Sphingomonas Negativus sp. nov., and *Sphingomonas Gyeonggiense* sp. nov., Bacteria Isolated From Soil in South Korea

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Abstract

Two novel Gram-staining-negative bacterial strains BT553^T and BT552^T were isolated from soil collected in Gyeonggi province, Korea. Phylogenetic analysis using 16S rRNA gene sequences revealed that the strains BT553^T and BT552^T both belong to a distinct lineage in the genus *Sphingomonas* (family *Sphingomonadaceae*, order *Sphingomonadales*, class *Alphaproteobacteria*). Strain BT553^T was closely related to *Sphingomonas melonis* DAPP-PG 224 ^{T} (98.1 % 16S rRNA gene similarity) and *Sphingomonas aquatilis* JSS7^T (98.1%). Strain BT553^T was closely related to *Sphingomonas melonis* DAPP-PG 224 ^{T} (98.2 %) and *Sphingomonas aquatilis* JSS7^T (98.1%). The genome size of strain BT553^T was 3,941,714 bp. Bacterial growth was observed at 10°C–30°C (optimum 25°C), pH 5.0–9.0 (optimum pH 7.0) in R2A agar and the presence of up to 2% NaCl. The genome size of strain BT552^T was 4,035,561 bp. Bacterial growth was observed at 10°C–30°C (optimum 25°C), pH 5.0–9.0 (optimum pH 7.0) in R2A agar and in the presence up to 2% NaCl. The major cellular fatty acids of strains BT553^T and BT552^T were Summed Feature 3 and (16:1 ω6*c* / 16:1 ω7*c*), Summed Feature 8 (18:1 ω7*c* / 18:1 ω6*c*), and 14:0. In addition, their predominant respiratory quinone was Q-10. The major polar lipids of strain BT553^T was identified to be diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC), and sphingoglycolipid (SL). The major polar lipids of strain BT552^T was identified to be diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC), phospholipid (PL), and sphingoglycolipid (SL). Based on the biochemical, chemotaxonomic, and phylogenetic analyses, strains BT553^T and BT552^T are novel bacterial species within the genus *Sphingomonas*. The type strain of *Sphingomonas negativus* is BT553^T (= KCTC 82095^T = NBRC XXXX^T) and the type strain of *Sphingomonas gyeonggiense* is BT552^T (= KCTC 82094^T = NBRC XXXX^T).

Introduction

The genus *Sphingomonas*, a member of the family *Sphingomonadaceae* in the phylum *Proteobacteria*, was first described by Holmes et al. (1977) and later was modified by Yabuuchi et al. (1990). At the time of writing (May 2021), the genus *Sphingomonas* comprised 161 species with validly published and correct names (https://lpsn.dsmz.de/genus/sphingomonas). The species in this genus are characterized as obligately aerobic, Gram-negative, non-spore forming, straight or slightly curved rods or ovoid cells, and motile of nonmotile. Mostly, colonies are lemon-yellow colored, and they produce catalase (Weisburg et al. 1991). They produce acids from pentoses, hexoses, and disaccharides by oxidation, but not from polyalcohols or inulin. They use ubiquinone 10 as a respiratory quinone. Cellular lipid contains two major fatty acids including cis-octadecenoic and 2- hydroxymyristic and it also contains sphingoglycolipid (SGL). The genus probably distributes widely in various environments and some species are opportunistic pathogen to humans. Usually, the nucleotide position 1290 (in *Escherichia coli*) is deleted in 16S rRNA and G + C contents of DNA are 64–66 mol% (Yabuuchi et al.1990).

During the soil sample screening, we found a strain designated as HKS19 that showed similar characteristics to the genus *Sphingomonas*. The results of our polyphasic taxonomic analysis were described in this paper.

