

Salinimicrobium soli sp. nov., isolated from soil of reclaimed land

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A Gram-stain-negative, facultatively anaerobic, non-gliding, rod-shaped bacterial strain, designated CAU 1287^T, was isolated from a soil sample of reclaimed land and its taxonomic position was investigated using a polyphasic approach. Strain CAU 1287^T grew optimally at 30 °C, at pH 5.5 and in the presence of up to 2 % (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain CAU 1287^T formed a distinct lineage within the genus *Salinimicrobium* and shared the highest 16S rRNA gene sequence similarity of 98.0 % with *Salinimicrobium gaetbulicola* KCTC 23579^T. Similar to other species of the genus *Salinimicrobium*, menaquinone-6 (MK-6) was the only isoprenoid quinone and iso-C_{15:0} and anteiso-C_{15:0} were the major cellular fatty acid detected in strain CAU 1287^T. The polar lipid pattern of strain CAU 1287^T consisted of phosphatidylethanolamine and various unidentified lipids including three aminolipids, one aminophospholipid, one phosphoglycolipid, one glycolipid, and three lipids. The DNA G + C content of CAU 1287^T was 43.4 mol%. On the basis of phenotypic, chemotaxonomic and phylogenetic data, strain CAU 1287^T represents a novel species of the genus *Salinimicrobium*, for which the name *Salinimicrobium soli* sp. nov. is proposed. The type strain is CAU 1287^T (=KCTC 32163^T=CCUG 64794^T).

The genus *Salinimicrobium*, a member of the family *Flavobacteriaceae* of the phylum *Bacteroidetes* (Bernardet & Nakagawa, 2006), was proposed by Lim *et al.* (2008) with the description of *Salinimicrobium catena* as the type species of the genus. The genus *Salinimicrobium* consists of aerobic, Gram-stain-negative, rod-shaped, oxidase-negative bacteria that are characterized by the presence of menaquinone-6 (MK-6) as the only isoprenoid quinone (Lim *et al.*, 2008). At the time of writing, the genus *Salinimicrobium* comprises six species with validly published names (Parte, 2014): *S. catena* (Ying *et al.*, 2007), *S. terrae* (Chen *et al.*, 2008), *S. xinjiangense* (Lim *et al.*, 2008), *S. marinum* (Nedashkovskaya *et al.*, 2010), *S. gaetbulicola* (Lee *et al.*, 2012) and *S. sediminis* (Subhash *et al.*, 2014). Members of the genus *Salinimicrobium* have been isolated from marine environments such as sediments and saline soil. In the course of the screening of bacteria with biotechnological potential from marine environmental samples, a bacterial strain, designated CAU 1287^T, was isolated from a reclaimed-land soil sample collected in Modo (37° 32' 12.28" N 126° 24' 51.47" E), Republic of

Korea on 3 March 2012. The purpose of the present study was to establish the taxonomic position of this bacterial strain by using a polyphasic characterization that included the determination of phenotypic and chemotaxonomic properties, and a detailed phylogenetic investigation based on 16S rRNA gene sequences.

Isolation was performed according to Gordon & Mihm (1962) using marine agar 2216 (MA; Difco). The crushed soil sample was serially diluted with sterilized 0.9 % sodium chloride solution, plated on MA and incubated under aerobic conditions at 30 °C for 7 days. Strain CAU 1287^T was purified by subculture plating on MA. The strain was preserved at –70 °C in marine broth 2216 (MB; Difco) supplemented with 25 % (v/v) glycerol. The type strains of closely related species, *S. gaetbulicola* KCTC 23579^T, *S. xinjiangense* KCTC 12883^T, *S. sediminis* KCTC 32444^T and *S. marinum* KCTC 12719^T, were used as reference strains in determination of DNA–DNA relatedness and fatty acid analysis.

