

Sphingomonas aestuarii sp. nov., isolated from tidal flat sediment

Seong Woon Roh,¹ Kyoung-Ho Kim,¹ Young-Do Nam,¹ Ho-Won Chang,¹ Min-Soo Kim,¹ Hee-Mock Oh² and Jin-Woo Bae¹

Correspondence
Jin-Woo Bae
baejw@kribb.re.kr

¹Department of Biology, Kyung Hee University, Seoul 130-701, Korea

²Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305-806, Korea.

A novel bacterium (strain K4^T) belonging to the genus *Sphingomonas* was isolated from tidal flat sediment in Korea. Its morphology, physiology, biochemical features and 16S rRNA gene sequence were characterized. Colonies of this strain are yellow in colour and the cells are rod-shaped, exhibiting negative Gram staining. The strain grows at 0–5% (w/v) NaCl and 20–35 °C, with optimal growth occurring at 0% (w/v) NaCl and 30 °C. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain K4^T is associated with the genus *Sphingomonas*. Within the phylogenetic tree, this novel strain shares a branching point with *Sphingomonas asaccharolytica* Y-345^T, with which it shares 97.3% 16S rRNA gene sequence similarity. The polyamine pattern predominantly contains the *Sphingomonas*-specific triamine *sym*-homospermidine. Combined analysis of 16S rRNA gene sequences, DNA–DNA relatedness, physiological and biochemical test results identified genotypic and phenotypic differences between strain K4^T and other *Sphingomonas* species. On the basis of these differentiating features, it is concluded that strain K4^T (=KCTC 22050^T=DSM 19475^T) represents a novel species of the genus *Sphingomonas*, for which the name *Sphingomonas aestuarii* sp. nov. is proposed.

The genus *Sphingomonas* was first proposed by Yabuuchi *et al.* (1990); it was amended by Takeuchi *et al.* (1993) and divided into four genera: *Sphingomonas sensu stricto*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* (Takeuchi *et al.*, 2001). Currently, more than 30 species with validly published names in the genus *Sphingomonas sensu stricto* have been isolated from various environments, including natural mineral water (Lee *et al.*, 2001), plants (Takeuchi *et al.*, 1995; Xie & Yokota, 2006) and soil (Ohta *et al.*, 2004; Reddy & Garcia-Pichel, 2007; Yoon *et al.*, 2006).

In this study, a novel strain was isolated from tidal flat sediment in Yeosu (34° 47' 26" N 127° 34' 01" E), South Korea, and cultured by dilution plating on R2A agar (Difco). Cell biomass for analysis of cellular composition and DNA extraction was collected from R2A agar plates incubated at 30 °C for 2 days. Chromosomal DNA was extracted by using a DNA extraction kit (iNtRON

Biotechnology). The 16S rRNA gene was PCR-amplified from chromosomal DNA by using PCR Pre-Mix (Solgent) with two bacterial universal primers (Baker *et al.*, 2003). The PCR product was purified with a PCR purification kit (Cosmo Genetech) and sequencing was performed as described previously (Roh *et al.*, 2008). Almost-full-length 16S rRNA gene sequences were assembled by using SeqMan software (DNASTAR). The 16S rRNA gene sequence of the novel isolate was aligned with those of related taxa obtained from GenBank by using the multiple sequence alignment program CLUSTAL_X (1.8) (Thompson *et al.*, 1997). The phylogenetic relationships of representative *Sphingomonas* species were determined by using the MEGA3 (Kumar *et al.*, 2004) and PAUP 4.0 (Swofford, 2003) software programs. Distance matrices were determined by following the assumptions described by Kimura (1980) and used to elaborate dendrograms by the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods. A bootstrap analysis evaluating the stability of the trees was performed by using a consensus tree based on 1000 randomly generated trees. DNA–DNA hybridization was performed by using the fluorometric method of Ezaki *et al.* (1989).

Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain K4^T is associated with the genus

Abbreviations: DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PDE, phosphatidylmethylethanolamine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, unknown phospholipid; SGL, sphingoglycolipid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain K4^T is EF660755.

