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Novosphingobium ginsenosidimutans sp. nov., with the Ability to Convert Ginsenoside

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A Gram-negative, strictly aerobic, non-motile, non-spore-forming, and rod-shaped bacterial strain designated FW-6^T was isolated from a freshwater sample and its taxonomic position was investigated by using a polyphasic approach. Strain FW-6^T grew optimally at 10–42°C and at pH 7.0 on nutrient and R2A agar. Strain FW-6^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 FW-6^T was shown to belong to the family Sphingomonadaceae and was related to *Novosphingobium aromaticivorans* DSM 12444^T (98.1% sequence similarity) and *N. subterraneum* IFO 16086^T (98.0%). The G+C content of the genomic DNA was 64.4%. The major menaquinone was Q-10 and the major fatty acids were summed feature 7 (comprising C$_{18:1}$ ω9*c*/ω12t/ω7*c*), summed feature 4 (comprising C$_{16:1}$ ω7*c*/iso-C$_{15:0}$ 2OH), C$_{16:0}$, and C$_{14:0}$ 2OH. DNA and chemotaxonomic data supported the affiliation of strain FW-6^T to the genus *Novosphingobium*. Strain FW-6^T could be differentiated genotypically and phenotypically from the recognized species of the genus *Novosphingobium*. The isolate that has ginsenoside converting ability therefore represents a novel species, for which the name *Novosphingobium ginsenosidimutans* sp. nov. is proposed, with the type strain FW-6^T (= KACC 16615^T = JCM 18202^T).

Key words: 16S rRNA gene, polyphasic taxonomy, ginsenoside, *Novosphingobium ginsenosidimutans*

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Ginseng is one of the most well-known medicinal plants in the world [3]. Ginsenosides are the major active components in the biological and pharmacological effects of ginseng [20, 23, 25]. Thus far, more than 180 kinds of naturally occurring ginsenosides have been isolated [8]. In Korean ginseng (*Panax ginseng* C. A. Meyer), six major ginsenosides (ginsenosides Rb$_1$, Rb$_2$, Rc, Rd, Re, and Rg$_1$) constitute the main portion of Korean and American ginsengs [30, 33]. However, the efficacy of ginsenoside increases with the extent of deglycosylation, which enhances its hydrophobicity and cell wall permeability. Biotransformation of ginsenoside (deglycosylation) is a 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 [1, 10, 18, 22, 29, 38, 41].

During the course of a study to screen ginsenoside converting aerobic bacterial strains isolated from fresh water or sediment of Duck Lake, KAIST, Daejeon, South Korea, several novel bacterial strains showing ginsenoside converting activity were screened. Among them, a strain designated FW-6^T, which could convert ginsenoside Rb$_1$ to Rd appeared to be a member of the genus *Novosphingobium* and became the subject of a taxonomic investigation.

The genus *Novosphingobium* was established as a consequence of the dissection of the genus *Sphingomonas* [40], which was based on phylogenetic and chemotaxonomic analyses [35]. At the time of writing, the genus consisted of 20 validly named species, with *N. capsulatum* as the type species [11]. Many novel species have been described in recent years from a variety of environmental sources (oil-contaminated soil, deep-sea water, subsurface water,

freshwater sediment); namely, *N. panipatense* [15], *N. sediminicola* [4], and *N. soli* [21].

In the present study, we conducted 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 FW-6^T should be placed in the genus *Novosphingobium* as the type strain of a novel species, *Novosphingobium ginsenosidimutans* sp. nov.

Strain FW-6^T was originally isolated from the freshwater of Duck Lake, KAIST, Daejeon, South Korea. This freshwater sample was thoroughly suspended in 50 mM phosphate buffer (pH 7.0) and spread on R2A agar (Difco, USA) plates. The plates were incubated at 30°C for 2 weeks. One isolate, FW-6^T, was cultured routinely on R2A agar or nutrient agar (Difco, USA) at 25°C and preserved as a suspension in nutrient broth with 20% (w/v) glycerol at −70°C. The strain FW-6^T was deposited to the Korean Agricultural Culture Collection (=KACC 16615^T), and Japan Collection of Microorganisms (=JCM 18202^T).

