Sphingobacterium cladoniae sp. nov., isolated from lichen, *Cladonia* sp., and emended description of *Sphingobacterium siyangense*

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A strictly aerobic, Gram-stain-negative bacterium, designated strain No.6^T, was isolated from a lichen (*Cladonia* sp.) collected in Geogeum Island, Korea, and its taxonomic status was established by a polyphasic study. Cells of strain No.6^T were non-motile, catalase- and oxidase-positive, non-spore-forming rods. Growth was observed at 15–35 °C (optimum, 25–30 °C), at pH 5.0–10.0 (optimum, pH 6.0–8.0) and with 0–3 % NaCl (optimum, 0–2 %). The predominant cellular fatty acids were summed feature 3 (comprising iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$, 41.5 %), iso-$C_{15:0}$ (26.7 %) and $C_{16:0}$ (9.6 %), and menaquinone MK-7 was the only respiratory quinone. The G+C content of the genomic DNA of strain No.6^T was 36.8 mol%. A phylogenetic tree based on 16S rRNA gene sequences showed that strain No.6^T fell within the evolutionary group encompassed by the genus *Sphingobacterium*. Levels of 16S rRNA gene sequence similarity between the novel strain and the type strains of recognized *Sphingobacterium* species ranged from 92.1 to 99.1 %, the highest values being with *Sphingobacterium siyangense* SY1^T (99.1 %) and *Sphingobacterium multivorum* IAM 14316^T (98.5 %). DNA–DNA relatedness between strain No.6^T and these two type strains were 32.0 and 5.7 %, respectively. The polar lipids found in strain No.6^T were phosphatidylethanolamine, two unidentified phospholipids, three unidentified aminophospholipids, one glycolipid and four unidentified lipids. One unidentified sphingolipid was also found. On the basis of phenotypic and genotypic data, strain No.6^T represents a novel species of the genus *Sphingobacterium*, for which the name *Sphingobacterium cladoniae* sp. nov. is proposed. The type strain is No.6^T (=KCTC 22613^T =JCM 16113^T). An emended description of *Sphingobacterium siyangense* is also proposed.

The genus *Sphingobacterium*, a member of the family *Sphingobacteriaceae* (Steyn *et al.*, 1998) in the phylum *Bacteroidetes*, was first proposed by Yabuuchi *et al.* (1983) through reclassification of two *Flavobacterium* species (Holmes *et al.*, 1981, 1982) as *Sphingobacterium multivorum* and *Sphingobacterium spiritivorum* (type species) and description of a novel species, *Sphingobacterium mizutae* [corrected to *S. mizutaii* by Holmes *et al.* (1988)]. Currently, the genus *Sphingobacterium* comprises 14 species with validly published names isolated from various habitats such as soil, compost and 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). In addition to the three original species, the genus contains *Sphingobacterium antarcticum* [Shivaji *et al.* (1992); specific epithet corrected by Euzéby (1998)], *S. thalpophilum* (previously *Flavobacterium thalpophilum*; Takeuchi & Yokota, 1992), *S. faecium* (Takeuchi & Yokota, 1992), *S. daejeonense* (Kim *et al.*, 2006), *S. composti* (Ten *et al.*, 2006), *S. canadense* (Mehnaz *et al.*, 2007), *S. siyangense* (Liu *et al.*, 2008), *S. kitahiroshimense* (Matsuyama *et al.*, 2008), *S. anhuiense* (Wei *et al.*, 2008), *S. bambusae* (Duan *et al.*, 2009) and *S. shayense* (He *et al.*, 2010). Members of the genus *Sphingobacterium* are Gram-stain-negative rods that are positive for catalase and oxidase activities and negative for heparinase and gelatinase activities and for indole production. They contain menaquinone 7 (MK-7) as the predominant

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Three supplementary figures are available with the online version of this paper.

isoprenoid quinone, and iso-$C_{15:0}$, iso-$C_{15:0}$ 2-OH, $C_{16:1}\omega7c$ and $C_{17:0}$ 3-OH as the main fatty acids. The range of DNA G+C content is approximately 35–44 mol% (Liu *et al.*, 2008; He *et al.*, 2010).