Materials And Methods

Strain isolation

Two strains BT553^T and BT552^T were isolated from soil collected in Gyeonggi province, Korea. The soil was stored in a sterile plastic bottle at 4°C as soon as it was collected and then transported to laboratory. Soil (g) was added to 10 mL sterilized seawater, shaken at 25°C for 2 h, and then diluted by tenfold; 100 µL of the diluent was spread on R2A agar (Difco) and incubated at 25°C for 7 days. The colonies are purified and identified by 16S rRNA gene sequences using the EzBioCloud server (https://www.ezbiocloud.net/).

Morphology, physiology, and biochemical analysis

Morphological properties were observed using light microscopy (B1 series; Motic) and scanning electron microscopy (JEOL, JEM1010). The Gram-staining-reaction was performed using a commercial kit (bioMérieux, France), following the manufacturer's instructions. Oxidase and catalase activities were determined using 3% (w/v) H_2O_2 solution and 1% (w/v) tetramethyl-p-phenylenediamine, respectively (Cappuccino and Sherman 2002). Growth of the strains were tested at 25°C under various pH conditions (4 to 10, 0.5 pH intervals) and various NaCl concentrations (0 to 5% [w/v%], 1% intervals). The growth on various media was tested on R2A agar plates in Luria–Bertani (LB) agar, nutrient agar (NA), MacConkey (MAC) agar, and trypticase soy agar (TSA) mediums (all purchased from Difco) and were observed for three days at different temperatures (4°C, 10°C, 25°C, 30°C). The biochemical and physiological tests were performed using API 20NE kits, and enzymic activities were tested using an API ZYM kit (bioMérieux) following the manufacturer's instructions.

Genome sequencing

The genomic DNA of strains BT553^T and BT552^T were extracted using a genomic DNA extraction kit (Solgent). The DNA sequencing library was prepared by the Nextera DNA Flex Library Prep Kit (Illumina). Whole-genome sequencing was done using iSeq 100 (150 bp paired-end). The sequence was assembled using SPAdes 3.10.1 (Algorithmic Biology Lab, St. Petersburg Academic University of the Russian Academy of Sciences). The whole-genome sequences of strains BT553^T and BT552^T were deposited in the GenBank (www.ncbi.nlm.nih.gov/) database. The genome sequences of strains BT553^T and BT552^T were annotated using the National Center for Biotechnology Information Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al. 2016).

Phylogenetic analysis

The 16S rRNA genes of strains BT553^T and BT552^T were amplified by 27F and 1492R universal bacterial primers and the sequencing was performed using the 337F, 518R, 785F and 926R universal primers (Macrogen).

The taxonomic positions of strains BT553^T and BT552^T were determined using 16S rRNA sequences of related taxa, obtained from EzBioCloud and compared using the EzEditor2 program (Jeon et al. 2014). The phylogenetic tree was constructed using the MEGAX program (Kumar et al. 2018) with the neighbor-joining (Saitou and Nei 1987), maximum-likelihood, and maximum-parsimony algorithms (Ziheng 1995). The evolutionary distances were calculated according to the Kimura two-parameter model (Kimura 1983). A bootstrap analysis was conducted with 1,000 replicates (Felsenstein 1985).

Chemotaxonomic characteristics

The polar lipids were extracted according to Minnikin et al. (1984) and identified by two-dimensional TLC. The separated spots were detected with detection reagents sprayed onto the lipids (Komagata and Suzuki 1987). The quinones were extracted and purified using Sep-Pak Vac cartridges (Waters) and were analyzed by high-performance lipid chromatography (HPLC) according to the method of Hiraishi et al. (1996). For cellular fatty acid analysis, strains BT553^T and BT552^T were grown on R2A agar plates for 3 days at 25°C, and 300 mg of cells were harvested and freeze-dried. Cellular fatty acids were extracted, methylated and analyzed according to the standard protocol of the Sherlock Microbial Identification System (version 6.0; MIDIdatabase TSBA6) (Sasser 1990).

