Salegentibacter salarius sp. nov., isolated from a marine solar saltern

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A Gram-negative, non-motile, rod-shaped bacterium, strain ISL-6^T, phenotypically resembling members of the genus *Salegentibacter*, was isolated from a marine solar saltern of the Yellow Sea in Korea and subjected to a polyphasic taxonomic investigation. Strain ISL-6^T grew optimally at pH 7.0–8.0 and 30 °C and in the presence of 8 % (w/v) NaCl. It contained MK-6 as the predominant menaquinone. The major fatty acids were iso-C_{15:0} and anteiso-C_{15:0}. The DNA G + C content was 37.5 mol%. A phylogenetic analysis based on 16S rRNA gene sequences showed that strain ISL-6^T belonged to the genus *Salegentibacter*. Strain ISL-6^T exhibited 16S rRNA gene sequence similarity values of 92.0–98.6 % with respect to the type strains of recognized *Salegentibacter* species. Low DNA–DNA relatedness values, differential phenotypic properties and phylogenetic distinctiveness demonstrated that strain ISL-6^T is distinguishable from the recognized *Salegentibacter* species. Therefore strain ISL-6^T represents a novel species of the genus *Salegentibacter*, for which the name *Salegentibacter salarius* sp. nov. is proposed. The type strain is ISL-6^T (=KCTC 12974^T =CCUG 54355^T).

The genus *Salegentibacter* was created by McCammon & Bowman (2000) to accommodate the organism previously known as [*Flavobacterium*] *salegens*. Subsequently, five other *Salegentibacter* species, isolated from marine environments or various marine invertebrates, have been described: *Salegentibacter holothuriorum* (Nedashkovskaya *et al.*, 2004), *S. mishustinae* (Nedashkovskaya *et al.*, 2005), *S. flavus* (Ivanova *et al.*, 2006), *S. agarivorans* (Nedashkovskaya *et al.*, 2006) and *S. catena* (Ying *et al.*, 2007). In this study, we report on the taxonomic characterization of a *Salegentibacter*-like bacterial strain, ISL-6^T, isolated from a marine solar saltern of the Yellow Sea in Korea.

Strain ISL-6^T was isolated, using the dilution plating technique, at 25 °C on marine agar 2216 (MA; Difco) supplemented with 8 % (w/v) NaCl. The type strains of five *Salegentibacter* species (*S. agarivorans* KCTC 12560^T, *S. flavus* CIP 107843^T, *S. holothuriorum* LMG 21968^T, *S. mishustinae* KCTC 12263^T and *S. salegens* LMG 13193^T) were used as reference strains for DNA–DNA hybridization. The morphological, physiological and biochemical characteristics of strain ISL-6^T were investigated using routine cultivation on MA supplemented with 6 % (w/v) NaCl at 30 °C. The cell morphology was examined by using light microscopy (E600; Nikon) and transmission electron microscopy (CM-20; Philips). Flagellation was investigated

by using transmission electron microscopy on cells from an exponentially growing culture negatively stained with 1 % (w/v) phosphotungstic acid. Grids were examined after being air-dried. Gliding motility was investigated as described by Bowman (2000). The Gram-reaction was determined by using the bioMérieux Gram stain kit according to the manufacturer's instructions. Growth under anaerobic conditions was determined after incubation of cells in a Forma anaerobic chamber on MA supplemented with 6 % (w/v) NaCl and on MA supplemented with 6 % (w/v) NaCl and 0.1 % (w/v) potassium nitrate, both of which had been prepared under nitrogen. 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 excluded. Growth at various NaCl concentrations (total 1–20 %, w/v, in increments of 1 %) was investigated in marine broth 2216 (MB; Difco) and trypticase soy broth (Difco). Growth at various temperatures (4, 10, 15, 20, 25 and 28 and from 30 to 45 °C in 1 °C increments) was measured on MA supplemented with 6 % (w/v) NaCl. The pH range for growth was determined in MB supplemented with 6 % (w/v) NaCl and adjusted to various pH values (pH 4.5–9.5, in increments of 0.5 pH units) after autoclaving. Catalase and oxidase activities were determined as described by Cowan & Steel (1965). DNase activity was examined by using DNase test agar with methyl green (Difco) supplemented with 8 % (w/v) NaCl. Hydrolysis of casein, starch, Tweens 20, 40, 60 and 80, hypoxanthine, tyrosine and

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain ISL-6^T is EF486353.