Genomic DNA of strain CAU 1287^T was isolated and purified using a genomic DNA extraction kit (Intron). The 16S rRNA gene was amplified by PCR using the universal primers 8F/1525R following established procedures (Lane, 1991). The amplified 16S rRNA gene was sequenced directly using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and an automatic DNA sequencer

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CAU 1287^T is JX624252.

Three supplementary figures are available with the online Supplementary Material.

(model 3730; Applied Biosystems). Multiple alignments with sequences of the six recognized members of the genus *Salinimicrobium* and other representative members of the family *Flavobacteriaceae*, and calculation of sequence similarities were carried out by using CLUSTAL X 2.1 software (Larkin *et al.*, 2007) and the EzTaxon server (<http://www.ezbiocloud.net/eztaxon>). Evolutionary distance matrices were created by the neighbour-joining method (Jukes & Cantor, 1969). Phylogenetic trees were generated by using the neighbour-joining (Saitou & Nei, 1987), least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) algorithms in the PHYLIP software package (Felsenstein, 1989). The tree topology was evaluated by the bootstrap resampling method (Felsenstein, 1985) with 1000 replicates of the neighbour-joining dataset with the SEQBOOT and CONSENSE programs from the PHYLIP package. The extent of DNA–DNA relatedness was determined using the fluorometric microplate method at 60 °C (Goris *et al.*, 1998). The mol% G + C content of the genomic DNA was determined using HPLC by the method of Tamaoka & Komagata (1984).

The nearly complete 16S rRNA gene sequence of strain CAU 1287^T (1515 bp) was determined and compared with the available reference sequences in the GenBank database (accessed April 2015). Phylogenetic analysis indicated that the strain fell into the genus *Salinimicrobium*. The neighbour-joining tree is shown in Fig. 1. The trees obtained with the three other treeing methods showed essentially the same topology (Fig. S1, available in the online Supplementary Material). Pairwise analysis showed that the most closely related species were *S. gaetbulicola* KCTC 23579^T, *S. xinjiangense* KCTC 12883^T, *S. sediminis* KCTC 32444^T, *S. marinum* KCTC 12719^T, *S. terrae* YIM-C338^T and *S. catena* HY1^T with 16S rRNA gene sequence similarities of 98.0, 97.9, 97.5, 97.0, 96.8 and 95.9 %, respectively. The mean DNA–DNA relatedness values between strain CAU 1287^T and the most closely related strains *S. gaetbulicola* KCTC 23579^T, *S. xinjiangense* KCTC 12883^T and *S. sediminis* KCTC 32444^T were 24.0 ± 0.5 %, 26.0 ± 0.5 % and 35.0 ± 0.5 %, respectively. These values are lower than the 70 % cut-off point recommended by Wayne *et al.* (1987) for the description of genomic species, supporting the proposal that strain CAU 1287^T represents a distinct species. The G + C content of the DNA of strain CAU 1287^T was 43.4 mol%, a value in the range reported for species of the genus *Salinimicrobium* (Chen *et al.*, 2008).

Strain CAU 1287^T was cultivated routinely on MA at 30 °C to investigate morphological, physiological and biochemical characteristics (Bernardet *et al.*, 2002), except for spore formation that was assessed on nutrient sporulation medium (Nicholson & Setlow, 1990). Cell morphology was examined by light microscopy (model DM 1000; Leica). Transmission electron microscopy (model JEM 1010; JEOL) was used to determine the presence of flagella using cells from an exponentially growing culture. Gram staining was carried out using the bioMérieux Gram staining kit. Gliding motility was evaluated as described by Bowman (2000). After

