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Sphingosinicella ginsenosidimutans sp. nov., with ginsenoside converting activity

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The Gram-reaction-negative, strictly aerobic, non-motile, non-spore-forming, and rod-shaped bacterial strain designated BS11^T was isolated from the compost and its taxonomic position was investigated by using a polyphasic approach. Strain BS11^T grew optimally at 30–37°C and at pH 7.0 in the absence of NaCl on nutrient agar. Strain BS11^T displayed β-glucosidase activity that was responsible for its ability to transform ginsenoside Rb_1 (one of the dominant active components of ginseng) to Rd. On the basis of 16S rRNA gene sequence similarity, strain BS11^T was shown to belong to the family *Sphingomonadaceae* and was related to *Sphingosinicella vermicomposti* YC7378^T (96.3% sequence similarity), *S. xenopeptidilytica* 3-2W4^T (96.2%), *S. microcystinivorans* Y2^T (96.1%), and *S. soli* KSL-125^T (95.9%). The G+C content of the genomic DNA was 64.9%. The major menaquinone was Q-10 and the major fatty acids were summed feature 7 (comprising $C_{18:1}$ ω7c/ω9t/ω12t; 40.6%), $C_{16:0}$ (22.5%), $C_{17:1}$ ω6c (13.7%) and $C_{17:0}$ (9.1%). DNA and chemotaxonomic data supported the affiliation of strain BS11^T to the genus *Sphingosinicella*. Strain BS11^T could be differentiated genotypically and phenotypically from the recognized species of the genus *Sphingosinicella*. The novel isolate therefore represents a novel species, for which the name *Sphingosinicella ginsenosidimutans* sp. nov. is proposed, with the type strain BS11^T (=KACC 16619^T =JCM 18201^T).

Keywords: 16S rRNA gene, polyphasic taxonomy, *Sphingosinicella ginsenosidimutans*, ginsenoside

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Introduction

Ginseng, a famous traditional herbal plant, was used for thousands of years to cure disease and stay healthy. In Asian countries, ginseng have been regarded to have various function due to its beneficial effects on the central nervous system, anti-diabetic, anticancer effects, and so on (Kim and Park, 2011; Lee *et al*., 2011; Jin *et al*., 2012; Bae *et al*., 2014; Lee and Kim, 2014). Ginsenosides are the major active components in the biological and pharmacological effects of ginseng (Park *et al*., 2005). In Korean ginseng (*Panax ginseng* C. A. Meyer), six major ginsenosides (ginsenosides Rb_1, Rb_2, Rc, Rd, Re, and Rg_1) constitutes more than 90% of all ginsenosides (Park, 2004). However, the efficacy of ginsenoside increases with the extent of deglycosylation, which enhances its hydrophobicity and cell wall permeability. Biotransformation of ginsenoside (deglycosylation) is promising way to achieve custom designed minor ginsenosides by hydrolyzing sugar moieties specifically from the major ginsenosides using bacterial and fungal strains or their enzymes (Zhao *et al*., 2009; An *et al*., 2010; Park *et al*., 2010; Cui *et al*., 2011; Hong *et al*., 2012; Kim *et al*., 2012; Wang *et al*., 2012).

During the course of a study to screen ginsenoside converting aerobic bacterial strains isolated from compost of Yesan province, South Korea, several novel bacterial strains showing ginsenoside converting activity were screened. Among them, a strain designated BS11^T, which could convert ginsenoside Rb_1 to Rd appeared to be a member of the genus *Sphingosinicella* and became the subject of a taxonomic investigation.

The genus *Sphingosinicella* was recently created by Maruyama *et al*. (2006) with the description of a single species, *Sphingosinicella microcystinivorans*, as a novel member of the family *Sphingomonadaceae*. At the time of writing, the genus consisted of 4 validly named species, with *S. microcystinivorans* as the type species (Euzéby, 1997) together with *S. soli* (Yoon *et al*., 2008) and *S. vermicomposti* (Yasir *et al*., 2010).

