Sphingobacterium kyonggiense sp. nov., isolated from chloroethene-contaminated soil, and emended descriptions of *Sphingobacterium daejeonense* and *Sphingobacterium mizutaii*

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A Gram-reaction-negative, strictly aerobic, rod-shaped, non-motile strain, designated 2-1-2^T, was isolated from perchloroethylene/trichloroethene-contaminated soil in Suwon, South Korea. A polyphasic approach was used to study the taxonomic position of strain 2-1-2^T. Strain 2-1-2^T showed highest 16S rRNA gene sequence similarities to *Sphingobacterium daejeonense* TR6-04^T (97.9 %) and *Sphingobacterium mizutaii* ATCC 33299^T (97.1 %); sequence similarities to other *Sphingobacterium* species were less than 93.0 %. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain 2-1-2^T belonged to the clade formed by members of the genus *Sphingobacterium* in the family *Sphingobacteriaceae*. The G+C content of the genomic DNA was 36.6 mol%. Strain 2-1-2^T showed the typical chemotaxonomic features of the genus *Sphingobacterium*, with the presence of a ceramide phosphorylethanolamine (CerPE-2) as the major ceramide, menaquinone 7 (MK-7) as the predominant respiratory quinone and iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and summed feature 3 (comprising iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$) as the major fatty acids. On the basis of phylogenetic inference, fatty acid profile and other phenotypic properties, and DNA–DNA relatedness, strain 2-1-2^T represents a novel species of the genus *Sphingobacterium*, for which the name *Sphingobacterium kyonggiense* sp. nov. is proposed; the type strain is 2-1-2^T (=KEMC 2241-005^T=JCM 16704^T). Emended descriptions of *Sphingobacterium daejeonense* and *Sphingobacterium mizutaii* are also proposed.

The genus *Sphingobacterium* (family *Sphingobacteriaceae*, phylum *Bacteroidetes*) was first described by Yabuuchi *et al.* (1983), with three species *Sphingobacterium spiritivorum* (type species), *S. multivorum* and *S. mizutae* (later corrected to *S. mizutaii*). The following species with validly published names were subsequently described: *S. antarcticum*, *S. canadense*, *S. composti*, *S. daejeonense*, *S. faecium*, *S. kitahiroshimense*, *S. siyangense*, *S. thalpophilum*, *S. anhuiense* and *S. shayense* (Shivaji *et al.*, 1992; Mehnaz *et al.*, 2007; Ten *et al.*, 2006; Yoo *et al.*, 2007; Kim *et al.*, 2006; Takeuchi & Yokota, 1992; Steyn *et al.*, 1998; Holmes *et al.*, 1983; Matsuyama *et al.*, 2008; Liu *et al.*, 2008; Wei *et al.*, 2008). The name *Sphingobacterium composti* was actually proposed for two different strains (Ten *et al.*, 2006; Yoo *et al.*, 2007). As the name *S. composti* Yoo *et al.* 2007 is considered a later homonym of *S. composti* Ten *et al.*, 2006 (http://www.bacterio.cict.fr/s/sphingobacterium.html), only the latter species was considered in this study. Most members of the genus *Sphingobacterium* occur in freshwater, soil and compost, but some strains also occur in clinical specimens.

Perchloroethylene (PCE, Perc or tetrachloroethene), used mainly for fabric dry cleaning and metal degreasing, is one of the most commonly detected solvents in groundwater (Moran *et al.*, 2007). PCE and its metabolite dichloroacetylene are recognized as human and animal neurotoxins. These fat-soluble substances have a high affinity for the lipophilic tissues of the central nervous system (Altmann *et al.*, 1995). PCE also readily crosses both the placental and blood–brain barriers (Klaassen, 2001). Chloroethenes are known to be reductively dechlorinated by anaerobic bacteria utilizing chloroethenes as electron acceptors in their respiration (Yoshida *et al.*, 2007). Microbial reductive dechlorination is an effective process to remediate environments contaminated with PCE and trichloroethene (TCE).

