Sphingobacterium daejeonense sp. nov., isolated from a compost sample

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A Gram-negative, strictly aerobic, rod-shaped, non-motile, non-spore-forming bacterial strain, designated TR6-04^T, was isolated from compost and characterized taxonomically by using a polyphasic approach. The organism grew optimally at 30 °C and at pH 6.5–7.0. The isolate was positive for catalase and oxidase tests but negative for gelatinase, indole and H₂S production. Comparative 16S rRNA gene sequence analysis showed that strain TR6-04^T fell within the radiation of the cluster comprising *Sphingobacterium* species and clustered with *Sphingobacterium mizutaii* ATCC 33299^T (96.7 % sequence similarity); the similarity to sequences of other species within the family *Sphingobacteriaceae* was less than 92.0 %. The G + C content of the genomic DNA was 38.7 mol%. The predominant respiratory quinone was MK-7. The major fatty acids were iso-C_{15:0}, iso-C_{17:0} 3-OH and summed feature 4 (iso-C_{15:0} 2-OH and/or C_{16:1} ω7c). These chemotaxonomic data supported the affiliation of strain TR6-04^T to the genus *Sphingobacterium*. However, on the basis of its phenotypic properties and phylogenetic distinctiveness, strain TR6-04^T (=KCTC 12579^T = LMG 23402^T = CCUG 52468^T) should be classified as the type strain of a novel species, for which the name *Sphingobacterium daejeonense* sp. nov. is proposed.

The genus *Sphingobacterium* was created by Yabuuchi *et al.* (1983), who reclassified two former *Flavobacterium* species (Holmes *et al.*, 1981, 1982) as *Sphingobacterium multivorum* and *Sphingobacterium spiritivorum* and proposed a novel species, *Sphingobacterium mizutae* [Holmes *et al.* (1988) corrected the spelling of the specific epithet to *mizutaii*]. At present, the genus *Sphingobacterium* also includes three other species, *Sphingobacterium antarcticum* (Shivaji *et al.*, 1992) [the original spelling of the specific epithet, *antarcticus*, was corrected by Euzéby (1998)], *Sphingobacterium thalpophilum* (previously classified as *Flavobacterium thalpophilum*) and *Sphingobacterium faecium* (Takeuchi & Yokota, 1992). Two other previously described species, [*Sphingobacterium*] *heparinum* and [*Sphingobacterium*] *pis-cium* (Takeuchi & Yokota, 1992), were reclassified in the genus *Pedobacter* (Steyn *et al.*, 1998). *Sphingobacterium* species have been isolated from Antarctic soil and from clinical specimens, including blood, urine and the uterus of human patients with opportunistic infections (Holmes *et al.*, 1982; Yabuuchi *et al.*, 1983; Shivaji *et al.*, 1992). Members of the genus are Gram-negative rods that are positive for catalase and oxidase, negative for heparinase, gelatinase and indole production and contain iso-C_{15:0},

iso-C_{15:0} 2-OH, C_{16:1} ω7c and C_{17:0} 3-OH as the main fatty acids (Takeuchi & Yokota, 1992; Steyn *et al.*, 1998).

In this study, we have characterized an aerobic, non-spore-forming strain, TR6-04^T, isolated from compost. Phenotypic, chemotaxonomic and phylogenetic analyses establish the affiliation of the isolate to the genus *Sphingobacterium*. The data obtained also suggest that the isolate represents a novel species of the genus.

Strain TR6-04^T was originally isolated from compost composed of cow dung and rice straw which was collected near Daejeon city in South Korea. This compost sample was thoroughly suspended in 50 mM phosphate buffer (pH 7.0) and the suspension, following serial dilution, was spread onto R2A agar (Difco) plates. The plates were incubated at 30 °C for 3 weeks. Single colonies on the plates were purified by transferring them onto new plates. The purified colonies were tentatively identified by analysis of partial 16S rRNA gene sequences. Strain TR6-04^T was one of the isolates that predominated on the R2A agar plates under aerobic conditions. Strain TR6-04^T was routinely cultured on R2A agar at 30 °C and maintained as a glycerol suspension (20 %, w/v) at –70 °C.