In the present study, we conducted a phylogenetic (16S rRNA gene), phenotypic, genotypic, and chemotaxonomic analyses to determine the precise taxonomic position of this strain. On the basis of the results obtained in this study, we propose that strain BS11^T should be placed in the genus *Sphingosinicella* as the type strain of a novel species, *Sphingosinicella ginsenosidimutans* sp. nov.

Materials and Methods

Isolation of bacterial strain and culture condition

Strain BS11^T was originally isolated from the compost of

Yesan province, South Korea. This sample was thoroughly suspended in 50 mM phosphate buffer (pH 7.0) and spread on nutrient agar (NA, Difco) plates. The plates were incubated at 30°C for 2 weeks. One isolate, BS11^T, was cultured routinely on R2A agar (Difco) or NA at 25°C and preserved as a suspension in nutrient broth with 20% (w/v) glycerol at -70°C. The strain BS11^T was deposited to the Korean Agricultural Culture Collection (=KACC 16619^T), and Japan Collection of Microorganisms (=JCM 18201^T).

Phenotypic and biochemical characteristics

The Gram reaction was determined using the non-staining method using 3% KOH, as described by Buck (1982). Cell morphology and motility was observed under a Nikon light microscope at ×1,000, with cells grown on R2A agar for 2 days at 30°C. Catalase and oxidase tests were performed as outlined by Cappuccino and Sherman (2002). Anaerobic growth was determined in serum bottles containing R2A broth supplemented with thioglycolate (1 g/L), in which the upper air layer had been replaced with nitrogen. In addition, biochemical phenotypic test were carried out using API 20NE, API ID 32GN and API ZYM test kits according to the instructions of the manufacturer (bioMérieux). Tests for degradation of DNA (using DNase agar from Scharlau, with DNase activity by flooding plates with 1 M HCl), casein, and starch (Atlas, 1993) were performed and evaluated after 7 days. Growth at different temperatures (4, 10, 18, 30, 37, 42, and 45°C) and various pH values (pH 4.5–10.0 at intervals of 0.5 pH units) was assessed after 7 days of incubation. Three different buffers (final concentration, 50 mM) were used to adjust the pH of R2A broth. Acetate buffer was used for pH 5.0–5.5, phosphate buffer was used for pH 6.0–8.0 and Tris buffer was used for pH 8.5–10.0. Salt tolerance was tested on R2A medium supplemented with 1–10% (w/v at intervals of 1% unit) NaCl after 7 days of incubation. Growth on nutrient agar, trypticase soy agar (TSA, Difco), and MacConkey agar (Difco) was also evaluated at 30°C. Susceptibility to antibiotics was tested on TSA plates using antibiotic discs containing the following: ampicillin, 10 μg; cephalothin, 30 μg; gentamycin, 10 μg; kanamycin, 30 μg; streptomycin, 10 μg; erythromycin, 15 μg; chloramphenicol, 30 μg; tetracycline, 30 μg; lincomycin, 15 μg; neomycin, 30 μg; penicillin, 10 U; vancomycin, 30 μg; cycloheximide, 10 μg; novobiocin, 10 μg; oleandomycin, 10 μg. The occurrence of genes *mlrA*, *mlrB*, and *puf* was investigated by using primers and conditions described previously (Geueke *et al.*, 2007); *S. microcystinivorans* KCTC 12019^T and *S. xenopeptidilytica* DSM 17130^T were used as positive or negative controls.

Biotransformation of ginsenosides

Ginsenosides Rb_1, Rc, Rd, F_2, and compound K were purchased from Nanjing Zelang Medical Technology Co., Ltd. The reaction mixture, containing 200 μl of 1 mM ginsenosides and 200 μl of a bacterial suspension inoculated in a nutrient broth, was incubated for 3 days, at 150 rpm and 30°C. During the reaction, a 50 μl aliquot was taken daily, extracted with an equal volume of water-saturated *n*-butanol, and subjected to TLC (Thin-layer chromatography) analysis. TLC was performed using $60F_{254}$ silica gel plates (Merck) with $CHCl_3$-CH_3OH-H_2O (65:35:10, v/v, lower phase) as the solvent. The spots on the TLC plates were detected by spraying with 10% (v/v) H_2SO_4 followed by heating at 110°C for 5 min.

