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

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A bacterial strain, ISL-4^T, comprising Gram-negative, non-motile, rod-shaped cells, was isolated from a marine solar saltern of the Yellow Sea in Korea and was subjected to a polyphasic taxonomic investigation. Phenotypically, the strain resembled members of the genus *Salegentibacter*. It 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 37.9 mol%. A phylogenetic analysis based on 16S rRNA gene sequences showed that strain ISL-4^T belonged to the genus *Salegentibacter*, exhibiting sequence similarity values of 95.9–98.6 % with respect to the type strains of recognized *Salegentibacter* species (with the exception of *Salegentibacter catena*). Low DNA–DNA relatedness values and differential phenotypic properties, together with its phylogenetic distinctiveness, demonstrated that strain ISL-4^T represents a novel species of the genus *Salegentibacter*, for which the name *Salegentibacter salinarum* sp. nov. is proposed. The type strain is ISL-4^T (=KCTC 12975^T =CCUG 54354^T).

The genus *Salegentibacter* was created by McCammon & Bowman (2000) from the reclassification of [*Flavobacterium*] *salegens* as *Salegentibacter salegens*. At the time of writing, seven *Salegentibacter* species have been described, having been isolated from hypersaline lake water, marine water, sediment and invertebrates: *S. salegens* (McCammon & Bowman, 2000), *Salegentibacter holothuriorum* (Nedashkovskaya *et al.*, 2004), *Salegentibacter mishustinae* (Nedashkovskaya *et al.*, 2005), *Salegentibacter flavus* (Ivanova *et al.*, 2006), *Salegentibacter agarivorans* (Nedashkovskaya *et al.*, 2006), *Salegentibacter catena* (Ying *et al.*, 2007) and *Salegentibacter salarius* (Yoon *et al.*, 2007). However, it has been noted that *S. catena* should be reclassified to another (or a novel) genus because of its very distinct phylogenetic position (Yoon *et al.*, 2007). In this study, we report on the taxonomic characterization of a *Salegentibacter*-like bacterial strain, ISL-4^T, isolated from a marine solar saltern in Korea.

Strain ISL-4^T was isolated by means of the dilution plating technique at 25 °C on marine agar 2216 (MA; Difco) supplemented with 8 % (w/v) NaCl. The type strains of all *Salegentibacter* species except *S. catena* were used as reference strains for DNA–DNA hybridization. *S. holothuriorum* LMG 21968^T and *S. salegens* LMG 13193^T were obtained from the Laboratorium voor Microbiologie Universiteit Gent (Ghent, Belgium), *S. mishustinae* KCTC 12263^T and *S. agarivorans* KCTC 12560^T were obtained

from the Korean Collection for Type Cultures (Taejeon, Korea), *S. flavus* CIP 107843^T was obtained from the Collection de l'Institut Pasteur (Paris, France) and *S. salarius* ISL-6^T was maintained in our laboratory (Yoon *et al.*, 2007). The morphological, physiological and biochemical characteristics of strain ISL-4^T were investigated using routine cultivation on MA supplemented with 6 % (w/v) NaCl at 30 °C. 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 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 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 excluded and 0.45 % (w/v) MgCl₂·6H₂O was added. Growth with NaCl at various concentrations (1–20 %, using 1 % increments) was investigated in marine broth 2216 (MB; Difco) and trypticase soy broth (Difco), both of which were supplemented with 0.45 % (w/v) MgCl₂·6H₂O. Any requirement for Mg²⁺ ions was investigated in the