Results And Discussion

Morphology, physiology, and biochemical analysis

Strains BT553^T and BT552^T were Gram-negative and rod-shaped (Fig. S1). Colonies of strain BT553^T were circular, convex, slimy, and yellow in color after incubation for three days at 25°C. Colonies of strain BT552^T were circular, flat, and yellow after incubation for three days at 25°C. Strain BT553^T could survive at 10°C–30°C (optimum 25°C) and pH 5.0–9.0 (optimum 7.0) in the R2A medium. Strain BT552^T could survive at 10°C–30°C (optimum 25°C) and pH 5.0–9.0 (optimum 7.0) in the R2A medium. In the API 20NE test, strain BT553^T was positive for *β*-glucosidase (esculin hydrolysis), *β*-galactosidase (PNPG), D-glucose, L-arabinose, and D-maltose and weakly positive for protease (gelatin hydrolysis), D-mannose, *N*-acetyl-D-glucosamine, and L-malate (malic acid). But negative for nitrate reduction, production of indole and acid production from glucose, arginine dihydrolase, urease, D-mannitol, gluconate, caprate, adipate, citrate, and phenylacetate. Strain BT552^T was positive for *β*-glucosidase (esculin hydrolysis), *β*-galactosidase (PNPG), D-glucose, and L-arabinose and weakly positive for D-maltose, D-mannose, and L-malate (malic acid). But negative for nitrate reduction, production of indole, production of acid from glucose, arginine dihydrolase, urease, protease (gelatin hydrolysis), D-mannitol, *N*-acetyl-D-glucosamine, gluconate, caprate, adipate, citrate, and phenylacetate. The result of the API ZYM test showed that strain

BT553^T was positive for alkaline phosphatase, leucine arylamidase, alkaline phosphatase, leucine arylamidase, *β*-galactosidase (ONPG), *α*-glucosidase (starch hydrolysis), *N*–acetyl-*β*-glucosaminidase and weakly positive for valine arylamidase, acid phosphatase, naphtol-AS-BI-phosphohydrolase, *α*-galactosidase, and *α*-mannosidase. Whereas it was negative for esterase (C4), esterase (C8), lipase (C14), cystine arylamidase, trypsin, *α*-chymotrypsin, *β*-glucuronidase, *β*-Glucosidase, and *α*-fucosidase. Strain BT552^T was positive for alkaline phosphatase, valine arylamidase, acid phosphatase, and *β*-galactosidase (ONPG) and weakly positive for leucine arylamidase cystine arylamidase *α*-chymotrypsin *N*-acetyl-*β*-glucosaminidase. Whereas it was negative for esterase (C4), esterase (C8), lipase (C14), trypsin, naphtol-AS-BI-phosphohydrolase, *α*-galactosidase, *β*-glucuronidase, *α*-glucosidase (starch hydrolysis), *β*-glucosidase, *α*-mannosidase, and *α*-fucosidase. Different features between two novel strains and reference strains are provided in Table 1.

Table 1

Different characteristics of *Sphingomonas negativus* sp. nov., *Sphingomonas gyeonggiense* sp. nov., and closely related species.

Characteristic	1	2	3	4
NaCl % range	0	1	1	3
Protease (gelatin hydrolysis)	w	-	-	-
Hydrolysis of				
D-Glucose	+	+	+	-
D-Mannose	w	w	+	+
N-Acetyl-D-glucosamine	w	-	+	+
D-Maltose	+	w	+	+
L-Malate	w	w	+	-
Phenyl acetate	-	-	+	+
G+C content	65.9	66.8	67.0	ND

Taxa: 1, strain BT553^T; 2, strain BT552^T; 3, *S. melonis* DAPP-PG 224 ^{T} (data from Buonaurio et al. 2002); 4, *S. aquatilis* JSS7^T (data from Lee et al. 2001).

+, positive; -, negative; w, weak positive

Genome sequence analysis and phylogenetic analysis

The draft genome of strain BT553^T consisted of 3,941,714 bp with a coverage of 55.3×. A total of 3,674 protein-coding genes and 45 tRNA genes were predicted by NCBI PGAP analysis. The genome sequence of the strain BT553^T was deposited in GenBank under accession number NZ_JAELXS000000000. The

draft genome of strain BT552^T consisted of 4,035,561 bp with a coverage of 54.1×. A total of 3,823 protein-coding genes and 47 RNA genes (3 rRNA genes, 44 tRNA genes) were predicted by NCBI PGAP analysis. The genome sequence of the strain BT552^T has been deposited in GenBank under accession number NZ_JAFEMC010000000. Ortho ANI values between strain BT553, BT552 and *S. melonis* DAPP-PG 224^T were 76.7% and 76.5%, respectively.