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) but with the modification that artificial seawater supplemented with 6 % (w/v) NaCl was used for the preparation of media. The artificial seawater contained the following (l^{-1} distilled water): 23.6 g NaCl, 0.64 g KCl, 4.53 g $MgCl_2 \cdot 6H_2O$, 5.94 g $MgSO_4 \cdot 7H_2O$ and 1.3 g $CaCl_2 \cdot 2H_2O$ (Bruns *et al.*, 2001). H_2S production was tested as described previously (Bruns *et al.*, 2001). The presence of flexirubin-type pigments was investigated as described by Reichenbach (1992). Susceptibility to antibiotics was investigated on MA plates supplemented with 6 % (w/v) NaCl by using antibiotic discs with the following amounts (μg , unless indicate otherwise): polymyxin B, 100 IU; streptomycin, 50; penicillin G, 20 IU; chloramphenicol, 100; ampicillin, 10; cephalothin, 30; gentamicin, 30; novobiocin, 5; tetracycline, 30; kanamycin, 30; lincomycin, 15; oleandomycin, 15; neomycin, 30; and carbenicillin, 100. Acid production from carbohydrates was tested as described by Leifson (1963). The utilization of various substrates for growth was determined as described by Yurkov *et al.* (1994). Enzyme activities were determined by using the API ZYM system (bioMérieux).

Cell biomass for DNA extraction and for isoprenoid quinone analysis was obtained from a culture grown at 30 °C in MB supplemented with 6 % (w/v) NaCl. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), with the modification that RNase T1 was used in combination with RNase A to minimize contamination by RNA. The 16S rRNA gene was PCR-amplified using 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). Isoprenoid quinones were investigated as described by Komagata & Suzuki (1987), using reversed-phase HPLC. For the cellular fatty acid analysis, cell mass of strain ISL-6^T was harvested after cultivation for 7 days at 30 °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 according to the method of Tamaoka & Komagata (1984), with the modification that the DNA was hydrolysed using nuclease P1 (Sigma) and the resultant nucleotides were analysed by reversed-phase HPLC. DNA–DNA hybridization was performed fluorometrically according to 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-6^T are given in the species