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presence and absence of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.45 %, w/v) at various NaCl concentrations (0–5 %, using 1 % increments) by using trypticase soy broth prepared according to the formula of the Difco medium except that NaCl was excluded. Growth at various temperatures (4, 10, 15, 20, 25 and 28 and 30–45 °C, using 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, using increments of 0.5 pH units) after autoclaving. Catalase and oxidase activities were determined as described by Cowan & Steel (1965). DNase activity was investigated by using DNase test agar with methyl green (Difco) and 8 % (w/v) NaCl. Hydrolysis of casein, starch, Tweens 20, 40, 60 and 80, hypoxanthine, tyrosine and xanthine was tested on MA supplemented with 6 % (w/v) NaCl by using the substrate concentrations described by Cowan & Steel (1965). Hydrolysis of aesculin, gelatin and urea and reduction of nitrate 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 (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). 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 concentrations: polymyxin B, 100 IU; streptomycin, 50 µg; penicillin G, 20 IU; 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), using supplementation with 7 % (w/v) NaCl. The utilization of various substrates for growth was determined as described by Yurkov *et al.* (1994), using supplementation with 6.5 % (w/v) NaCl. 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 amplified with a 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). Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987), using reversed-phase HPLC. For cellular fatty acid analysis, cell mass of strain ISL-4^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 the fatty acid methyl esters 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 DNA was hydrolysed using nuclease P1 (Sigma) and the resultant nucleotides were analysed by reversed-phase HPLC. DNA–DNA hybridization was performed fluorometrically with 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 for 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 ISL-4^T are given in the species description and in Table 1. The almost-complete 16S rRNA gene sequence of strain ISL-4^T comprised 1485 nt, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. In the neighbour-joining phylogenetic tree constructed on the basis of 16S rRNA gene sequences, strain ISL-4^T fell within the cluster comprising all *Salegentibacter* species except *S. catena* (Fig. 1). The overall topology of the trees generated with the maximum-likelihood and maximum-parsimony algorithms was similar. Strain ISL-4^T exhibited 16S rRNA gene sequence similarity values of 98.6, 98.6, 98.2, 97.8, 97.5, 95.9 and 93.0 % with respect to the type strains of *S. agarivorans*, *S. salegens*, *S. mishustinae*, *S. salarius*, *S. holothuriorum*, *S. flavus* and *S. catena*, respectively. The sequence similarity values with respect to other species used in the phylogenetic analysis were below 93.2 %. The predominant isoprenoid quinone detected in strain ISL-4^T was MK-6, at a peak area ratio of approximately 92 %. The fatty acid profile of strain ISL-4^T was composed of the following (each constituting >0.5 % of total fatty acids): branched fatty acids iso-C_{15:0} (15.9 %), anteiso-C_{15:0} (12.6 %), iso-C_{16:0} (5.7 %), iso-C_{15:1} (5.4 %), iso-C_{16:1} (3.1 %), anteiso-C_{17:1}ω9c (3.5 %), iso-C_{17:1}ω9c (2.8 %), iso-C_{14:0} (1.3 %) and anteiso-C_{15:1} (0.8 %); hydroxy fatty acids iso-C_{17:0} 3-OH (7.7 %), C_{17:0} 2-OH (6.5 %), iso-C_{16:0} 3-OH (6.2 %), C_{15:0} 2-OH (2.4 %), iso-C_{15:0} 3-OH (2.0 %) and C_{16:0} 3-OH (0.6 %); summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, 7.8 %); straight-chain fatty acids C_{15:0} (6.5 %) and C_{16:0} (0.7 %); unsaturated fatty acids C_{17:1}ω6c (3.1 %), C_{15:1}ω6c (2.7 %) and C_{18:1}ω5c (0.7 %); and an unknown fatty acid with an equivalent chain length of 16.582 (0.8 %). This fatty acid profile was similar to those of *Salegentibacter* species, although there were slight differences in the proportions of some fatty acids. These differences might result partly from different cultivation conditions and extraction procedures (Ivanova *et al.*, 2006; Nedashkovskaya *et al.*, 2004, 2005, 2006; Yoon *et al.*, 2007). The DNA G+C content of strain

Table 1. Differential phenotypic characteristics of strain ISL-4^T and *Salegentibacter* type strains

Strains: 1, ISL-4^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); 7, *S. salarius* ISL-6^T (Yoon *et al.*, 2007). *S. catena* was not included because of its very distinct phylogenetic position. All of the strains were positive for catalase and oxidase activities, hydrolysis of Tween 20, Tween 40 (not determined for *S. flavus*) and starch and for growth at 34 °C and in the presence of 8 % NaCl. All of the strains were negative for the production of flexirubin-type pigments and for the production of acid from sorbitol, inositol and mannitol. +, Positive; –, negative; w, weakly positive; ND, no data available.