The similarity of the 16S rRNA gene sequence between the BT553^T and BT552^T strains was 98.5% and the ANI value was 78.4%, indicating that they represent different species. The novel isolate BT553^T was closely related to *Sphingomonas melonis* DAPP-PG 224^T (98.1 % 16S rRNA gene similarity) and *Sphingomonas aquatilis* JSS7^T (98.1%). The novel isolate BT552^T was closely related to *Sphingomonas melonis* DAPP-PG 224 ^{T} (98.2 % 16S rRNA gene similarity) and *Sphingomonas aquatilis* JSS7^T (98.1%). According to Chun et al (2018), we could conclude that BT553^T would be a new species as its 16S rRNA similarity was 98.7% or less. In the neighbor-joining tree, strain BT553^T and strain BT552^T formed an independent cluster (Fig. 1) and clearly showed that strain BT553^T and strain BT552^T are novel species of the genus *Sphingomonas*.

Chemotaxonomic characterization

The fatty acid profiles of strains BT553^T and BT552^T were compared with those of the closely related two species of the genus *Sphingomonas* (Table 2). The major fatty acids of strains BT553^T and BT552^T were Summed Feature 3 and (16:1 ω6c / 16:1 ω7c), Summed Feature 8 (18:1 ω7c / 18:1 ω6c), and 14:0. The fatty acid profiles of strains BT553^T and BT552^T were similar to those of the two most closely related *Sphingomonas* species (Table 2). The polar lipids of strain BT553^T consisted of one diphosphatidylglycerol (DPG), one phosphatidylethanolamine (PE), one phosphatidylglycerol (PG), one phosphatidylcholine (PC), two phospholipids (PL), one sphingolipid (SL), and one lipid (Fig. S2). Polar lipids of strain BT552^T consisted of one diphosphatidylglycerol (DPG), one phosphatidylethanolamine (PE), one phosphatidylglycerol (PG), one phosphatidylcholine (PC), two phospholipids (PL), one sphingolipid (SL), two aminolipids (AL), one aminophospholipid (APL), and two one lipids (Fig. S3). The dominant respiratory quinone of strain BT553^T and BT552^T was Q-10.

Table 2

Cellular fatty acid profiles of *Sphingomonas negativus* sp. nov., *Sphingomonas gyeonggiense* sp. nov., and closely related species.

Fatty acids	1	2	3	4
Saturated				
14:0	1.3	3.4	tr	tr
14:0 2OH	21.6	13.7	5.5	10.9
16:0	12.2	8.5	18.4	20.6
18:0	tr	tr	1.2	1.0
Unsaturated				
16:1 *ω* 5*c*	6.17	1.2	1.0	tr
17:1 *ω* 6*c*	tr	1.3	tr	tr
18:1 *ω* 5*c*	tr	tr	1.5	1.5
18:1 *ω* 7*c* 11-methyl	5.9	2.7	5.4	tr
Summed Feature 3 (16:1 *ω* 6*c* / 16:1 *ω* 7*c*)	14.3	20.2	4.3	tr
Summed Feature 4 (17:1 iso I / 17:1 anteiso B)	tr	tr	tr	4.6
Summed Feature 8 (18:1 *ω* 7*c* / 18:1 *ω* 6*c*)	34.9	45.4	60.3	59.9

Taxa: 1, strain BT553^T (data from this study); 2, strain BT552^T (data from this study); 3, *S. melonis* DAPP-PG 224 ^{T} (data from Buonaurio et al. 2002); 4, *S. aquatilis* JSS7^T (data from Lee et al. 2001). For unsaturated fatty acids, the double bond location was presented by counting the number from the methyl (*ω*) end of the carbon chain.

tr, trace (<1 %); -, not detected.

