INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION
Zhang *et al.*, *Int J Syst Evol Microbiol* 2018;68:2551–2556
DOI 10.1099/ijsem.0.002875

MICROBIOLOGY SOCIETY

Sphingomicrobium arenosum sp. nov., isolated from marine sediment

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Abstract

A Gram-stain-negative, strictly aerobic, motile by one single flagellum, dark-orange pigmented and rod-shaped bacterial strain, designated CAU 1457^T, was isolated from marine sediment in the Republic of Korea and its taxonomic position was investigated by using a polyphasic approach. The isolate grew optimally at 30 °C, at pH 6.0 and in the presence of 2 % (w/v) NaCl. Based on 16S rRNA gene sequences similarity, strain CAU 1457^T belonged to the genus *Sphingomicrobium* and was related most closely to *Sphingomicrobium astaxanthinifaciens* JCM 18551^T (98.2 % similarity). Strain CAU 1457^T contained ubiquinone-10 as the predominant isoprenoid quinone and 11-methyl $C_{18:1}\omega7c$ and summed feature 8 ($C_{18:1}\omega7c/\omega6c$) as the major cellular fatty acids. Triamine *sym*-homospermidine was detected as the major compound in the polyamine pattern. The polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, sphingoglycolipid, four unidentified glycolipids, one unidentified aminophospholipid, two unidentified phospholipids, one unidentified aminolipid and one unidentified lipid. DNA–DNA relatedness between strain CAU 1457^T and the closely related strains, *Sphingomicrobium astaxanthinifaciens* JCM 18551^T and *Sphingomicrobium aestuariivivum* KCTC 42286^T were 32.7 and 28.4 %, respectively. The DNA G+C content of strain was 68.8 mol%. The phenotypic, chemotaxonomic and phylogenetic data indicated that strain CAU 1457^T represents a novel species of the genus *Sphingomicrobium*, for which the name *Sphingomicrobium arenosum* sp. nov. is proposed. The type strain is CAU 1457^T (=KCTC 62233^T=NBRC 113094^T).

The genus *Sphingomicrobium* was first purposed by Kämpfer *et al.* [1] with the description of *Sphingomicrobium lutaoense* isolated from water of a coastal hot spring of Green Island. Members of the genus *Sphingomicrobium* are Gram-stain-negative, rod-shaped, strictly aerobic, catalase- and oxidase-positive, and contain Q-10 as the major respiratory quinone [1–4]. At the time of writing, the genus *Sphingomicrobium* consists of five recognized species with validly published names, including *S. lutaoense* [1], *Sphingomicrobium astaxanthinifaciens* [2], *Sphingomicrobium marinum* and *Sphingomicrobium flavum* isolated from coastal surface seawater in Taiwan [3], and *Sphingomicrobium aestuariivivum* isolated from a tidal flat in Aphae Island located in the southwestern sea of the Republic of Korea [4]. In the course of screening bacteria from a marine environment, a strain designated CAU 1457^T was isolated from marine sediment collected in Modo (37° 32′ 12.28″ N, 126° 24′ 51.47″ E) located in the Republic of Korea. The aim of the present study was to determine the taxonomic position of strain CAU 1457^T by using a polyphasic approach, which included the determination of phenotypic, chemotaxonomic properties and a detailed phylogenetic investigation based on 16S rRNA gene sequences.

The marine sediment sample was collected from Modo, Republic of Korea. One gram of sample was serially diluted with 10 ml sterilized 0.85 % (w/v) sodium chloride solution and 100 µl of each serial dilution was plated on marine agar 2216 (MA; BD, Becton, Dickinson and Company) supplemented with cycloheximide (50 mg^{-1}) and nalidixic acid (20 mg^{-1}) against fungi. MA agar plates were incubated under aerobic conditions at 30 °C for 7 days as described by Gordon and Mihm [5]. A single colony was selected and purified by subculturing on MA incubated at 30 °C for 48 h. The purified bacterial cells were maintained in marine broth 2216 (MB; BD) supplemented with 25 % (v/v) glycerol at −70 °C. The type strains of closely related species *S. astaxanthinifaciens*

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Keywords: *Sphingomicrobium arenosum*; Proteobacteria; marine sediment.
Abbreviations: FAME, fatty acid methyl ester; HPLC, high-performance liquid chromatography; Q-10, ubiquinone-10.
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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CAU 1457^T is MH091576.
Four supplementary figures are available with the online version of this article.