PCR amplification, 16S rRNA gene sequencing, and phylogenetic analysis

The genomic DNA of strain BS11^T was extracted using a commercial genomic DNA-extraction kit (Solgent). The 16S rRNA gene was amplified from the chromosomal DNA using the universal bacterial primer pair 9F and 1512R and the purified PCR products were sequenced by Solgent Co. Ltd. (Im *et al.*, 2010). Full sequences of the 16S rRNA gene were compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from GenBank and EzTaxon-e server (Kim *et al.*, 2012). Multiple alignments were performed by Clustal_X program (Thompson *et al.*, 1997) and gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). The phylogenetic trees were constructed by using the neighbor-joining (Saitou and Nei, 1987), maximum-parsimony (Fitch, 1971), and maximum-likelihood methods (Felsenstein, 1981) with the MEGA6 Program (Tamura *et al.*, 2013) with bootstrap values based on 1,000 replications (Felsenstein, 1985).

Isoprenoid quinone, cellular fatty acids, and polar lipid analysis

Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under a vacuum and re-extracted in *n*-hexane-water (1:1, v/v). The crude quinone in *n*-hexane solution was purified using Sep-Pak Vac Cartridges Silica (Waters) and subsequently analyzed by HPLC, as described by Hiraishi *et al.* (1996). Cellular fatty acid profiles were determined for strains grown on nutrient agar for 48 h at 30°C. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acid methyl esters were then analyzed by gas chromatography (model 6890; Hewlett Packard) using the Microbial Identification software package (Sasser, 1990). Polar lipids were extracted for freeze-dried cells grown on nutrient broth for 48 h at 30°C, and examined by two-dimensional TLC and identified as described by Minnikin *et al.* (1977).

Determination of DNA G + C content

For measurement of the G+C content of chromosomal DNA, the genomic DNA of strain BS11^T was extracted and purified as described by Moore and Dowhan (1995) and enzymatically degraded into nucleosides. The G+C content was then determined as described by Mesbah *et al.* (1989), using reverse-phase HPLC.

Results and Discussion

Morphological and phenotypic characteristics

Cells of strain BS11^T were Gram-reaction-negative, strictly aerobic, non-spore-forming, non-motile, rod shaped, oxidase-

Table 1. Differentiating characteristics of strain BS11^T and the type strains of related *Sphingosinicella* species. Strains: 1, *Sphingosinicella ginsenosidimutans* BS11^T; 2, *Sphingosinicella vermicomposti* KCTC 22446^T; 3, *Sphingosinicella xenopeptidilytica* DSM 17130^T; 4, *Sphingosinicella microcystinivorans* KCTC 12019^T; 5, *Sphingosinicella soli* KCTC 12482^T.
All data from this study, except the DNA G+C contents of the reference strains (taken from Maruyama *et al*., 2006; Geueke *et al*., 2007; Yoon *et al*., 2008; Yasir *et al*. 2010). All strains are Gram-reaction-negative, yellowish rods, negative for hydrolysis of DNA, xylan, starch, tyrosine, and skim milk. In API 20 NE and API ID 32 GN kits all strains are positive for 3-hydroxy-butyrate. All strains are negative for reduction of nitrates to nitrogen, indole production, glucose acidification, protease, and assimilation of the following substrates: β-galactosidase, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-D-glucosamine, D-maltose, gluconate, caprate, adipate, malate, salicin, D-melibiose, L-fucose, D-sorbitol, 2-ketogluconate, 4-hydroxy-benzoate, D-ribose, inositol, D-sucrose, itaconate, malonate, 5-ketogluconate, and 3-hydroxy-benzoate. In API ZYM kits, all the strains are positive for alkaline phosphatase, leucine arylamidase, valine arylamidase, crystine arylamidase, acid phosphatase, and naphtol-AS-BI-phosphohydrolase but negative for lipase (C14), α-galactosidase, β-glucuronidase, α-glucosidase, α-mannosidase, and α-fucosidase.
+, Positive; –, negative.