In the course of collecting micro-organisms from PCE/TCE-contaminated soil, a Gram-reaction-negative, non-motile,

Abbreviations: AL, aminolipid; APL, aminophospholipid; CerPE, ceramide phosphorylethanolamine; CerPI, ceramide phosphoryl-*myo*-inositol; CerPM-1, ceramide phosphoryl-1-β-mannose; GL, glycolipid; GPL, glycophospholipid; PCE, perchloroethylene; PE, phosphatidylethanolamine; PL, phospholipid; TCE, trichloroethene.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 2-1-2^T is GU358699.

A supplementary figure is available with the online version of this paper.

aerobic, non-spore-forming strain, named 2-1-2^T, was isolated. On the basis of 16S rRNA gene sequence analysis, strain 2-1-2^T was considered to belong to the genus *Sphingobacterium*. Strain 2-1-2^T was subjected to a polyphasic taxonomic investigation and the results indicated it represents a novel *Sphingobacterium* species.

Strain 2-1-2^T was originally isolated from PCE/TCE-contaminated soil collected near Suwon city (37° 16′ N 127° 03′ E) in South Korea. One gram of soil sample was mixed with 50 ml saline solution (0.85 % NaCl), vortexed and serially diluted; 100 µl of each dilution was spread onto 0.1 × R2A agar (Difco) and incubated at 30 °C for 1 week. Single colonies of strain 2-1-2^T were transferred onto new plates and the purified colonies were tentatively identified by analysis of partial 16S rRNA gene sequences. The isolate was routinely cultured on nutrient agar (NA; Difco) at 30 °C and preserved as a suspension in Luria–Bertani (LB) broth (Difco) with 20 % glycerol (w/v) at −70 °C.

The genomic DNA of strain 2-1-2^T was extracted using a commercial genomic DNA extraction kit (Solgent) and PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to Kim *et al.* (2005). The nearly complete sequence of the 16S rRNA gene (1467 bp) was compiled with SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from GenBank and edited using the program BioEdit (Hall, 1999). Multiple alignments were performed with the program CLUSTAL_X (Thompson *et al.*, 1997). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods in the program MEGA3 (Kumar *et al.*, 2004) with bootstrap values based on 1000 replications (Felsenstein, 1985) (Fig. 1).

On the basis of 16S rRNA gene sequence similarities, the closest relatives to strain 2-1-2^T were *S. daejeonense* TR6-04^T (97.9 %) and *S. mizutaii* ATCC 33299^T (97.1 %). Sequence similarities to other *Sphingobacterium* species were ⩽93 %. Consequently, the type strains of *S. daejeonense* and *S. mizutaii* were obtained and grown under the same conditions as strain 2-1-2^T and used as reference strains for DNA–DNA hybridization experiments and phenotypic tests.

