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NOTE

Microlunatus terrae sp. nov., a Bacterium Isolated from Soil

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Strain BS6^T, a Gram-positive non-motile bacterium, was isolated from soil in South Korea and characterized to determine its taxonomic position. Phylogenetic analyses based on the 16S rRNA gene sequence revealed that strain BS6^T belonged to the family *Propionibacteriaceae* in the class *Actinobacteria*. Strain BS6^T showed the highest 16S rRNA gene sequence similarity with *Microlunatus soli* CC-012602^T (98.6%) and high sequence similarities with *Microlunatus* species (94.5–98.6%). Chemotaxonomic data revealed that the predominant fatty acids were anteiso-$C_{17:0}$, anteiso-$C_{15:0}$, summed feature 8 ($C_{18:1}$ *ω7c*/*ω6c*), and iso-$C_{16:0}$. The cell wall peptidoglycan contained $_{LL}$-diaminopimelic acid, and the major polar lipids were diphosphatidylglycerol, and phosphatidylglycerol. Based on these data, BS6^T (=KCTC 19858^T =JCM 17661^T =CCARM 9244^T =KEMC 9004-079^T) should be classified as a type strain of a novel species, for which the name *Microlunatus terrae* sp. nov. is proposed.

Keywords: *Actinobacteria*, *Microlunatus*, *Propionibacteriaceae*, taxonomy

The genus *Microlunatus* was first proposed by Nakamura *et al.* (1995), and *Microlunatus phosphovorus* was the type species. The genus *Microlunatus* is a aerobic, Gram-positive, non-motile coccoid containing $_{LL}$-diaminopimelic acid as a cell-wall peptidoglycan, and anteiso-$C_{15:0}$, iso-$C_{15:0}$, and iso-$C_{16:0}$ as major fatty acids. After proposing the genus *Microlunatus*, *Microlunatus ginsengisoli* was isolated from a ginseng field (Cui *et al.*, 2007), *Microlunatus aurantiacus* was isolated from rhizosphere soil (Wang *et al.*, 2008), *Microlunatus panaciterrae* was isolated from a ginseng field (An *et al.*, 2008), *Microlunatus soli* was isolated from a spawn used for growing edible mushrooms (Kämpfer *et al.*, 2010a), and *Microlunatus parietis* was isolated from indoor wall material (Kämpfer *et al.*, 2010b). The genus *Microlunatus* presently contains six validated species.

Strain BS6^T was originally isolated from soil in which many trunks were rotting in a forest near Daejeon city, by direct plating onto ten-fold diluted R2A agar (Difco, USA) and a 30°C incubation. The purified colonies were identified tentatively by partial 16S rRNA gene sequences and preserved in a glycerol solution (20%, w/v) at -70°C. Strain BS6^T was deposited at the Korean Collection for Type Cultures (KCTC), Japan Collection of Microorganisms (JCM), Culture Collection of Antimicrobial Resistant Microbes (CCARM), and the Korea National Environmental Microorganism Bank (KEMB).

The 16S rRNA gene of strain BS6^T was amplified from chromosomal DNA using the 9F and 1512R universal bacterial primer set (Weisburg *et al.*, 1991). The purified PCR product was sequenced by Genotech (Korea) (Kim *et al.*, 2005). The full sequence of the 16S rRNA gene was compiled with SeqMan software (DNASTAR Inc., USA). The 16S rRNA gene sequences of related taxa were obtained from GenBank and edited with the BioEdit program (Hall, 1999). Multiple alignments were performed with the CLUSTAL X program (Thompson *et al.*, 1997). Pairwise distances for the neighbor-joining algorithm (Saitou and Nei, 1987) were calculated according to the Kimura two-parameter model (Kimura, 1983), and a phylogenetic tree was constructed using the MEGA 3 program (Kumar *et al.*, 2004). A bootstrap analysis with 1,000 replicates was also conducted to obtain confidence levels for the branches (Felsenstein, 1985). The min-mini heuristic method with a search factor of one was applied in a maximum-parsimony analysis using the MEGA 3 Program, and a maximum-likelihood analysis was performed with Phylip 3.69.