During studies on the diversity of lichen-associated and/or heavy-metal-resistant micro-organisms, a bacterium designated strain No.6^T was isolated from lichen and investigated using a polyphasic taxonomic approach. On the basis of this study, we propose that strain No.6^T represents a novel species of the genus *Sphingobacterium*.

A lichen (*Cladonia* sp.) was collected in Geogeum Island (34° 26′ N 127° 10′ E) near the Southern Sea in Korea and transported to the laboratory in a sterile plastic bag. Lichen samples were washed three times in 45 ml of sterile 0.85 % NaCl with shaking, dried on a UV-sterilized bench and homogenized in a laboratory blender. Lichen homogenates (5 g) were transferred to a conical tube and resuspended in 5 ml of sterile 0.85 % NaCl. Part of the suspension (0.1 ml) was inoculated on Luria–Bertani (LB) agar (Difco) and incubated at 28 °C. Bacterial colonies were picked up and transferred to LB agar containing heavy metals such as $CdCl_2$ (40 μg ml^{-1}), $FeCl_2$ (40 μg ml^{-1}), $HgCl_2$ (40 μg ml^{-1}) and $PbCl_2$ (40 μg ml^{-1}) which were purchased from Sigma Chemical Co. and incubated at 28 °C. Some of the bacterial colonies that grew in the presence of heavy metals were selected. Strain No.6^T was resistant to all the heavy metals tested (data not shown). It was subcultivated on LB agar at 28 °C and stored at −80 °C in LB broth (Difco) supplemented with 20 % (v/v) glycerol.

Genomic DNA was extracted and purified by using a commercial genomic DNA extraction kit (Promega), and the nearly complete 16S rRNA gene sequence was amplified using bacterial universal primers (Weisburg *et al.*, 1991). Sequencing of the 16S rRNA gene of strain No.6^T was carried out as described previously (Lane, 1991). The resulting sequence was compared with 16S rRNA gene sequences available in GenBank, using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/) to determine the approximate phylogenetic affiliation and using the CLUSTAL X software (Thompson *et al.*, 1997) to align the sequences of closely related organisms. Sequence similarity values were computed using the EzTaxon server (http://www.eztaxon.org/; Chun *et al.*, 2007). Gaps at the 5′ and 3′ ends and ambiguous bases were removed from the alignment using BioEdit (Hall, 2007). Phylogenetic trees based on a comparison of 1283 bases were constructed using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) algorithms in MEGA 5 software (Kumar *et al.*, 2008), with bootstrap values based on 1000 replications (Felsenstein, 1985). Evolutionary distance matrices were calculated according to the algorithm of the Kimura two-parameter model (Kimura, 1983).

The 16S rRNA gene sequence of strain No.6^T comprised 1421 nt. It shared highest similarity with *S. siyangense* SY1^T (99.1 %), *S. multivorum* IAM14316^T (98.5 %), *S. canadense* CR11^T (97.9 %), and with other members of the genus *Sphingobacterium* (92.1–96.3 %). The neighbour-joining tree showed that strain No.6^T belonged to the genus *Sphingobacterium* and formed a tight cluster with *S. siyangense* SY1^T with a high bootstrap value (99 %) (Fig. 1). The maximum-likelihood and maximum-parsimony trees showed essentially the same topology (data not shown).

For comparative studies, *S. siyangense* KCTC 22131^T and *S. multivorum* KACC 12158^T were obtained from the Korean Collection for type cultures (KCTC) and the Korean Agricultural Culture Collection (KACC), respectively. To further clarify the taxonomic status of strain No.6^T, DNA–DNA hybridization experiments were carried out with these two reference strains by using the membrane filter hybridization method described by Brown (2005). Probe labelling, hydridization and detection were conducted by using the non-radioactive DIG-High Prime system and hybridized DNA was visualized using the DIG luminescent detection kit (Roche) according to the manufacturer's instructions (Fig. S1, available in IJSEM Online). DNA–DNA relatedness was quantified by using a densitometer (Bio-Rad). The signal produced by self-hybridization was taken as 100 %, and percentage relatedness values were calculated from duplicate samples. Strain No.6^T showed 32.0 and 5.7 % DNA–DNA relatedness to *S. siyangense* KCTC 22131^T and *S. multivorum* KACC 12158^T, respectively. These values were well below the threshold value of 70 % accepted for the delineation of a bacterial species (Wayne *et al.*, 1987; Stackebrandt & Ebers, 2006). Phylogenetic inference and DNA–DNA hybridization data demonstrated that strain No.6^T cannot be allocated to any described *Sphingobacterium* species.