Cell morphology and motility were observed with an Olympus light microscope (1000 × magnification) using cells grown for 24 h in nutrient broth at 30 °C. For electron microscopy, cells were gently resuspended in 1 drop deionized H_2O and samples were placed on carbon- and Formvar-coated nickel grids for 30 s. Grids were floated on 1 drop of 0.1 % (w/v) aqueous uranyl acetate, blotted dry and then viewed with a Carl Zeiss LEO912AB EM at 100 kV under standard operating conditions. The Gram reaction was performed by using the non-staining method, as described by Buck (1982). Catalase activity was assessed by bubble production in 3 % (v/v) H_2O_2 and oxidase activity was assessed using 1 % (w/v) tetramethyl *p*-phenylenediamine (Cappuccino & Sherman, 2002). Anaerobic growth was examined in serum bottles with sodium thioglycolate (1 g l^{-1}) added to nutrient broth and the upper air layer was replaced with nitrogen. Physiological characteristics were determined with API 20E, API 20NE, API ID 32 GN and API ZYM galleries (bioMérieux) according to the instructions of the manufacturer. Growth at 4, 10, 20, 25, 28, 30, 37, 40, 42 and 45 °C and at pH 3.0–12.0 (at 1.0 pH unit intervals) was assessed in LB broth (Difco) for 5 days. The pH of the medium was adjusted using diluted NaOH and HCl. Salt tolerance was tested in R2A broth supplemented with 0–10 % (w/v) NaCl incubated for 5 days at 30 °C. Degradation of DNA (DNase agar; Scharlau), casein, chitin, starch (Atlas, 1993), xylan and CM-cellulose (Ten *et al.*, 2004) was also investigated; reactions were read after 5 days. Growth on trypticase soy agar (TSA; Difco), R2A agar and LB agar was also evaluated at 30 °C. The phenotypic characteristics of strain 2-1-2^T are given in the species description and those that differentiated strain 2-1-2^T from the two most closely related *Sphingobacterium* species are listed in Table 1.

Isoprenoid quinones were extracted from bacterial cells of strain 2-1-2^T 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). Strain 2-1-2^T contained menaquinone 7 (MK-7) as the major respiratory quinone, in line with all members of the family *Sphingobacteriaceae*. The presence of sphingolipids is listed in the formal descriptions of the genus *Sphingobacterium* (Yabuuchi *et al.*, 1983) and of the family *Sphingobacteriaceae* (Steyn *et al.*, 1998). *S. spiritivorum*, the type species of the genus *Sphingobacterium*, was shown to contain the sphingophospholipids ceramide phosphorylethanolamine (CerPE-1 and CerPE-2), ceramide phosphoryl-*myo*-inositol (CerPI-1 and CerPI-2) and ceramide phosphoryl-1-β-mannose (CerPM-1). These sphingophospholipids were also found among the cellular lipids of several other *Sphingobacterium* species (Naka *et al.*, 2003). For detection of alkaline-stable sphingolipids, the total lipids were extracted from strain 2-1-2^T, *S. daejeonense* KCTC 12579^T and *S. mizutaii* KACC 12159^T using Folch's method (Folch *et al.*, 1957). One portion of the crude lipids was treated with 0.5 M KOH in chloroform/methanol (1 : 1, v/v) at 37 °C for 2 h. The reaction was stopped by neutralization with 2 M HCl. Alkaline-stable lipids were dried, redissolved in chloroform/methanol (2 : 1 v/v) and separated by TLC on silica gel with the acidic solvent system chloroform/methanol/acetic acid/water (100 : 20 : 12 : 5, by vol.) (Naka *et al.*, 2003). Lipids were observed after staining the TLC plates with ninhydrin and 10 % ethanolic molybdophosphoric acid. Sphingolipids of strain 2-1-2^T were identified by comparing the R_f values in the TLC with those of the reference strains. The TLC of the alkaline-stable sphingophospholipids (Fig.