The Gram reaction was determined using the non-staining method, as described by Buck [6]. 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 [7]. 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 tests were carried out using API 20NE, API ID 32GN, and API ZYM test kits according to the instructions of the manufacturer (bioMérieux, France). Tests for degradation of DNA (using DNase agar from Scharlau, with DNase activity by flooding plates with 1M HCl), casein, pullulan, laminarin, and starch [2] were performed and evaluated after 7 days. Growth at different temperatures (4°C, 10°C, 18°C, 30°C, 37°C, 42°C, 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 R2A plates using antibiotic discs containing the following: ampicillin, 10 μg; cephalothin, 30 μg; gentamicin, 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; and oleandomycin, 10 μg.

Ginsenosides Rb_1, Rc, Rd, F_2, and compound K were purchased from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China). 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 7 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 analysis. TLC was performed using $60F_{254}$ silica gel plates (Merck, Germany) 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.

The genomic DNA of strain FW-6^T was extracted using a commercial genomic DNA-extraction kit (Solgent, Korea). 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 (Daejeon, Korea) [19]. Full sequences of the 16S rRNA gene were compiled using SeqMan software (DNASTAR, USA). The 16S rRNA gene sequences of related taxa were obtained from the GenBank and EzTaxon servers [9]. Multiple alignments were performed by the Clustal_X program [37] and gaps were edited in the BioEdit program [16]. Evolutionary distances were calculated using the Kimura two-parameter model [24]. The phylogenetic trees were constructed by using the neighbor-joining [31], maximum-parsimony [14], and maximum-likelihood methods with the MEGA5 program [36] with bootstrap values based on 1,000 replications [13].

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 the *n*-hexane solution was purified using Sep-Pak Vac Cartridges Silica (Waters) and subsequently analyzed by HPLC, as described by Hiraishi *et al*. [17]. Cellular fatty acid profiles were determined for strains grown on R2A 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) using a gas chromatograph (6890; Hewlett Packard) and the TSBA60 database [32]. Polar lipids were extracted for freeze-dried cells grown on R2A broth for 48 h at 30°C, and examined by two-dimensional TLC, and the total lipids was detected using the 5% ethanolic molybdophosphoric acid spray reagent as described by Minnikin *et al*. [27].

For measurement of the G+C content of chromosomal DNA, the genomic DNA of strain FW-6^T was extracted and purified as described by Moore [28] and enzymatically degraded into nucleosides. The G+C content was then determined as described by Mesbah *et al.* [26], using reverse-phase HPLC.

DNA–DNA hybridization experiments were performed between strain FW-6^T, and 2 reference strains (*N.*

Table 1. Differentiating characteristics of strain FW-6^T and the type strains of related *Novosphingobium* species.

Characteristics	1	2	3
Isolation source	Freshwater	Sediments	Sediments
Temperature range (°C)	10–42	10–42	10–42
pH range	5.5–10	5–10	5.5–10
NaCl range (%)	0–1	0–2	0–2
Oxidase	+	-	-
Catalase	-	+	+
Decomposition of			
Xylan	-	-	+
Carbon assimilation of			
L-Arabinose	-	-	+
D-Mannose	-	+	-
N-Acetyl-D-glucosamine	-	+	-
Salicin	-	+	+
D-Melibiose	w	-	-
L-Fucose	w	+	-
L-Arabinose	w	-	+
Propionate	-	+	+
Valerate	-	+	+
Citrate	+	-	-
L-Histidine	+	-	-
4-Hydroxybenzoate	-	+	+
L-Proline	-	+	+
L-Rhamnose	-	+	+
Lactate	w	+	-
Glycogen	w	-	-
API ZYM test results			
Esterase (C4)	+	-	+
Esterase lipase(C8)	+	-	-
α-Chymotrypsin	+	-	-
α-Galactosidase	-	+	+
α-Glucuronidase	-	+	+
N-Acetyl-β-glucosaminidase	-	+	-
G+C content (mol%)	64.4	62.9–65.0	60

Strains: 1, FW-6^T; 2, *N. aromaticivorans* KCTC 2888^T; 3, *N. subterraneum* KCTC 2889^T.