A nearly complete 16S rRNA gene sequence of strain TR6-04^T was determined as follows. DNA was extracted using

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TR6-04^T is AB249372.

a commercial genomic DNA extraction kit (Core Biosystem) and amplification of the 16S rRNA gene by PCR and sequencing of purified PCR product were carried out according to Kim *et al.* (2005). Partial 16S rRNA gene sequences were assembled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Multiple alignments were performed using the CLUSTAL_X program (Thompson *et al.*, 1997). Gaps were edited in BioEdit (Hall, 1999). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were constructed by using the neighbour-joining method (Saitou & Nei, 1987) and the maximum-parsimony method (Fitch, 1972) using the MEGA3 program (Kumar *et al.*, 2004) with bootstrap values based on 1000 replications (Felsenstein, 1985).

The 16S rRNA gene sequence of strain TR6-04^T was a continuous stretch of 1472 bp. Comparative 16S rRNA gene sequence analyses showed that strain TR6-04^T is phylogenetically affiliated to the genus *Sphingobacterium*. The phylogenetic tree based on the neighbour-joining algorithm showed that strain TR6-04^T fell within the radiation of the cluster comprising *Sphingobacterium* species and joined *Sphingobacterium mizutaii* ATCC 33299^T (96.7% similarity) with a bootstrap resampling value of 100% (Fig. 1). Strain TR6-04^T exhibited less than 92.0% 16S rRNA gene sequence similarity with respect to the type strains of other recognized *Sphingobacterium* species. The phylogenetic definition of a species generally includes strains with approximately 70% or greater DNA–DNA relatedness (Wayne *et al.*, 1987). According to the available compilation of data, organisms that have less than 97.0% 16S rRNA gene sequence similarity will not reassociate to more than 60%, irrespective of the hybridization method applied (Stackebrandt & Goebel, 1994; Keswani & Whitman, 2001). This phylogenetic result demonstrated that strain TR6-04^T was not related to any of the previously described *Sphingobacterium* taxa at the species level.

A 16S rRNA gene sequence of the type strain of *S. antarcticum* was not available. Instead, we noticed a 16S rRNA gene sequence from *S. antarcticum* strain 6B1Y (strain 6BY in the original paper of Shivaji *et al.*, 1992),

which had shown 100% DNA–DNA relatedness with strain 4BY^T, the type strain of *S. antarcticum* (Shivaji *et al.*, 1992; S. Shivaji, personal communication). Thus this sequence can represent the species *S. antarcticum*. However, neighbour-joining trees showed that strain 6B1Y clustered in the genus *Pedobacter* and showed close affiliation with *Pedobacter piscium* DSM 11725^T (Fig. 1). Strain 6B1Y and *P. piscium* DSM 11725^T showed high 16S rRNA gene sequence similarity (99.9%) in a pairwise comparison. The 16S rRNA gene of strain 6B1Y was sequenced only after the original description of the genus *Pedobacter* was published. Taxonomic rearrangement is required for this species.

The Gram reaction was performed by the non-staining method as described by Buck (1982). Cell morphology was observed under a Nikon light microscope at $\times 1000$, with cells grown for 3 days at 30 °C on R2A agar. Catalase activity was determined by bubble production in 3% (v/v) H₂O₂ and oxidase activity was determined using 1% (w/v) tetramethyl *p*-phenylenediamine. For single-carbon-source assimilation studies, a defined liquid medium containing basal salts was used (l⁻¹): K₂HPO₄ 1.8 g, KH₂PO₄ 1.08 g, NaNO₃ 0.5 g, NH₄Cl 0.5 g, KCl 0.1 g, MgSO₄ 0.1 g, CaCl₂ 0.05 g. To this medium, a vitamin solution (Widdel & Bak, 1992), trace element solution SL-10 (Widdel *et al.*, 1983) and selenite/tungstate solution (Tschech & Pfennig, 1984) were added and the pH of the medium was adjusted to 6.8. This liquid medium was aliquotted into 96-well trays and filter-sterilized carbon sources were added into each well (individually at 0.1% w/v). The plates were incubated at 30 °C for 7 days and growth in was examined visually. A negative control well did not contain an added carbon source, and a positive control well contained R2A broth. Some physiological characteristics were determined with API 20E galleries according to the instructions of the manufacturer (bioMérieux). Anaerobic growth was examined in serum bottles with sodium thioglycolate (1 g l⁻¹) added to R2A broth and the upper air layer replaced with nitrogen gas. For examination of the reduction of nitrate as the final electron acceptor under anaerobic conditions, 10 mM KNO₃ was added to the medium for anaerobic growth and the reduction of nitrate was monitored by using an ion chromatograph (model 790 personal IC;

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strain TR6-04^T among strains of the genera *Sphingobacterium* and *Pedobacter*. GenBank accession numbers are shown in parentheses. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at branch points. Dots indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. Bar, 0.01 substitutions per nucleotide position.