The morphological, physiological and biochemical characteristics of strain No.6^T and the two reference strains were investigated using routine cultivation on LB agar at 30 °C. Cell morphology and size were studied using phase-contrast and transmission electron microscopy (JEM-1010; JEOL) as described previously (Bernardet *et al.*, 2002). Growth temperature range and optimum were measured from 5 to 45 °C (at 5 °C intervals) on LB agar, and the pH range and optimum for growth were examined at pH 4.0–11.0 (at 0.5 pH unit intervals) in LB broth, the pH of which was adjusted with 1 M HCl or 1 M NaOH. The requirement for and tolerance to NaCl were determined in the presence of 0–10 % (w/v) NaCl (at 1 % intervals) in LB broth. Flagellar motility was examined by using wet mounts made from fresh LB broth cultures grown at 30 °C for 1 day. The Gram reaction was determined by using the bioMérieux Gram stain kit according to the manufacturer's instructions. Catalase activity was evaluated by the production of oxygen bubbles in 3 % (v/v) H_2O_2 and oxidase activity was tested by oxidation of a 1 % (w/v) *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine solution. Growth under anaerobic conditions was tested on LB agar using the GasPak anaerobic system (BBL) following incubation at 30 °C for 20 days. Hydrolysis of

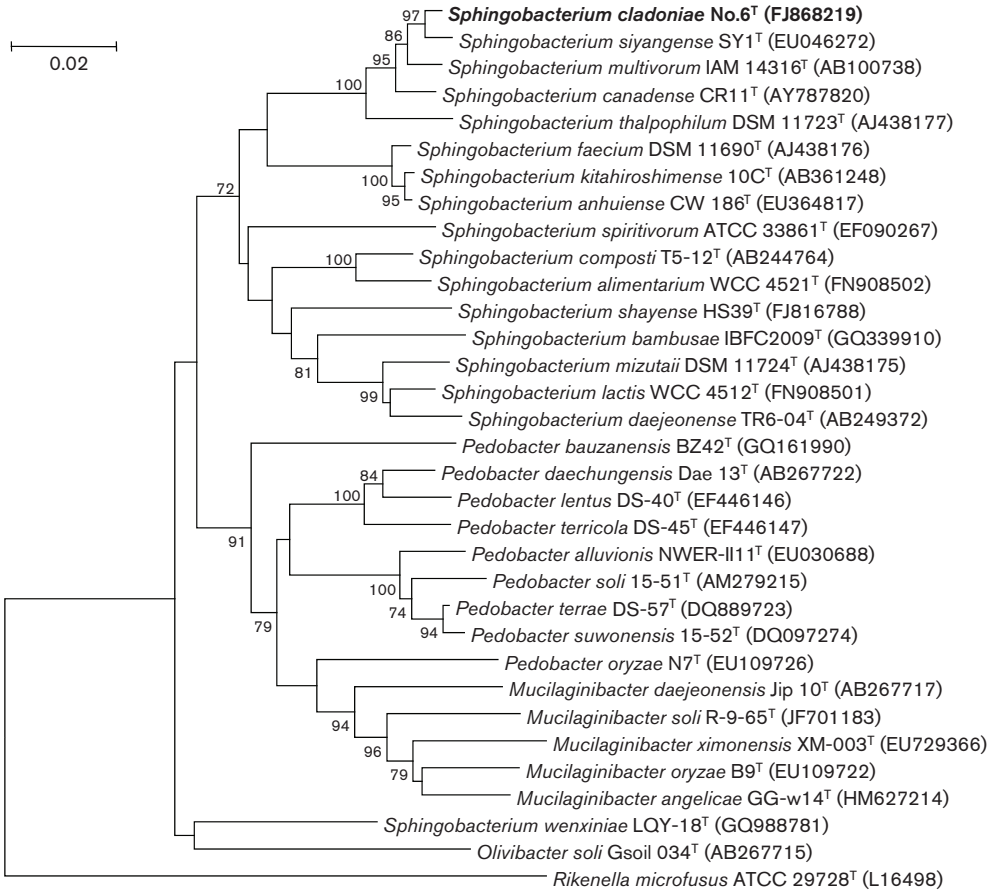

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships of strain No.6^T and related taxa. Bootstrap percentages (based on 1000 replicates) greater than 70% are shown at branch points. *Rikenella microfusus* ATCC 29728^T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

casein, chitin, starch, carboxymethyl (CM)-cellulose, DNA, and Tweens 20, 40, 60 and 80 was examined using LB agar supplemented with 1% of each substrate as described previously (Høvik Hansen & Sørheim, 1991; Smibert & Krieg, 1994). Other biochemical and enzyme characteristics of the three strains were determined using API 20E, API 20NE, API 50CH (all incubated at 30 °C for 2 days) and API ZYM (incubated at 30 °C for 8 h) test kits (bioMérieux) and utilization of different carbon sources was tested with GN2 MicroPlates (Biolog) incubated at 30 °C for 2 days. Antibiotic sensitivity of strain No.6^T was tested after 