growth for 7 days, endospore formation was determined by staining with malachite green as described previously by Conn *et al.* (1957). The temperature range for growth of strain CAU 1287^T in MA at 4, 10, 20, 30, 37, 45 and 55 °C in an aerobic incubator (model MIR-253; Sanyo) and in an anaerobic chamber (model Bactron; Sheldon) was determined after 72 h by measuring the turbidity of the broth. Growth was tested at 30 °C in MB adjusted to pH 4.5–11.0 (at 0.5 pH unit intervals); the pH of the media was adjusted by using the following buffers: sodium acetate/acetic acid (for pH <6), Tris/HCl (for pH 6–9) or sodium carbonate (for pH >9). The salinity range for growth was evaluated in MY medium (Moraine & Rogovin, 1966) using 0–15 % (w/v) NaCl as described by Rodríguez-Valera *et al.* (1981). Catalase activity was determined by bubble production in a 3 % (v/v) hydrogen peroxide solution. Oxidase activity was determined from the oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Cappuccino & Sherman, 2002). Hydrolysis of casein, gelatin and aesculin, and nitrate reduction were determined according to Smibert & Krieg (1994). Acid production from carbohydrates, enzyme activity, and other physiological and biochemical features were tested using the API ZYM and API 50CH systems (bioMérieux) at 37 °C and 30 °C, respectively, according to the manufacturer's instructions. API ZYM strips were read after 24 h and API 50CH strips after 48 h.

Detailed phenotypic features of strain CAU 1287^T are given in Table 1 and in the species description. Strain CAU 1287^T was Gram-stain-negative, facultatively anaerobic, and non-gliding. Endospores were not observed. Cells are rod-shaped, approximately 0.4–0.6 µm in diameter and 1.0–2.4 µm in length. Colonies were yellow, circular and convex with entire margins after 3 days on MA at 30 °C. Flagella were not observed (Fig. S2). Strain CAU 1287^T grew at temperatures between 20 and 37 °C (optimum 30 °C) and at NaCl concentrations between 0 and 4 % (optimum 2 %). Visible growth occurred at pH 5.5–8.0 and strain CAU 1287^T showed the greatest visible growth at pH 5.5. Strain CAU 1287^T hydrolysed gelatin and had enzyme activities of lipase (C14), α-chymotrypsin and acid phosphatase. However, strain CAU 1287^T differed from type strains of closely related species, namely *S. gaetbulicola* KCTC 23579^T, *S. xinjiangense* KCTC 12883^T, *S. sediminis* KCTC 32444^T and *S. marinum* KCTC 12719^T by 37 °C (maximal growth temperature), by the negative reaction for *N*-acetyl-β-glucosaminidase and by positive results for α-chymotrypsin and acid phosphatase activities. These differences are sufficient to suggest that strain CAU 1287^T is different from other species of the genus *Salinimicrobium*.

For fatty acid analysis, the cell mass of strain CAU 1287^T and the type strains of the most closely related species of the genus *Salinimicrobium* (*S. gaetbulicola* KCTC 23579^T, *S. xinjiangense* KCTC 12883^T, *S. sediminis* KCTC 32444^T and *S. marinum* KCTC 12719^T) were harvested from MA in late-exponential growth phase after cultivation for 3 days at 37 °C according to a standard MIDI protocol (Sherlock Microbial Identification System version 6.1).