The morphological, biochemical, and chemotaxonomic characters of strains BT553^T and BT552^T were consistent with descriptions of the genus *Sphingomonas*. On the basis of the phylogenetic differences between strain BT553^T, strain BT552^T and species of the genus *Sphingomonas*, a novel species, *Sphingomonas negativus* sp. nov., has been proposed, with BT553^T as the type strain and *Sphingomonas negativus* sp. nov., has been proposed, with BT552^T as the type strain.

Description of Sphingomonas negativus **sp. nov.**

Sphingomonas negativus (ne.g.,a.ti'vus. L. masc. adj. *negativus*, negative, because of the Gram-negative staining reaction).

Cells are Gram-staining-negative and rod-shaped. Colonies are irregular, convex, and yellow in color after the three-day incubation at 25°C. The cell size of strain BT553^T is approximately 1.5 μm in diameter and approximately 5.8 μm in length. Growth occurs at 10–30°C (optimum 25°C) and pH 5.0–9.0 (optimum 7.0). Cells grow on R2A agar, TSA, NA, LB agar, and Macconkey agar. Cells are oxidase activity negative and catalase activity positive. The major respiratory quinone is Q-10. The dominant cellular fatty acids are Summed Feature 3 and (16:1 $\omega 6c$ / 16:1 $\omega 7c$), Summed Feature 8 (18:1 $\omega 7c$ / 18:1 $\omega 6c$), and 14:0. The major polar lipids are diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC), and sphingoglycolipid (SL). The whole genome sequence of strain BT553^T have been deposited in GenBank under accession number NZ_JAELXS000000000. The GenBank accession number for the 16S rRNA gene sequence of strain BT553^T is MT893357. The strain type BT553^T (= KCTC 82095^T = NBRC XXXX^T) was isolated from a soil sample collected in Korea.

Description of Sphingomonas gyeonggiense **sp. nov.**

Sphingomonas gyeonggiense (gyeong.gi.ense. L. masc. n. *gyeonggiense*, isolation source was collected in Gyeonggi province).

Cells are Gram-staining-negative and rod-shaped. Colonies are irregular, convex, and yellow in color after the three-day incubation at 25°C. The cell size of strain BT552^T is approximately 1.5 μm in diameter and approximately 5.7 μm in length. Growth occurs at 10–30°C (optimum 25°C) and pH 5.0–9.0 (optimum 7.0). Cells grow on R2A agar, TSA, and NA and LB agar but not on Macconkey agar. Cells are oxidase activity negative and catalase activity positive. The major respiratory quinone is Q-10. The dominant cellular fatty acids are Summed Feature 3 and (16:1 $\omega 6c$ / 16:1 $\omega 7c$), Summed Feature 8 (18:1 $\omega 7c$ / 18:1 $\omega 6c$), and 14:0. The major polar lipids are diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC), phospholipid (PL), and sphingoglycolipid (SL). The whole genome sequences of strain BT552^T have been deposited in GenBank under accession numbers NZ_JAFEMC000000000. The GenBank accession number for the 16S rRNA gene sequence of strain BT552^T is MT893356. The strain type BT552^T (= KCTC 82094^T = NBRC XXXX^T) was isolated from a soil sample collected in Korea.

Declarations

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Conflicts of interest:

All authors certify that there is no conflict of interest.

Availability of data and material:

The GenBank accession numbers for the 16S rRNA gene sequences of strain BT553^T and strain BT552 are MT815535 and MT893355, respectively. The whole-genome sequences of strain BT553^T and strain BT552 have been deposited in GenBank under accession numbers NZ_JAEDAE000000000 and NZ_JAEDAD000000000, respectively.