Characteristic	1	2	3	4	5	6	7
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, cellobiose	+	–§	–	–	–	+	–
Sucrose, raffinose	+	–	–	+	ND	+	+
D-Galactose, D-glucose	+	+	+	–	ND	+	–
Lactose	+	–	+	–	ND	+	–
Maltose	+	+	+	+	ND	+	–
L-Rhamnose	+	–	–	–	–	–	–
Utilization of:							
L-Arabinose	+	–	–	–	–	+	–
D-Glucose	+	+	+	+	–	+	–
Sucrose	+	+	–	+	–	+	–
Susceptibility to:							
Ampicillin	–	+	+	+	ND	–	+
Benzylpenicillin, lincomycin	+	+	+	+	ND	–	+
Carbenicillin	+	+	+	–	ND	–	+
Kanamycin, neomycin	–	–	–	–	ND	+	–
Streptomycin	–	+	–	–	ND	+	–
Tetracycline	–	+	+	+	ND	–	–
DNA G + C content (mol%)	37.9	41¶	36.8	37.5	40–41	39.2	37.5

*Reported as negative by McCammon & Bowman (2000) and Nedashkovskaya *et al.* (2005, 2006), but positive by Nedashkovskaya *et al.* (2004) and Ivanova *et al.* (2006).

†Reported as positive by Nedashkovskaya *et al.* (2004, 2005, 2006), but negative by Ivanova *et al.* (2006).

‡Reported as negative by Dobson *et al.* (1993), but positive by Nedashkovskaya *et al.* (2004, 2005, 2006) and Ivanova *et al.* (2006).

§Reported as positive for L-arabinose by Dobson *et al.* (1993), but negative by Nedashkovskaya *et al.* (2004, 2005, 2006) and Ivanova *et al.* (2006).

||No data available for cellobiose.

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

ISL-4^T was 37.9 mol%. The results of chemotaxonomic analyses were consistent with the phylogenetic data. Accordingly, strain ISL-4^T should be considered as a member of the genus *Salegentibacter* (Ivanova *et al.*, 2006; Nedashkovskaya *et al.*, 2004, 2005, 2006; Yoon *et al.*, 2007).

Strain ISL-4^T exhibited DNA–DNA relatedness values of 11–24 % with respect to the type strains of the six more closely related *Salegentibacter* species, indicating that it represents a separate genomic species (Wayne *et al.*, 1987). Moreover, strain ISL-4^T was distinguishable from these six *Salegentibacter* species on the basis of several phenotypic differences (Table 1). Hence, the phylogenetic and genetic distinctiveness and differential phenotypic properties of strain ISL-4^T support its description as a representative of a novel species within the genus *Salegentibacter*, for which the name *Salegentibacter salinarum* sp. nov. is proposed.

Description of *Salegentibacter salinarum* sp. nov.

Salegentibacter salinarum (sa.li.na'rum. L. pl. gen. n. *salinarum* of salterns).

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of strain ISL-4^T, *Salegentibacter* species and some other related taxa. Bootstrap values (expressed as percentages of 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.

Cells are Gram-negative, non-motile rods, 0.5–0.8 µm in diameter and 1.0–5.5 µm in length. Colonies on MA supplemented with 6% (w/v) NaCl are circular, convex, smooth, glistening, yellow-coloured and 1.0–1.5 mm in diameter after 7 days incubation at 30 °C. Growth occurs at 4–41 °C and pH 5.5 and in the presence of 2–17% (w/v) NaCl. Optimal growth is observed at 30 °C and pH 7.0–8.0 and with 8% (w/v) NaCl. The Mg²⁺ ion is required for growth. Growth does not occur under anaerobic conditions. Flexirubin-type pigments are not produced. Aesculin, L-tyrosine, Tween 60 and Tween 80 are hydrolysed, but hypoxanthine and xanthine are not. Maltose, D-mannose, trehalose, pyruvate and salicin are utilized, but cellobiose, D-fructose, D-galactose, D-xylose, acetate, citrate, succinate, benzoate, L-malate, formate and L-glutamate are not. Acid is produced from D-fructose, D-mannose, melezitose and trehalose, but not from melibiose or D-ribose. 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 37.9 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, ISL-4^T (=KCTC 12975^T =CCUG 54354^T), was isolated from a marine solar saltern of the Yellow Sea, Korea.

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