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Paenibacillus pini sp. nov., a Cellulolytic Bacterium Isolated from the Rhizosphere of Pine Tree

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Strain S22^T, a novel cellulolytic bacterium was isolated from the rhizosphere of pine trees. This isolate was Gram-reaction positive, motile and rods, and formed terminal or subterminal ellipsoidal spores. S22^T represented positive activity for catalase, oxidase, esterase (C4), esterase lipase (C8), β-galactosidase, leucine arylamidase, and hydrolysis of esculin. It contained *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall. The predominant isoprenoid quinone was menaquinone 7 (MK-7), and the major cellular fatty acids were anteiso-$C_{15:0}$ (52.9%), iso-$C_{16:0}$ (11.3%), and iso-$C_{15:0}$ (10.0%). The DNA G+C content was 43.3 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that this isolate belonged to the family *Paenibacillaceae*. S22^T exhibited less than 97.0% 16S rRNA gene similarity with all relative type strains in the genus *Paenibacillus*, and the most closely related strains were *Paenibacillus anaericanus* MH21^T and *Paenibacillus ginsengisoli* Gsoil 1638^T, with equal similarities of 95.8%. This polyphasic evidence suggested that strain S22^T should be considered a novel species in the genus *Paenibacillus*, for which the name, *Paenibacillus pini* sp. nov., is proposed. The type strain is S22^T (=KCTC 13694^T =KACC 14198^T =JCM 16418^T)

***Keywords*:** *Paenibacillus pini*, cellulose, pine tree, rhizosphere

The rhizosphere, the soil adjacent to and influenced by the plant root, is a habitat of diverse bacterial strains (Sørensen, 1997). Plant growth and health are influenced by rhizosphere microorganisms with regard to nutrient solubilization or N_2 fixation (Höflich *et al*., 1994). The genus *Paenibacillus* is proposed for rRNA group 3 bacilli according to comparative 16S rRNA sequence analysis (Ash *et al*., 1993). Some reported paenibacilli have been isolated from the rhizosphere of a diverse plant species for instance: *Paenibacillus brasilensis* from maize (von der Weid *et al*., 2002), *Paenibacillus filicis* from the fern (Kim *et al.*, 2009), *Paenibacillus forsythiae* from *Forsythia mira* (Ma and Chen, 2008), *Paenibacillus naphthalenovorans* from salt marsh plants (Daane *et al*., 2002), *Paenibacillus rhizosphaerae* from *Cicer arietinum* (Rivas *et al*., 2005), *Paenibacillus riograndensis* from *Triticum aestivum* (Beneduzi *et al*., 2009), *Paenibacillus sonchi* from *Sonchus oleraceus* (Hong *et al*., 2009), and *Paenibacillus zanthoxyli* from *Zanthoxylum simulans* (Ma *et al*., 2007).

A new cellulolytic bacterial strain was isolated among biopolymer degrading bacteria from the rhizosphere of pine trees (*Pinus densiflora*). This bacterial strain was belonged to the family *Paenibacillaceae*; however, it was clearly different from reported species of the genus *Paenibacillus* on the basis of a 16S rRNA gene sequence analysis. In this study, we presented detailed taxonomic characterization of strain S22^T.

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Materials and Methods

Isolation of the bacteria

Biopolymer-degrading bacteria were isolated from rhizosphere samples of plants. Collected soil samples were serially diluted with sterile 0.85% (w/v) NaCl solution and plated onto R2A agar medium (BBL, USA). The plates were then incubated at 25°C for 6 days. Single colonies from the R2A plates were selected, transferred onto R2A agar plates containing biopolymers such as carboxymethyl (CM)-cellulose or pectin, and the plates were incubated at 25°C for 6 days. CM-cellulose degrading bacteria were screened by staining the plates with 1% Congo red (Rivas *et al*., 2003). Pectinase activity was confirmed by staining the plates with 1% *n*-hexadecyltrimethylammonium bromide. Strain S22^T, isolated from the rhizosphere of a pine tree collected from Mt. Geyjok in Daejeon, Republic of Korea (36° 22′ 56.4″ N, 127° 26′ 21.2″ E), showed cellulolytic activity. S22^T was routinely cultured on R2A or TSA agar plates (BBL) and maintained at 4°C or as a glycerol suspension (20%, w/v) at -70°C. This isolate was deposited into the Korean Collection for Type Cultures (KCTC) as KCTC 13694^T, the Korean Agricultural Culture Collection (KACC) as KACC 14198^T, and the Japan Collection of Microorganisms (JCM) as JCM 16418^T. Closely related *Paenibacillus* strains, *Paenibacillus anaericanus* MH21^T (KACC 11533^T), *Paenibacillus barengoltzii* SAFN-016^T (KCTC 13674^T), *Paenibacillus chibensis* NRRL B-142^T (KCTC 3758^T), *Paenibacillus cookii* LMG 18419^T (KCTC 3999^T), *Paenibacillus ginsengisoli* Gsoil 1638^T (KCTC 13931^T), and *Paenibacillus*