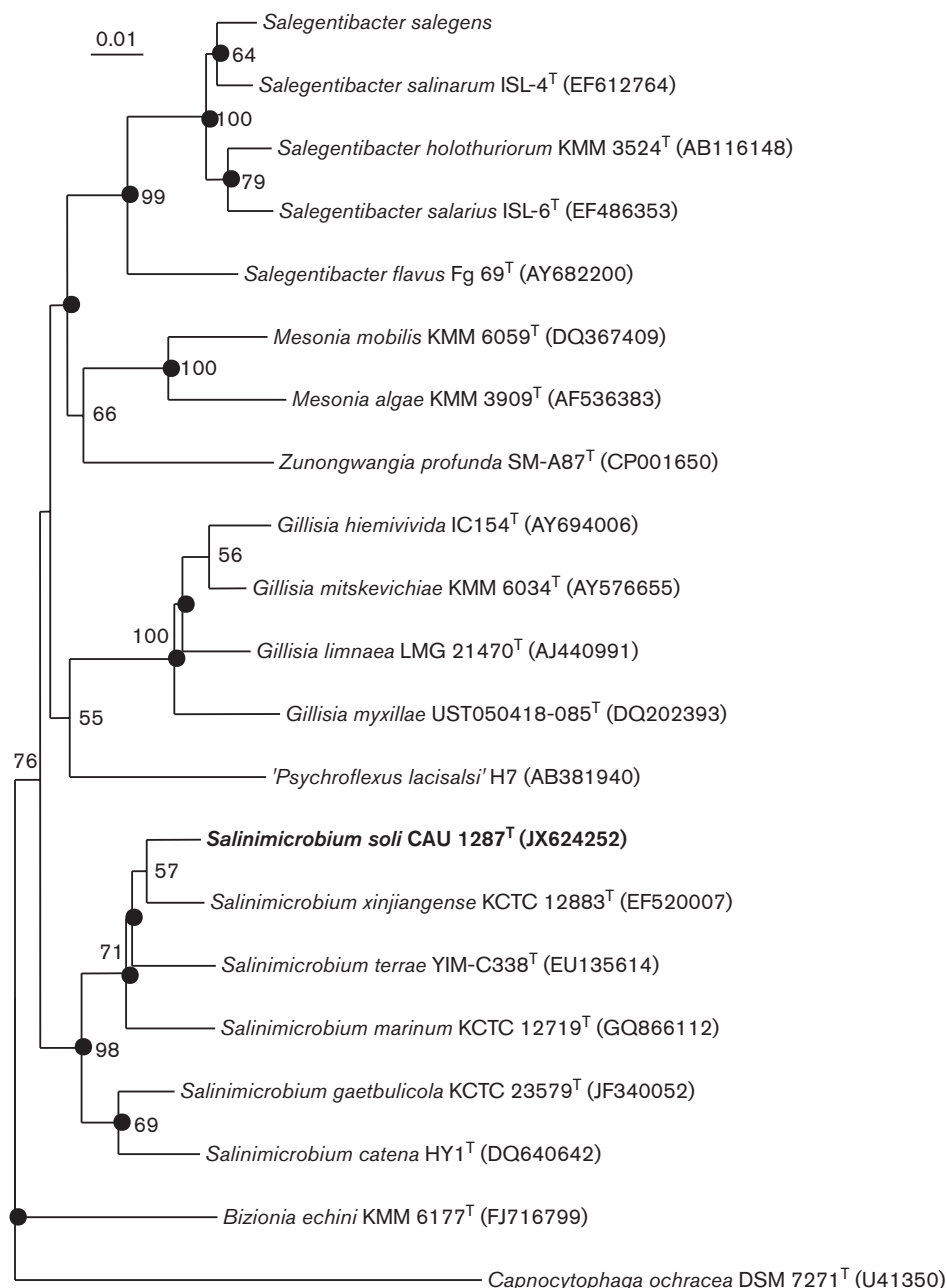


Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationships between strain CAU 1287^T and the type strains of recognized species of the genus *Salinimicrobium*. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the least-squares, maximum-likelihood and maximum-parsimony algorithms. Numbers at nodes are bootstrap values based on a neighbour-joining analysis of 1000 resampled datasets; only values >70 % are given. *Capnocytophaga ochracea* DSM 7271^T (GenBank accession no. U41350) was used as an outgroup organism. Bar, 0.01 substitutions per nucleotide position.

Fatty acid methyl esters were obtained as described previously (Minnikin *et al.*, 1980), and were separated using an automated gas chromatography system (model 6890N and 7683 autosampler; Agilent). The peaks were identified by using the Microbial Identification software package (MOORE library v. 5.0; MIDI database TSBA6). The

polar lipids of strain CAU 1287^T were identified using two-dimensional thin-layer chromatography by the method of Minnikin *et al.* (1984). The plates were sprayed with 10 % ethanolic molybdophosphoric acid (for total lipids), molybdenum blue (for phospholipids), ninhydrin (for aminolipids), and α -naphthol/sulphuric acid reagent