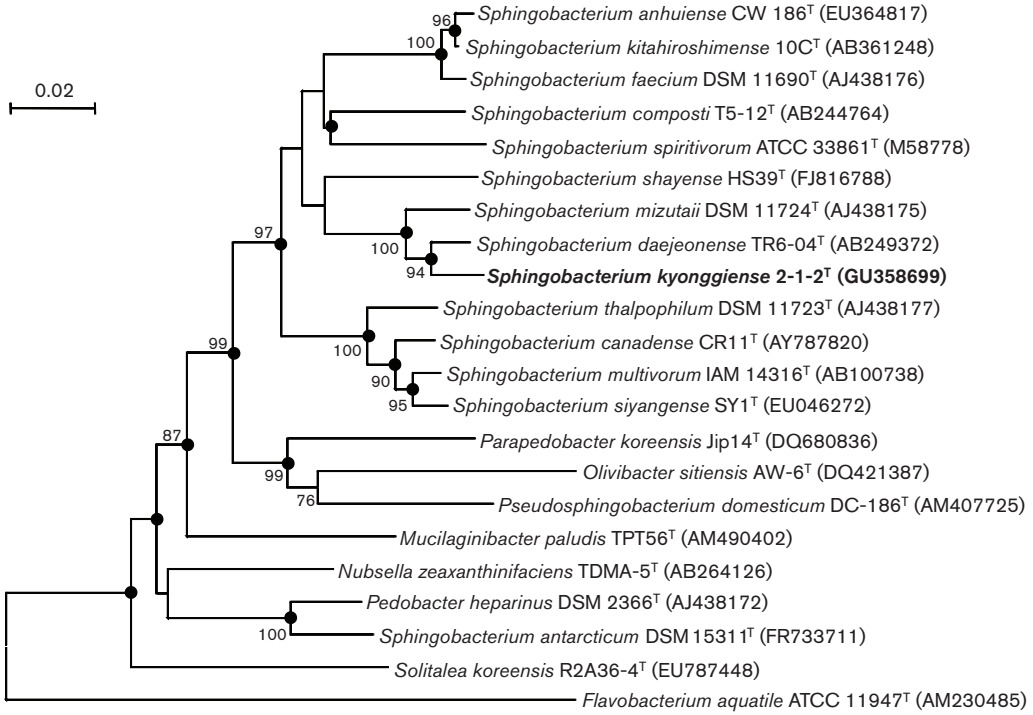

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain 2-1-2^T, other *Sphingobacterium* species and representatives of other members of the family *Sphingobacteriaceae*. Bootstrap values >70 % (percentages of 1000 replications) are shown at branching points. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. Bar, 0.02 substitutions per nucleotide position. *Flavobacterium aquatile* ATCC 11947^T was used as an outgroup.

Table 1. Differential characteristics of strain 2-1-2^T and closely related members of the genus *Sphingobacterium*

Strains: 1, *S. kyonggiense* sp. nov. 2-1-2^T; 2, *S. daejeonense* KCTC 12579^T; 3, *S. mizutaii* KACC 12159^T. All data are from this study, except the DNA G+C contents of the two reference strains (taken from Kim *et al.*, 2006). All strains are Gram-reaction-negative and non-motile. None of them reduce nitrate or produce acid from glucose. +, Positive reaction; −, negative reaction; w, weakly positive reaction.

Characteristic	1	2	3
Growth at pH 5.0	−	+	−
Growth at 45 °C	−	+	−
Production of indole	+	−	−
Voges–Proskauer test	+	−	+
Enzyme activity (API ZYM)			
N-Acetyl-β-glucosaminidase	+	+	−
Cystine arylamidase	+	+	−
Esterase (C4)	+	−	w
Trypsin	w	w	+
Assimilation of (API ID 32GN):			
Potassium 5-ketogluconate	−	w	−
L-Arabinose	−	+	−
Maltose	w	+	+
L-Rhamnose	−	+	−
L-Proline	w	−	−
DNA G+C content (mol%)	36.6	38.7	39.3–40.0

2) showed that strain 2-1-2^T displays a ceramide pattern similar to that of *S. mizutaii* KACC 12159^T, i.e. CerPE-1, CerPE-2, CerPI-1, CerPI-2 and CerPM-1 were detected, which is in line with the profile reported by Naka *et al.* (2003) for *S. mizutaii*. In *S. daejeonense* KCTC 12579^T, CerPE-1 was not detected but the other four ceramides were present. For polar lipid analysis of strain 2-1-2^T, *S. daejeonense* KCTC 12579^T and *S. mizutaii* KACC 12159^T, the total cellular polar lipids were extracted and examined by two-dimensional TLC (Minnikin *et al.*, 1977). The total lipids were revealed by spraying one plate with 5 % ethanolic molybdophosphoric acid and the different spots were identified by spraying specific reagents on other plates (Fig. S1, available in IJSEM Online). The results of TLC showed that strain 2-1-2^T contained a major amount of phosphatidylethanolamine (PE) and moderate to minor amounts of four unknown aminolipids (AL_1–AL_4), one unknown glycolipid (GL), one unknown phospholipid (PL), five unknown aminophospholipids (APL_1–APL_5), one unknown glycophospholipid (GPL) and two unknown polar lipids (L_1–L_2). *S. daejeonense* KCTC 12579^T and *S. mizutaii* KACC 1259^T also showed PE as the major polar lipid, but most minor polar lipids differed from those of strain 2-1-2^T.