All data are from this study, except the DNA G+C contents of the reference strains (taken from Balkwill *et al*. [5]). All strains are Gram-negative, yellowish rods, negative for hydrolysis of DNA, starch, and skim milk. In API 20 NE and API ID 32 GN kits, all strains are positive for β-galactosidase, D-glucose, D-maltose, adipate, 3-hydroxybutyrate, D-sucrose, suberate, and acetate activities. All strains are negative for nitrate reduction, indole production, acid production from glucose, arginine dihydrolase, urease, and assimilation of the following substrates: D-mannitol, gluconate, caprate, malate, citrate, phenyl-acetate, D-mannitol, D-sorbitol, 2-ketogluconate, D-ribose, inositol, itaconate, malonate, L-alanine, 5-ketogluconate, 3-hydroxybenzoate, and L-serine. In API ZYM kits, all the strains are positive for alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, and β-glucosidase activities but negative for lipase (C14), α-mannosidase, and α-fucosidase.

+, Positive; –, negative; w, weakly positive.

aromaticivorans KCTC 2888^T and *N. subterraneum* KCTC 2889^T) with the method described by Ezaki *et al*. [12] using photobiotin-labeled DNA probes and microdilution wells. Hybridization was performed at 50°C with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values were converted to percentage DNA–DNA relatedness values.

Cells of strain FW-6^T were Gram-negative, strictly aerobic, non-spore-forming, non-motile, rod shaped, oxidase-positive, and catalase-negative. 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, FW-6^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 strain FW-6^T from other *Novosphingobium* species are listed in Table 1. A time-course study of the biotransformation of the ginsenosides Rb_1 was conducted. As shown in Fig. 1, ginsenoside Rb_1 was gradually transformed into ginsenoside Rd, and almost completely hydrolyzed after 7 days.

The 16S rRNA gene sequences of the strain FW-6^T determined in this study were continuous stretches of 1407 bp (base position 28–1491 with respect to the *Escherichia coli* numbering system), which were deposited in the GenBank database (Accession No. JQ349046). A sequence similarity calculation using the EzTaxon server [http://www.eztaxon.org/] [9] indicated that the closest relatives

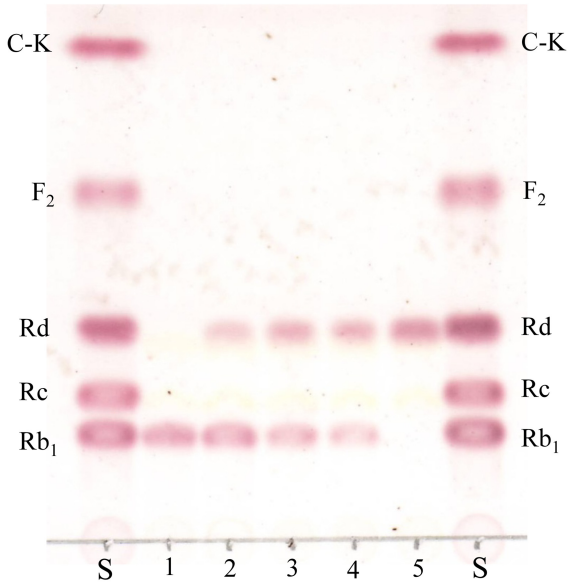

Fig. 1. Thin layer chromatography analyses of time-course transformation of ginsenoside Rb_1 by strain FW-6^T.

