Salinihabitans flavidus gen. nov., sp. nov., isolated from a marine solar saltern

Jung-Hoon Yoon, So-Jung Kang, Soo-Young Lee and Tae-Kwang Oh

Correspondence

Jung-Hoon Yoon
jhyoon@kribb.re.kr

Korea Research Institute of Bioscience and Biotechnology (KRIIBB), PO Box 115, Yusong, Taejeon, Republic of Korea

A Gram-negative, non-motile and rod-shaped bacterial strain, ISL-46^T, belonging to the *Alphaproteobacteria*, was isolated from a marine solar saltern in Korea, and subjected to a polyphasic taxonomic study. Strain ISL-46^T grew optimally at pH 7.0–8.0 and 30 °C and in the presence of 7 % (w/v) NaCl. It contained Q-10 as the predominant ubiquinone and C_{19:0} cyclo ω8c, C_{18:1}ω7c and 11-methyl C_{18:1}ω7c as the major fatty acids. The DNA G+C content was 63.5 mol%. Strain ISL-46^T exhibited 16S rRNA gene sequence similarity values of 94.1–95.3 % to members of the phylogenetically related genera *Roseivivax*, *Salipiger*, *Citricella*, *Yangia* and *Citrimonas*. Strain ISL-46^T could be differentiated from the above-mentioned genera by differences in compositions of the major fatty acids and in some phenotypic properties. On the basis of phenotypic, chemotaxonomic and phylogenetic data, strain ISL-46^T is considered to represent a novel genus and species, for which the name *Salinihabitans flavidus* gen. nov., sp. nov. is proposed. The type strain is ISL-46^T (=KCTC 22485^T=CCUG 56758^T).

In the course of screening micro-organisms from a marine solar saltern in the Yellow Sea, Korea, many novel bacterial strains have been isolated and characterized taxonomically. One of these isolates, ISL-46^T, which phylogenetically is most closely related to the class *Alphaproteobacteria*, is the subject of this study. The aim of the present work was to determine the exact taxonomic position of strain ISL-46^T by using a polyphasic characterization that included the determination of the phenotypic and chemotaxonomic properties and a detailed phylogenetic investigation based on 16S rRNA gene sequences.

Strain ISL-46^T was isolated by means of the standard dilution plating technique at 30 °C on marine agar 2216 (MA; Difco) supplemented with 8 % (w/v) NaCl. The morphological, physiological and biochemical characteristics of strain ISL-46^T were investigated using routine cultivation at 30 °C on MA (5 % NaCl MA) supplemented with 5 % (w/v) NaCl. The 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. 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 5 % NaCl MA and on 5 % NaCl MA supplemented with potassium nitrate (0.1 %, w/v), both of which had been prepared anaerobically under 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. Growth at various NaCl concentrations (0.5 %, w/v, and 1.0–20.0 %, w/v, at increments of 1.0 %) was investigated in marine broth 2216 (MB; Difco) or trypticase soy broth (Difco). Growth at various temperatures (4, 10, 20, 25, 28, 30, 35, 37, 40 and 45 °C) was measured on 5 % NaCl MA. The pH range for growth was determined in 5 % NaCl MB that was adjusted to various pH values (pH 4.5–9.5, using increments of 0.5 pH units). 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 5 % NaCl MA using the substrate concentrations described by Cowan & Steel (1965). Hydrolysis of aesculin, gelatin and urea and nitrate reduction were investigated as described previously (Lányi, 1987) with the modification that artificial seawater supplemented with 5 % (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 5 % NaCl MA plates by using antibiotic discs with the following concentrations; polymyxin B (100 U), streptomycin (50 µg), penicillin G (20 U), chloramphenicol (100 µg),

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ISL-46^T is FJ265707.

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). Utilization of various substrates for growth was determined as described by Baumann & Baumann (1981), using supplementation with 5.8 % (w/v) NaCl and 0.01 % (w/v) yeast extract. The carbon sources were added at a concentration of 0.2 % (w/v) after sterilization by filtration. Enzyme activities were determined by using the API ZYM system (bioMérieux).