In order to perform fatty acid methyl ester analysis, strain 2-1-2^T, *S. daejeonense* KCTC 12579^T and *S. mizutaii* KACC 12159^T were grown on TSA for 48 h at 30 °C. Two loopfuls

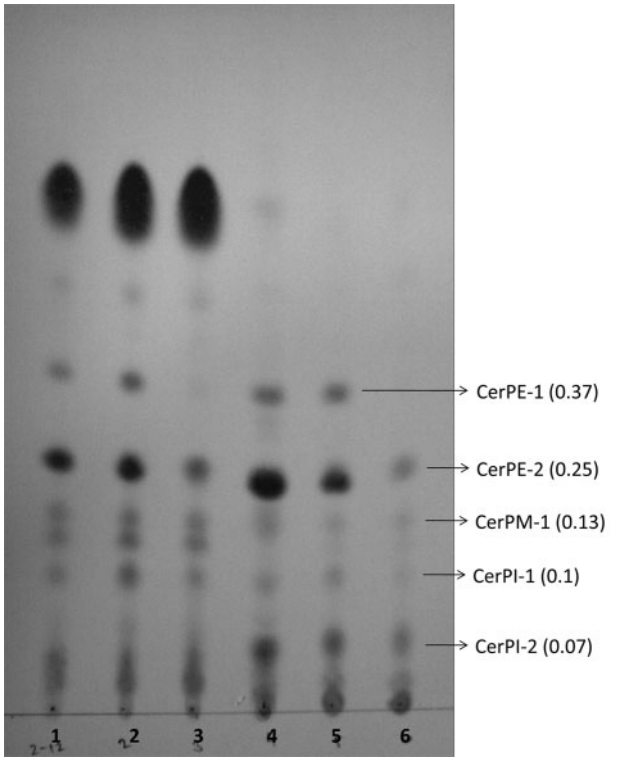

Fig. 2. TLCs of the total lipids and alkaline-stable lipids. Lanes; 1 and 4, strain 2-1-2^T; 2 and 5, *S. mizutaii* KACC 12159^T; 3 and 6, *S. daejeonense* KCTC 12579^T. Lanes 1–3 contain crude total lipids and lanes 4–6 contain alkaline-stable lipids. The TLC plate was stained with ninhydrin and 10 % ethanolic molybdophosphoric acid. Abbreviations: CerPE-1 and CerPE-2, ceramide phosphoryl ethanolamine; CerPI-1 and CerPI-2, ceramide phosphoryl-*myo*-inositol; CerPM-1, ceramide phosphoryl-1-β-mannose. R_f values are given in parentheses.

Table 2. Fatty acid composition (%) of strain 2-1-2^T and closely related members of the genus *Sphingobacterium*

Strains: 1, *S. kyonggiense* sp. nov. 2-1-2^T; 2, *S. daejeonense* KCTC 12579^T; and 3, *S. mizutaii* KACC 12159^T. All strains were incubated on TSA for 2 days at 30 °C. For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain. The *cis* and *trans* isomers are indicated by the suffixes *c* and *t*, respectively. Fatty acids amounting to <1 % of the total fatty acids in the three strains are not shown. tr, Traces (<1 %); ND, not detected; The major components (>10 %) are indicated in bold.

