. nov. is proposed.

Description of *Alkalibacillus flavidus* sp. nov.

Alkalibacillus flavidus (fla'vi.dus. L. masc. adj. *flavidus* pale yellow).

Cells are rod-shaped (0.2–0.4 × 0.8–9.5 µm), but a few cells that are longer than 10.0 µm do occur. Gram-stain-positive. Motile by means of peritrichous flagella. Terminal spherical endospores are observed in swollen sporangia. Colonies on 8 % NaCl MA are circular, raised, glistening, smooth, light yellow in colour and 1.0–1.5 mm in diameter after incubation for 3 days at 37 °C. Optimal temperature for growth is 37 °C. Growth occurs at 10

(weak) and 55 °C, but not at 4 or 60 °C. Optimal pH for growth is between 8.5 and 9.0; growth occurs at pH 5.5 and 9.5, but not at pH 5.0 or 10.0. Optimal growth occurs in the presence of approximately 10 % (w/v) NaCl. Growth occurs in the presence of 4 and 26 % (w/v) NaCl, but not in the presence of 3 or 27 % (w/v) NaCl. Anaerobic growth does not occur on 8 % NaCl MA or on 8 % NaCl MA supplemented with potassium nitrate. Catalase- and oxidase-positive. Nitrate reduction is negative. Tweens 20, 40, 60 and 80 are hydrolysed, but aesculin, casein, gelatin, hypoxanthine, starch, tyrosine and xanthine are not. H₂S is not produced. Acid is produced from maltose and D-ribose, but not from L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, lactose, D-mannitol, D-mannose, melezitose, melibiose, raffinose, L-rhamnose, sucrose, trehalose, D-xylose, *myo*-inositol or D-sorbitol. Susceptible to ampicillin, carbenicillin, cephalothin, chloramphenicol, gentamicin, kanamycin, lincomycin, novobiocin, oleandomycin, penicillin G and streptomycin, but not to neomycin, polymyxin B or tetracycline. In assays with the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8) and α-glucosidase are present, but lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. The predominant menaquinone is MK-7. The major fatty acids (>10 % of total fatty acids) are iso-C_{15:0}, anteiso-C_{15:0} and iso-C_{16:0}.

The type strain is ISL-17^T (=KCTC 13258^T =CCUG 56753^T), isolated from a marine solar saltern of the Yellow Sea, Korea. The DNA G+C content of the type strain is 48.1 mol% (determined by HPLC).

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