motobuensis MC10^T (KCTC 3992^T), were received from KCTC or KACC for comparison of physiological characteristics.

Physiological characteristics

The isolate and related type strains of the genus *Paenibacillus* were grown on R2A or TSA plates at 30°C. The colony morphology was observed after culturing the strain on nutrient agar (NA; BBL), R2A, and TSA plates for 4 days at 25°C. Cell size and morphology were observed using transmission electron microscopy (H-7600 transmission electron microscope; Hitachi, Japan). For spore formation the isolate was cultured on TSA plate for 3 days and observed using a light microscope (Nikon E600; Nikon, Japan) after staining with malachite green (Schaeffer and Fulton, 1933). The Gram stain kit (Difco, USA) was used for Gram staining. Anaerobic growth was tested with the GasPak EZ Anaerobe Pouch System (BD, USA). Motility was tested by culturing in TSB medium (BBL) that contained 0.4% agar. Oxidase activity was assessed colorimetrically using an Oxidase Reagent (bioMérieux, France), and catalase activity was determined by bubble production using 3% (v/v) H_2O_2. Growth under diverse conditions, including temperatures of 10~40°C, pH 4.0~10.0 (0.5 unit increments), and NaCl concentrations of 0~4% (w/v), was examined using batch cultures grown in TSB medium. Growth was measured by OD_{600} over a period of 3 days using a DU 730 UV/Vis Scanning Spectrophotometer (Beckman Coulter, USA). MacConkey agar plates (BBL) were used to assess the growth of the isolate on that medium. Standard microbiological methods were used to assess the hydrolysis of casein and starch (Atlas, 1993), and the method described by Chakrabarty *et al*. (1970) was used to assess the hydrolysis of Tween 80. Other enzyme activities of the isolate and closely related type strains were measured with API ZYM test strips (bioMérieux) after 6 h incubation at 30°C. Other biochemical and physiological traits of the isolate and closely related type strains were examined using API 20 NE and API 20 E test strips (bioMérieux) over a period of 3 days at 30°C. The API 50CH kit and 50CHB medium (bioMérieux) was used to assess carbon source oxidation over a period of 3 days at 30°C.

Chemotaxonomy

Quinone was determined according to the method described by Collins and Jones (1981) and Komagata and Suzuki (1987) using freeze-dried cells previously grown in tryptic soy broth at 30°C for 2 days. The quinone was purified by preparative TLC (silica gel F254; Merck) and identified by HPLC (Hitachi L-5000; Hitachi) using a reversed-phase column (YMC pack ODS-AM; YMC, Japan). Diaminopimelic acid isomers of the peptidoglycan were extracted from freeze-dried cells grown in TSB and analyzed using the method of Komagata and Suzuki (1987). Cellular fatty acid compositions of strain S22^T and closely related type strains of the genus *Paenibacillus* were analyzed with cells cultured on TSA for 2 days at 30°C. Fatty acids were extracted according to the standard protocol of the Microbial Identification System (MIDI; Sasser, 1990) and identified with the Sherlock software package (MIDI, USA) after separation by a gas chromatograph (HP 6890N; Agilent, USA).

Determination of G+C content

The HPLC method described by Mesbah *et al*. (1989) was used for measuring the G+C DNA content. As a reference strain for G+C content analysis, *Escherichia coli* KCTC 2441^T was obtained from KCTC. Strain S22^T and *E. coli* KCTC 2441^T were cultured in TSB medium, and genomic DNA was extracted according to the method described by Sambrook and Russell (2001). Extracted DNA was treated with nuclease P1 for hydrolysis and with alkaline phosphatase for dephosphorylation. Each nucleoside was separated from the resultant mixture of nucleosides by an HPLC equipped with a reversed-phase column (Supelcosil LC-18-S; Supelco, Germany).