Fatty acid	1	2	3
Straight-chain saturated			
$C_{14:0}$	1.2	ND	ND
$C_{16:0}$	3.2	1.4	tr
$C_{14:0}$ 2-OH	1.0	tr	ND
$C_{16:0}$ 3-OH	6.7	tr	tr
$C_{17:0}$ 2-OH	1.6	3.2	ND
Branched saturated			
iso-$C_{14:0}$	1.0	tr	1.1
iso-$C_{15:0}$	**27.8**	**31.4**	**29.3**
iso-$C_{15:0}$ 3-OH	1.2	1.4	2.0
iso-$C_{16:0}$ 3-OH	1.7	1.0	ND
iso-$C_{17:0}$ 3-OH	**12.5**	**17.2**	**21.4**
anteiso-$C_{15:0}$	5.0	5.9	1.5
Monounsaturated			
iso-$C_{15:1}$ G	ND	1.1	ND
$C_{15:1}\omega6c$	ND	1.4	ND
Summed features*			
3	**29.6**	**28.8**	**27.5**
9	2.4	2.8	3.8

*Summed feature 3 comprised iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$ and summed feature 9 comprised iso-$C_{17:1}\omega9c$ and/or 10-methyl $C_{16:0}$.

of bacterial mass were collected and subjected to saponification, methylation and extraction using the methods of Kuykendall *et al.* (1988). The fatty acid methyl ester mixtures were separated using the Sherlock Microbial Identification System (TSBA, version 6.0; MIDI), and then analysed by GC (Hewlett Packard 6890) and identified by the Microbial Identification software package (Sasser, 1990). The overall fatty acid compositions of the three strains (Table 2) were very similar, dominated by 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 3 (comprising iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$), in line with members of the genus *Sphingobacterium* (Takeuchi & Yokota, 1992; Steyn *et al.*, 1998). However, there were some qualitative and quantitative differences in the fatty acid contents of the three strains. For instance, strain 2-1-2^T contained a significant amount of $C_{16:0}$ 3-OH in contrast to the two reference strains.

For the determination of the DNA G+C content, genomic DNA was extracted as mentioned above and enzymically degraded into nucleosides. The nucleosides were analysed using HPLC as described previously (Tamaoka & Komagata, 1984; Mesbah *et al.*, 1989). DNA–DNA hybridization experiments were performed fluorometrically, according to the method developed by Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and micro-dilution wells. Hybridization was performed at 39.3 °C in the presence of 50 % (v/v) formamide with the ionic strength of 0.3 M NaCl and carried out reciprocally with five replications per sample. The highest and lowest values obtained for each sample were excluded and the remaining three values were utilized for calculation of relatedness; DNA relatedness values are expressed as means of these three values. The DNA G+C content of strain 2-1-2^T was 36.6 mol%, a value within the range reported for *Sphingobacterium* species, i.e. from 36.3 mol% (*S. anhuiense* CW 186^T) to 44.2 mol% (*S. thalpophilum* ATCC 43320^T) (Takeuchi & Yokota, 1992; Steyn *et al.*, 1998; Wei *et al.*, 2008). Strain 2-1-2^T exhibited low levels of DNA relatedness with *S. daejeonense* KCTC 12579^T (15.0 %) and *S. mizutaii* KACC 12159^T (8.0 %),

indicating that it is not related to them at the species level (Wayne *et al.*, 1987).

The results of the polyphasic analyses clearly showed that strain 2-1-2^T represents a novel species within the genus *Sphingobacterium*, for which the name *Sphingobacterium kyonggiense* sp. nov. is proposed. On the basis of new data obtained in this study, emended descriptions of *S. daejeonense* and *S. mizutaii* are also proposed.

Description of *Sphingobacterium kyonggiense* sp. nov.