JCM 18551^T, *S. aestuariivivum* KCTC 42286^T and *S. lutaoense* DSM 24194^T were obtained from the Japan Collection of Microorganisms (JCM), the Korean Collection for Type Cultures (KCTC) and the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSM) for use as reference strains.

Extraction of genomic DNA of strain CAU 1457^T was performed using a genomic DNA extraction kit (Intron). The 16S rRNA gene was amplified by PCR using the universal primers 8F/1525R as described by Lane [6]; and the PCR products were purified and cloned in *Escherichia coli* DH5α component cells using a RBC T&A cloning vector kit (RBC Bioscience). The plasmid DNA was extracted, purified using a plasmid DNA purification kit (Intron) and sequenced using primer pair M13F (5′-GTTTTCCCAGTCACGAC-3′) and M13R (5′-TCACCAGGAAACAGGCTATGAC-3′) by an automatic DNA sequencer (model 3730; Applied Biosystems). The sequence was compared with available sequences in the EzBioCloud database (www.ezbiocloud.net) [7], after multiple alignment by using CLUSTAL_X 2.1 software [8]. Distance matrices were calculated by the neighbour-joining method [9]. Phylogenetic trees were reconstructed by using the neighbour-joining [10], maximum-likelihood [11] and maximum-parsimony [12] algorithms available within the MEGA7 program [13]. The topology of tree was evaluated by the bootstrap resampling method [14] based on 1000 replicates. The extent of DNA–DNA relatedness was observed using the fluorometric microplate method [15], as modified by Goris *et al.* [16]. The genomic DNA G+C measurement was determined as according to Tamaoka and Komagata [17] using reversed-phase high-performance liquid chromatography (HPLC).

Phylogenetic analysis was carried out using the almost-complete sequence of the 16S rRNA gene of strain CAU 1457^T (1480 bp) with the available reference sequences in the GenBank database. In the neighbour-joining tree, strain CAU 1457^T clustered with the genus *Sphingomicrobium* and shared highest sequence similarity with *S. astaxanthinifaciens* JCM 18551^T (similarity, 98.2 %), *S. aestuariivivum* KCTC 42286^T (98.0 %) and values for other species in the genus *Sphingomicrobium* were 93.4–96.4 % (Fig. 1). The trees generated with other treeing methods showed a similar topology (Figs S1 and S2, available in the online version of this article). The G+C content of the DNA of strain CAU 1457^T was 68.8 mol%, a value in the range reported for member within the genus *Sphingomicrobium* (63.4–70.6 mol%) [1–4]. The result of DNA–DNA hybridization between CAU 1457^T with the most closely reference strains, *S. astaxanthinifaciens* JCM 18551^T and *S. aestuariivivum* KCTC 42286^T were 32.7 and 28.4 %, respectively. The value is well below the <70 % cut-off point suggested by Wayne *et al.* [18] for the description of genomic species.

Colonies of strain CAU 1457^T were examined for morphological characteristics such as colony appearance, pigment, size, shape and texture. Endospore formation was assessed on nutrient sporulation medium as described by Nicholson and Setlow [19] and observed by staining with malachite green after 7 days of growth [20]. Cell morphology was determined under DM 1000 light microscopy (Leica) using cells grown routinely at 30 °C on MA [21] and presence of flagella was investigated under JEM 1010 transmission electron microscopy (JEOL) using an exponentially growing culture. Gram staining was performed using the bioMérieux Gram staining kit according to the manufacturer's instructions. Motility was evaluated in semisolid agar and turbidity was observed as described by Leifson [22]. Growth at various temperature (4, 10, 20, 30, 37, 45 and 55 °C) of strain CAU 1457^T was assessed by growing on MA for 48 h in an aerobic incubator (model MIR-253; Sanyo). Growth on MA, glucose yeast extract agar (GYE; comprising per litre: 10 g yeast extract, 10 g glucose and 15 g agar), Reasoner's 2A agar (R2A; BD), nutrient agar (NA; BD), brain heart infusion agar (BHI; BD) and tryptic soy agar (TSA; BD) was observed after incubated at the optimal temperature for 48 h. The pH range for growth was tested in MB adjusted to pH 4.5–11.0 (at 0.5 pH unit intervals) by using phosphate–citrate buffer (pH 4.0–6.5), Tris-HCl buffer (pH 7.0–9.0) and $NaHCO_3$/NaOH (pH 9.5–11.0). The NaCl requirement was evaluated in marine broth 2216 (MB; BD) prepared according to the formula of BD medium except that NaCl was included and then supplemented with 0–15% NaCl before sterilization [23]. Anaerobic growth was examined on MA incubated in an anaerobic chamber (Bactron) at the optimal temperature for 1 week. Carbon source utilization was performed by growing the strain in the basal medium supplemented with filter-sterilized carbon compounds at a final concentration of 1 % (w/v). The basal medium contained (per litre) 4 g $(NH_4)_2SO_4$, 2 g K_2HPO_4, 0.5 g $MgSO_4 \times 7H_2O$ and 2 g KH_2PO_4 at optimum pH and NaCl concentration. Activities of catalase and oxidase were determined using 3 % (v/v) hydrogen peroxide solution and 1 % (w/v) tetramethyl-*p*-phenylenediamine, respectively [24]. Nitrate reduction was examined in nitrate broth (sulfanilic acid/a-naphthylamine test) inoculated with strains. Hydrolysis of casein, gelatin, aesculin and starch was determined according to Smibert and Krieg [25]. Biochemical properties and enzyme activities were analysed using the API ZYM, API 20E, API 20NE, and API 50CH systems (bioMérieux) according to the manufacturer's recommendations.

