INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION
Lee et al., Int J Syst Evol Microbiol 2017;67:4632–4638
DOI 10.1099/ijsem.0.002345

MICROBIOLOGY SOCIETY

Sphingobacterium humi sp. nov., isolated from soil

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Abstract

A Gram-stain-negative, facultatively aerobic bacterium, designated strain D1^T, was isolated from soil in South Korea. Cells of strain D1^T were non-motile rods with oxidase- and catalase-positive activities. Growth was observed at 15–40 °C (optimum, 30–37 °C), at pH 5.5–9.0 (optimum, pH 7.0–8.0) and in the presence of 0.0–5.0 % (w/v) NaCl (optimum, 0.0–1.0 %). The only respiratory quinone detected was menaquinone 7 (MK-7), and iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and summed feature 3 (comprising $C_{16:1}\omega7c$/$C_{16:1}\omega6c$) were identified as the major fatty acids. Phosphatidylethanolamine was the major polar lipid, and two unidentified glycophospholipids and four unidentified lipids were also detected as minor polar lipids. Sphingolipids, a typical chemotaxonomic feature of the genus *Sphingobacterium*, were detected. The G+C content of the genomic DNA was 43.6 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain D1^T formed a phyletic lineage with *Sphingobacterium hotanense* XH4^T within the genus *Sphingobacterium*. Strain D1^T was most closely related to *S. hotanense* XH4^T (98.1 % 16S rRNA gene sequence similarity) and *Sphingobacterium cellulitidis* R-53603^T (97.2 %), and the DNA–DNA relatedness level between strain D1^T and the type strain of *S. cellulitidis* was 43.1±0.7 %. Based on the phenotypic, chemotaxonomic and molecular features, strain D1^T clearly represents a novel species of the genus *Sphingobacterium*, for which the name *Sphingobacterium humi* sp. nov. is proposed. The type strain is D1^T (=KACC 18595^T=JCM 31225^T).

The genus *Sphingobacterium*, the type genus of the family *Sphingobacteriaceae* in the phylum *Bacteroidetes*, was first proposed by Yabuuchi *et al.* [1] with three species, *Sphingobacterium spiritivorum* (the type species), *Sphingobacterium multivorum* and *Sphingobacterium mizutae* (corrected to *Sphingobacterium mizutaii* [2]). Among species of the genus *Sphingobacterium*, *Sphingobacterium heparinum*, *Sphingobacterium piscium*, *Sphingobacterium antarcticum* and *S. mizutaii*, which were initially reported as members of the genus *Sphingobacterium*, were reclassified as *Pedobacter heparinus*, *Pedobacter piscium*, *Pedobacter antarcticus* and *Flavobacterium mizutaii*, respectively [1–6]. However, *F. mizutaii* is currently generally accepted as *S. mizutaii* of the genus *Sphingobacterium* [7]. At the time of writing, the genus *Sphingobacterium* comprises 43 species with validly published names, including the recently described species of the genus *Sphingobacterium*, *Sphingobacterium soli* [8], *Sphingobacterium alkalisoli* [9], *Sphingobacterium cellulitidis* [10] and *Sphingobacterium chuzhouense* [11]. Most members of the genus *Sphingobacterium* are Gram-stain-negative, catalase- and oxidase-positive rods without flagella. They contain a high content of sphingophospholipids and iso-$C_{15:0}$, iso-$C_{15:0}$ 3-OH, $C_{16:1}\omega7c$ and $C_{17:0}$ 3-OH as the major fatty acids and menaquinone-7 (MK-7) as the main respiratory quinone [1]. Species of the genus *Sphingobacterium* with diverse physiological and functional properties have been isolated from diverse habitats such as farmland soil, tree bark, compost and rhizosphere soil [1, 11–15], suggesting that members of the genus *Sphingobacterium* may play important roles in environmental habitats. In this study, another presumably novel strain belonging to the genus *Sphingobacterium* was isolated from soil and its taxonomic properties were characterized using a polyphasic approach.