Sphingomonas. Comparison of the 16S rRNA gene sequence of strain K4^T with those of *Sphingomonas* species with validly published names by using FASTA (EMBL) showed that the sequence was related most closely to that of *Sphingomonas asaccharolytica* Y-345^T (97.3%), followed by *Sphingomonas mali* Y-351^T (96.9%), *Sphingomonas pruni* Y-250^T (96.7%), *Sphingomonas desiccabilis* CP1D^T (96.4%), *Sphingomonas aquatilis* JSS7^T (96.3%), *Sphingomonas koreensis* JSS26^T (96.2%), *Sphingomonas melonis* DAPP-PG 224^T (96.2%), *Sphingomonas soli* T5-04^T (96.2%), *Sphingomonas insulae* DS-28^T (96.1%) and *Sphingomonas panni* C52^T (96.1%). In the phylogenetic tree created by using the maximum-likelihood method (Fig. 1), the novel strain shares a branching point with *S. asaccharolytica* Y-345^T. DNA–DNA homology studies were performed to determine the genomic relationship between the isolate and its closest phylogenetic relatives: *S. asaccharolytica* Y-345^T, which has the highest 16S rRNA gene sequence similarity (97.3%) to the novel strain, and *S. soli* T5-04^T, which clusters with the novel strain in the phylogenetic tree based on the neighbour-joining method (data not shown). The result revealed 46.0 and 29.1% DNA–DNA homology between strain K4^T and *S. asaccharolytica* Y-345^T and *S. soli* T5-04^T, respectively. The characteristics of 16S rRNA gene sequence similarity and DNA–DNA relatedness values below the threshold of 70% (Wayne *et al.*, 1987) indicate that strain K4^T represents a distinct genospecies.

Cell morphology was examined by light microscopy (Eclipse 80i; Nikon) and electron microscopy, and Gram staining was performed by the non-staining method described by Buck (1982). Motility was examined by the wet-mount method and spore formation was determined by using the staining method (Schaeffer & Fulton, 1933). Growth on tryptic soy agar (TSA), marine agar (MA), nutrient agar (NA) and Luria agar (LA) (all from Difco) was also determined. Enzyme activities and substrate utilization as sole carbon source were determined by using API 20NE, API 50CH and API ZYM test strips according to the manufacturer's instructions (bioMérieux). Growth at different temperatures (4, 10, 15, 17, 20, 25, 30, 35, 37, 40 and 50 °C) was assessed on R2A agar. Various NaCl concentrations (0.0, 1.0, 3.0, 5.0, 7.5, 10.0, 15.0 and 20.0%, w/v) and pH conditions (pH 3.0–12.0) were examined for

optimal growth using R2A broth. Analyses of gelatin and starch hydrolysis were conducted as described by Smibert & Krieg (1994). Catalase activity was determined by observing bubble production in 3% (v/v) hydrogen peroxide solution and oxidase activity was determined by using an oxidase reagent (bioMérieux).

Cells of strain K4^T are non-spore-forming, Gram-negative, non-motile and rod-shaped. Colonies of strain K4^T are yellow-pigmented, like those of other *Sphingomonas* strains. Growth occurs on R2A agar plates containing up to 5% (w/v) NaCl, but not at 7.5% (w/v) NaCl. Strain K4^T cannot reduce nitrate to nitrite. It can grow on complex media such as TSA, LA, MA or NA plates. Strain K4^T is catalase- and oxidase-positive. Gelatin hydrolysis occurs, but starch hydrolysis does not. The optimal temperature for growth is 30 °C. A detailed species description is presented below and Table 1 lists the characteristics that differentiate strain K4^T from related members of the genus *Sphingomonas*.

For quantitative analysis of cellular fatty acid composition, a loop of cell mass was harvested after 2 days; cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification system (MIDI). Fatty acids were analysed by gas chromatography (Hewlett Packard 6890) and identified by the Microbial Identification software package (Sasser, 1990). The predominant cellular fatty acids in strain K4^T were C_{18:1}ω7c (44.9%), 11-Me-C_{18:1}ω7c (17.7%) and C_{16:0} (17.6%). Minor amounts of 2-hydroxy fatty acids C_{14:0} 2-OH (4.7%), summed feature 3 comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH (3.9%) and C_{15:0} 2-OH (0.6%) were also present. The detailed fatty acid composition is given in the species description. No 3-hydroxy fatty acids were identified. This composition profile, showing octadecenoic and hexadecanoic acid isomers as the major fatty acids, with a minor presence of 2-hydroxy fatty acids and the absence of 3-hydroxy fatty acids, is characteristic of members of the genus *Sphingomonas* (Takeuchi *et al.*, 2001).