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 1 day; 3, 3 days; 4, 5 days; 5, 7 days. Abbreviations: C-K, compound K.

of strain FW-6^T were *Novosphingobium aromaticivorans* DSM 12444^T (98.1% sequence similarity) and *N. subterraneum* IFO 16086^T (98.0%). This relationship between strain FW-6^T and other members of the genus *Novosphingobium* was also evident in the phylogenetic tree, which used over 1,350 nt (Fig. 2). Strain FW-6^T, *N. aromaticivorans* DSM 12444^T, and *N. subterraneum* IFO 16086^T formed a monophyletic group with a high bootstrap

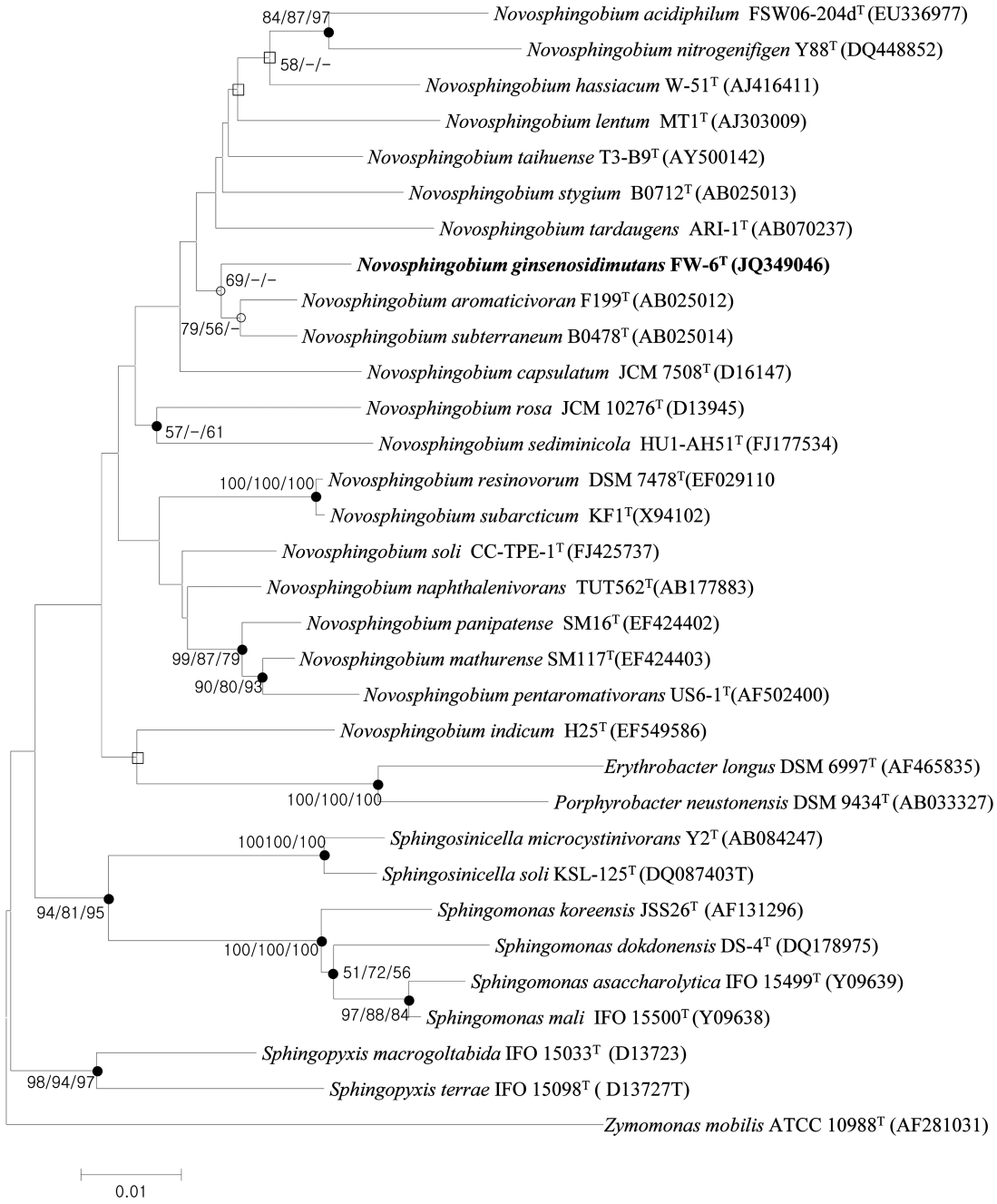

Fig. 2. Phylogenetic tree constructed from a comparative analysis of 16S rRNA gene sequences showing the relationships of *Novosphingobium ginsenosidimutans* FW-6^T with other related species.