Strain D1^T was isolated from a soil sample of Pyeongtaek, South Korea (36° 56′ 29.3″ N 126° 59′ 34.7″ E), using a previously described procedure with some modifications [16, 17]. Briefly, the soil sample (less than 5 cm depth) was serially diluted with 0.85 % (w/v) saline, and aliquots of each serial dilution were spread on R2A agar (BD). After aerobic incubation at 30 °C for 5 days, the 16S rRNA genes of colonies grown on R2A agar were PCR-amplified using the universal primers F1 and R13, and the PCR amplicons were double-digested with *Hae*III and *Hha*I [16]. Representative PCR amplicons showing distinct fragment patterns were

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Keywords: *Sphingobacterium humi*; *Bacteroidetes*; new taxa; species novel.
Abbreviations: DDH, DNA–DNA hybridization; MK, menaquinone; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.
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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain D1^T is KU668559.
One supplementary table and three supplementary figures are available with the online Supplementary Material.

partially sequenced using primer F1. The resulting 16S rRNA gene sequences were compared with those of type strains available in the EzTaxon-e server [18]. From the analysis, a putatively novel strian representing the genus *Sphingobacterium*, designated strain D1^T, was selected for further phenotypic and phylogenetic analysis. Strain D1^T was routinely incubated aerobically on tryptic soy agar (TSA; BD) at 30 °C for 3 days, unless otherwise stated, and stored at −80 °C in tryptic soy broth (TSB; BD) containing 15 % (v/v) glycerol for long-time preservation. The type strains of *S. cellulitidis* (LMG 28764^T), *S. mizutaii* (KACC 12159^T), *Sphingobacterium lactis* (DSM 22361^T), *Sphingobacterium kyonggiense* (KACC 15594^T), *Sphingobacterium daejeonense* (KACC 11693^T) and *S. spiritivorum* (KACC 10895^T) were purchased from their culture collections and used as reference strains for the comparison of phenotypic properties and fatty acid compositions and DNA–DNA hybridization; the type strain of *Sphingobacterium hotanense* that was most closely related to strain D1^T in 16S rRNA gene sequence similarity was not available in the culture collections.

The 16S rRNA gene of strain D1^T was cloned into the pCR2.1 vector using a TOPO cloning kit (Invitrogen) according to the manufacturer's instructions and sequenced using the M13 reverse and T7 primers of the TOPO cloning kit at Macrogen (Korea) to obtain an almost-complete 16S rRNA gene sequence. The resulting 16S rRNA gene sequence (1449 nucleotides) of strain D1^T was compared with those of all reported type strains using the Nucleotide Similarity Search program [18]. The 16S rRNA gene sequences of strain D1^T and closely related type strains were aligned using the fast secondary-structure aware Infernal aligner of the Ribosomal Database Project (RDP) [19]. Phylogenetic trees based on the neighbor-joining (NJ) and maximum-parsimony (MP) algorithms were reconstructed using the PHYLIP software (version 3.695; [20]), and their tree topologies were evaluated through bootstrap analyses using a 1000-resampled dataset. Maximum-likelihood (ML) analysis with bootstrap values was performed using RAxML-HPC BlackBox (version 8.2.10) in the Cyber-Infrastructure for Phylogenetic Research project (CIPRES, www.phylo.org; [21]). DNA–DNA hybridization (DDH) between strain D1^T and *S. cellulitidis* LMG 28764^T was performed using a genome-probing microarray method, as described previously [22], which was confirmed by reciprocally interchanging DDH between probe and target DNA.