Polar lipids were extracted, separated by two-dimensional thin-layer chromatography on 20 × 20 cm silica gel glass plates (Merck) with the two solvents chloroform/methanol/water (65:25:4, v/v) for the first dimension (from

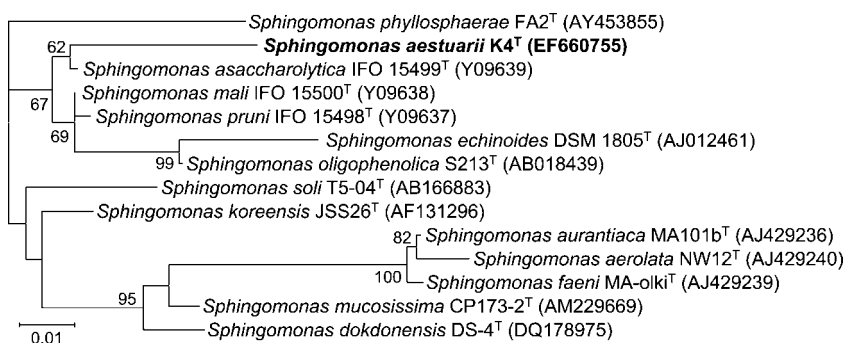


Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences. The position of strain K4^T is shown with respect to other species of the genus *Sphingomonas*. The phylogenetic tree was generated by the maximum-likelihood method. GenBank accession numbers are shown in parentheses. Numbers at nodes indicate bootstrap values >50%. Bar, 0.01 accumulated change per nucleotide.

Table 1. Differentiating characteristics of *Sphingomonas aestuarii* sp. nov. and closely related species

Taxa: 1, *Sphingomonas aestuarii* K4^T; 2, *S. asaccharolytica* IFO 15499^T (data from Takeuchi *et al.*, 1995); 3, *S. mali* IFO 15500^T (Takeuchi *et al.*, 1995); 4, *S. pruni* IFO 15498^T (Takeuchi *et al.*, 1995); 5, *S. echinoides* DSM 1805^T (Denner, 1999); 6, *S. oligophenolica* S213^T (Ohta *et al.*, 2004); 7, *S. koreensis* JSS26^T (Lee *et al.*, 2001); 8, *S. soli* T5-04^T (Yang *et al.*, 2006). +, Positive; –, negative; NR, not reported; PNPG, *p*-nitrophenyl- β -D-galactopyranoside.

Characteristic	1	2	3	4	5	6	7	8
Growth at 37 °C	–	NR	NR	NR	NR	+	+*	NR
Motility	–	+	+	+	+	–	+	–
Reduction of nitrate	–	–	–	–	+†	+	–	NR
Starch hydrolysis	–	+	–	+	NR	NR	NR	NR
Enzyme activities:								
Lipase	–	+	+	+	NR	NR	+	NR
Protease (gelatin hydrolysis)	+	–	–	–	–	+	–	+
β -Galactosidase (PNPG hydrolysis)	+	+	+	+	+	NR	+	–
β -Glucosidase (aesculin hydrolysis)	+	+	+	+	+	NR	–	+
Assimilation of:								
5-Ketogluconate	+	–	+	–	NR	NR	–	–
Gluconate	–	–	+	+	–	+	–	–
Malate	–	–	+	–	+	–	+	–
Arabinose	–	+	+	+	+	+	–	+
L-Fucose	–	NR	+	+	NR	NR	NR	–
D-Mannose	+	+	+	+	–	+	–	–
Melibiose	–	+	+	–	–	NR	+	–
L-Rhamnose	–	+	+	+	–	NR	+	–
Sucrose	–	–	+	+	–	NR	+	+
N-Acetylglucosamine	–	+	+	+	NR	NR	+	–
Salicin	–	–	+	+	–	NR	+	–

*Data from Busse *et al.* (2005).

