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# *Sphingomonas humi* sp. nov., Isolated from Soil

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**A Gram-negative, non-motile, non-spore-forming, small, orange, rod-shaped bacterium was isolated from soil in South Korea and characterized to determine its taxonomic position. Phylogenetic analysis based on 16S rRNA gene sequence examination revealed that strain PB323$^T$ belongs to the family *Sphingomonadaceae*. The highest degree of sequence similarity was found with *Sphingomonas kaistensis* PB56$^T$ (98.9%), followed by *Sphingomonas astaxanthinifaciens* TDMA-17$^T$ (98.3%). Chemotaxonomic characteristics (the G+C content of the genomic DNA 69.0 mol%, Q-10 quinone system, $C_{18:1}\omega7c/\omega9t/\omega12t$, $C_{16:1}\omega7c/C_{15:0}$ iso 2OH, $C_{17:1}\omega6c$, and $C_{16:0}$ as the major fatty acids) corroborated assignment of strain PB323$^T$ to the genus *Sphingomonas*. Results of physiological and biochemical tests clearly demonstrate that strain PB323$^T$ represents a distinct species and support its affiliation with the genus *Sphingomonas*. Based on these data, PB323$^T$ (=KCTC 12341$^T$ =JCM 16603$^T$ =KEMB 9004-003$^T$) should be classified as a type strain of a novel species, for which the name *Sphingomonas humi* sp. nov. is proposed.**

***Keywords*:** taxonomy, 16S rRNA gene, *S. humi*

Yabuuchi *et al.* (1990) first proposed the genus *Sphingomonas* in order to identify Gram-negative, rod-shaped, yellow-pigmented bacteria. The genus *Sphingomonas* belongs to the class *Alphaproteobacteria* (Takeuchi *et al.*, 2001; Busse *et al.*, 2003; Li *et al.*, 2004; Rivas *et al.*, 2004; Kim *et al.*, 2007).

In a series of studies, we attempted to isolate microorganisms from soil to investigate community structure based on a culture-dependent method (Duan *et al*., 2009; Kim *et al*., 2009) as well as the antimicrobial activity of plants against microorganisms growing in soil and food (Moon *et al*., 2009). In this study, an orange-colored *Sphingomonas*-like strain was isolated from soil near a pond located on the campus of the Korea Advanced Institute of Science and Technology (KAIST) in Daejeon city, South Korea. In order to determine its precise taxonomic position, strain PB323$^T$ was characterized by a polyphasic approach, which included phylogenetic analysis of 16S rRNA gene sequences, genomic relatedness, and chemotaxonomic and phenotypic properties. Results obtained in this study indicate that PB323$^T$ should be assigned as a new member of the genus *Sphingomonas*.

## Materials and Methods

### Isolation of bacterial strains and culture conditions

The strain PB323$^T$ was isolated from soil near a pond via direct plating onto ten-fold diluted R2A agar (Difco, USA). Single colonies on these plates were purified by transferring them onto new plates and subjecting them to an additional incubation period of 5 days at 30°C. The purified colonies were tentatively identified using partial 16S rRNA gene sequences cultured routinely on R2A agar (Difco) at 30°C and preserved in a glycerol solution (20%, w/v) at -70°C. This organism was then submitted to the Korean Collection for Type Cultures, Japan Collection of Microorganisms and Korea National Environmental Microorganism Bank (KCTC 12341$^T$ =JCM 16603$^T$ =KEMB 9004-003$^T$).

### Phenotypic and biochemical characteristics

Cell morphology and motility were observed using a Nikon light microscope (1,000× magnification), with the cells being allowed to grow for 3 days at 30°C on R2A agar. Gram reactions were conducted according to the non-staining method described by Buck (1982). Oxidase activity was evaluated via the oxidation of 1% (w/v) tetramethyl-p-phenylene diamine. Catalase activity was determined by measurement of bubble production after the application of 3% (v/v) hydrogen peroxide solution. Growth at different temperatures (4, 25, 30, 32, 37, 40, 42, and 45°C) was assessed on TSA and LB agar for 5 days. Growth at various pHs (5, 7, 9, and 11) was assessed in TSB and LB broth at 30°C. Growth on different media was assessed on TSA, LB, NA, and R2A agar. The API 20NE, API ID32 GN, and API ZYM microtest systems were employed, according to the recommendations of the manufacturer (bioMérieux, France) for studying carbon source utilization and assaying the enzyme activities of strain PB323$^T$.