Comparative analysis based on the 16S rRNA gene sequences revealed that strain D1^T was most closely related to *S. hotanense* XH4^T, *S. cellulitidis* R-53603^T, *S. mizutaii* DSM 11724^T, *S. lactis* WCC 4512^T, *S. kyonggiense* KEMC 2241-005^T and *S. daejeonense* TR6-04^T with 98.1, 97.2, 96.7, 96.6, 96.0 and 95.7 % sequence similarities, respectively. A phylogenetic analysis based on the NJ algorithm showed that strain D1^T formed a phylogenetic lineage with *S. hotanense* XH4^T within the genus *Sphingobacterium* (Fig. 1). Phylogenetic trees reconstructed by the ML and MP algorithms supported that strain D1^T formed a phylogenetic lineage with *S. hotanense* XH4^T within the genus *Sphingobacterium* (data not shown). The DNA–DNA relatedness level between strain D1^T and the type strain of *S. cellulitidis* was 43.1±0.7 %, which was clearly below the 70 % threshold generally accepted for species delineation [23]. This result suggests that strain D1^T represents a novel species of the genus *Sphingobacterium*.

Growth of strain D1^T was tested at 30 °C for 3 days on TSA, marine agar (MA; BD), laboratory-prepared Luria–Bertani (LB) agar, R2A agar and nutrient agar (NA; BD). Growth of strain D1^T at different temperatures (10, 15, 20, 25, 30, 37, 40, 43 and 45 °C) was checked in TSB for 3 days. Growth of strain D1^T at different pH values (pH 5.0–10.0 at 0.5 pH unit intervals) was assessed in TSB at 30 °C for 3 days. TSB media with pH values below pH 8.0 and of pH 8.0–10.0 were prepared using Na_2HPO_4/NaH_2PO_4 and Tris/HCl buffers, respectively, as described previously [24], and their pH values were adjusted again after sterilization (121 °C for 15 min). Salt tolerance of strain D1^T was evaluated in TSB containing different concentrations of NaCl (0–6 %, w/v, at 1 % intervals), which was prepared in the laboratory according to the BD formula. Gram-staining was conducted using a Gram stain kit (bioMérieux), according to the manufacturer's instructions. Anaerobic growth was tested on TSA at 30 °C for 21 days under anaerobic conditions (with 4–10 % CO_2) using the GasPak Plus system (BBL). Cell morphology and the presence of flagella were investigated using transmission electron microscopy (JEM-1010, JEOL) and phase-contract microscopy with cells grown on TSA at 30 °C for 2 days. Gliding motility of strain D1^T was determined using the hanging-drop technique as described by Bernardet *et al.* [25]. Nitrate reduction was examined according to the method of Lányi [26]. Catalase and oxidase activities of strain D1^T were tested by the production of oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution and the oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck), respectively [27]. The following properties of strain D1^T and the six reference strains were assessed in parallel under the same conditions at 30 °C. Hydrolysis of tyrosine, aesculin, starch, casein, Tween 20 and Tween 80 was tested on TSA, according to the procedures described by Lányi [26] and Smibert and Krieg [27]. Additional enzymic activities, biochemical features and oxidation of carbon sources were determined with the API ZYM and API 20E kits (bioMérieux) and GN2 MicroPlate system (Biolog), respectively, according to the instructions of the manufacturers.

Strain D1^T grew on TSA, R2A agar, LB agar, NA and MA (optimum, TSA). Cells were Gram-stain-negative rods without a flagellum (1.0–2.1 µm in length and 0.7–0.8 µm in width) (Fig. S1, available in the online Supplementary Material). Slow growth under anaerobic conditions occurred on TSA after 21 days of incubation at 30 °C. In the Biolog GN2 MicroPlate, strain D1^T oxidized dextrin, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, cellobiose, D-fructose,