To determine G+C content, genomic DNA was extracted and purified with the Qiagen Genomic-Tip System 100/G (Japan) and enzymatically degraded into nucleosides. The nucleosides were then analyzed using reverse-phase high performance liquid chromatography, as described previously (Tamaoka and Komagata, 1984; Mesbah *et al.*, 1989). DNA-DNA hybridization was performed fluorometrically, according to the method developed by Ezaki *et al.* (1989), using photobiotin-labeled DNA probes and micro-dilution wells. Hybridization was performed with five replications per sample. The highest and lowest values obtained for each sample were excluded, and the remaining three values were utilized to calculate hybridization values. DNA relatedness was expressed as the mean of these three values.

Gram reactions were conducted according to the non-staining method described by Buck (1982). Cell morphology was examined by light microscopy (Nikon E600) and transmission electron microscopy (Carl Zeiss LEO912AB) after the cells had grown for 3 days at 30°C on R2A agar. Oxidase

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activity was evaluated by the oxidation of 1% (w/v) tetramethyl-p-phenylene diamine. Catalase activity was determined by measuring bubble production after applying 3% (v/v) hydrogen peroxide solution. Growth on different media was assessed on trypticase soy agar (TSA), Luria-Bertani agar (LB), nutrient agar (NA), and R2A agar. The API 20NE, API ID32GN, API 50CH, and API ZYM microtest systems were employed according to the recommendations of the manufacturer (bioMérieux, France) to study carbon source utilization and enzyme activities of the strains. Growth at different temperatures (4, 15, 20, 30, 37, 40, and 42°C) was assessed on NA agar for 5 days. Growth at various pH levels (4, 5, 6, 7, 8, 9, and 10) was assessed in LB broth at 30°C.

Cells were allowed to grow on R2A agar for 5 days at 30°C to perform the fatty acid methyl ester analysis, and then two loops of well-grown cells were harvested. Fatty acid methyl esters were prepared, separated, and identified with the Sherlock Microbial Identification System (Sherlock version 6.01; data base TSBA6; MIDI, Inc., USA) (Sasser, 1990).

The amino acid composition of the cell-wall peptidoglycan was determined by thin-layer chromatography TLC after hydrolysis with 6 M HCl at 100°C for 18 h as described by Komagata and Suzuki (1987).

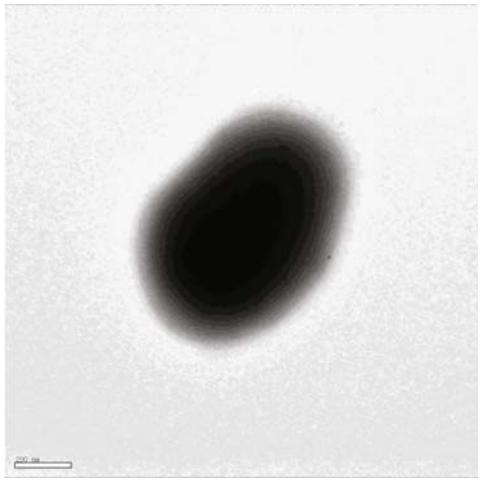

Fig. 1. Cell morphology of strain BS6^T as determined by transmission electron microscopy after growth on R2A for 3 days at 30°C. Bar, 0.2 μm.

Polar lipids were extracted according to the procedures described by Minnikin *et al.* (1984) and identified using

Table 1. Differential characteristics between strain BS6^T and all *Microlunatus* species

Strains: 1, strain BS6^T; 2, *M. aurantiacus* YIM 45721^T; 3, *M. ginsengisoli* Gsoil 633^T; 4. *M. panaciterrae* Gsoil 954^T; 5, *M. parietis* 12-Be-011^T; 6, *M. phosphovorus* DSM 10555^T; 7, *M. soli* CC-012602^T. All data were obtained in this study.

All strains produced β-galactosidase (ONPG), *β*-galactosidase (PNPG), *α*-glucosidase, *β*-glucosidase, and leucine arylamidase. None of the strains produced arginine dihydrolase, lipase (C14), or urease. They did not assimilate caprate, citrate, or phenyl acetate.