Characteristics	1	2	3	4	5
Isolation source	Compost	Vermicompost	Wastewater	Eutrophic lake	Alkaline soil
Cell morphology	Thin rods	Rod	Rod	None spore forming rods	Short rods or rods
Motility	-	-	ND	+	+
Colony color	Yellow	Yellow	Pale yellow	Yellow	Yellow
Temperature range (°C)	10–42	10–37	10–37	10–42	10–30
pH range	5–10	5.5–10	5.5–10	5.5–10	5.5–10
NaCl range (%)	1–2	1–2	1–2	1–3	1–2
Oxidase	+	+	+	-	-
Catalase	+	-	+	+	+
Detection (by PCR) of:					
mlrA	-	-	-	+	-
mlrB	-	-	-	+	-
puf	-	-	-	+	-
Urease	-	-	+	+	-
Decomposition of tween 80	-	-	-	+	-
Nitrate reduction	-	+	+	+	-
Arginine dihydrolase	-	-	+	+	-
β-Glucosidase	+	+	-	-	-
Carbon utilization of					
D-Glucose	+	+	-	-	-
Citrate	-	+	+	+	-
Phenyl-acetate	-	-	+	+	-
D-Glucose	+	+	-	+	-
Propionate	-	-	+	+	-
Valerate	+	-	+	+	-
Citrate	-	-	+	+	-
L-Histidine	-	-	+	-	+
L-Proline	-	-	+	-	-
L-Rhamnose	-	-	+	-	-
D-Maltose	-	+	-	-	-
Suberate	-	-	+	+	+
Acetate	-	-	+	+	-
Lactate	-	-	+	+	-
L-Alanine	-	-	+	+	-
Glycogen	-	+	-	-	-
L-Serine	-	-	+	-	-
API ZYM test results					
Esterase (C4)	-	+	-	+	+
Esterase lipase(C8)	-	+	+	+	+
Trypsin	-	+	+	+	+
α-Chymotrypsin	-	-	+	+	+
β-Glucosidase	+	-	-	-	-
N-Acetyl-β-glucosaminidase	-	-	+	+	+
G+C content (mol%)	64.9	59.4	65	63.6-63.7	65.1

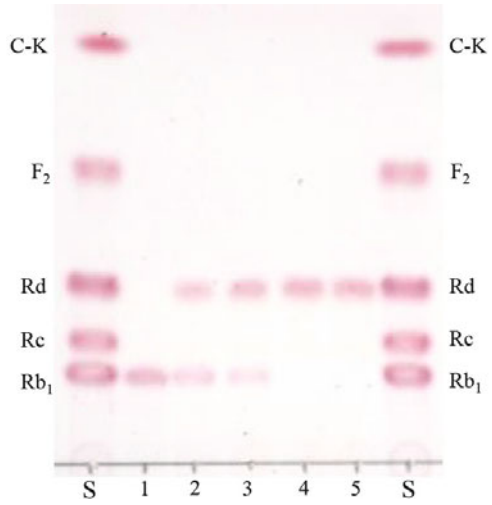

Fig. 1. TLC analyses of time-course transformation of ginsenoside Rb_1 by strain BS11^T Developing solvent: $CHCl_3$/MeOH/H_2O (65:35:10, v/v, lower phase). S, saponin standards; 1, ginsenoside Rb_1; 2, reaction mixture of Rb_1 after 12 h; 3, 24 h; 4, 2 days; 5, 3 days.

positive and catalase-positive. The colonies grown on R2A agar plates for 2 days were smooth, circular, transparent, yellowish in color, convex, and 2–3 mm in diameter. On R2A agar, BS11^T was able to grow at 10–42°C, but not at 45°C. The isolate grew on nutrient agar and TSA, but not on MacConkey agar. The phenotypic and chemotaxonomic characteristics that differentiate the strain BS11^T from other *Sphingosinicella* species are listed in Table 1.