Metrohm) equipped with a conductivity detector and an anion-exchange column (Metrosep Anion Supp 4; Metrohm). Degradation of DNA (using DNase agar from Scharlau, supplemented with 1 M HCl), casein, chitin and starch (Atlas, 1993), lipid (Kouker & Jaeger, 1987) and xylan and cellulose (Ten *et al.*, 2004) was also investigated; reactions were read after 5 days. Growth at different temperatures (4, 15, 25, 30, 37 and 42 °C) and various pH values (pH 5.0–10.0 at intervals of 0.5 pH units) was assessed after 5 days incubation. Salt tolerance was tested on R2A medium supplemented with 1–10 % (w/v) NaCl after 5 days incubation. Growth on nutrient agar, trypticase soy agar (TSA; Difco) and MacConkey agar was also evaluated at 30 °C.

Strain TR6-04^T is a Gram-negative, non-spore-forming, non-motile and rod-shaped bacterium. After 3 days incubation at 30 °C, colonies on R2A agar medium are round, slightly yellowish, convex and 1.0–2.0 mm in diameter. On R2A agar, the optimal growth temperature for TR6-04^T was 30 °C. Strain TR6-04^T was able to grow at 42 °C, but not at 10 or 45 °C. The pH growth range was between pH 5.0 and 9.0, with an optimum between pH 6.5 and 7.0. Growth occurred in the absence of NaCl and in the presence of 5.0 % (w/v) NaCl, but not 7 % (w/v) NaCl. Phenotypic and chemotaxonomic characteristics that differentiate strain TR6-04^T from related *Sphingobacterium* species are listed in Table 1. In contrast to all other *Sphingobacterium* species, strain TR6-04^T assimilates L-xylose, does not

Table 1. Differential phenotypic characteristics of strain TR6-04^T and related type strains of *Sphingobacterium* species

Strains: 1, *S. daejeonense* sp. nov. TR6-04^T (data from the present study); 2, *S. spiritivorum* NBRC 14948^T; 3, *S. multivorum* NBRC 14947^T; 4, *S. mizutaii* NBRC 14946^T; 5, *S. thalpophilum* NBRC 14963^T; 6, *S. faecium* NBRC 15299^T (data in columns 2–6 taken from Takeuchi & Yokota, 1992; Steyn *et al.*, 1998); 7, *S. antarcticum* MTCC 675^T (data from Shivaji *et al.*, 1992). All strains are positive for aerobic growth at 30 °C, catalase and oxidase activities, assimilation of D-glucose, D-mannose, D-raffinose, D-fructose, L-lactose, D-maltose and sucrose and acid production from D-glucose. All strains are negative for Gram staining, sporulation, indole production, motility, assimilation of acetate and acid production from inositol. With the exception of *S. antarcticum* MTCC 675^T, all strains are positive for assimilation of D-cellobiose, D-trehalose, amygdalin, N-acetyl-D-glucosamine and salicin and acid production from D-melibiose and amygdalin, and all strains are negative for assimilation of D-fucose, L-sorbose, gluconate, dulcitol, xylitol, propionate, valerate, caprate, phenylacetate, benzoate, 3-hydroxybenzoate, maleate, DL-lactate, glutarate, tartrate, itaconate, adipate, suberate, oxalate, glycine, L-alanine, L-leucine and L-valine (data are not reported for *S. antarcticum*). +, Positive; –, negative; V, variable; ND, no data available.

Characteristic	1	2	3	4	5	6	7
Growth at:							
5 °C	–	–	–	–	–	+	+
42 °C	+	–	–	–	+	–	–
Hydrolysis of:							
DNA	–	+	+	+	–	+	ND
Starch	–	+	+	+	+	+	–
Aesculin	–	+	+	+	+	+	+
Gelatin	–	–	–	–	–	–	+
Urease	–	+	+	+	+	+	+
Assimilation of:							
L-Rhamnose	–	+	+	–	+	+	+
L-Arabinose	–	–	+	V	+	+	+
D-Mannitol	–	+	–	–	–	–	+
D-Melibiose	+	+	+	+	+	+	–
Glycerol	–	V	–	–	+	+	+
Inulin	–	V	V	–	+	+	+
Pyruvate	–	–	–	V	–	–	+
L-Glutamate	–	–	+	+	–	+	+
Acid production from:							
L-Rhamnose	–	–	V	–	+	–	–
L-Arabinose	–	–	+	+	+	+	–
Sucrose	–	+	+	+	+	+	–
DNA G + C content (mol%)	38.7	39.0	39.9–40.5	39.3–40.0	44.0–44.2	37.3	39.3