Phylogenetic analysis of isolate based on 16S rRNA gene sequence

The DNA encoding the 16S rRNA gene of the isolate was amplified by PCR using the universal primers 27f and 1492r previously described by Lane (1991). Sequencing of the amplified gene was performed using an ABI prism BigDye Terminator Cycle Sequencing Ready Reaction kit v.3.1 and an ABI 3730XL capillary DNA Sequencer (Applied Biosystems, USA) at SolGent Co., Republic of Korea. The resultant sequences were assembled into a nearly complete 16S rRNA gene sequence with Vector NTI software (Invitrogen, USA). The 16S rRNA gene sequence of strain S22^T was compared with available 16S rRNA gene sequences from GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/), and closely related sequences were extracted using the Eztaxon server (http://www.eztaxon.org/; Chun *et al*., 2007). Alignment of the 16S rRNA gene sequences of strain S22^T and related type strains was conducted using CLUSTAL X software (Thompson *et al*., 1997). Phylogenetic trees were constructed using neighbor-joining, maximum parsimony and maximum-likelihood methods implemented by the PHYLIP package (Felsenstein, 1993). Tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1,000 resamplings. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain S22^T is GQ423056 (http://www.ncbi.nlm.nih.gov).

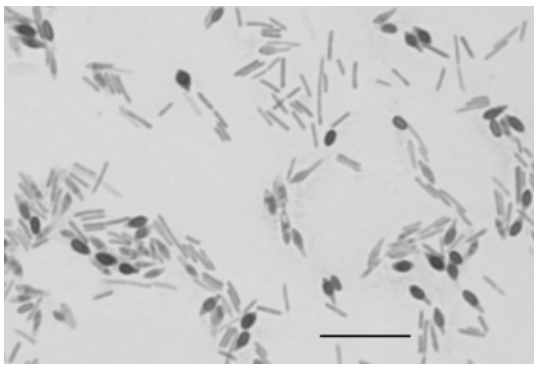

Fig. 1. Light photo micrograph showing strain S22^T spores stained with malachite green. Bar, 10 μm.

Results and Discussion

Morphology and physiological characteristics

$S22^T$ was Gram-reaction-positive, strictly aerobic, motile, catalase-positive, and oxidase-positive. Strain could grow in TSB medium that contained 0~3% NaCl but not in medium containing ≥4% (w/v) NaCl. Growth of strain $S22^T$ occurred between 10°C and 37°C (optimal growth at 20~30°C) but not below 10°C or above 40°C. The initial medium pH range that allowed growth of strain $S22^T$ was 5.0~8.0; the optimal pH was 7.0~8.0. Single cells of strain $S22^T$ were observed as motile rods that measured 2.9~3.6 μm in length and 0.6~0.8 μm in width. Terminal or subterminal ellipsoidal spores were observed in the swollen sporangia (Fig. 1). Colony morphology is circular, smooth in texture, and undulate in margin on NA, R2A, and TSA plates. Colony elevation is umbonate on NA/R2A and convex on TSA. Colony colors on NA, R2A, and TSA plates are cream, citron yellow, and banana yellow, respectively. The colony diameters on NA, R2A, and TSA plates were 2.5, 2.0, and 3.5 mm, respectively, after 4 days at 25°C. The isolate could not grow on MacConkey agar. The physiological and biochemical properties differentiating $S22^T$ from other related *Paenibacillus* strains are shown in Table 1. The other phenotypic characteristics of strain $S22^T$ are presented in the species description.