This tree was made using the neighbor-joining method (Saitou and Nei [31]) with a Kimura [24] two-parameter distance matrix and pairwise deletion. GenBank accession numbers are shown in parentheses. Filled circles, open circles, and a square indicate generic branches that were also recovered using the maximum-parsimony and maximum-likelihood algorithms, the maximum-parsimony algorithm, and maximum-likelihood algorithm, respectively. Numbers at nodes indicate bootstrap values as calculated by neighbor-joining/ maximum-parsimony/maximum-likelihood probabilities in percent. Bootstrap values greater than 50% are shown at the branch points. Bar, 0.01 substitutions per 1 nucleotide position.

value (98%), which was supported by both tree-making methods used in this study. DNA–DNA hybridization tests between strain FW-6^T and its nearest phylogenetic neighbors was attempted, since strains differing by <3.0% at the 16S rRNA gene level should be compared at the whole-genome level [34].

The DNA G+C content of strain FW-6^T was 64.9 mol%, similar to those of *N. aromaticivorans* DSM 12444^T and *N. subterraneum* IFO 16086^T, which were in the range of 60.0–64.9 mol%. DNA–DNA relatedness values between strain FW-6^T and *N. aromaticivorans* DSM 12444^T and *N. subterraneum* IFO 16086^T were 33% (reciprocal, 28%) and 21% (reciprocal, 19%), respectively.

The major respiratory quinone of strain FW-6^T was ubiquinone 10 (Q-10), in line with all other members of the family Sphingomonadaceae. The cellular fatty acids of strain FW-6^T and related type strains are listed in Table 2. The predominant fatty acid of all the 3 compared strains was summed feature 7 (comprising $C_{18:1}$ ω9*c*/ω12t/ω7*c*), which ranged from 28.5–47.5% of the total fatty acids. According to Table 2, qualitative and quantitative differences in fatty acid content could be observed between strain FW-6^T and its phylogenetically closest relatives. The polar lipid profile of strain FW-6^T contained the phosphatidylglycerol, phosphatidylethanolamine, sphingoglycolipid, phosphatidylcholine, diphosphatidylglycerol, and unknown lipids (Fig. 3).

Table 2. Cellular fatty acid profiles of strain FW-6^T and phylogenetically related species of the genus *Novosphingobium.*

Fatty acid	1	2	3
Saturated			
$C_{14:0}$	5.0	3.9	6.3
$C_{15:0}$	5.1	-	-
$C_{16:0}$	18.3	9.5	11.8
$C_{17:0}$	1.5	-	-
Unsaturated			
$C_{16:1}$ ω5*c*	-	0.6	0.7
$C_{17:1}$ ω6*c*	6.1	1.0	1.3
$C_{17:1}$ ω8*c*	0.7	-	-
$C_{18:1}$ ω5*c*	-	0.6	0.5
Branched -chain fatty acid			
anteiso-$C_{17:1}$ ω9*c*	0.5	-	-
Hydroxy fatty acids			
$C_{14:0}$ 2OH	9.8	12.7	10.4
$C_{15:0}$ 2OH	1.2	-	-
Summed feature			
4; $C_{16:1}$ ω7*c*/iso-$C_{15:0}$ 2OH	23.4	24.2	23.4
7; $C_{18:1}$ ω9*c*/ω12*t*/ω7*c*	28.5	47.5	45.6

Summed feature represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature consists of 4, $C_{16:1}$ ω7*c* and/or iso-$C_{15:0}$ 2OH; 7, $C_{18:1}$ ω9*c* and/or ω12*t* and/or ω7*c*.

Strains: 1, FW-6^T; 2, *N. aromaticivorans* KCTC 2888^T; 3, *N. subterraneum* KCTC 2889^T.