Morphological, physiological and biochemical characteristics of strain CAU 1457^T are given in the species description and in Table 1. Strain CAU 1457^T was Gram-stain-negative, strictly aerobic and motile. After 48 h incubation at 30 °C, colonies on MA were $\leq$0.5 mm in diameter, dark-orange pigmented, circular and convex with entire margin. During the exponential growth phase, cells of strain CAU 1457^T formed a rod shape with a single polar flagellate (Fig. S3), which were 0.7–1.9 μm longth and 0.1–0.4 μm wide. Endospores were not observed. Strain CAU 1457^T grew well on MA and weakly grew on R2A; however, no growth was observed on GYE, NA, BHI or TSA. Strain CAU 1457^T exhibited growth at temperatures between 20 and 37 °C (optimum, 30 °C), NaCl

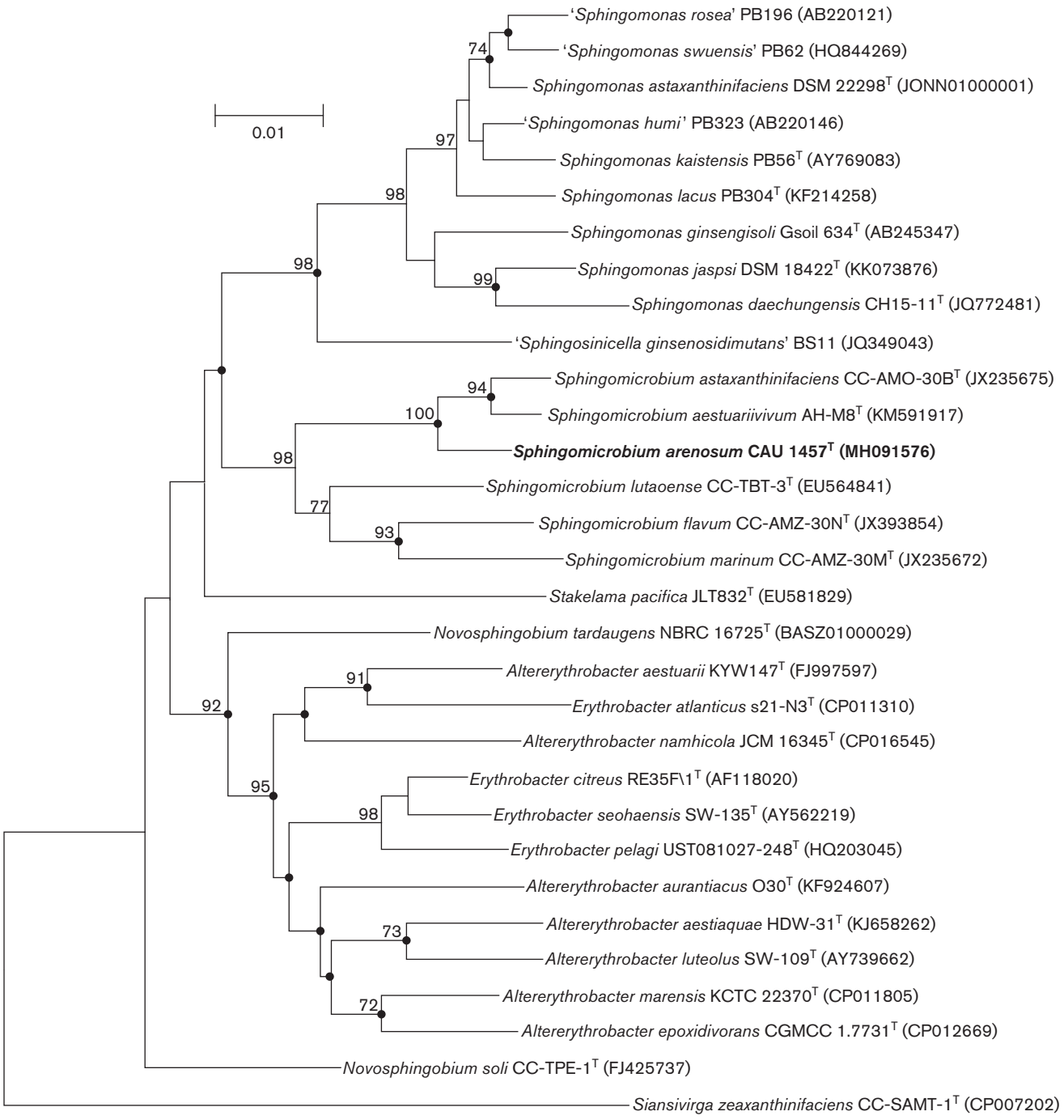

Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the phylogenetic relationships of strain CAU 1457^T and the related strain of *Sphingomicrobium* species. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. Bootstrap values >70 %, based on a neighbour-joining analysis of 1000 resampled datasets, are shown at nodes. *Siansivirga zeaxanthinifaciens* CC-SAMT-1^T (CP007202) is used as an outgroup.

concentrations between 0 and 7 % (2 %) and pH 5.5–8.5 (pH 6.0). Adonitol, L-arabinose, D-fructose, D-galactose, α-D-glucose, m-inositol, lactose, maltose, D-mannitol, melibiose, raffinose, L-rhamnose, D-sorbitol, sucrose, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proline and L-threonine were utilized. Strain CAU 1457^T showed positive reactions for catalase, oxidase, hydrolysis of aesculin, and potassium 5-ketogluconate. Nitrate was