Chemotaxonomy

Strain $S22^T$ possessed anteiso-$C_{15:0}$ (52.9%), iso-$C_{16:0}$ (11.3%), and iso-$C_{15:0}$ (10.0%) as the major cellular fatty acids. The fatty acid content clearly discriminated $S22^T$ from closely related *Paenibacillus* type strains (Table 2). The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The predominant isoprenoid quinone of strain $S22^T$ was MK-7. In

Table 1. Phenotypic characteristics of strain $S22^T$ and closely related *Paenibacillus* strains
Strains: 1, *P. pini* $S22^T$; 2, *P. anaericanus* $MH21^T$ (data from Horn *et al*., 2005); 3, *P. ginsengisoli* Gsoil 1638^T (Lee *et al*., 2007); 4, *P. chibensis* NRRL $B\text{-}142^T$ (Shida *et al*., 1997b); 5, *P. barengoltzii* $SAFN\text{-}016^T$ (Osman *et al*., 2006); 6, *P. cookii* LMG $SSI018^T$ (Logan *et al*., 2004); and 7, *P. motobuensis* $MC10^T$ (Iida *et al*., 2005). All strains are positive for catalase, esterase lipase (C8), β-galactosidase and leucine arylamidase, hydrolyze esculin and starch, acetoin production and produce acid from glucose, esculin, cellobiose, maltose, lactose, trehalose, starch, and glycogen. All strains are negative for arginine dihydrolase, β-glucuronidase, lipase (C14), lysine decarboxylase, ornithine decarboxylase, tryptophane deaminase, indole production, hydrolysis of casein and gelatin, assimilation of citrate, capric acid, adipate, malate, trisodium citrate and phenylacetate and acid production from erythritol, D-arabinose, L-xylose, adonitol, sorbose, dulcitol, inositol, sorbitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-keto-gluconate, and 5-keto-gluconate. +, Positive; -, negative; w, weakly positive.

Characteristic	1	2	3	4	5	6	7
Oxidase	+	+	+	-	+	+	+
Nitrate reduction[a]	-	-	+	+	+	+	-
Anaerobic growth	-	+	+	-	-	+	+
Temp. range (°C)	10-37	5-40	20-30	10-50	10-50	20-50	20-55
pH range	5.0-8.0	5.8-8.5	5.0-8.5	4.5-9.0	4.5-9.0	4.5-9.0	6.0-8.0
Hydrolysis of[a]:							
Cellulose	+	+	+	+	-	-	+
Pectin	-	-	-	-	-	+	-
Tween 80	-	-	-	+	+	-	-
API test[a]:							
α-Galactosidase	-	-	-	+	+	+	-,w
α-Glucosidase	+	-	-	+	+	+	+
α-Fucosidase	-	-	-	+	-	+	-
Assimilation of:							
D-Glucose	+	-	-	+	-	+	+
L-Arabinose	-	-	-	+	-	-,w	+
D-Mannitol	-	-	-	+	-	-	+
N-Acetyl-glucosamine	+	-	-	+	-	-	+
D-Maltose	+	-	-	+	-	+	+
Potassium gluconate	+	-	-	+	+	+	+
Acid production from:							
D-Xylose	-	+	+	+	+	+	+
Galactose	-	+	+	+	+	+	+
Fructose	-	+	+	+	+	-	+
Mannitol	-	-	-	+	+	-	+
Methyl-α-D-Glucoside	-	+	+	+	+	-	+
Melibiose	-	+	+	+	+	+	+
Sucrose	-	-	+	+	+	+	+
Inulin	-	+	+	-	+	-	-

[a] data from this study

Table 2. Fatty acid compositions of strain S22^T and closely related strains
Strains: 1, *P. pini* S22^T; 2, *P. anaericanus* MH21^T; 3, *P. ginsengisoli* Gsoil 1638^T; 4, *P. chibensis* NRRL B-142^T; 5, *P. barengoltzii* SAFN-016^T; 6, *P. cookii* LMG 18419^T; and 7, *P. motobuensis* MC10^T. All data are taken from this study. Values are the percentages of total fatty acid. -, Not detected. Fatty acids comprising less than 0.5% of the total in all strains were excluded.

Fatty acid	1	2	3	4	5	6	7
Saturated							
$C_{12:0}$	-	1.1	0.6	-	3.9	-	0.7
$C_{14:0}$	2.5	12.6	9.2	0.9	12.1	0.8	3.3
$C_{15:0}$	3.2	1.3	1.8	-	0.4	0.3	-
$C_{16:0}$	8.8	28.0	25.6	8.8	23.0	4.5	-
Branched							
iso-$C_{13:0}$	0.3	-	-	-	-	-	0.5
iso-$C_{14:0}$	3.6	5.2	3.3	0.9	1.0	1.3	3.6
iso-$C_{15:0}$	10.0	4.5	4.6	7.8	3.4	7.1	27.6
iso-$C_{16:0}$	11.3	12.1	7.7	9.6	11.4	19.4	-
iso-$C_{17:0}$	1.9	1.2	1.5	6.9	3.7	5.6	-
anteiso-$C_{13:0}$	0.6	-	0.8	-	0.8	-	0.6
anteiso-$C_{15:0}$	52.9	31.6	40.8	47.1	25.7	36.4	63.4
anteiso-$C_{17:0}$	4.1	2.0	3.0	17.7	12.7	23.7	-
Others							
Unknown 14.502[a]	-	-	-	-	0.7	-	-