+, positive; -, negative; w, weak positive; ND, not determined

Characteristic	1	2	3	4	5	6	7
Nitrate reduction							
Nitrate reduction to NO_2	-	w	w	+	-	+	-
Nitrate reduction to N_2	-	ND	ND	ND	-	ND	-
β-Glucosidase (esculin hydrolysis)	w	+	+	+	+	+	+
Protease (gelatin hydrolysis)	+	-	+	-	-	+	+
D-Glucose	+	w	+	w	w	+	+
L-Arabinose	+	w	+	w	w	w	+
D-Mannose	+	w	+	w	w	+	+
D-Mannitol	+	w	+	-	w	+	+
N-Acetyl-D-glucosamine	+	w	w	-	w	+	+
D-Maltose	+	w	+	w	w	+	+
Gluconate	w	-	w	-	-	-	-
Adipate	w	-	-	-	-	-	-
L-Malate	-	w	+	-	-	w	w
Alkaline phosphatase	-	+	w	-	-	+	-
Esterase (C4)	w	w	+	w	w	w	+
Esterase (C8)	+	w	w	w	w	w	+
Valine arylamidase	+	+	w	-	-	+	+
Cystine arylamidase	w	+	-	-	-	+	w
Trypsin	w	+	+	+	+	+	w
α-Chymotrypsin	+	-	-	-	-	-	+
Acid phosphatase	+	+	+	+	-	+	+
Naphtol-AS-BI-phosphohydrolase	-	w	+	w	-	+	w
α-Galactosidase	+	+	+	+	+	+	-
β-Glucuronidase	-	w	+	-	-	w	-
N–Acetyl-*β*-glucosaminidase	w	+	+	+	+	+	+
α-Mannosidase	+	w	-	+	+	+	+
α-Fucosidase	-	-	-	+	-	-	w

two-dimensional TLC followed by spraying with the appropriate detection reagents (Minnikin *et al*., 1984; Komagata and Suzuki, 1987). The mobile phase for TLC development was chloroform/methanol/water (65:25:4, v/v/v), and the second mobile phase was chloroform/methanol/acetic acid/water (80:12:15:4, v/v/v/v). The total lipid profile was detected by spraying with molybdophosphoric acid solution (Sigma-Aldrich, USA) followed by heating at 150°C; aminolipids were detected by spraying with 0.2% (w/v) ninhydrin solution followed by heating at 105°C for 10 min; glycolipids were detected with 0.5% 1-naphthol in methanol/water (1:1, v/v) and sulfuric acid/ethanol (1:1, v/v) followed by heating at 120°C for 5–10 min; phospholipids were detected by spraying with Zinzadze reagent; and phosphatidylcholine was detected by spraying with Dragendorff reagent (Sigma-Aldrich).

Strain BS6^T was pale-yellow in color when routinely cultured on R2A agar at 30°C. It was a Gram-positive, aerobic, non-motile, coccus-shaped cell (Fig. 1) and was able to grow at temperatures of 4–42°C. Optimal growth occurred at 30°C. Strain BS6^T grew well at pHs of 4–9. Other physiological characteristics of strain BS6^T are summarized in the species description. The differential characteristics between strain BS6^T and the most closely related type strain, *Microlunatus soli* CC-012602^T, are shown in Table 1.

The 16S rRNA gene sequence of strain BS6^T was a continuous stretch of 1441 nucleotides. Strain BS6^T belonged to Class *Actinobacteria*, Order *Actinomycetales*, Family *Propionibacteriaceae*. The highest degrees of sequence similarity of strain BS6^T were found with *Microlunatus* species, *Microlunatus soli* CC-012602^T (98.6%), *Microlunatus ginsengisoli* Gsoil 633^T (95.6%), *Microlunatus panaciterrae* Gsoil 954^T (95.3%), *Microlunatus aurantiacus* YIM 45721^T (95.0%), *Microlunatus phosphovorus* DSM 10555^T (94.7%), and *Microlunatus parietis* 12-Be-011^T (94.5%). In the phylogenetic tree (Fig. 2), strain BS6^T clearly belonged to the *Microlunatus* lineage (ML, MP, and NJ trees) in the Family *Propionibacteriaceae* as evidenced by the high bootstrap value of 71%.

The predominant cellular fatty acids of strain BS6^T were iso-$C_{16:0}$ (25.4%), anteiso-$C_{17:0}$ (20.4%), anteiso-$C_{15:0}$ (17.0%), summed feature 7 ($C_{18:1}$ $\omega7c$/$C_{18:1}$ $\omega9t$/$C_{18:1}$ $\omega12t$) (16.8%), summed feature 8 ($C_{18:1}$ $\omega7c$) (9.6%), and $C_{17:1}$ $\omega6c$ (5.5%). Minor fatty acids of strain BS6^T were iso-$C_{15:0}$ (2.2%), iso-$C_{17:0}$ (1.2%), iso-$C_{14:0}$ 3OH (0.5%), $C_{14:0}$ 2OH (0.4%), summed feature 2 ($C_{16:1}$ iso I / $C_{14:0}$ 3OH) (0.4%), $C_{16:0}$ (0.3%), feature 4 ($C_{17:1}$ iso I/anteiso B) (0.2%), $C_{14:0}$ (0.1%), and $C_{17:0}$ (0.1%). Strain BS6^T showed a fatty acid profile most similar to that of *M. soli* CC-012602^T, which agreed with the result that the 16S rRNA gene sequence of strain BS6^T was most similar to that of *M. soli* CC-012602^T. But, strain BS6^T was distinguished from members of the genus *Microlunatus* by exhibiting a different fatty acid profile (Table 2). For example,