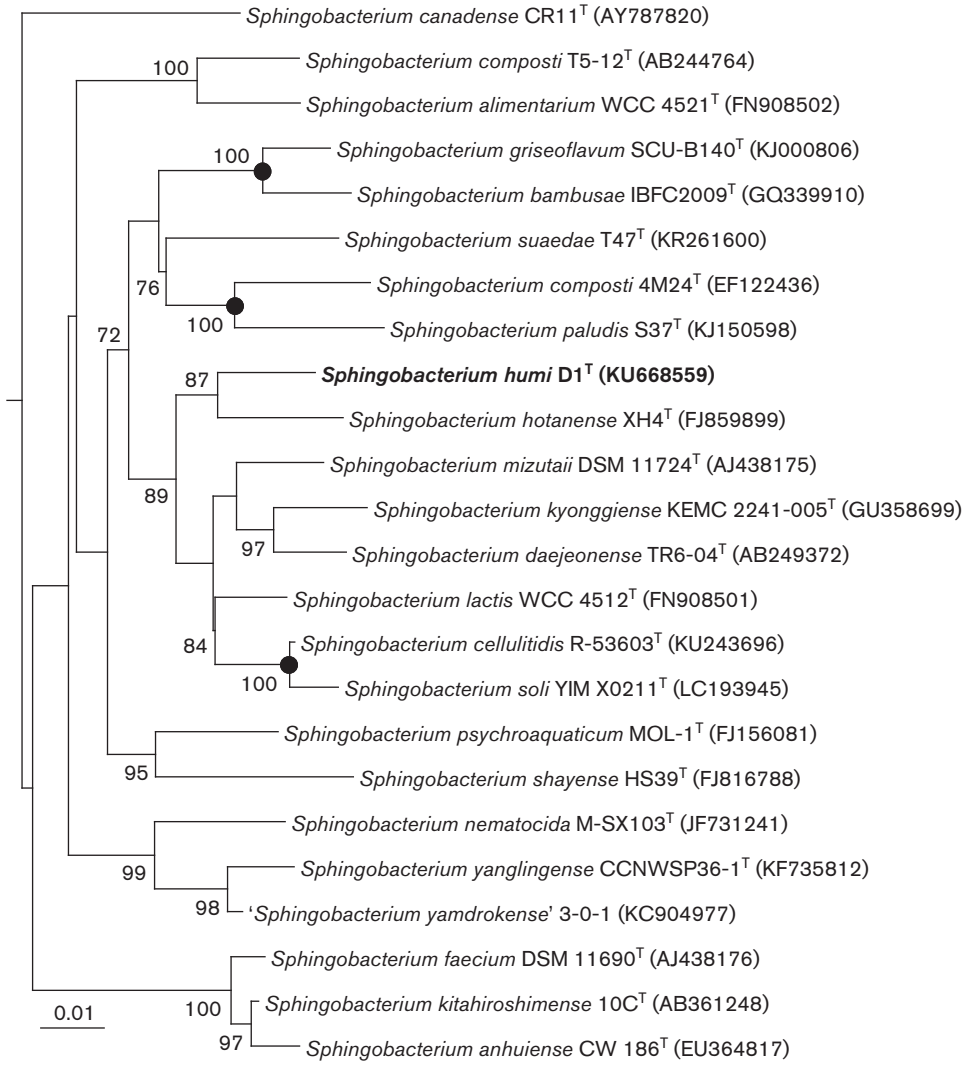

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strain D1^T and the type strains of closely related species of the genus *Sphingobacterium*. Bootstrap values are shown on nodes in percentages of 1000 replicates; only values over 70 % are indicated. Filled circles (●) indicate the corresponding nodes that were also recovered by the ML and MP algorithms. *Flavobacterium granuli* Kw05^T (AB180738) was used as the outgroup (not shown). Bar, 0.01 changes per nucleotide position.

gentiobiose, α-D-glucose, lactose, lactulose, maltose, D-mannose, melibiose, methyl β-D-glucoside, D-psicose, sucrose, trehalose, turanose, pyruvic acid methyl ester, succinamic acid monomethyl ester, acetic acid, D,L-lactic acid, L-alanylglycine, L-glutamic acid, glycyl-L-glutamic acid, L-histidine, L-threonine, uridine, phenyethylamine and glycerol, but strain D1^T did not oxidize other carbon compounds in the Biolog GN2 MicroPlate. The phenotypic characteristics of strain D1^T are presented in the species description and compared with those of the closely related type strains of species of the genus *Sphingobacterium* in Tables 1 and S1. Many phenotypic characteristics including the ranges of temperature, NaCl and pH for growth, catalase and oxidase activities and the absence of nitrate reduction ability were in common with those of closely related species of the genus *Sphingobacterium*, whereas others such as facultatively anaerobic growth and oxidation of uridine allowed the differentiation of strain D1^T from closely related species of the genus *Sphingobacterium* (Tables 1 and S1) [1, 10, 28–31].