2 days of incubation at 30 °C on LB agar by the disc diffusion method using the following commercial antibiotic-impregnated discs (BBL Becton Dickson): ampicillin (10 μg), carbenicillin (100 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), lincomycin (15 μg), nalidixic acid (30 μg), neomycin (30 μg), novobiocin (5 μg), ofloxacin (5 μg), oleandomycin (15 μg), penicillin (10 IU), polymyxin B (300 μg) and tetracycline (30 μg). The results were interpreted according to the guidelines set out by the Clinical and Laboratory Standards Institute. The phenotypic characteristics of strain No.6^T are detailed in Table 1, and in the species description. Strain No.6^T notably differed from *S. siyangense* KCTC 22131^T and *S. multivorum* KACC 12158^T by its ability to hydrolyse casein, gelatin and Tween 80 (Table 1).

For analysis of the fatty acid composition, strain No.6^T and the two reference strains were grown aerobically on trypticase soy agar (TSA) at 30 °C for 2 days. Fatty acid methyl esters were obtained by saponification, methylation and extraction by using the standard protocol of MIDI (Sherlock Microbial Identification System, version 4.5). Fatty acids were analysed by GC (Agilent 6890N) and identified by using the TSBA50 database of the Microbial Identification System (Sasser, 1990). Polar lipids of strain No.6^T and *S. siyangense* KCTC 22131^T were extracted according to the procedures described by Minnikin *et al.* (1984) and separated by two-dimensional TLC using 10×10 cm silica gel 60 F_{254} plates. Individual polar lipids were identified by spraying the plates with ethanolic molybdophosphoric acid, molybdenum blue, ninhydrin and α-naphthol reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987) and co-migration with authentic standards

Table 1. Characteristics that differentiate strain No.6^T, *Sphingobacterium siyangense* KCTC 22131^T and *Sphingobacterium multivorum* KACC 12158^T

Strains: 1, No.6^T; 2, *S. siyangense* KCTC 22131^T; 3, *S. multivorum* KACC 12158^T. +, Positive; −, negative; w, weakly positive. All data from this study except the DNA G+C contents of *S. siyangense* KCTC 22131^T and *S. multivorum* KACC 12158^T, which were taken from Liu *et al.* (2008) and Mehnaz *et al.* (2007), respectively.