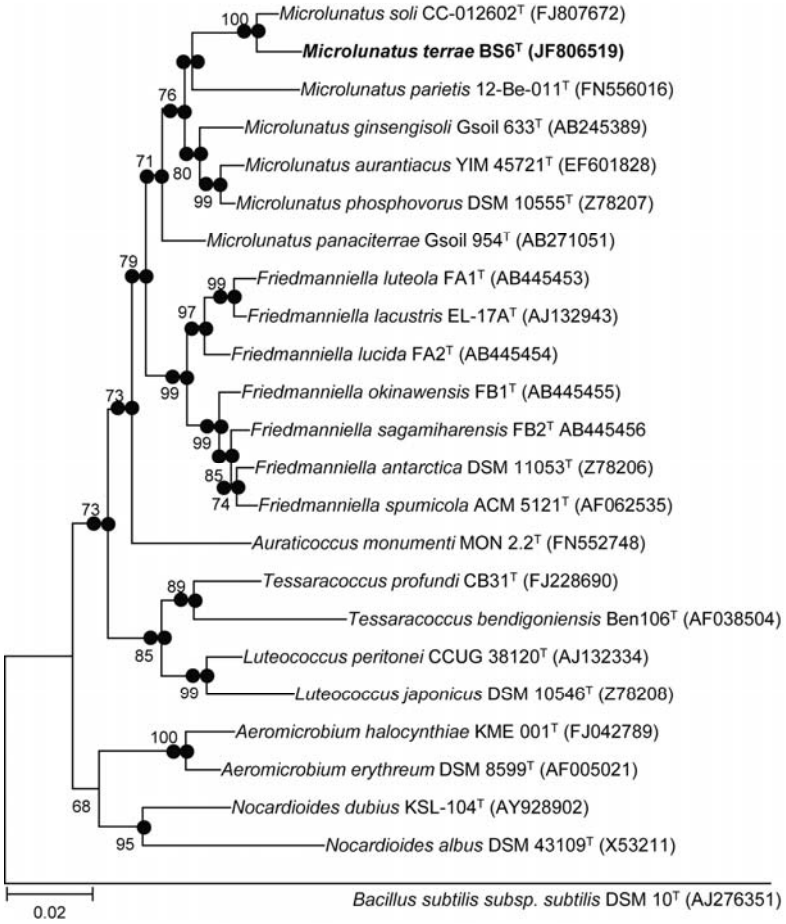

Fig. 2. A phylogenetic tree based on the 16S rRNA gene sequences of strain BS6^T and representatives of related taxa. The neighbor-joining method was used. Bar represents 0.02 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1,000 replications) >50% are shown at branch points. Black circles indicate the common nodes recovered from either the maximum-parsimony algorithm or the maximum-likelihood tree. Black double-circles indicate that the corresponding nodes were recovered in both the maximum-parsimony tree and the maximum likelihood tree.

Table 2. Cellular fatty acid profiles of strain BS6^T and *Microlunatus* species

Strains: 1, BS6^T; 2, *M. aurantiacus* YIM 45721^T; 3, *M. ginsengisoli* Gsoil 633^T; 4, *M. panaciterrae* Gsoil 954^T; 5, *M. phosphovorus* DSM 10555^T; 6, *M. soli* CC-012602^T.

All strains were grown on R2A agar at 30°C for 3 days. *M. parietis* 12-Be-011^T was not grown on R2A agar. The double bond position was located by counting from the methyl (*ω*) end of the carbon chain for unsaturated fatty acids.