The isoprenoid quinone of strain D1^T was analysed with a HPLC (model LC-20A, Shimadzu) system equipped with a reversed-phase column (250×4.6 mm; Kromasil, Akzo Nobel) and a diode array detector (SPD-M20A, Shimadzu) using methanol/isopropanol (2 : 1, v/v) as an eluent (1 ml min^{-1}), as described previously [32]. For the cellular fatty acid analysis, strain D1^T and the six reference strains were cultivated in TSB at 30 °C and microbial cells were harvested

Table 1. Comparisons of phenotypic characteristics of strain D1^T and the type strains of closely related species of the genus *Sphingobacterium*

Strains: 1, D1^T (this study); 2, *S. hotanense* XH4^T [28]; 3, *S. cellulitidis* LMG 28764^T [10]; 4, *S. mizutaii* KACC 12159^T [1]; 5, *S. lactis* DSM 22361^T [29]; 6, *S. kyonggiense* KACC 15594^T [30]; 7, *S. daejeonense* KACC 11693^T [31]; 8, *S. spiritivorum* KACC 10895^T [1]. All strains are positive for the following characteristics: oxidase*, catalase*, enzyme activity of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arlyamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and *N*-acetyl-β-glucosaminidase. All strains are negative for the following characteristics: gliding motility*, nitrate reduction*, hydrolysis of casein and gelatin, H_2S production, enzyme activity of arginine dihydrolase, lysine decarboxylase and tryptophan deaminase and acid production from inositol, D-sorbitol and amygdalin. With the exception of *S. hotanense* XH4^T (data not available), all strains are positive for acetoin production. All strains are negative for hydrolysis of tyrosine and enzyme activity of ornithine decarboxylase. +, Positive; −, negative; NA, not available. All results except data indicated were obtained from this study.

Characteristic	1	2*	3	4	5	6	7	8
Growth under/at/with:*								
Anaerobic conditions	+	−	−	−	−	−	−	−
Temperature (°C)	15–40	20–45	15–37	10–40	10–41	15–42	15–42	5–42
NaCl (%)	0–5	0–3	0–6	NA	0–5	0–4	0–5	NA
pH	5.5–9.0	6.0–9.0	7.0–9.0	5.0–9.0	6.0–9.0	6.0–10.0	5.0–9.0	NA
Hydrolysis of:								
Starch	−	−	+	+	+	−	−	+
Tween 20	+	+	+	+	+	−	−	+
Tween 80	−	+	−	−	−	−	−	+
Aesculin	+	−	+	+	+	+	+	+
Production of indole	+	−	−	−	−	+	−	−
Enzyme activity of (API ZYM):								
Lipase (C14)	−	+	−	−	−	−	−	−
Trypsin	−	+	−	+	+	−	+	+
α-Chymotrysin	+	−	−	+	−	−	−	−
β-Glucuronidase	+	−	+	+	−	−	−	−
β-Glucosidase	+	+	−	+	+	+	+	+
α-Galactosidase, β-galactosidase	+	−	+	+	+	+	+	−
α-Mannosidase	+	+	+	+	−	−	+	−
α-Fucosidase	−	−	−	−	−	−	−	+
Acid production from (API 20E):								
D-Glucose	+	−	−	+	+	+	+	+
D-Mannitol, sucrose	+	−	−	−	−	−	−	+
L-Rhamnose	−	−	−	−	−	−	−	+
Melibiose	−	−	−	+	+	+	+	+
L-Arabinose	−	−	−	+	+	−	−	+
DNA G+C content (mol%)*	43.6	41.2	37.3	39.3–40.0	44.2	36.6	38.7	40.0

*Data from the literature cited.