Cell biomass for DNA extraction and for isoprenoid quinone analysis was obtained from cultures grown in 5 %

NaCl MB at 30 °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 PCR using two universal primers, 5'-GAGTTTGATCCTGGCTCAG-3' (positions 9–27 in *Escherichia coli* 16S rRNA numbering) and 5'-AGAAAGGAGGTGATCCAGCC-3' (positions 1542–1525 in *E. coli* 16S rRNA numbering), 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)

Table 1. Differential phenotypic characteristics of *Salinihabitans* gen. nov. and the genera *Roseivivax*, *Salipiger*, *Citricella*, *Yangia* and *Citreimonas*

Genera: 1, *Salinihabitans* gen. nov.; 2, *Roseivivax* (data from Suzuki *et al.*, 1999); 3, *Salipiger* (Martínez-Cánovas *et al.*, 2004); 4, *Citricella* (Sorokin *et al.*, 2005; Choi & Cho, 2006); 5, *Yangia* (Dai *et al.*, 2006); 6, *Citreimonas* (Choi & Cho, 2006). +, Positive reaction; –, negative reaction; w, weakly positive reaction; ND, not determined or not described; v, variable reaction. All genera are aerobic and positive for catalase activity. All genera are negative for Gram-staining.

Characteristic	1	2	3	4	5	6
Motility	+	+	–	–	+	–
Presence of BChl <i>a</i>	–	+	–	–	–	–
Requirement for Na ⁺	+	v	+	+	+	+
Optimum temperature for growth (°C)	30	27–30	20–40	25–28	37	30–35
Optimum NaCl concentration for growth (%)	7	ND	9–10	2	5	5–6
Growth at:						
4 and 10 °C	+	ND	–	–	–	–
37 °C	+	ND	+	–	+	+
Oxidase	+	+	+	+	w	–
Nitrate reduction	–	v	–	–	–	+
Hydrolysis of:						
Gelatin	–	v	–	–	–	+
Urea	–	v	+	+	+	–
Utilization of:						
L-Arabinose	–	ND	–	+	–	+
Cellobiose	–	ND	–	+	ND	+
D-Fructose	–	ND	–	+	–	+
D-Galactose	–	v	–	–	ND	–
D-Glucose	–	+	–	+	–	+
Maltose	–	ND	–	+	+	ND
D-Mannose	–	ND	–	ND	ND	+
Sucrose	–	ND	ND	+	–	+
L-Glutamate	–	+	ND	+	+	–
Acetate	+	+	–	+	ND	+
Citrate	–	+	–	+	ND	+
Succinate	+	+	–	+	ND	+
Formate	–	ND	–	–	ND	–
L-Malate	+	+	ND	+	+	ND
Pyruvate	+	+	ND	+	ND	+
Major fatty acid(s)	C _{19:0} cyclo ω8c, C _{18:1} ω7c, 11-methyl C _{18:1} ω7c	C _{18:1}	C _{18:1} ω7c, C _{16:0}	C _{18:1} ω7c, C _{16:0}	C _{18:1}	C _{18:1} ω7c, C _{19:0} cyclo ω8c
DNA G + C content (mol%)	63.5	59.7–64.4	64.5	67.5–69.2	63.3	67.3

using reversed-phase HPLC. For cellular fatty acid analysis, cell mass of strain ISL-46^T was harvested from 5 % NaCl MA plates after cultivation for 7 days at 30 °C. 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 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.

Morphological, cultural, physiological and biochemical characteristics of strain ISL-46^T are given in the genus and species descriptions (see below) or are shown in Table 1. The almost complete 16S rRNA gene sequence of strain ISL-46^T determined in this study comprised 1421 nt, representing approximately 96 % of the *E. coli* 16S rRNA gene sequence. In the phylogenetic tree based on the neighbour-joining algorithm, strain ISL-46^T joined the clade comprising the genera *Roseivivax* (Suzuki *et al.*, 1999), *Salipiger* (Martínez-Cánovas *et al.*, 2004), *Citricella* (Sorokin *et al.*, 2005), *Yangia* (Dai *et al.*, 2006) and *Citrimonas* (Choi & Cho, 2006) (Fig. 1). This topology was also found in the tree constructed using the maximum-parsimony algorithm (Fig. 1). Strain ISL-46^T exhibited 16S rRNA gene sequence similarity values of 94.6, 94.1, 95.0, 94.2 and 95.3 % to the type strains of two *Roseivivax* species, *Salipiger mucosus* A3^T, *Citricella thiooxidans* CHLG 1^T, *Yangia pacifica* DX5-10^T and *Citrimonas salinaria* CL-SP20^T, respectively.

The predominant isoprenoid quinone detected in strain ISL-46^T was ubiquinone-10 (Q-10). The fatty acid profile of strain ISL-46^T was composed of the following (each constituting >0.5 % of total fatty acids): cyclo fatty acids C_{19:0}ω8c (46.2 %) and C_{17:0} (2.2 %); unsaturated fatty acid C_{18:1}ω7c (18.4 %); 11-methyl C_{18:1}ω7c (15.1 %); straight-chain fatty acids C_{16:0} (8.7 %) and C_{17:0} (0.5 %); hydroxy fatty acid C_{12:1} 3-OH (3.2 %); unknown fatty acid ECL 11.799 (2.2 %); and summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, 2.0 %). The DNA G+C content of strain ISL-46^T was 63.5 mol%.