†Summed feature contained fatty acids that could not be separated by GLC with the Microbial Identification System. ND, not detected; tr, trace (<1.0%)

Fatty acids	1	2	3	4	5	6
Saturated						
14:0	0.1					
14:0 iso		5.9	5.4	3.5	9.5	0.5
15:0		3.2				
15:0 iso	2.2	8.1	24.7	16.6	19.8	6.4
15:0 anteiso	17.0	59.6	42.4	53.6	34.9	16.4
16:0	0.3					0.1
16:0 iso	25.4	12.4	23.8	10.7	25.2	18.5
17:0	0.1			6.7		
17:0 iso	1.2			3.0	6.9	0.4
17:0 anteiso	20.4		3.7	6.0	3.6	4.1
Unsaturated						
17:1 *ω6c*	5.5					10.7
17:1 *ω9c*						1.8
18:3 *ω6c* (6, 9, 12)						0.1
Hydroxy						
14:0 2OH	0.4					0.9
14:0 iso 3OH	0.5	6.9				3.7
Summed Feature 2 (16:1 iso I / 14:0 3OH)	0.4					0.6
Summed Feature 4 (16:1 *ω7c* / 15:0 iso 2OH)		4.0				
Summed Feature 4 (17:1 iso I / anteiso B)	0.2					
Summed Feature 7 (18:1 *ω7c* / *ω*9t / *ω*12t)	16.8					21.0
Summed Feature 8 (18:1 *ω7c*)	9.6					14.9

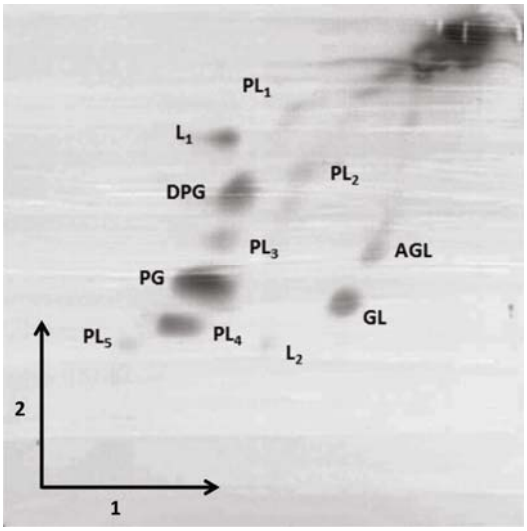

Fig. 3. Two dimensional thin-layer chromatogram sprayed with molybdophosphoric acid reagent to identify total polar lipids of strain BS6^T. Ascending solvent system: (I) chloroform/methanol/water (65:25:4, v/v/v); (II) chloroform/methanol/acetic acid/water (80:12:15:4, v/v/v/v). Molybdophospholic acid (PE, PG, and PL), ninhydrin (PE), α-naphthol (GL), and Zinzadze reagent (PE, PG, and PL) were applied to detect the polar lipids. Abbreviations: DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; PL1-PL5, unknown phospholipids; AGL, unknown aminoglycolipid; GL, unknown glycolipid; L, unknown polar lipid (not stainable with any of the specific spray reagents applied, indicating that it does not contain a phosphate group, an amino group, or a sugar moiety).

strain BS6^T has larger amounts of anteiso-$C_{17:0}$ (20.4%), $C_{17:1}$ *ω6c* (5.5%), summed feature 7 ($C_{18:1}$ *ω7c*/*ω*9t/*ω*12t) (16.8%), and summed feature 8 ($C_{18:1}$ *ω7c*) (9.6%), whereas most *Microlunatus* species have smaller amounts of corresponding fatty acids. Strain BS6^T has smaller amounts of iso-$C_{15:0}$ (2.2%) and anteiso-$C_{15:0}$ (17.0%), and no iso-$C_{14:0}$ (0.0%).

Strain BS6^T contained $_{LL}$-diaminopimelic acid, $_{D}$-alanine, and glycine in the cell wall peptidoglycans, and these amino acids are common in *Microlunatus* species of which the cell-wall peptidoglycan type is A3$_\gamma$. Major polar lipids found in strain BS6^T were diphosphatidylglycerol (DPG) and phosphatidylglycerol (PG). Minor amounts of unknown phospholipids (PL1–PL5) were also found (Fig. 3). Strain BS6^T showed a polar lipid profile similar to that of other *Microlunatus* species (Kämpfer *et al*., 2010a, 2010b). The major quinone of strain BS6^T was menaquinone MK-9(H_4).

The G+C content of genomic DNA from strain BS6^T was 66.5 mol%.