after approximately 2 days, showing the same growth phase (exponential phase, optical density at 600 nm of 0.8). The cellular fatty acids of microbial cells were saponified, methylated and extracted using the standard MIDI protocol. The fatty acid methyl esters were analysed by gas chromatography (model 6890, Hewlett Packard) and identified by using the TSBA6 database of the Microbial Identification System (Sherlock version 6.0B, MIDI; [32]). The polar lipids of strain D1^T were analysed by TLC using cells harvested during the exponential growth phase, as described by Minnikin *et al.* [33]. The following reagents were used to detect different polar lipids: 10 % ethanolic molybdatophosphoric acid reagent (for total polar lipids), ninhydrin (for aminolipids), Dittmer–Lester reagent (for phospholipids) and α-naphthol/sulfuric acid (for glycolipids). To detect the presence of sphingolipids, the sphingolipids of strain D1^T and two reference strains were analysed as described by Choi and Lee [30]. The DNA G+C content of strain D1^T was determined by the fluorometric method [34] using SYBR green I and a real-time PCR thermocycler (Bio-Rad).

The only respiratory quinone detected from strain D1^T was menaquinone-7 (MK-7). The major cellular fatty acids (>10 % of the total fatty acids) of strain D1^T were iso-$C_{15:0}$ (35.3 %), summed feature 3 (comprising $C_{16:1}\omega7c/C_{16:1}\omega6c$, 29.9 %) and iso-$C_{17:0}$ 3-OH (14.0 %). The overall fatty acid profile of strain D1^T was almost similar to those of other reference strains of the genus *Sphingobacterium* although there were some differences in the respective proportions of some components (Table 2). Phosphatidylethanolamine was identified as the major polar lipid, and two unidentified glycophospholipids and four unidentified lipids were also detected as minor

Table 2. Cellular fatty acid contents (percentages) of strain D1^T and the type strains of closely related species of the genus *Sphingobacterium*

Strains: 1, D1^T; 2, *S. hotanense* XH4^T [28]; 3, *S. cellulitidis* LMG 28764^T; 4, *S. mizutaii* KACC 12159^T; 5, *S. lactis* DSM 22361^T; 6, *S. kyonggiense* KACC 15594^T; 7, *S. daejeonense* KACC 11693^T; 8, *S. spiritivorum* KACC 10895^T. All results except the data for *S. hotanense* XH4^T were obtained from this study. Data are expressed as percentages of the total fatty acids, and fatty acids amounting to less than 1.0 % in all strains are not shown. Major components (>10.0 %) are highlighted in bold type; TR, trace amount (<1.0 %); —, not detected.

Fatty acid	1	2	3	4	5	6	7	8
Saturated								
$C_{14:0}$	TR	–	3.4	TR	TR	TR	TR	2.5
$C_{16:0}$	1.6	1.8	4.7	3.0	4.0	1.4	2.6	3.9
Unsaturated								
$C_{16:1}\omega5c$	TR	–	TR	TR	TR	TR	–	1.4
Branched								
iso-$C_{15:0}$	**35.3**	**34.0**	**53.2**	**48.2**	**39.4**	**37.2**	**39.8**	**34.3**
anteiso-$C_{15:0}$	2.9	5.5	TR	1.5	3.5	3.6	8.0	TR
iso-$C_{15:1}$ F	TR	–	TR	TR	TR	1.1	–	1.5
Hydroxy								
iso-$C_{15:0}$ 3-OH	2.0	1.3	1.4	1.3	1.8	1.6	2.0	3.9
$C_{16:0}$ 3-OH	1.0	TR	1.1	1.6	1.9	1.3	TR	3.4
iso-$C_{16:0}$ 3-OH	TR	–	–	TR	TR	TR	1.1	TR
$C_{17:0}$ 2-OH	TR	1.5	–	TR	TR	TR	1.1	–
iso-$C_{17:0}$ 3-OH	**14.0**	**14.7**	8.5	**14.8**	**10.5**	8.7	8.3	5.9
Summed features*								
1	TR	TR	TR	TR	TR	1.1	TR	2.3
3	**29.9**	**20.7**	**20.3**	**18.9**	**31.6**	**30.4**	**31.1**	**30.8**
4	1.7	1.2	TR	1.6	TR	1.2	TR	2.5
9	5.3	5.9	TR	5.0	1.9	6.6	1.7	2.9