### Isoprenoid quinones and cellular fatty acids

Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v) purified via thin-layer chromatography, evaporated under vacuum conditions, and re-extracted in n-hexane:water (1:1, v/v). The crude n-hexane quinine solution was purified and subsequently analyzed by HPLC, as previously described (Collins and Jones, 1981; Shin *et al*., 1996). In order to perform the fatty acid methyl ester analysis, the strain was allowed to grow on TSA for 3 days at 30°C, and then 2

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loops of well-grown cells were harvested. Fatty acid methyl esters were prepared, separated, and identified with the Sherlock Microbial Identification System (MIS), produced by MIDI, Inc., Newark, DE, USA (Sasser, 1990).

### Determination of DNA G+C content

For determination of G+C content, genomic DNA was extracted and purified with the QIAGEN Genomic-tip system 100/G (QIAGEN, Japan) and enzymatically degraded into nucleosides. The nucleosides were then analyzed using reverse-phase HPLC as previously described (Tamaoka and Komagata, 1984; Mesbah *et al*., 1989).

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

The 16S rRNA gene was amplified from chromosomal DNA using the universal bacterial primer set, 9F and 1512R (Weisburg *et al*., 1991). Purified PCR product was sequenced by Genotech, Daejeon, Korea (Kim *et al*., 2005). The full sequence of the 16S rRNA gene was compiled with SeqMan software (DNASTAR Inc.). The 16S rRNA gene sequences of related taxa were obtained from GenBank and edited using the BioEdit program (Hall, 1999). Multiple alignments were performed with the CLUSTAL X program (Thompson *et al*., 1997). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983) and the phylogenetic tree constructed via the neighbor-joining method (Saitou and Nei, 1987) in the MEGA 3 Program (Kumar *et al.*, 2004). Bootstrap analysis with 1,000 replicates was also conducted to obtain confidence levels for the branches (Felsenstein, 1985).

### Analysis of *puf*L and *puf*M gene sequences and phylogenetic analysis

The photochemical reaction center complex is a membrane protein that converts light energy into electrochemical energy through electron transfer and proton pumping processes. The reaction center complex of the purple bacteria is composed of at least 3 subunits: L, M, and H. To conclude the phylogenetic position of the strain PB323$^T$, genes encoding the typical type II photosynthetic reaction centre of *Proteobacteria*, the *puf*L and *puf*M genes, were amplified and sequenced as described by Nagashima *et al*. (1997). Sequences of related taxa were obtained from GenBank and the phylogenetic tree constructed as previously described for the 16S rRNA gene sequence.

### DNA-DNA hybridization

DNA-DNA hybridization was performed fluorometrically, according to the method developed by Ezaki *et al*. (1989), using photobiotin-labelled DNA probes and micro-dilution wells. Hybridization was performed with 5 replications per sample. The highest and lowest values obtained for each sample were excluded and the remaining 3 values were utilized in the calculation of hybridization values. The DNA relatedness values quoted are expressed as means of these 3 values.

### Nucleotide sequence accession number

The NCBI GenBank/EMBL/DDBJ accession number for 16S rRNA gene sequence of strain PB323$^T$ (=KCTC 12341$^T$ =JCM 16603$^T$ =KEMB 9004-003$^T$) is AB220146

## Results and Discussion

### Morphological and phenotypic characteristics

Strain PB323$^T$, when cultured on R2A agar (Difco) at 30°C, yielded orange-colored, circular colonies. The cells were

**Table 1.** Differential phenotypic characteristics between strain PB323$^T$ and closely related *Sphingomonas* species

Strains: 1, *S. humi* PB323$^T$; 2, *S. kaistensis* PB56$^T$; 3, *S. Astaxanthinifaciens* TDMA-17$^T$.