Characteristic	1	2	3
Growth at:			
5 °C	−	+	−
42 °C	−	+	−
Hydrolysis of:			
Casein	+	−	−
Gelatin	+	−	−
Tween 80	+	−	−
Acid production from:			
L-Arabinose	−	−	+
Cellobiose	+	−	+
Glucose	+	−	+
Maltose	+	−	+
Mannose	+	−	+
Sucrose	+	−	+
Utilization of:			
Dextrin	+	+	−
L-Arabinose	w	+	+
D-Galactose	+	+	−
Melibiose	+	+	−
D-Psicose	−	+	+
L-Rhamnose	+	+	−
Succinic acid	+	+	−
L-Histidine	−	+	+
DNA G+C content (mol%)	36.8	38.5	42.2

(Sigma). For the analysis of sphingolipids, cell mass of strain No.6^T and *S. siyangense* KCTC 22131^T was harvested from TSA after incubation for 2 days at 30 °C. The sphingolipids were extracted and analysed by TLC using the method of Yano *et al.* (1982). The respiratory quinones of strain No.6^T were analysed by the Korean Culture Center of Microorganisms (KCCM; Seoul, Korea). The respiratory quinones were extracted and purified according to Komagata & Suzuki (1987), and separated by using reversed-phase HPLC with a Spherisorb 5 μm ODS2 column (250 × 4.6 mm; Waters) using an elution of methanol and isopropyl ether (4 : 1) as described previously (Tamaoka, 1986). The DNA G+C content of strain No.6^T was determined using the thermal denaturation method (Marmur & Doty, 1962) using an Ultrospec 2100 spectrophotometer (Pharmacia Biotech). DNA from *Escherichia coli* K-12 was used as a control.

The major cellular fatty acids of strain No.6^T were summed feature 3 (comprising iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$, 41.5 %), iso-$C_{15:0}$ (26.7 %) and $C_{16:0}$ (9.6 %). Small amounts of iso-$C_{15:0}$ 3-OH (4.2 %), $C_{14:0}$ (3.0 %) and iso-$C_{17:0}$ 3-OH (2.7 %) were also present. This fatty acid profile was very similar to those of the two reference strains grown under the same conditions, although there were slight differences in the presence and proportions of some fatty acids (Table 2). The polar lipid profile of strain No.6^T comprised phosphatidylethanolamine, two unidentified phospholipids, three unidentified aminophospholipids, one unidentified glycolipid and four unidentified lipids. (Fig. S2). The polar lipid profile of strain No.6^T was very similar to that of *S. siyangense* KCTC 22131^T except that strain No.6^T contained one supplementary unidentified phospholipid and one supplementary unidentified lipid. Both strains contained one unidentified sphingolipid, which is a distinct feature of members of the genus *Sphingobacterium* (Fig. S3). The only respiratory quinone of strain No.6^T was menaquinone 7 (MK-7); this menaquinone is the major or only one in all members of the family *Sphingobacteriaceae*. The G+C content of the genomic DNA of strain No.6^T was 36.8 mol%, a value within the range reported for *Sphingobacterium* species (35.4–44.2 mol%) (Liu *et al.*, 2008; He *et al.*, 2010). Hence, chemotaxonomic and genomic data support allocation of strain No.6^T to the genus *Sphingobacterium*.

In conclusion, on the basis of phenotypic characterization and phylogenetic inference, strain No.6^T represents a novel species of the genus *Sphingobacterium*, for which the name *Sphingobacterium cladoniae* sp. nov. is proposed. In addition, an emended description of *S. siyangense* is proposed on the basis of new data obtained in this study.

Table 2. Fatty acid composition (%) of strain No.6^T, *Sphingobacterium siyangense* KCTC 22131^T and *Sphingobacterium multivorum* KACC 12158^T

Strains: 1, No.6^T; 2, *S. siyangense* KCTC 22131^T; 3, *S. multivorum* KACC 12158^T. Fatty acids amounting to less than 0.5 % in all strains tested are not listed. tr, Trace (<0.5 %); −, not detected. All data from this study.

Fatty acid	1	2	3
$C_{12:0}$	−	−	1.5
$C_{13:1}$ AT 12–13	1.0	tr	1.7
$C_{14:0}$	3.0	3.6	5.5
iso-$C_{15:0}$	26.7	29.3	20.9
iso-$C_{15:0}$ 3-OH	4.2	3.5	3.3
$C_{16:0}$	9.6	11.4	14.7
$C_{16:0}$ 3-OH	1.9	2.0	4.4
iso-$C_{17:1}\omega9c$	0.9	0.9	0.6
iso-$C_{17:0}$ 3-OH	2.7	2.4	2.4
Summed feature 3*	41.5	38.0	38.3
ECL 13.565†	2.4	1.6	1.2

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$.
†ECL, Unknown fatty acid designated by its equivalent chain-length.