Biotransformation of ginsenosides

A time course study of the biotransformation of the ginsenosides Rb_1 was conducted. As the TLC results are shown in Fig. 1, 0.5 mM of ginsenoside Rb_1 was transformed into ginsenoside Rd and almost completely hydrolyzed after 2 days.

Phylogenetic analysis

The 16S rRNA gene sequences of the strain BS11^T determined in this study were continuous stretches of 1,409 bp (base position 28–1491 with respect to the *Escherichia coli* numbering system), which were deposited in a GenBank database (accession numbers JQ349043). A sequence similarity calculation from using the EzTaxon-e server (Kim *et al.*, 2012) indicated that the closest relatives of strain BS11^T were *Sphingosinicella vermicomposti* YC7378^T (96.3% sequence similarity), *S. xenopeptidilytica* 3-2W4^T (96.2%), *S. microcystinivorans* Y2^T (96.1%), and *S. soli* KSL-125^T (95.9%). This relationship between strain BS11^T and other members of the genus *Sphingosinicella* was also evident in the phylogenetic tree, which used over 1380 nt (Fig. 2). Strain BS11^T, *S. vermicomposti* YC7378^T, *S. soli* KSL-125^T, *S. microcystinivorans* Y2^T, and *S. xenopeptidilytica* 3-2W4^T formed a monophyletic group with a bootstrap high value (99%), which was supported by both tree making methods used in this study. DNA-DNA hybridization tests between strain BS11^T and its nearest phylogenetic neighbors was not attempted since strains differing by >3.0% at the 16S rRNA gene level are unlikely to exhibit >70% relatedness at the whole-genome level (Stackebrandt and Goebel, 1994).

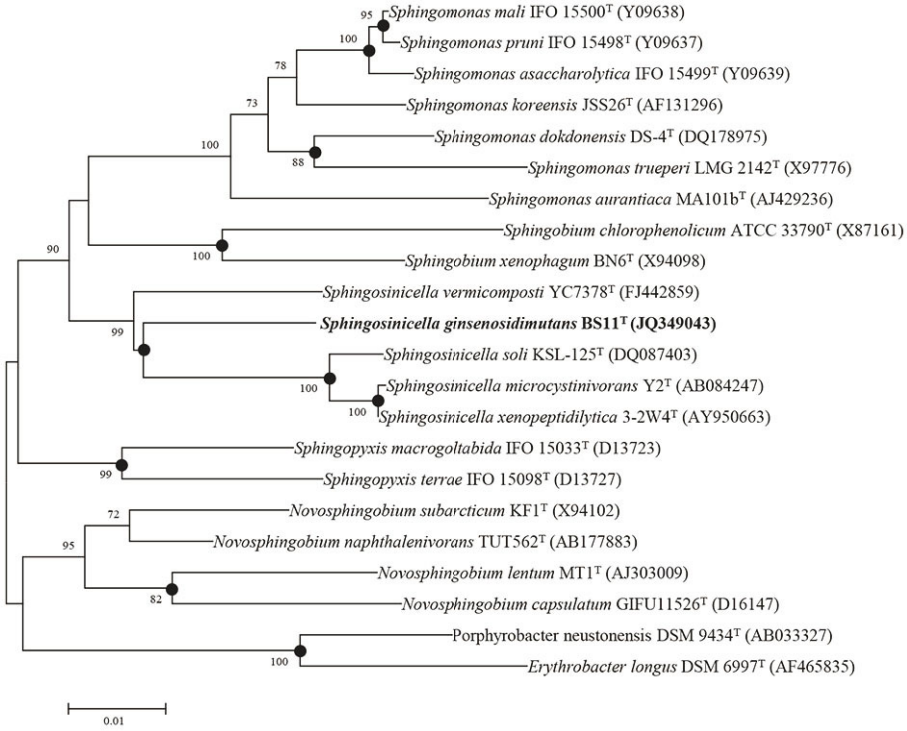

Fig. 2. Phylogenetic tree constructed from a comparative analysis of 16S rRNA gene sequences showing the relationships of strain BS11^T with other related species. This tree was made using the neighbor-joining method (Saitou and Nei, 1987) with a Kimura (1983) two-parameter distance matrix and pairwise deletion. Dots indicate generic branches that were also recovered by using maximum-parsimony algorithms. Bootstrap values (expressed as percentages of 1,000 replications) greater than 70% are shown at the branch points. Bar, 0.01 substitutions per 1 nucleotide position.