Table 1. Differential properties of strain CAU 1457^T and the type strains of the most closely related *Sphingomicrobium* species

Strains: 1, CAU 1457^T; 2, *S. astaxanthinifaciens* JCM 18551^T; 3, *S. aestuariivivum* KCTC 42286^T; 4, *S. lutaoense* DSM 24194^T. Data were taken from this study unless otherwise indicated. All strains were positive for catalase and oxidase; acid production from aesculin and potassium 5-ketogluconate; activity of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase. All strains were negative for nitrate reduction, hydrolysis of casein, gelatin, starch, activity of β-glucoronidase, β-glucosidase, α-mannosidase and α-fucosidase. +, Positive; −, negative; w, weakly.

Characteristic	1	2	3	4
Cell size (μm)	0.7–1.9×0.1–0.4	1.1–2.8×0.5–0.7[a]	0.2–10.0×0.1–0.5[b]	1.5–2.0×1.0–1.1[c]
Temperature range for growth (°C)	20–37	10–40[a]	15–40[b]	20–36[c]
Optimum	30	30[a]	30[b]	28[c]
pH range for growth	5.5–8.5	6.5–10.5	6–9	6.5–10.5
Optimum	6.0	6.5	6.5	6.5
NaCl tolerance (%)	1–7	1–4	1–6	1–4
Optimum	2	3	3	1
Motility	+	+	−	−
Urease	+	−	−	−
Arginine dihydrolase	+	−	−	−
Ornithine decarboxylase	+	−	−	−
Carbon source utilization:				
Citrate	+	−	−	−
Melibiose	+	−	−	−
Sucrose	+	−	−	−
Acid production from:				
L-Arabinose	+	−	−	−
D-Galactose	+	−	−	−
Raffinose	+	−	−	−
Potassium gluconate	+	−	−	−
Enzyme activity:				
Lipase (C14)	+	−	−	−
Crystine arylamidase	+	+	w	−
Trypsin	+	+	+	−
α-Chymotrypsin	+	−	+	−
Acid phosphatase	+	+	−	−
Naphthol-AS-BI-phosphohydrolase	+	+	−	+
α-Galactosidase	−	−	−	w
β-Galactosidase	−	−	−	w
α-Glucosidase	−	−	w	w
β-Glucosidase	−	−	−	w
N-acetyl-β-glucosaminidase	−	+	−	−
DNA G+C content (mol%)	68.8	70.6[a]	66.7[b]	63.4[c]