The phylogenetic analyses based on 16S rRNA gene sequences showed that strain ISL-46^T did not fall within the radiation encompassed by a recognized genus but forms an independent evolutionary lineage within the *Alphaproteobacteria* (Fig. 1). The predominant ubiquinone type (Q-10) of strain ISL-46^T was identical to those of the phylogenetically related genera *Roseivivax*, *Salipiger* and *Yangia* (Suzuki *et al.*, 1999; Martínez-Cánovas *et al.*, 2004; Dai *et al.*, 2006). However, the differences in major fatty acids, C_{19:0} cyclo ω8c and 11-methyl C_{18:1}ω7c, distinguished strain ISL-46^T from the genera *Roseivivax*, *Salipiger*, *Citricella*, *Yangia* and *Citrimonas*, although these differences may partly result from different culture conditions and extraction procedures (Table 1). Strain ISL-46^T could be also differentiated from the above-mentioned genera by differences in phenotypic properties, as listed in

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain ISL-46^T (*Salinihabitans flavidus* gen. nov., sp. nov.) and some other related taxa. Bootstrap values (expressed as percentages of 1000 replications) of >40 % are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. Open circles indicate that the corresponding nodes were recovered in the tree generated with the maximum-parsimony algorithm. *Stappia stellulata* IAM 12621^T (GenBank accession number D88525) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Table 1. The phylogenetic data and differential chemotaxonomic and phenotypic properties suggest that strain ISL-46^T constitutes a new genus and novel species within the *Alphaproteobacteria*, for which the name *Salinihabitans flavidus* gen. nov., sp. nov. is proposed.

Description of *Salinihabitans* gen. nov.

Salinihabitans (Sa.li.ni.ha'bi.tans. L. fem. pl. n. *salinae* salterns, salt-works; L. part. adj. *habitans* inhabiting; N.L. part. adj. *Salinihabitans* inhabitant of salt-works).

Cells are Gram-negative and rod-shaped. The predominant ubiquinone is Q-10. The type species is *Salinihabitans flavidus*.

Description of *Salinihabitans flavidus* sp. nov.

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

Exhibits the following properties in addition to those given in the genus description. Cells are $0.3\text{--}0.6 \times 0.8\text{--}4.5\ \mu\text{m}$ and motile by means of a single polar flagellum. Colonies on 5% NaCl MA are circular, convex, smooth, glistening, pale-yellow in colour and $0.5\text{--}0.8\ \text{mm}$ in diameter after incubation for 7 days at $30\ ^\circ\text{C}$. Growth occurs at 4 and $40\ ^\circ\text{C}$, but not at $45\ ^\circ\text{C}$. Optimal pH for growth is between 7.0 and 8.0; growth occurs at pH 6.0, but not at pH 5.5. Growth occurs in the presence of 17% (w/v) NaCl, but not in the absence of NaCl or in the presence of more than 18% (w/v) NaCl. Growth does not occur under anaerobic conditions. Hypoxanthine, xanthine and L-tyrosine are hydrolysed, but aesculin, casein, starch and Tweens 20, 40 and 60 are not. H_2S is not produced. Trehalose, D-xylene, benzoate and salicin are not utilized as sole carbon and energy sources. Susceptible to ampicillin, carbenicillin, cephalothin, chloramphenicol, gentamicin, kanamycin, neomycin, novobiocin, oleandomycin, penicillin G and streptomycin, but not to lincomycin, polymyxin B or tetracycline. In assays with the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8) and leucine arylamidase are present, but lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, 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. Major fatty acids ($>10\%$ of total fatty acids) are $\text{C}_{19:0}$ cyclo $\omega 8c$, $\text{C}_{18:1}$ $\omega 7c$ and 11-methyl $\text{C}_{18:1}$ $\omega 7c$. The DNA G+C content of the type strain is 63.5 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, ISL-46^T (=KCTC 22485^T=CCUG 56758^T), was isolated from a marine solar saltern of the Yellow Sea, Korea.

Acknowledgements

This work was supported by the 21C Frontier program of Microbial Genomics and Applications (grant MG05-0401-2-0) from the Ministry of Education, Science and Technology (MEST) of the Republic of Korea.

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