All data were obtained from this study. All strains produced catalase and esterase (C4) and were Gram-negative. They did not reduce nitrate and did not produce indole from tryptophan, nor did they produce oxidase, N-acetyl-$\beta$-glucosaminidase, arginine dihydrolase, $\alpha$-fucosidase, $\alpha$-galactosidase, $\beta$-galactosidase, $\beta$-glucuronidase, lipase (C14), $\alpha$-mannosidase or urease. All strains did not assimilate acetate, caprate, 3-hydroxybenzoate, itaconate, 5-ketogluconate, D,L-lactate, malonate, phenyl acetate, suberate, L-arabinose, L-fucose, D-melibiose, D-ribose, D-sucrose, myo-inositol, D-sorbitol, L-alanine, or salicin. +, positive; -, negative; w, weak positive.

| Characteristic | 1 | 2 | 3 |
|---|---|---|---|
| Motility | - | - | + |
| Cell morphology | short rod | short rod | oval/short rod |
| Cell size (μm) | 0.6-1.2 × 0.5-1.3 | 0.5-1.0 × 0.7-1.5 | 0.3-0.5 × 0.5-0.9 |
| Colony Color | Orange | Pinkish-red | Deep Red |
| Optimum growth temperature (°C ) | 30 | 30 | 40 |
| Acid from glucose | w | w | - |
| Enzyme activity | | | |
| Acid phosphatase | + | w | w |
| Alkaline phosphatase | + | + | - |
| Cystine arylamidase | w | - | w |
| Esterase Lipase (C8) | + | + | - |
| Leucine arylamidase | + | + | - |
| Lipase (C14) | - | - | + |
| Naphtol-AS-BI-phosphohydrolase | + | w | + |
| Trypsin | + | w | w |
| Valine arylamidase | w | + | + |
| α-Chymotrypsin | + | - | + |
| α-Glucosidase | w | - | + |
| β-Glucosidase (esculin hydrolysis) | - | - | w |
| Assimilation test | | | |
| 2-Ketogluconate | + | - | - |
| 3-Hydroxybutyrate | - | + | w |
| 4-Hydroxybenzoate | - | + | - |
| Adipate | + | + | - |
| Citrate | + | - | - |
| D-Glucose | - | + | + |
| D-Mannose | - | + | - |
| Gluconate | w | w | - |
| Glycogen | - | + | w |
| L-Histidine | + | - | - |
| L-Proline | - | + | w |
| L-Rhamnose | + | + | - |
| L-Serine | + | + | w |
| Malate | + | + | - |
| Maltose | + | + | - |
| Mannitol | + | + | - |
| N-Acetyl-glucosamine | - | + | - |
| Propionate | - | - | w |
| Valerate | + | - | w |
| G+C content | 69.0 | 69.9 | 67.7 |

found to be Gram-negative, non-motile, short rod-shapes. The strain PB323$^T$ was able to grow at a temperature range of 10-40°C but not at 45°C. The optimum growth temperature was 30-32°C. Results regarding the physiological characteristics of strain PB323$^T$ are summarized in the species description, and a comparison of selective characteristics with closely related type strains of *Sphingomonas* species is shown in Table 1.

## Cellular fatty acids and isoprenoid quinones

Strain PB323$^T$ showed a cellular fatty acid profile with large amounts of saturated and unsaturated fatty acids, including: summed feature 7 ($C_{18:1}\omega7c/\omega9t/\omega12t$) (42.5%), and summed feature 4 ($C_{16:1}\omega7c/C_{15:0}$ iso 2-OH) (33.5%), $C_{17:1}\omega6c$ (8.2%) and $C_{16:0}$ (7.6%). The fatty acid profile of strain PB323$^T$ is actually similar to that of 2 other closely related *Sphingomonas* species, *S. kaistensis* PB56$^T$ and *S. astaxanthinifaciens* TDMA-17$^T$. Comparative cellular fatty acid profiles of strain PB323T are shown in Table 2.