†Data from Takeuchi *et al.* (2001).

left to right) and chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, v/v) for the second dimension (from bottom to top), and detected by spraying the plate with specific reagents, as described by Tindall (1990). The designations of all spots were as referred to by Busse *et al.* (1999). Polar lipids of strain K4^T comprised diphosphatidylglycerol (DPG), phosphatidylcholine (PC), phosphatidyl-dimethyl-ethanolamine (PDE), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), sphingoglycolipid (SGL) and unknown phospholipids (PL). The polar lipid profile of strain K4^T resembles those of *Sphingomonas* species, as it contains PE, PG, DPG and SGL, which all sphingomonads contain (Busse *et al.*, 1999), as well as PDE, PC and PL.

For analysis of polyamines, cells were cultivated aerobically in PYE medium (DSM medium 655) at 30 °C. Polyamines were extracted according to Busse & Auling (1988) and analysed by using the instrumentation described by Stolz *et al.* (2007). The polyamine pattern of strain K4^T consisted of the major compound *sym*-homospermidine [17.9 μmol (g dry weight)^{–1}] and only minor amounts of putrescine [0.1 μmol (g dry weight)^{–1}], spermidine [1.7 μmol (g dry weight)^{–1}] and spermine [1.4 μmol (g dry weight)^{–1}]. This polyamine pattern is in agreement with the traits of the genus *Sphingomonas* (Busse *et al.*, 1999, 2003; Takeuchi *et al.*, 2001).

Results from 16S rRNA gene sequence analysis, DNA–DNA relatedness, physiological and biochemical tests indicated genotypic and phenotypic differences between strain K4^T and other *Sphingomonas* species. For these reasons, it is concluded that strain K4^T (=KCTC 22050^T=DSM 19475^T) represents a novel species of the genus *Sphingomonas*, for which the name *Sphingomonas aestuarii* sp. nov. is proposed.

Description of *Sphingomonas aestuarii* sp. nov.

Sphingomonas aestuarii (a.es.tu.a'ri.i. L. gen. n. *aestuarii* of a tidal flat).

Cells are Gram-negative, non-motile, non-spore-forming and rod-shaped, forming yellow, round colonies with a diameter of 1.0 mm after 2 days incubation on R2A agar plates at 30 °C. Growth also occurs on TSA, LA, MA and NA. Growth occurs at 0–5% (w/v) NaCl and 20–35 °C, with optimal growth occurring at 0% (w/v) NaCl and 30 °C. Nitrate is not reduced to nitrite, indole is not produced and glucose fermentation does not occur. Catalase- and oxidase-positive and arginine dihydrolase- and urease-negative. Gelatin hydrolysis occurs, but starch hydrolysis does not. According to the API 20NE and API

50CH systems, D-glucose, D-fructose, D-mannose, amygdalin, arbutin, aesculin, cellobiose, maltose, starch, gentiobiose, turanose and 5-ketogluconate are assimilated, but glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl- β -D-xyloside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl- α -D-mannoside, methyl- α -D-glucoside, N-acetylglucosamine, salicin, D-lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate, potassium gluconate, capric acid, adipic acid, malate, trisodium citrate and phenylacetic acid are not assimilated. Assays using the API ZYM system demonstrated activities for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -galactosidase, α -glucosidase and β -glucosidase. In contrast, lipase (C14), trypsin, α -chymotrypsin, α -galactosidase, β -glucuronidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase activities were not observed. Predominant fatty acids are C_{18:1} ω 7c, 11-Me-C_{18:1} ω 7c and C_{16:0}. Also present are C_{14:0}, C_{15:0}, C_{14:0} 2-OH, summed feature 3 (containing C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH), C_{16:1} ω 5c, C_{15:0} 2-OH, C_{17:1} ω 6c, C_{17:0}, C_{18:1} ω 5c and C_{18:0}. The polar lipid fraction consists of DPG, PC, PDE, PE, PG, SGL and PL. The polyamine pattern contains the predominant compound *sym*-homospermidine.

The type strain is K4^T (=KCTC 22050^T=DSM 19475^T), isolated from tidal flat sediment in Yeosu (34° 47' 26" N 127° 34' 01" E), Korea.

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