Sphingobacterium kyonggiense (ky.ong.gi.en′se. N.L. neut. adj. *kyonggiense* of or belonging to Kyonggi, named after Kyonggi University in the Republic of Korea.)

Cells are Gram-reaction-negative, strictly aerobic, non-motile, non-spore-forming rods, 0.5–1.0 μm in diameter and 1.2–2.0 μm in length. After 3 days of incubation at 30 °C on R2A, colonies are 1–2 mm in diameter, pale yellow, smooth, convex and circular with regular edges. Growth occurs at 15–42 °C (optimum, 30 °C). The pH range for growth is pH 6.0–10.0 (optimum, pH 7.0–8.0). Growth occurs in 0–4 % (w/v) NaCl, but not in 6 % (w/v) NaCl. Grows on TSA, LB agar, NA and R2A agar. Positive for catalase and oxidase. Nitrate is not reduced. Chitin, starch, CM-cellulose, DNA, xylan, casein and aesculin are not hydrolysed. In the API ZYM and API 20E strips, *N*-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphate, cystine arylamidase, esterase, esterase lipase, β-galactosidase, α-glucosidase, β-glucosidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase activities are present; weak α-galactosidase and trypsin activities are also present. Arginine dihydrolase, α-chymotrypsin, α-fucosidase, β-glucuronidase, lipase, lysine decarboxylase, α-mannosidase, ornithine decarboxylase, tryptophan deaminase, urease and gelatinase activities are absent. In the API ID32GN strip, *N*-acetylglucosamine, maltose, glycogen, D-glucose, melibiose and L-proline are assimilated but L-rhamnose, D-ribose, inositol, sucrose, itaconic acid, suberic acid, sodium malonate, sodium acetate, lactic acid, L-alanine, potassium 5-ketogluconate, 3-hydroxybenzoic acid, L-serine, D-mannitol, salicin, L-fucose, D-sorbitol, L-arabinose, L-histidine, propionic acid, capric acid, valeric acid, trisodium citrate, potassium 2-ketogluconate, 3-hydroxybutyric acid and 4-hydroxybenzoic acid are not assimilated. In the API 20E strip, acid is produced from D-glucose, melibiose and amygdalin but not from L-arabinose, D-mannitol, inositol, D-sorbitol, L-rhamnose or sucrose. The predominant respiratory quinone is MK-7. The major fatty acids (>12 %) are iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and summed feature 3 (comprising iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$). Contains CerPE-2 as the major sphingophospholipid. Minor amounts of the following ceramides are also present: CerPE-1, CerPI-1, CerPI-2 and CerPM-1. The polar lipid profile includes predominantly PE; moderate to minor amounts of several unknown GLs, ALs, PLs, APLs, GPLs and polar lipids are also present.

The type strain, 2-1-2^T (=KEMC 2241-005^T=JCM 16704^T), was isolated from PCE/TCE-contaminated soil at Suwon city in South Korea. The genomic DNA G+C content is 36.6 mol%.

Emended description of *Sphingobacterium daejeonense* Kim *et al.* 2006

The description is as given by Kim *et al.* (2006) with the following additions. The major polar lipid is PE; moderate to minor amounts of several unknown ALs, PLs, APLs, GPLs and polar lipids are also present. Contains CerPE-2 and CerPI-2 as the major sphingophospholipids; minor amounts of CerPI-1 and CerPM-1 are also present.

Emended description of *Sphingobacterium mizutaii* Yabuuchi *et al.* 1983

The description is as given by Yabuuchi *et al.* (1983) with the following additions. The major polar lipid is PE; moderate to minor amounts of several unknown GLs, ALs, PLs, APLs, GPLs and polar lipids are also present. Contains CerPE-2 as the major sphingophospholipid; minor amounts of CerPE-1, CerPI-1, CerPI-2 and CerPM-1 are also present.

Acknowledgements

This research work was supported by the Korea Ministry of Environment as The GAIA Project (173-092-012).

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