Strain PB323$^T$ contained ubiquinone Q-10 as the predominant respiratory lipoquinone, which is commonly found in the *Sphingomonas* species.

## Phylogenetic analysis

The 16S rRNA gene sequence of strain PB323$^T$ was found to be a continuous stretch of 1419 nucleotides. The 16S rRNA gene sequences of related taxa were obtained from GenBank. Strain PB323$^T$ belonged to the class *Alphaproteobacteria*, the order *Sphingomonadales*, and the family *Sphingomonadaceae*. The highest degree of sequence similarity was found with *Sphingomonas kaistensis* PB56$^T$ (98.9%) and *Sphingomonas astaxanthinifaciens* TDMA-17$^T$ (98.3%). Lower sequence similarities were found with other described *Sphingomonas* species (<95.9%). In the phylogenetic tree (Fig. 1), strain PB323$^T$ clearly belongs to the genus *Sphingomonas* lineage. Based on 16S rRNA gene sequencing, the phylogenetic position of strain PB323$^T$ among members of the genus *Sphingomonas* was determined to be unique and distinct.

Using the 1.5 kb amplification product obtained for the *puf*LM genes, respective nucleotide sequences were determined and a phylogenetic tree constructed as described for the 16S rRNA gene sequences. The *puf*LM gene sequences of strain PB323$^T$ were found to be most closely related to those of *S. kaistensis* PB56$^T$ and to form a distinct lineage separate from all other species of the family *Sphingomonadaceae*. These results are supported by high bootstrap resampling values (Fig. 2).

## DNA-DNA hybridization

The G+C content of genomic DNA from strain PB323$^T$ was 69.0 mol%. Strain DCY$^T$ exhibited relatively low levels of DNA-DNA relatedness values with the type strains *S. kaistensis* PB56$^T$ (37%) and *S. astaxanthinifaciens* TDMA-17$^T$ (28%). DNA-DNA hybridization levels with other type strains were determined to be less than 70% (Wayne *et al*., 1987; Stackebrandt and Goebel, 1994), which is the threshold delineating a genomic species. Thus, our results support the placement of strain PB323$^T$ as a representative of a separate and previously unrecognized species.

## Taxonomic conclusion

Based on the phenotypic, chemotaxonomic, and phylogenetic data, we conclude that strain PB323$^T$ is representative of a novel species of the genus *Sphingomonas*, for which the name *Sphingomonas humi* sp. nov. is proposed.

**Table 2.** Cellular fatty acid profiles of strain PB323$^T$ and closely related *Sphingomonas* species

Strains: 1, *S. humi* PB323$^T$; 2, *S. kaistensis* PB56$^T$; 3, *S. Astaxanthinifaciens* TDMA-17$^T$.

All type strains were grown in this study on TSA at 30°C for 3 days for fatty acid analysis. For unsaturated fatty acids, the position of the double bond was located by counting from the methyl ($\omega$) end of the carbon chain. The *cis* and *trans* isomers are indicated by the suffixes *c* and *t*, respectively. †Summed feature contained fatty acids, which could not be separated by GLC with the Microbial Identification System (MIDI). nd, not detected; -, trace (<1.0%).

| Fatty acid | 1 | 2 | 3 |
|---|---|---|---|
| Saturated fatty acids | | | |
| 14:0 | - | nd | - |
| 15:0 | 1.4 | nd | nd |
| 16:0 | 7.6 | 8.6 | 12.6 |
| 17:0 | - | 1.1 | - |
| 18:0 | nd | nd | - |
| Unsaturated fatty acids | | | |
| 16:1 $\omega5c$ | 2.0 | 4.0 | 2.6 |
| 17:1 $\omega6c$ | 8.2 | 7.6 | 3.6 |
| 17:1 $\omega8c$ | 1.6 | 2.0 | - |
| 18:1 $\omega5c$ | - | 1.2 | - |
| 18:1 $\omega7c$ 11-methyl | nd | nd | 3.1 |
| Hydroxy fatty acids | | | |
| 14:0 2OH | 1.2 | - | - |
| 18:1 2OH | - | 1.3 | 1.2 |
| †Summed feature 4 (16:1$\omega7c$ / 15:0 iso 2OH) | 33.5 | 29.9 | 30.2 |
| †Summed feature 7 (18:1$\omega7c/\omega9t/\omega12t$) | 42.5 | 43.2 | 44.2 |