Description of *Sphingobacterium cladoniae* sp. nov.

Sphingobacterium cladoniae (cla.do.ni′a.e. N.L. gen. n. *cladoniae* of a *Cladonia*, the lichen that was the source of the type strain).

Cells are Gram-stain-negative, non-motile, non-spore-forming, strictly aerobic rods, 0.8–1.5 μm in length and 0.7–1.0 μm in diameter. Colonies on LB agar are 1.0–3.0 mm in diameter, convex, circular and smooth with entire margins, initially white and turning yellow after 48 h of incubation on LB agar. Growth occurs at 15–35 °C (optimum, 25–30 °C), at pH 5.0–10.0 (optimum, pH 6.0–8.0) and with 0–3 % NaCl (optimum, 0–2 %). Catalase and oxidase activities are present. Casein, gelatin, starch, DNA, aesculin, and Tweens 20, 40, 60 and 80 are hydrolysed, but CM-cellulose and chitin are not. In the API 20E and API 20NE kits, positive reactions are observed for acetoin (Voges–Proskauer) production and glucose fermentation, while negative reactions are observed for nitrate reduction, and indole and H_2S production; β-galactosidase, arginine dihydrolase and urease activities are present, while lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase activities are absent. In the API ZYM kit, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase and *N*-acetyl-β-glucosaminidase activities are present, while lipase (C14), trypsin, α-chymotrypsin, β-glucuronidase, α-mannosidase and α-fucosidase activities are absent. Acid is produced from D-arabinose, glucose, fructose, mannose, rhamnose, methyl α-D-mannoside, methyl α-D-glucoside, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch and gentiobiose, but not from any other substrate in the API 50CH kit. The following compounds are utilized as sole carbon sources in the GN2 MicroPlate: α-cyclodextrin, dextrin, glycogen, Tween 40, Tween 80, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, L-arabinose, cellobiose, D-fructose, L-fucose, D-galactose, α-D-glucose, α-lactose, lactulose, maltose, D-mannose, melibiose, methyl β-D-glucoside, raffinose, L-rhamnose, sucrose, trehalose, turanose, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, D-galacturonic acid, D-glucuronic acid, α-hydroxybutyric acid, α-ketobutyric acid, α-ketovaleric acid, DL-lactic acid, propionic acid, succinic acid, L-alaninamide, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-leucine, L-proline, L-serine, L-threonine, uronic acid, uridine, glycerol, DL-α-glycerol phosphate, α-D-glucose 1-phosphate and D-glucose 6-phosphate. None of the other carbon sources in the GN2 MicroPlate is utilized. Resistant to ampicillin (10 μg), carbenicillin (100 μg), gentamicin (10 μg), kanamycin (30 μg), lincomycin (15 μg), nalidixic acid (30 μg), neomycin (30 μg), novobiocin (5 μg), oleandomycin (15 μg), penicillin (10 IU), polymyxin B (300 μg) and tetracycline (30 μg), but sensitive to erythromycin (15 μg) and ofloxacin (5 μg). The predominant fatty acids are summed feature 3 (comprising iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$), iso-$C_{15:0}$ and $C_{16:0}$. The polar lipid profile consists of phosphatidylethanolamine, two unidentified phospholipids, three unidentified aminophospholipids, one unidentified glycolipid and four unidentified lipids. One unidentified sphingolipid is also present. The only respiratory quinone is menaquinone MK-7.

The type strain, No.6^T (=KCTC 22613^T=JCM 16113^T), was isolated from a lichen (*Cladonia* sp.) collected in Geogeum Island, Korea. The G+C content of the DNA of the type strain is 36.8 mol%.

Emended description of *Sphingobacterium siyangense* Liu *et al.* 2008

The description is as given by Liu *et al.* (2008) with the following additions. The polar lipid profile consists of phosphatidylethanolamine, one unidentified phospholipid, three unidentified aminophospholipids, one unidentified glycolipid and three unidentified lipids. One unidentified sphingolipid is also present.

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