Table 2. Cellular fatty acid profiles of strain BS11^T and phylogenetically related species of the genus *Sphingosinicella*. Strains: 1, *Sphingosinicella ginsenosidimutans* BS11^T; 2, *Sphingosinicella vermicomposti* KCTC 22446^T; 3, *Sphingosinicella xenopeptidilytica* DSM 17130^T; 4, *Sphingosinicella microcystinivorans* KCTC 12019^T; 5, *Sphingosinicella soli* KCTC 12482^T. All data from this study. All strains were cultured on nutrient agar for 2 days at 30°C. Fatty acids amounting to <0.5% of the total fatty acids in all strains are not listed. -, not detected. tr, trace amount below 0.5%.

	1	2	3	4	5
Saturated					
$C_{14:0}$	-	1.5	1.3	1.6	1.4
$C_{15:0}$	2.0	-	-	-	3.0
$C_{16:0}$	22.5	11.1	8.6	5.9	9.3
$C_{17:0}$	9.1	-	-	-	-
$C_{18:0}$	-	5.5	-	-	-
Unsaturated					
$C_{16:1}$ *ω5c*	-	2.0	1.1	2.3	-
$C_{17:1}$ *ω6c*	13.7	1.8	1.5	-	9.8
$C_{17:1}$ *ω8c*	3.4	-	-	-	-
Hydroxy fatty acids					
$C_{14:0}$ 2OH	-	12.2	13.9	11.6	9.5
$C_{15:0}$ 2OH	4.1	1.1	-	-	4.1
$C_{16:0}$ 2OH	4.7	-	-	-	1.2
Cyclo fatty acid					
$C_{19:0}$ cyclo *ω8c*	-	-	5.5	1.2	4.3
Summed feature					
4; $C_{16:1}$ *ω7c*/iso-$C_{15:0}$ 2OH	-	28.2	38.8	35.8	27.0
7; $C_{18:1}$ *ω7c*/*ω9t*/*ω12t*	40.6	32.5	29.3	41.6	30.4
Methyl ester					
$C_{18:1}$ *ω7c* 11-methyl	-	4.3	-	-	-

*Summed feature represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature consist of: 4, $C_{16:1}$ *ω7c*/iso-$C_{15:0}$ 2OH; 7, $C_{18:1}$ *ω7c*/*ω9t*/*ω12t*.

Cellular fatty acid, quinone, and polar lipid composition

The major respiratory quinone of strain BS11^T was ubiquinone 10 (Q-10), in line with all other members of the family *Sphingomonadaceae*. The cellular fatty acids of strain BS11^T and related type strains are listed in the Table 2. The predominant fatty acid of all the 4 compared strains was summed feature 7 (comprising $C_{18:1}$ *ω7c*/*ω9t*/*ω12t*), which ranged from 29.3–41.6% of the total fatty acids. According to the Table 2, qualitative and quantitative differences in fatty acid content could be observed between strain BS11^T and its phylogenetically closest relatives. In particular, our strain differed from other *Sphingosinicella* species by the presence of $C_{17:0}$ and iso-$C_{16:0}$ 2OH and it could be differentiated from other *Sphingosinicella* species by the absence of $C_{16:1}$ *ω7c*/iso-$C_{15:0}$ 2OH and $C_{14:0}$ 2OH.

The polar lipids detected were phosphatidylglycerol, phosphatidylethanolamine, sphingoglycolipid, phosphatidylcholine, diphosphatidylglycerol, and unknown lipids (Fig. 3).

DNA G+C content

The DNA G+C content of strain BS11^T was 64.9 mol%, similar to those of *S. vermicomposti* YC7378^T, *S. soli* KSL-125^T, *S. microcystinivorans* Y2^T, and *S. xenopeptidilytica* 3-2W4^T, which were in the range of 59.4–65.1 mol%.