## Description of *Sphingomonas humi* sp. nov.

***Sphingomonas humi*** (húmi. L. gen. n. *humi* of/from soil)

PB323$^T$ is a Gram-negative, non-motile, short rod when grown on R2A agar (Difco) at 30°C for 5 days. The colonies are circular, entire, low convex, smooth, opaque, and orange in color. Optimal growth temperature is 30°C and optimum pH is 6.0-8.0. They are oxidase negative and catalase positive. Acid is weakly produced from D-glucose and indole is not produced. There is no reduction of nitrate to nitrite or nitrogen.

Strain PB323$^T$ assimilates adipate, citrate, 2-ketogluconate, malate, valerate, maltose, L-rhamnose, mannitol, L-histidine, and L-serine, and weakly assimilates gluconate. Strain PB323$^T$ does not assimilate acetate, caprate, 3-hydroxybenzoate, 4-hydroxybenzoate, 3-hydroxybutyrate, itaconate, 5-ketogluconate, D,L-lactate, malonate, phenyl acetate, propionate, suberate, L-arabinose, L-fucose, D-glucose, glycogen, D-mannose, D-melibiose, D-ribose, D-sucrose, myo-inositol, D-sorbitol, *N*-acetyl-D-glucosamine, L-alanine, L-proline or salicin.

Strain PB323$^T$ produces acid phosphatase, alkaline phosphatase, $\alpha$-chymotrypsin, esterase (C4), esterase lipase (C8), leucine arylamidase, naphtol-AS-BI-phosphohydrolase,