description (see below) and in Table 1. The almost-complete 16S rRNA gene sequence of strain ISL-6^T comprised 1485 nt, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. In the phylogenetic tree constructed using the neighbour-joining algorithm, strain ISL-6^T fell within the cluster comprising all of the *Salegentibacter* species (except *S. catena*) (Fig. 1). Similar topology was found in trees constructed using the maximum-likelihood and maximum-parsimony algorithms (Fig. 1). Strain ISL-6^T exhibited 16S rRNA gene sequence similarity of 98.6, 98.3, 97.7, 96.8, 94.8 and 92.0 % with respect to the type strains of *S. mishustinae*, *S. holothuriorum*, *S. salegens*, *S. agarivorans*, *S. flavus* and *S. catena*, respectively. Sequence similarities with respect to other species used in the phylogenetic analysis were below 93.4 %. Chemotaxonomic properties confirm the phylogenetic data, showing that strain ISL-6^T is affiliated with the genus *Salegentibacter*. The predominant isoprenoid quinone detected in strain ISL-6^T was MK-6. The fatty acid profile of strain ISL-6^T comprised the following (each constituting >0.5 % of total fatty acids): branched fatty acids iso-C_{15:0} (17.0 %), anteiso-C_{15:0} (11.6 %), iso-C_{15:1} (8.5 %), iso-C_{16:0} (6.4 %), iso-C_{16:1} (2.7 %), iso-C_{17:1}ω9c (2.1 %), anteiso-C_{17:1}ω9c (1.6 %), iso-C_{14:0} (1.4 %) and anteiso-C_{15:1} (1.0 %); hydroxy fatty acids iso-C_{17:0} 3-OH (8.9 %), iso-C_{16:0} 3-OH (7.3 %), C_{17:0} 2-OH (5.2 %), iso-C_{15:0} 3-OH (2.5 %), C_{15:0} 2-OH (2.5 %) and C_{16:0} 3-OH (0.5 %); summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, 9.2 %); unsaturated fatty acids C_{17:1}ω6c (3.7 %) and C_{15:1}ω6c (1.3 %); straight-chain fatty acids C_{15:0} (3.1 %) and C_{16:0} (0.9 %); and unknown fatty acids with equivalent chain-lengths of 16.582 (0.6 %) and 13.565 (0.6 %). This fatty acid profile was similar to those of the *Salegentibacter* species, although there were differences in the proportions of some fatty acids, probably because of differences in cultivation conditions and extraction procedures (Ivanova *et al.*, 2006; Nedashkovskaya *et al.*, 2004, 2005, 2006). The DNA G+C content of strain ISL-6^T was 37.5 mol%. Strain ISL-6^T exhibited DNA–DNA relatedness values of 7–21 % with respect to the type strains of the five phylogenetically related *Salegentibacter* species, *S. mishustinae* KCTC 12263^T (21 %), *S. holothuriorum* LMG 21968^T (20 %), *S. salegens* LMG 13193^T (15 %), *S. agarivorans* KCTC 12560^T (13 %) and *S. flavus* CIP 107843^T (7 %). These values indicate that strain ISL-6^T represents a genomic species that is distinct from these five *Salegentibacter* species (Wayne *et al.*, 1987). Strain ISL-6^T differed from these species in terms of several phenotypic characteristics, as shown in Table 1. The phylogenetic and genetic distinctiveness and differential phenotypic properties of ISL-6^T support the description of this strain as a novel species within the genus *Salegentibacter* (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994; Table 1), for which the name *Salegentibacter salarius* sp. nov. is proposed. In this study, *S. catena* was shown to be phylogenetically distantly related to the clade comprising the other *Salegentibacter* species. The separate position of *S. catena* was also found in trees generated with the

Table 1. Differential phenotypic characteristics of strain ISL-6^T and *Salegentibacter* species

Strains: 1, ISL-6^T (this study); 2, *S. salegens* ACAM 48^T (Dobson *et al.*, 1993; Nedashkovskaya *et al.*, 2004, 2005, 2006); 3, *S. holothuriorum* KMM 3524^T (Nedashkovskaya *et al.*, 2004, 2006); 4, *S. mishustinae* KMM 6049^T (Nedashkovskaya *et al.*, 2005); 5, *S. flavus* KMM 6000^T (Ivanova *et al.*, 2006); 6, *S. agarivorans* KMM 7019^T (Nedashkovskaya *et al.*, 2006). *S. catena* was not included because of its very distinct phylogenetic position. All strains were positive for catalase and oxidase activities, for hydrolysis of Tweens 20 and 40 (not determined for *S. flavus*) and starch and for growth at 34 °C and in the presence of 8 % NaCl. All strains were negative for the production of flexirubin-type pigments and for acid production from L-rhamnose, sorbitol, inositol and mannitol. +, Positive; –, negative; w, weakly positive; ND, no data available.

Characteristic	1	2	3	4	5	6
Gliding motility	–	–	–	–	–	+
NaCl requirement for growth	+	–*	+	+	+	+
Nitrate reduction	–	+	–	–	–	+
Production of H ₂ S	–	–†	+	+	–	+
Growth at/with:						
37 °C	+	–	+	–	–	+
41 °C	+	–	–	–	–	+
12 % (w/v) NaCl	+	+	–	+	–	+
18 % (w/v) NaCl	–	+	–	+	–	+
Hydrolysis of:						
Agar	–	–	–	–	–	+
Casein	+	–	–	+	–	–
DNA	+	+	+	–	ND	+
Gelatin	–	+	+	+	W	+
Urea	–	–	–	–	+	–
Acid production from:						
L-Arabinose, D-xylose, D-cellobiose	–	–‡	–	–	–§	+
Sucrose, L-raffinose	+	–	–	+	ND	+
D-Galactose, D-glucose	–	+	+	–	ND	+
Lactose	–	–	+	–	ND	+
Maltose	–	+	+	+	ND	+
Utilization of:						
L-Arabinose	–	–	–	–	–	+
D-Glucose	–	+	+	+	–	+
Sucrose	–	+	–	+	–	+
Susceptibility to:						
Ampicillin, benzylpenicillin, lincomycin	+	+	+	+	ND	–
Carbenicillin	+	+	+	–	ND	–
Kanamycin, neomycin	–	–	–	–	ND	+
Streptomycin	–	+	–	–	ND	+
Tetracycline	–	+	+	+	ND	–
DNA G + C content (mol%)	37.5	41	36.8	37.5	40–41	39.2