[a] Unknown fatty acid; this compound does not have a listed name in the Peak Library File of the MIDI system and therefore were designated by their equivalent chain-length (ECL).

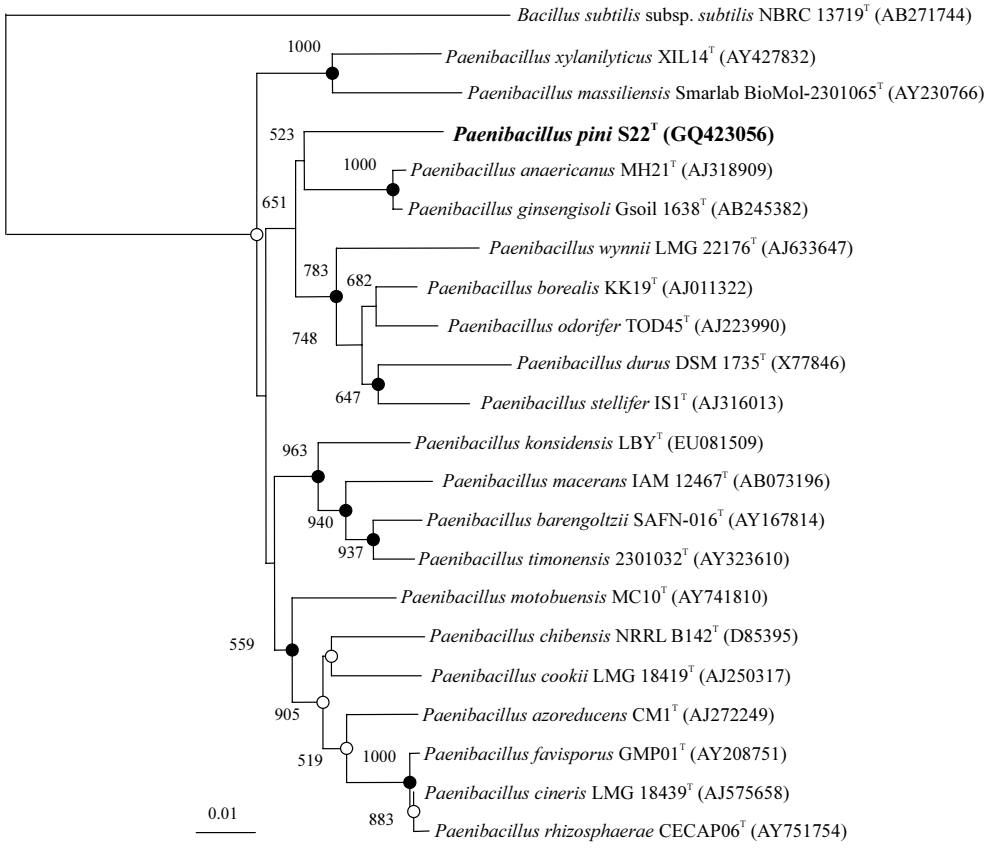

Fig. 2. Neighbor-joining tree showing the phylogenetic position of strain S22^T within related *Paenibacillus* strains. Closed circles represent that the corresponding nodes are also recovered in both maximum-likelihood and maximum-parsimony methods. Open circles represent that the corresponding nodes are recovered either in maximum-likelihood or maximum-parsimony methods. GenBank accession nos. of the 16S rRNA gene sequences are given in parentheses. Numbers at nodes indicate bootstrap values (based on 1,000 resamplings) if they are more than 500. Bar, 0.01 substitutions per nucleotide position. *Bacillus subtilis* subsp. *subtilis* NBRC 13719^T was used as outgroup.

a previous study, it has been reported that anteiso-$C_{15:0}$ and MK-7 were the characteristic major fatty acid and respiratory quinone in the genus *Paenibacillus* (Ash *et al.*, 1993; Shida *et al*., 1997a).