Strain BS6^T exhibited low DNA-DNA relatedness with the closely related *M. soli* CC-012602^T (54.75±5.91%). Reciprocally, *M. soli* CC-012602^T exhibited low DNA-DNA relatedness with strain BS6^T (38.67±4.94%). DNA-DNA hybridization levels between strain BS6^T and strain *M. soli* CC-012602^T were determined to be <70%, which is the threshold for delineating a genomic species (Wayne *et al*., 1987; Stackebrandt and Goebel, 1994). Thus, our results support the placement of strain BS6^T as a representative of a sepa-

rate and previously unrecognized genomic species.

Based on the phylogenetic, chemotaxonomic, and phenotypic data, we conclude that strain BS6^T is a representative novel species, for which the name *Microlunatus terrae* sp. nov. is proposed.

Description of *Microlunatus terrae* sp. nov.

Microlunatus terrae (ter'ra.e. L. gen. n. *terrae* of the Earth) Strain BS6^T is 0.4–0.6 μm wide and 0.7–0.9 μm long, Gram-positive, aerobic, non-motile, and coccus-shaped when grown on NA agar (Difco) at 30°C for 4 days. Colonies grown on NA agar for 4 days are circular and pale creamy white in color. Growth occurred on TSA, LB, NA, and R2A. Growth occurred at temperatures of 4–40°C. The optimum growth occurred at 30°C. Strain BS6^T grew well at pH of 5–9 and tolerated up to 6% NaCl (w/v). No reduction of nitrate to nitrite or nitrogen was evident. The bacterium was oxidase and catalase-positive. Acid was not produced from D-glucose, and indole was not produced.

Growth was observed in L-arabinose, D-glucose, glycogen, D-maltose, D-mannose, D-melibiose, D-ribose, D-sucrose, acetate, adipate, gluconate, 2-ketogluconate, D,L-lactate, n-valerate, *N*-acetyl-D-glucosamine, myo-inositol, D-mannitol, D-sorbitol, and salicin. Growth was not observed in L-fucose, L-rhamnose, caprate, citrate, 3-hydroxybenzoate, 4-hydroxybenzoate, D,L-3-hydroxybutyrate, itaconate, 5-ketogluconate, L-malate, malonate, phenyl acetate, propionate, suberate, L-alanine, L-histidine, L-proline, or L-serine.

Acid was produced in the presence of arbutin, D-cellobiose, esculin ferric citrate, D-fructose, gentiobiose, D-lactose, maltose, D-melobiose, α-methyl-D-glucopyranoside, α-methyl-D-mannopyranoside, D-raffinose, L-rhamnose, salicin, D-sucrose, D-trehalose, D-xylose, *N*-acetyl-glucosamine, D-adonitol, erythritol, mannitol, and sorbitol. Acid was not produced in the presence of amygdalin, D-arabinose, L-arabinose, D-fucose, L-fucose, D-galactose, glycogen, inulin, D-lyxose, D-mannose, melezitose, β-methyl-D-xylose, ribose, L-sorbose, starch, D-tagatose, turanose, L-xylose, gluconate, 2-ketogluconate, 5-ketogluconate, D-arabitol, L-arabitol, dulicitol (galactitol), glycerol, inositol, or xylitol.

In tests with the API ZYM system, enzyme production was positive for *N*-acetyl-β-glucosaminidase, acid phosphatase, α-chymotrypsin, cysteine arylamidase, esterase (C4), esterase (C8), α-galactosidase, β-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-glucosidase (esculin hydrolysis), leucine arylamidase, α-mannosidase, protease (gelatin), trypsin, and valine arylamidase. Enzyme production was negative for alkaline phosphatase, arginine dihydrolase, α-fucosidase, β-glucuronidase, lipase (C14), naphtol–AS–BI–phosphohydrolase, and urease.

The predominant cellular fatty acids of strain BS6^T are anteiso-$C_{17:0}$, anteiso-$C_{15:0}$, summed feature 8 ($C_{18:1}$ ω7c/ω6c), and iso-$C_{16:0}$. The cell-wall peptidoglycans contain LL-diaminopimelic acid. The major polar lipids are DPG and PG. The G + C content of genomic DNA from strain BS6^T was 66.5 mol%.

The type strain, BS6^T (=KCTC 23577^T =JCM 17661^T =CCARM 9244^T =KEMC 9004-079^T), was isolated from soil in South Korea.

The NCBI GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BS6^T (=KCTC 19858^T =JCM 17661^T =CCARM 9244^T =KEMC 9004-079^T) is JF806519.

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