*Negative according to McCammon & Bowman (2000) and Nedashkovskaya *et al.* (2005, 2006), but positive according to Nedashkovskaya *et al.* (2004) and Ivanova *et al.* (2006).

†Negative according to Dobson *et al.* (1993), but positive according to Nedashkovskaya *et al.* (2004, 2005, 2006) and Ivanova *et al.* (2006).

‡Result for L-arabinose is positive according to Dobson *et al.* (1993), but negative according to Nedashkovskaya *et al.* (2004, 2005, 2006) and Ivanova *et al.* (2006).

§No data available for D-cellobiose.

||Data from Dobson *et al.* (1993); 37–38 mol% according to McCammon & Bowman (2000), 36.7 mol% according to Nedashkovskaya *et al.* (2004) and Ivanova *et al.* (2006) and 37.8 mol% according to Nedashkovskaya *et al.* (2005, 2006).

maximum-likelihood and maximum-parsimony algorithms (data not shown). *S. catena* exhibited low levels of 16S rRNA gene sequence similarity with respect to the other *Salegentibacter* species (91.9–93.0 %) and with respect to strain ISL-6^T (92.0 %). Consequently, *S. catena* may have to be reclassified within another genus or within a novel genus.

Description of *Salegentibacter salarius* sp. nov.

Salegentibacter salarius (sa.la'ri.us. L. masc. adj. *salarius* of, or belonging to, salt).

Cells are Gram-negative rods that are 0.4–0.6 µm in diameter and 0.7–4.0 µm in length and devoid of gliding motility. Colonies on MA supplemented with 6 % (w/v)

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of strain ISL-6^T, *Salagentibacter* species and some other related taxa. Bootstrap percentages (based on 1000 replications) >50% are shown at branching points. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Flavobacterium aquatile* ATCC 11947^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

NaCl are circular, convex, smooth, glistening, yellow in colour and 0.8–1.2 mm in diameter after 7 days incubation at 30 °C. Optimal growth occurs at 30 °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 8% (w/v) NaCl; growth occurs in the presence of 2–15% (w/v) NaCl. Growth does not occur under anaerobic conditions. Flexirubin-type pigments are not produced. Aesculin, L-tyrosine and Tween 60 are hydrolysed and Tween 80 is hydrolysed weakly, but hypoxanthine and xanthine are not. L-Malate is utilized, but D-cellobiose, D-fructose, D-galactose, maltose, D-mannose, trehalose, D-xylose, acetate, citrate, succinate, benzoate, pyruvate, salicin, formate and L-glutamate are not. Acid is produced from D-fructose, but not from D-mannose, D-melezitose, melibiose, D-ribose or trehalose. Susceptible to cephalothin, chloramphenicol, novobiocin and oleandomycin, but not to gentamicin or polymyxin B. In the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -glucosidase and N-acetyl- β -glucosaminidase activities are present, but lipase (C14), cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, β -glucosidase, α -mannosidase and α -fucosidase activities are absent. The predominant menaquinone is MK-6. The major fatty acids (>10% of total fatty acids) are iso-C_{15:0} and anteiso-C_{15:0}. The DNA G+C content of the type strain is 37.5 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, ISL-6^T (=KCTC 12974^T =CCUG 54355^T), was isolated from a marine solar saltern of the Yellow Sea, Korea.

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