G+C content and phylogenetic analysis

The G+C content of strain S22^T was 43.3 mol%. The 1,477 bp 16S rRNA gene sequence of strain S22^T was determined. The 16S rRNA gene sequence of strain S22^T is clearly differentiated from that of the *Paenibacillus* type species. The 16S rRNA gene sequence similarity values between strain S22^T and the other *Paenibacillus* species ranged from 95.8% to 90.3%. A phylogenetic tree, constructed using the neighbor-joining method, showed that strain S22^T belongs to the genus *Paenibacillus* (Fig. 2). Similar phylogenetic relationships were also observed in the trees constructed with maximum parsimony and maximum-likelihood algorithms. Strains with approximately 70% or greater DNA-DNA relatedness were generally considered to be new species (Wayne *et al*., 1987) and organisms that have less than 97.0% 16S rRNA gene sequence similarity will not reassociate to more than 60% (Stackebrandt and Goebel, 1994).

On the basis of the data presented, strain S22^T represents a novel species within the genus *Paenibacillus*, for which the name *Paenibacillus pini* sp. nov. is proposed.

Description of *Paenibacillus pini* sp. nov.

Paenibacillus pini (pi'ni. L. gen. n. *pini* of a pine tree)

Cells are Gram-reaction positive, strictly aerobic, catalase-positive, oxidase-positive, motile, and rod-shaped, measuring 2.9~3.6 μm long and 0.6~0.8 μm wide. Terminal or subterminal ellipsoidal spores are formed in the swollen sporangia. Colonies are umbonate on NA/R2A plates and convex on TSA plates. The colony colors are cream, citron yellow, and banana yellow on NA, R2A, and TSA plates, respectively. Strain grows in the presence of 0~3% NaCl, at pH ranging from 5.0~8.0 (optimally at pH 7.0~8.0), and at temperatures between 10°C and 37°C (optimally at 20~30°C). Starch, CM-cellulose, and Tween 80 are hydrolyzed, but casein, gelatin, and pectin are not. In API 50CH, 20E, 20NE, and ZYM tests, strain is positive for esculin hydrolysis, alkaline phosphatase, esterase (C4), esterase lipase (C8), β-galactosidase, and leucine arylamidase, assimilation of D-glucose, *N*-acetyl-glucosamine, D-maltose, and potassium gluconate, production of acetoin, weakly positive for acid phosphatase and negative for arginine dihydrolase, α-chymotrypsin, crystine arylamidase, α-fucosidase, lipase (C14), α-galactosidase, β-glucuronidase, lysine decarboxylase, α-mannosidase, ornithine decarboxylase, tryptophane deaminase, urease, and valine arylamidase activities. Strain is negative for production of H_2S and indole, assimilation of adipate, L-arabinose, capric acid, malate, D-mannitol, D-mannose, phenylacetate, and trisodium citrate. Strain produces acid from amygdalin, cellobiose, esculin, glucose, *N*-acetyl-glucosamine, glycogen, lactose, maltose, ribose, starch, and trehalose, weakly produces acid from gentiobiose and not produce acid from adonitol, D-arabinose, L-arabinose, D-arabitol, L-arabitol, arbutin, dulcitol, erythritol, D-fucose, L-fucose, fructose, galactose, gluconate, 2-keto-gluconate, 5-keto-gluconate, methyl-α-D-glucoside, inositol, inulin, D-lyxose, mannitol, methyl-α-D-mannopyranside, mannose, melibiose, melezitose, raffinose, rhamnose, salicin, sorbitol, sorbose, sucrose, D-tagatose, D-turanose, xylitol, methyl-β-D-xylopyranside, D-xylose, and L-xylose. The major isoprenoid quinone is MK-7. The cell wall peptidoglycan contains *meso*-diaminopimelic acid. The predominant fatty acid is anteiso-$C_{15:0}$. The DNA G+C content is 43.3 mol%. The type strain is S22^T (=KCTC 13694^T =KACC 14198^T =JCM 16418^T) and has been isolated from the rhizosphere of a pine tree in Daejeon, Korea.

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