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Figures

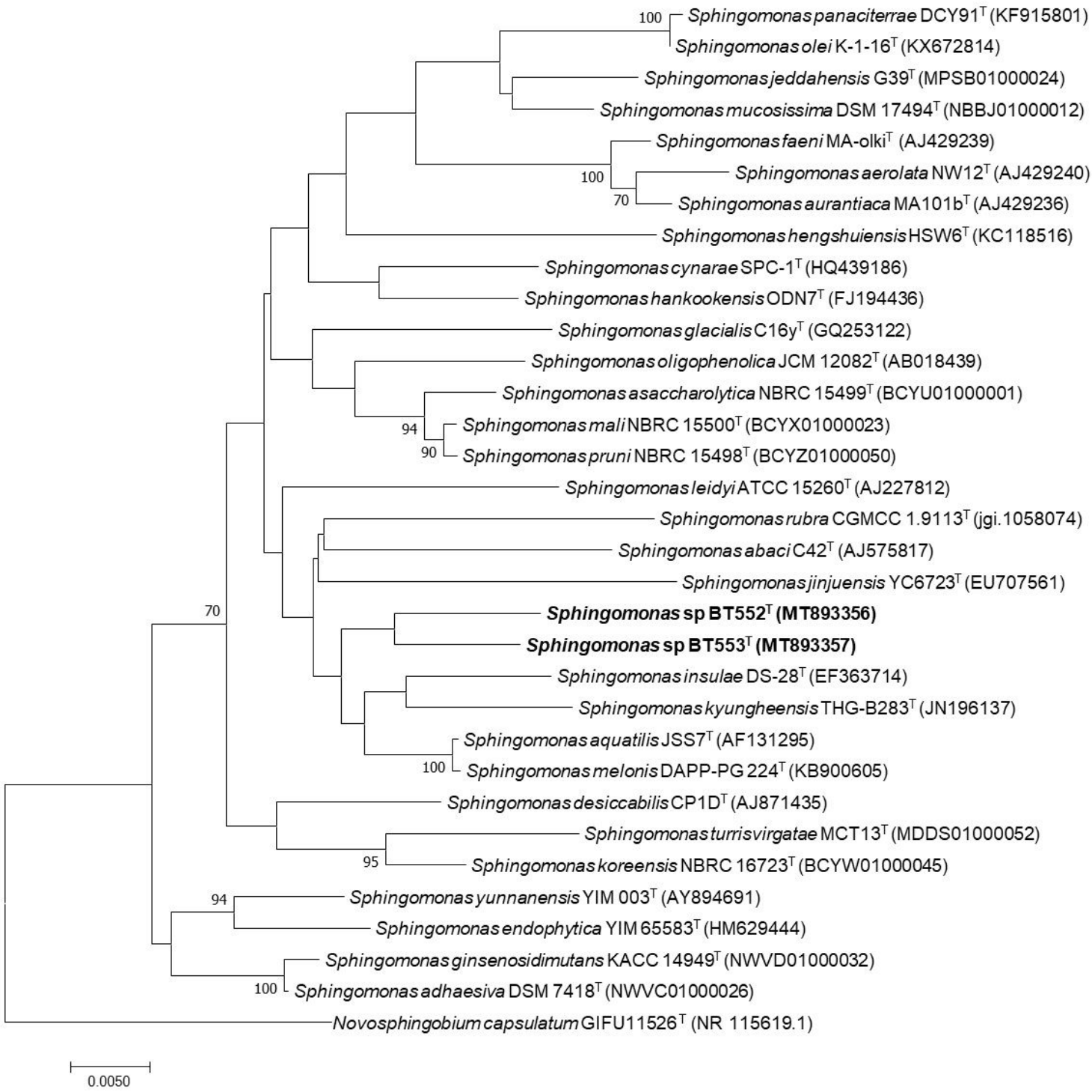

Figure 1

Neighbor-joining phylogenetic tree reconstructed from a comparative analysis of 16S rRNA gene sequences showing the relationships of strains BT553T and BT552T with closely related validly published species. Bootstrap values (>70 %) based on the neighbor-joining method are shown at the branch nodes. Bar, 0.0050 substitutions per nucleotide position. Novosphingobium capsulatum GIFU11526T was used as the outgroup.

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