*Summed features represent groups of two or three fatty acids that cannot be separated by gas/liquid chromatography with the MIDI system. Summed features 1, 3, 4 and 9 comprise iso-$C_{15:1}$ H and/or $C_{13:0}$ 3-OH, $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$, iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B, and iso-$C_{17:1}$ $\omega9c$ and/or $C_{16:0}$ 10-methyl, respectively.

polar lipids, which was generally consistent with the reference strains of the genus *Sphingobacterium* (Fig. S2) [10, 28–31]. Sphingolipids, which are a typical chemotaxonomic feature of the genus *Sphingobacterium* [1], were detected after a mild alkaline hydrolysis (Fig. S3). The DNA G+C content of strain D1^T was 43.6 mol%, which was in the range of the DNA G+C contents of the previously reported species of the genus *Sphingobacterium* (35–44 mol%) [35, 36]. In conclusion, the phylogenetic inference and the physiological and biochemical properties of strain D1^T support its assignment as a representative of a novel species of the genus *Sphingobacterium*.

Characteristics of strain D1^T such as phosphatidylethanolamine as the major polar lipid, the presence of sphingophospholipids, MK-7 as the major respiratory quinone and iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and summed feature 3 ($C_{16:1}\omega7c$/$C_{16:1}\omega6c$) as the major fatty acids were in common with those of species of the genus *Sphingobacterium*, but other characteristics of strain D1^T including DNA–DNA relatedness level, anaerobic growth, respective compositions of fatty acids and many phenotypic properties shown in Table 1 allowed differentiation of it from species of the genus *Sphingobacterium*. In conclusion, the phylogenetic inference and the physiological and biochemical properties of strain D1^T support its assignment as a representative of a novel species of the genus *Sphingobacterium*, for which the name *Sphingobacterium humi* sp. nov. is proposed.

DESCRIPTION OF *SPHINGOBACTERIUM HUMI* SP. NOV.

Sphingobacterium humi (hu′mi. L. gen. n. *humi* of soil).

Cells are Gram-stain-negative, facultatively aerobic rods without a flagellum; no gliding motility is observed. Colonies on TSA are circular, convex and smooth. Growth occurs at 15–40 °C (optimum, 30–37 °C), at pH 5.5–9.0 (optimum, pH 7.0–8.0) and in the presence of 0–5.0 % (w/v) NaCl (optimum, 0–1.0 %). Oxidase- and catalase-positive. Tween 20 and aesculin are hydrolysed, but Tween 80, tyrosine, casein, gelatin and starch are not. Nitrate is not reduced to nitrite. Production of H_2S is negative. Indole is produced. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arlyamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase and α-mannosidase activities are positive, but lipase (C14), trypsin, α-fucosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease and tryptophan deaminase activities are negative. Acid is

produced from trisodium citrate, D-mannitol and sucrose, but not from D-glucose, inositol, D-sorbitol, L-rhamnose, melibiose, amygdalin or L-arabinose. Phosphatidylethanolamine is the major polar lipid. Sphingolipids are present. The major cellular fatty acids are iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and summed feature 3 (comprising $C_{16:1}\omega7c/C_{16:1}\omega6c$). The only respiratory quinone is MK-7.

The type strain is D1^T (=KACC 18595^T=JCM 31225^T), isolated from soil in Pyeongtaek, South Korea. The DNA G+C content of the type strain is 43.6 mol%.

Funding information
This work was supported by the Program for Collection of Domestic Biological Resources from the National Institute of Biological Resources (NIBR no. 2017-02-001) of the Ministry of Environment (MOE) and the Strategic Initiative for Microbiomes in Agriculture and Food, Ministry of Agriculture, Food and Rural Affairs (as part of the multi-ministerial) Genome Technology to Business Translation Program, Republic of Korea.

Acknowledgements
The authors would like to thank a nomenclature reviewer for the support regarding the nomenclature of the micro-organism.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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