Table 1. Differential properties of strain CAU 1287^T and the type strains of the most closely related species of the genus *Salinimicrobium*

Strains: 1, CAU 1287^T; 2, *S. gaetbulicola* KCTC 23579^T; 3, *S. xinjiangense* KCTC 12883^T; 4, *S. sediminis* KCTC 32444^T; 5, *S. marinum* KCTC 12719^T. All data are from this study unless otherwise indicated. All strains were positive for hydrolysis of aesculin. +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5
Temperature for growth(°C)					
Range	20–37	10–40 ^a	10–48 ^b	10–40 ^c	4–41 ^d
Optimum	30	35–37 ^a	32–35 ^b	25–30 ^c	28–32 ^d
pH for growth					
Range	5.5–8.0	5.5–7.5 ^a	6.0–9.0 ^b	6.0–8.8 ^c	ND
Optimum	5.5	7.0–7.5 ^a	7.5–8.0 ^b	7.0–7.5 ^c	ND
NaCl tolerance (%)					
Range	0–4	0.5–10 ^a	0.5–10 ^b	0–9 ^c	0.5–15 ^d
Optimum	2	2 ^a	2–3 ^b	ND	4 ^d
Hydrolysis of:					
Casein	–	+	+	+	–
Gelatin	+	+	–	–	+
Starch	–	+	+	–	+
Acid production from:					
Gentiobiose	–	–	+	–	–
D-Tagatose	–	+	–	–	–
Potassium 5-ketogluconate	+	+	–	+	+
Enzyme activities					
Lipase (C14)	+	+	–	+	–
Leucine arylamidase	–	+	+	+	–
α -Chymotrypsin	+	–	–	–	–
Acid phosphatase	+	–	–	–	–
α -Glucosidase	–	–	+	+	+
β -Glucosidase	–	–	–	–	+
N-Acetyl- β -glucosaminidase	–	+	+	+	+
DNA G + C content (mol%)	43.4	45.1 ^a	42.1 ^b	47.5 ^c	40–41 ^d

*Data from: a, Lee *et al.* (2012); b, Lim *et al.* (2008); c, Subhash *et al.* (2014); d, Nedashkovskaya *et al.* (2010).

(for glycolipids) (Sigma-Aldrich). Isoprenoid quinones were analysed as described previously (Komagata & Suzuki, 1987) using reversed-phase HPLC. The quinones were eluted by an isocratic solvent system [methanol/iso-propyl ether (3 : 1, v/v)] using a flow rate of 1 ml min^{–1}.

The only respiratory quinone detected in strain CAU 1287^T was MK-6. This character is in agreement with those of numerous species of the genus *Salinimicrobium*, including the type species of the genus, *S. catena*. Phosphatidylethanolamine was the only polar lipid identified in strain CAU 1287^T. The other unidentified polar lipids were three unidentified aminolipids, one unidentified amino-phospholipid, one unidentified phosphoglycolipid, one unidentified glycolipid and three unidentified lipids (Fig. S3). The cellular fatty acid profile of strain CAU 1287^T contained unsaturated, branched-chain and hydroxy fatty acids (Table 2). The cellular fatty acids amounting >10 % were iso-C_{15:0} and anteiso-C_{15:0}, which are characteristic components of the fatty acid profiles of the type strains of species of the genus *Salinimicrobium*

(Table 2). Despite similarities in the fatty acid composition, there were differences in the amounts of some fatty acids. Furthermore, strain CAU 1287^T could be distinguished from closely related species by the absence of the branched-fatty acid anteiso-C_{15:1} A.

In conclusion, these data provide sufficient evidence to recognize strain CAU 1287^T as representing a novel species of the genus *Salinimicrobium*, for which the name *Salinimicrobium soli* sp. nov. is proposed.

Description of *Salinimicrobium soli* sp. nov.

Salinimicrobium soli sp. nov. (so'li. L. gen. n. *soli* of the soil).