hydrolyse aesculin and does not produce urease. Strain TR6-04^T could be distinguished from *S. antarcticum* MTCC 675^T on the basis of its inability to assimilate L-rhamnose, L-arabinose, D-ribose, pyruvate, formate, DL-3-hydroxybutyrate, lactate, malate, succinate, inositol, D-mannitol, D-sorbitol, glycerol and L-glutamate, to produce gelatinase and hydrogen sulphide, to grow at temperatures below 15 °C and to grow on MacConkey agar.

For the measurement of G + C content of the chromosomal DNA, genomic DNA was extracted and purified as described by Moore (1995) and was then enzymically degraded into nucleosides. G + C content was determined as described by Mesbah *et al.* (1989) using a reverse-phase HPLC. Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under vacuum and re-extracted in n-hexane/water (1:1, v/v). The crude n-hexane quinone solution was then purified using silica Sep-Pak Vac

cartridges (Waters) and subsequently analysed by HPLC, as described previously (Hiraishi *et al.*, 1996).

Cellular fatty acid profiles were determined for strains grown on TSA for 2 days. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI) and then analysed by a gas chromatograph (Hewlett Packard 6890) and identified by the Microbial Identification software package (Sasser, 1990). The cellular fatty acid profiles of strain TR6-04^T and related *Sphingobacterium* species are presented in Table 2. Strain TR6-04^T contained large amounts of iso-branched and iso-branched hydroxy fatty acids; the major components were iso-C_{15:0}, iso-C_{17:0} 3-OH and summed feature 4 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c), typical of members of the genus *Sphingobacterium* (Takeuchi & Yokota, 1992; Steyn *et al.*, 1998). Some qualitative and quantitative differences in fatty acid

Table 2. Fatty acid compositions of strain TR6-04^T and related *Sphingobacterium* species

Strains: 1, *S. daejeonense* TR6-04^T (data from the present study); 2, *S. spiritivorum* NBRC 14948^T; 3, *S. multivorum* NBRC 14947^T; 4, *S. mizutaii* NBRC 14946^T; 5, *S. thalpophilum* NBRC 14963^T; 6, *S. faecium* NBRC 15299^T (data in columns 2–6 were taken from Steyn *et al.*, 1998); 7, *S. antarcticum* MTCC 675^T (data from Shivaji *et al.*, 1992). Values are percentages of total fatty acids. +, Fatty acid detected but its content was not reported; tr, trace (<1.0%); –, not detected; ECL, equivalent chain-length (for unknown fatty acids with no name listed in the peak library file of the MIDI system which therefore cannot be identified). Major components (>15%) are indicated in bold.

Fatty acid	1	2	3	4	5	6	7
Straight-chain saturated							
C _{14:0}	—	1.0	2.7	tr	3.2	tr	+
C _{16:0}	3.4	3.5	7.8	tr	6.0	4.5	+
C _{16:0} 2-OH	—	—	tr	—	3.2	—	—
C _{16:0} 3-OH	—	2.7	5.3	tr	6.3	2.1	—
Branched saturated							
iso-C _{15:0}	45.6	30.1	22.2	30.0	17.7	24.6	29.0
iso-C _{17:0}	tr	—	—	—	—	—	—
iso-C _{15:0} 2-OH	—*	21.5	17.4	25.6	24.6	15.9	tr
iso-C _{15:0} 3-OH	1.5	2.2	3.2	3.0	4.3	3.7	tr
iso-C _{17:0} 3-OH	16.6	12.5	7.1	22.1	10.0	10.0	tr
anteiso-C _{15:0}	2.6	tr	—	tr	—	tr	—
C _{16:0} 10-methyl	—	—	—	—	—	1.4	—
Summed feature 4†	23.8	—	—	—	—	—	—
Monounsaturated							
iso-C _{15:1} G	tr	—	—	—	—	—	—
C _{16:1} ω5c	tr	tr	—	tr	—	1.5	—
C _{16:1} ω7c	—*	21.1	31.6	9.5	23.2	32.2	56.0
iso-C _{17:1} ω9c	2.9	1.7	tr	3.7	—	—	—
C _{17:1}	—	—	—	—	—	—	+
Unknown							
ECL 13:566	1.0	tr	—	1.3	1.4	tr	—
ECL 16:580	tr	tr	tr	tr	—	tr	—

*Components included in summed feature 4.