All data are from this study. All strains were cultured on R2A 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%.

In conclusion, the characteristics of strain FW-6^T were consistent with the description of the genus *Novosphingobium* with regard to morphological, biochemical, and chemotaxonomic properties. However, the phylogenetic distance between strain FW-6^T and recognized *Novosphingobium* species, the unique phenotypic characteristics (Table 1), and the low level of DNA–DNA relatedness values [39] warrant assignment of strain FW-6^T to the genus *Novosphingobium* as the type strain of a novel species, for which the name *Novosphingobium ginsenosidimutans* sp. nov. is proposed.

Description of *Novosphingobium ginsenosidimutans* sp. nov.

Novosphingobium 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-negative, strictly aerobic, non-motile, and non-spore-forming rods (0.3–0.5 μm in diameter and 1.3–2.1 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. Grows on R2A at 10–42°C and at pH 5.5–10, but not at 45°C.

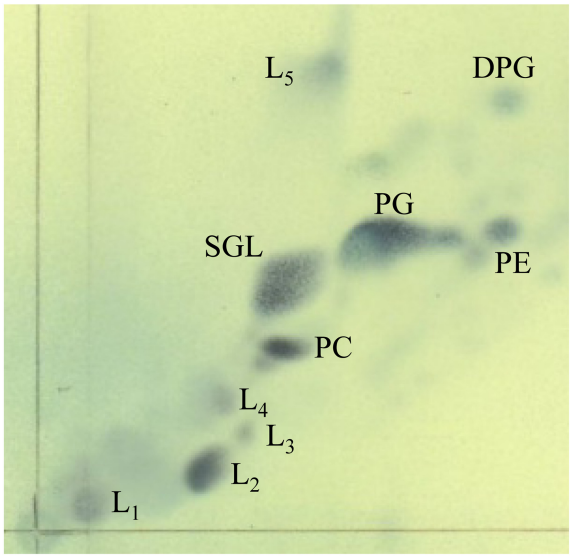

Fig. 3. Two-dimensional thin-layer chromatography of polar lipids of strain FW-6^T.

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 molybdophosphoric acid spray reagent was used to detect total lipids. Abbreviations: PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SGL, sphingoglycolipid; DPG, diphosphatidylglycerol; L1–L5, unknown lipids.

Optimum growth occurs at 25−37°C and at pH 7.0. Growth occurs well without NaCl supplement and in the presence of 1.0% (w/v) NaCl. Oxidase is positive but catalase is negative. Does not hydrolyze casein, DNA, xylan, skim milk, and starch. Susceptible to cephalothin, gentamicin, kanamycin, erythromycin, chloramphenicol, tetracycline, neomycin, vancomycin, and novobiocin, but not to ampicillin, streptomycin, lincomycin, penicillin, cycloheximide, and oleandomycin. Carbon assimilation tests as 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 quinone, and summed feature 7 (comprising $C_{18:1}$ ω9*c*/ω12t/ω7*c*), summed feature 4 (comprising $C_{16:1}$ ω7*c*/iso-$C_{15:0}$ 2OH), $C_{16:0}$, and $C_{14:0}$ 2OH are the major cellular fatty acids (>9%). The G + C content of the genomic DNA is 64.9 mol%. The polar lipids detected were phosphatidylglycerol, phosphatidylethanolamine, sphingoglycolipid, phosphatidylcholine, diphosphatidylglycerol, and unknown lipids.

The type strain, FW-6^T (= KACC 16615^T = JCM 18202^T), was isolated from the freshwater of Duck Lake, KAIST, Daejeon, South Korea. The 16S rRNA gene sequence of strain FW-6^T has been deposited in NCBI GenBank under the accession number JQ349046.

Acknowledgments

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Abbreviations

Rb_1, {3-*O*-[β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl]-20-*O*-[β-D-glucopyranosyl-(1-6)-β-D-glucopyranosyl]-20(*S*)-protopanaxadiol}; Rd, {3-*O*-[β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl]-20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol}

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