*Data taken from: *a*, Shahina *et al.* [2]; *b*, Park *et al.* [4]; *c*, Kämpfer *et al.* [1].

not reduced. Tests for the presence of the following were positive the API ZYM system: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase and naphtol-AS-BI-phosphohydrolase; acid production from L-arabinose, D-galactose, aesculin, raffinose, potassium gluconate and potassium 5-ketogluconate was positive in the API 50CH test; urease, arginine dihydrolase, ornithine decarboxylase, citrate utilization and assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, maltose, potassium gluconate adipic acid, malic acid and trisodium citrate in the API 20E and 20NE tests were observed in strain CAU 1457^T. However, strain CAU 1457^T differed from closely related species, namely *S. astaxanthinifaciens* JCM 18551^T and *S. aestuariivivum* KCTC 42286^T, by acid production ability from L-arabinose, D-galactose, raffinose, potassium gluconate, urease, arginine dihydrolase and ornithine decarboxylase as well as citrate, melibiose and sucrose utilization. These differences could be used to distinguish strain CAU 1457^T from other related *Sphingomicrobium* species.

For cellular fatty acid analysis, the fatty acid methyl esters (FAMEs) of strain CAU 1457^T and the type strains of the

most closely related *Sphingomicrobium* species, *S. astaxanthinifaciens* JCM 18551^T, *S. aestuariivivum* KCTC 42286^T and *S. lutaoense* DSM 24194^T, were extracted from cells cultivated on MA at 30 °C for 48 h. Cells were harvested at the late-exponential growth phase according to the standard MIDI protocol (Sherlock Microbial Identification System version 6.1) and extracted as previously described by Minnikin *et al.* [26]. The analysis of FAMEs was performed using an automated gas chromatography system (model 6890 N and 7683 autosampler; Agilent). Peaks were automatically integrated and identified using the Microbial Identification software package (MOORE library version 5.0; MIDI database TSBA6). The polar lipids of strain CAU 1457^T were extracted from freeze-dried cell and analysed by using two-dimensional thin-layer chromatography (TLC) as described by Minnikin *et al.* [27]. A mixture of chloroform–methanol–water (65 : 25 : 4, by vol.) was used as the solvent for the first dimension and chloroform–methanol–acetic acid–water (80 : 12 : 15 : 4, by vol.) was used as the solvent for the second dimension. Total lipids on the TLC plates were sprayed with 10 % ethanolic molybdatophosphoric acid, phospholipids with molybdenum blue, amino-containing lipids with ninhydrin and glycolipids with α- naphthol/sulphuric acid reagent (Sigma-Aldrich). Polyamines were extracted according the method of Busse and Auiling [28] and analysed by HPLC [29]. Isoprenoid quinones were extracted according to the method of Minnikin *et al.* [27] and determined by reversed-phase HPLC using an isocratic solvent system [methanol/isopropyl ether (3 : 1, v/v)] and a solvent flow rate of 1 ml min^{-1} [30].

The predominant respiratory quinone detected in strain CAU 1457^T was Q-10. This character is similar to those of members of the genus *Sphingomicrobium*. Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and sphingoglycolipid were the identified polar lipids in strain CAU 1457^T. The other unidentified polar lipids were four unidentified glycolipids, one unidentified aminophospholipid, two unidentified phospholipids, one unidentified aminolipid and one unidentified lipid (Fig. S4). The polyamines exhibited similar patterns to other members of the genus *Sphingomicrobium*, which indicated that triamine *sym*-homospermidine was a predominant polyamine in strain CAU 1457^T. The novel isolate contained branched-chain, hydroxy, saturated and unsaturated fatty acids (Table 2). The major fatty acids (amounting >10 %) were 11-methyl $C_{18:1}\omega7c$ and summed feature 8 ($C_{18:1}\omega7c/\omega6c$), which is consistent with the genus *Sphingomicrobium*, however there were differences in the amounts of some fatty acids.

In conclusion, it is evident from genotypic, phenotypic and chemotaxonomic data that strain CAU 1457^T represents a novel species of the genus *Sphingomicrobium*, for which the name *Sphingomicrobium arenosum* sp. nov. is proposed.

DESCRIPTION OF *SPHINGOMICROBIUM ARENOSUM* SP. NOV.