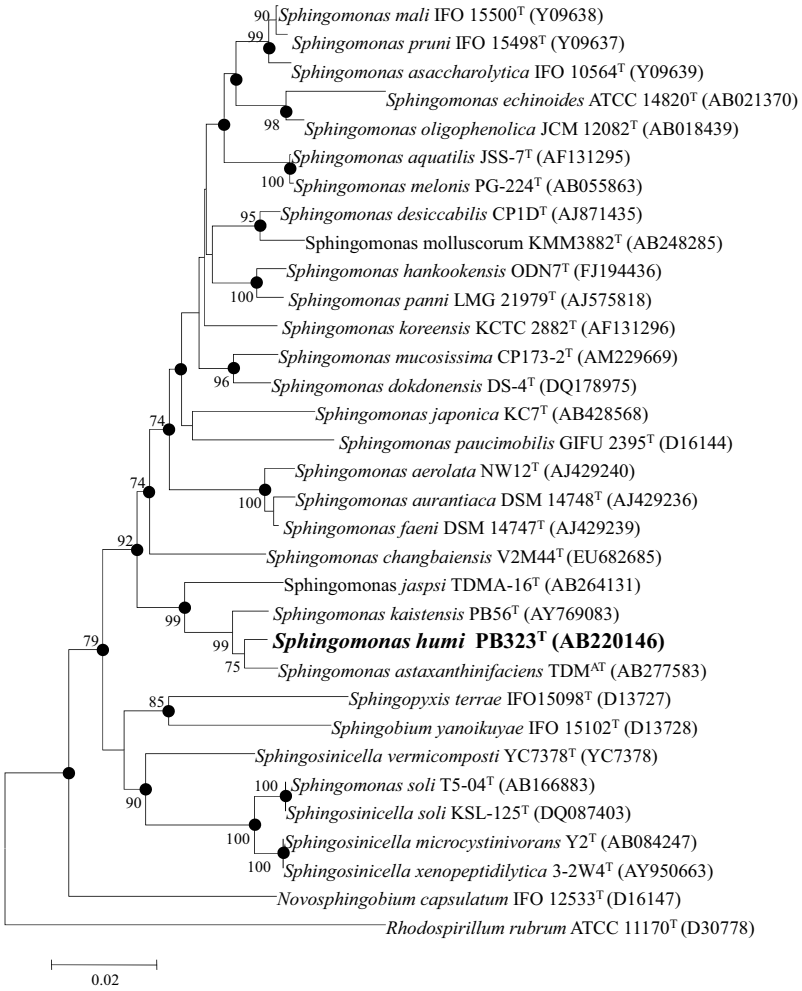


**Fig. 1.** A phylogenetic tree based on the 16S rRNA gene sequences of strain PB323$^T$ and representatives of related taxa. The neighbor-joining method was used. A bar represents 0.02 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1,000 replications) >50% are shown at the branch points. Filled circles indicate common nodes recovered from a maximum-parsimony algorithm.

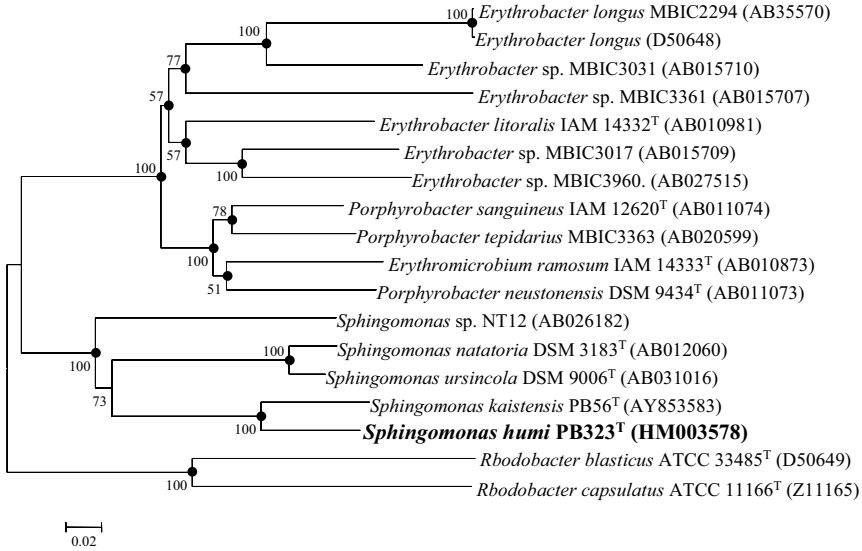


**Fig. 2.** A phylogenetic tree based on the *puf*L and *puf*M gene sequences of strain PB323$^T$ and representatives of some related taxa. The neighbor-joining method was used. A bar represents 0.02 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1,000 replications) >50% are shown at the branch points. Filled circles indicate common nodes recovered from a maximum-parsimony algorithm.

and trypsin, and weakly produces cystine arylamidase, α-glucosidase, and valine arylamidase. Strain PB323$^T$ does not produce N-acetyl-β-glucosaminidase, arginine dihydrolase, α-fucosidase, α-galactosidase, β-galactosidase, β-glucosidase (esculin hydrolysis), β-glucuronidase, lipase (C14), α-mannosidase, protease (gelatin hydrolysis) or urease.

The cellular fatty acids include: summed feature 7 ($C_{18:1}\omega7c/\omega9t/\omega12t$) (42.5%), summed feature 4 ($C_{16:1}\omega7c/C_{15:0}$ iso 2OH) (33.5%), $C_{17:1}\omega6c$ (8.2%) and $C_{16:0}$ (7.6%). The strain PB323$^T$ contains ubiquinone Q-10 as the predominant respiratory lipoquinone and the DNA G+C content is 69.0 mol%.

The type strain, PB323$^T$ was isolated from soil near a pond on the campus of KAIST in Daejeon, South Korea and deposited at KCTC (KCTC 12341$^T$), JCM (JCM 16603$^T$) and the Korea National Environmental Microorganism Bank (KEMB 9004-003$^T$).

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