†Summed features represent groups of two or three fatty acids that can not be separated by GLC with the MIDI system. Summed feature 4 contains iso-C_{15:0} 2-OH and/or C_{16:1}ω7c.

contents could be observed between strain TR6-04^T and the phylogenetically closest relative, *S. mizutaii* ATCC 33299^T. In comparison with this strain, strain TR6-04^T contained larger amounts of iso-C_{15:0} and smaller amounts of iso-C_{15:0} 2-OH and/or C_{16:1}ω7c and it did not contain C_{14:0} or C_{16:0} 3-OH. The G+C content of genomic DNA for members of the genus *Sphingobacterium* ranges from 37.3 mol% (reported for *S. faecium*) to 44.2 mol% (reported for *S. thalpophilum*) (Takeuchi & Yokota, 1992; Steyn *et al.*, 1998). The G+C content of the DNA of strain TR6-04^T was 38.7 mol% and falls within this range.

All of the characteristics determined for strain TR6-04^T are in accordance with those of the genus *Sphingobacterium*. On the basis of phylogenetic distance from established *Sphingobacterium* species, also indicated by relatively low 16S rRNA gene sequence similarities (<97%) and the combination of unique phenotypic characteristics, it is demonstrable that TR6-04^T is not affiliated with any species of this genus. Therefore, on the basis of the data presented, strain TR6-04^T should be placed in the genus *Sphingobacterium* as the type strain of a novel species, for which the name *Sphingobacterium daejeonense* sp. nov. is proposed.

Description of *Sphingobacterium daejeonense* sp. nov.

Sphingobacterium daejeonense (dae.jeon.en'se. N.L. neut. adj. *daejeonense* pertaining to Daejeon, a city in Korea, where the type strain was isolated).

Cell are Gram-negative, strictly aerobic, non-spore-forming, non-motile and rod-shaped (0.5–1.0 µm wide and 1.2–1.8 µm long). After 3 days incubation at 30 °C on R2A, colonies are 1–2 mm in diameter, smooth, convex, round and slightly yellowish. Growth occurs between 15 and 42 °C; the optimum temperature for growth is 30 °C. The pH range for growth is pH 5.0–9.0, with an optimum between pH 6.5 and 7.0. Tolerates 5% (w/v) NaCl, but not 7%. Growth occurs on TSA, but not on MacConkey agar. Positive for catalase and oxidase. Negative for hydrolysis of chitin, starch, cellulose, DNA, xylan, casein and aesculin. The following substrates are utilized for growth: D-glucose, D-galactose, D-mannose, D-fructose, D-arabinose, D-xylose, D-xylose, L-xylose, N-acetyl-D-glucosamine, salicin, D-cellobiose, D-lactose, D-maltose, D-melibiose, sucrose, D-trehalose, D-raffinose, D-adonitol and amygdalin. The following substrates are not utilized for growth: D-fucose, ethanol, L-rhamnose, L-sorbose, L-arabinose, D-ribose, pyruvate, formate, acetate, propionate, DL-3-hydroxybutyrate, valerate, caprate, maleate, fumarate, phenylacetate, benzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, citrate, lactate, malate, malonate, succinate, glutarate, tartrate, itaconate, adipate, suberate, oxalate, gluconate, dulcitol, inositol, D-mannitol, D-sorbitol, xylitol, glycerol, methanol, glycogen, inulin, dextran, L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, L-histidine, glycine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine,

L-threonine, L-tryptophan, L-tyrosine and L-valine. In API 20E tests, β-galactosidase and the Voges–Proskauer test are positive; tests for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, urease, gelatinase, hydrogen sulphide and indole production are all negative. Acid is produced from D-glucose, D-melibiose and amygdalin, but not from L-arabinose, D-mannitol, inositol, D-sorbitol, L-rhamnose or sucrose. MK-7 is the predominant menaquinone, and major fatty acids are iso-C_{15:0}, summed feature 4 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c) and iso-C_{17:0} 3-OH. The G+C content of genomic DNA of the type strain is 38.7 mol% (as determined by HPLC).

The type strain, TR6-04^T (=KCTC 12579^T=LMG 23402^T=CCUG 52468^T), was isolated from compost that was collected near Daejeon city in South Korea.

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