Taxonomic conclusions

The characteristics of strain BS11^T were consistent with the description of the genus *Sphingosinicella* with regard to morphological, biochemical and chemotaxonomic properties. However, the phylogenetic distance between strain BS11^T and recognized *Sphingosinicella* species, and the unique phenotypic characteristics (Table 1) warrant assignment of strain BS11^T to the genus *Sphingosinicella* as the type strain of a novel species, for which the name *Sphingosinicella ginsenosidimutans* sp. nov. is proposed.

Description of *Sphingosinicella ginsenosidimutans* sp. nov.

Sphingosinicella ginsenosidimutans (gin.se.no.si.di.mu'tans. N.L. n. *ginsenosidum*, ginsenoside; L. part. adj. *mutans*, transforming, converting; N.L. part. adj. *ginsenosidimutans*, ginsenoside-converting)

Cells are Gram-reaction-negative, strictly aerobic, non-motile, and non-spore-forming rods (0.3–0.5 μm in diameter and 1.5–2.2 μm in length) after culture on R2A agar for 2 days. Colonies are smooth, transparent, convex, circular with regular margins, yellowish in color, and 2–3 mm in diameter after two days on R2A agar. Growth also occurs on nutrient agar and TSA, but not on MacConkey. Cells Grow on R2A at 10–42°C and at pH 5–10, but not at 45°C. Optimum growth occurs at 30–37°C and at pH 7.0. Growth occurs well without NaCl supplement and in the presence of 1.0–2.0% (w/v) NaCl. Catalase and oxidase are positive. It does not hydrolyze casein, DNA, xylan, skim milk, and starch. The cells are susceptible to cephalothin, gentamycin, kanamycin, erythromycin, chloramphenicol, tetracycline, neomycin, vancomycin and novobiocin, but not to ampicillin, streptomycin, lincomycin, penicillin, cycloheximide, and oleandomycin. Carbon assimilation tests as a sole carbon sources (API ID 32 GN, API 20 NE) and the enzyme activities (API ZYM) are listed in Table 1. Q-10 is the predominant respiratory

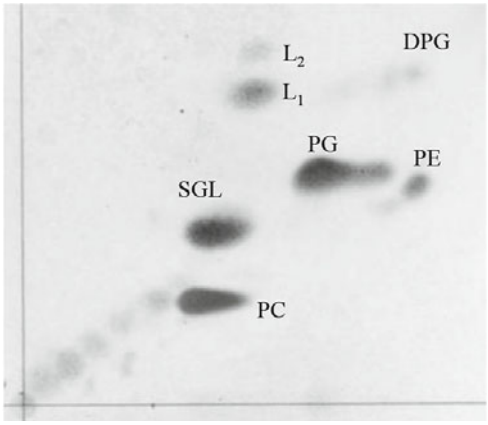

Fig. 3. Two-dimensional thin-layer chromatography of polar lipids of strain BS11^T. Chloroform/methanol/water (65:25:4, by vol.) was used in the first direction, followed by chloroform/acetic acid/methanol/water 80:15:12:4, by vol.) in the second direction. The 5% ethanolic molybdatophosphoric acid spray reagents were used to detect total lipids; Abbreviations: PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SGL, sphingoglycolipid; DPG, diphosphatidylglycerol; L1-L2, unknown lipids.

quinone, and summed feature 7 (comprising $C_{18:1}$ *ω7c*/*ω9t*/*ω12t*), $C_{16:0}$, $C_{17:1}$ *ω6c*, and $C_{17:0}$ are the major cellular fatty acids (>10%). The G + C content of the genomic DNA is 64.9 mol%. The polar lipids detected were phosphatidyl glycerol, phosphatidyl ethanolamine, sphingoglycolipid, diphosphatidyl glycerol, phosphatidyl choline, and unknown lipids.

The type strain, BS11^T (=KACC 16619^T =JCM 18201^T) was isolated from compost of Yesan province, South Korea.

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