Sphingomicrobium arenosum sp. nov. (a.re.no′sum. L. neut. adj. *arenosum* sandy, dwelling in marine sediment sand).

Table 2. Cellular fatty acid compositions (%) of strain CAU 1457^T and the type strains of *Sphingomicrobium* species

Strains: 1, CAU 1457^T; 2, *S. astaxanthinifaciens* JCM 18551^T; 3, *S. aestuariivivum* KCTC 42286^T; 4, *S. lutaoense* DSM 24194^T. Data were from this study and all strains were cultured under same conditions. Fatty acids amounting to >0.5 % of total fatty acids in all strains are shown. –, Not detected; TR, trace (<0.5 %)

Fatty acid	1	2	3	4
Saturated:				
$C_{14:0}$	–	0.7	TR	–
$C_{16:0}$	8.8	6.2	11.7	5.6
$C_{17:0}$	1.5	2.8	0.6	0.8
$C_{18:0}$	5.1	0.8	TR	1.2
Unsaturated:				
$C_{16:1}\omega5c$	1.0	1.1	1.9	2.0
$C_{17:1}\omega6c$	3.8	11.8	4.5	5.2
$C_{17:1}\omega8c$	–	1.6	1.5	1.8
$C_{18:1}\omega5c$	3.5	1.4	1.0	2.1
$C_{20:1}\omega7c$	0.9	–	–	–
Hydroxy:				
$C_{14:0}$ 2-OH	TR	0.5	1.6	–
$C_{18:1}$ 2-OH	4.6	8.6	5.7	15.7
iso-$C_{13:0}$ 3-OH	–	–	–	1.1
11-methyl-$C_{18:1}\omega7c$	10.1	9.3	7.5	6.4
Summed features:*				
3	8.2	13.7	27.5	14.3
7	0.6	–	–	–
8	51.7	41.1	35.7	43.2

*Summed features represent groups of two or three fatty acids that could not be separated by gas–liquid chromatography with the MIDI system. Summed feature 3 consisted of $C_{16:1}\omega7c/\omega6c$. Summed feature 7 consisted of unknown 18.846/$C_{19:1}\omega6c$/$C_{19:0}$ cyclo $\omega10c$. Summed feature 8 consisted of $C_{18:1}\omega7c/\omega6c$.

Cells are Gram-stain-negative, strictly aerobic, non-spore-forming, rod-shaped approximately 0.7–1.9 µm long and 0.1–0.4 µm wide, and motile with a single polar flagellate. After 3 days of incubation at 30 °C, colonies on MA are dark-orange coloured, transparent, circular, smooth, convex with entire margins and ≤0.5 mm in diameter. Cells grow well on MA, weakly on R2A, but not on GYE, BHI, NA and TSA. Growth occurs at 20–37 °C (optimum, 30 °C), at pH 5.5–8.5 (6.0) and in the presence of 0–7 % NaCl (2 %). The following carbon sources are utilized: adonitol, L-arabinose, D-fructose, D-galactose, α-D-glucose, m-inositol, lactose, maltose, D-mannitol, melibiose, raffinose, L-rhamnose, D-sorbitol, sucrose, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proline and L-threonine. Catalase and oxidase are present. Nitrate reduction and hydrolysis of gelatin, casein and starch are absent. In the API 50CH strip, L-arabinose, D-galactose, raffinose, aesculin, potassium gluconate and potassium 5-ketocluconate were positive. In the API ZYM strip, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid

phosphatase and naphtol-AS-BI-phosphohydrolase are present, but α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. In the API 20E and 20NE srips, positive for urease, arginine dihydrolase, ornithine decarboxylase and citrate utilization, and assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, maltose, potassium gluconate adipic acid, malic acid and trisodium citrate. The predominant isoprenoid quinone is Q-10 and the major polyamine is triamine *sym*-homospermidine. The polar lipid pattern comprises diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, sphingoglycolipid, four unidentified glycolipids, one unidentified aminophospholipid, two unidentified phospholipids, one unidentified aminolipid and one unidentified lipid. The major cellular fatty acids are 11-methyl $C_{18:1}\omega7c$ and summed feature 8 ($C_{18:1}\omega7c/\omega6c$). The DNA G+C content is 68.8 mol%. The type strain, CAU 1457^T (=KCTC 62233^T=NBRC 113094^T), was isolated from marine sand collected from Modo in the Republic of Korea.

Funding information
This research 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) and the Chung-Ang University Research Grants in 2017.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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