Cells are Gram-stain-negative, facultatively anaerobic and rod-shaped, approximately 0.4–0.6 µm in diameter and 1.0–2.4 µm in length. Colonies on MA are yellow, circular and convex with entire margins after incubation for 3 days at 30 °C. Growth occurs at 20–37 °C (optimum 30 °C) and

Table 2. Cellular fatty acid contents (%) of strain CAU 1287^T and the type strains of the most closely related species of the genus *Salinimicrobium*

Strains: 1, CAU 1287^T; 2, *S. gaetbulicola* KCTC 23579^T; 3, *S. xinjiangense* KCTC 12883^T; 4, *S. sediminis* KCTC 32444^T; 5, *S. marinum* KCTC 12719^T. All data were obtained in this study. Only those fatty acids amounting to >1.0 % in all strains are shown. –, Not detected.

Fatty acid	1	2	3	4	5
Unsaturated					
C ₁₅ :1 ω 6c	1.1	1.9	1.5	5.8	5.6
C ₁₇ :1 ω 6c	–	1.9	2.8	2.4	–
C ₁₇ :1 ω 8c	–	–	1.2	1.3	–
Branched-chain					
anteiso-C ₁₅ :0	12.8	8.0	13.3	12.7	11.0
anteiso-C ₁₅ :1 A	–	1.5	2.7	2.1	1.6
anteiso-C ₁₇ :1 ω 9c	2.1	2.3	5.0	5.3	3.7
iso-C ₁₄ :0	2.6	1.2	1.7	–	–
iso-C ₁₃ :0	2.8	2.6	–	–	–
iso-C ₁₅ :0	28.5	23.9	10.7	17.9	16.2
iso-C ₁₆ :0	6.9	2.9	9.3	5.7	4.3
iso-C ₁₅ :0 2-OH	–	–	–	–	3.0
iso-C ₁₅ :0 3-OH	4.0	4.5	2.6	2.3	2.7
iso-C ₁₅ :1 G	4.7	8.9	4.5	10.0	8.9
iso-C ₁₆ :0 3-OH	4.6	3.7	4.2	2.6	4.2
iso-C ₁₆ :1 H	2.1	1.9	3.7	3.4	3.3
iso-C ₁₇ :0 2-OH	–	3.8	–	–	–
iso-C ₁₇ :0 3-OH	9.4	13.2	9.0	6.6	10.8
iso-C ₁₇ :1 ω 9c	3.6	5.7	6.7	5.9	5.6
iso-C ₁₇ :1 ω 8c	–	–	–	–	1.0
iso-C ₁₇ :1 ω 6c	–	–	–	–	2.0
Hydroxy					
C ₁₅ :0 2-OH	2.6	2.7	3.5	2.2	–
C ₁₇ :0 2-OH	3.2	–	9.1	5.4	5.3
Summed feature 3*	3.2	8.7	4.5	4.9	7.3

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained C₁₆:1 ω 7c/ ω 6c.

at pH 5.5–8.0 (optimum pH 5.5). NaCl is not required for growth but up to 4.0 % (w/v) NaCl is tolerated. Catalase and oxidase are present. Gelatin and aesculin are hydrolysed. In the API 50CH strip, maltose and potassium 5-ketogluconate were positive. In the API ZYM strip, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase are present, but leucine arylamidase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase are absent. The major isoprenoid quinone is MK-6. The polar lipid pattern consists of phosphatidylethanolamine, three unknown aminolipids, one unknown aminophospholipid, one unknown phosphoglycolipid, one unknown glycolipid and three

unknown lipids. The major cellular fatty acids are iso-C₁₅:0 and anteiso-C₁₅:0.

The type strain CAU 1287^T (=KCTC 32163^T=CCUG 64794^T) was isolated from a sample of reclaimed-land soil collected from Modo in the Republic of Korea. The DNA G+C content of the type strain is 43.4 mol%.

Acknowledgements

This work was supported by the project on Survey of Indigenous Species of Korea of the National Institute of Biological Resources (NIBR) under the Ministry of Environment (MOE).

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