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Paenibacillus pinihumi sp. nov., a Cellulolytic Bacterium Isolated from the Rhizosphere of *Pinus densiflora*

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A novel cellulolytic bacterium, strain S23^T, was isolated from the rhizosphere of the pine trees in Daejeon, Republic of Korea. This isolate was Gram-positive, strictly aerobic, rod-shaped, catalase-negative, oxidase-positive, motile by means of peritrichous flagella, and tested positive for alkaline phosphatase, esterase lipase, leucine arylamidase, α-galactosidase, and β-galactosidase activities. The DNA G+C content was 49.5 mol%. The main cellular fatty acids were anteiso-$C_{15:0}$ (51.9%), iso-$C_{16:0}$ (14.7%), and iso-$C_{15:0}$ (13.2%). The major isoprenoid quinone was menaquinone 7 (MK-7). Diagnostic diamino acid in the cell-wall peptidoglycan was *meso*-diaminopimelic acid. Comparative 16S rRNA gene sequence analysis showed that this strain clustered with *Paenibacillus* species. The 16S rRNA gene sequence similarity values between S23^T and other *Paenibacillus* species were between 89.9% and 95.9%, and S23^T was most closely related to *Paenibacillus tarimensis* SA-7-6^T. On the basis of phylogenetic and phenotypic properties of strain S23^T, the isolate is considered as a novel species belonging to the genus *Paenibacillus*. Therefore, the name, *Paenibacillus pinihumi* sp. nov., is proposed for the rhizosphere isolate; the type strain is S23^T (=KCTC 13695^T =KACC 14199^T =JCM 16419^T)

***Keywords*:** cellulose, novel bacterium, pine tree, rhizosphere, *Paenibacillus pinihumi*

The genus *Paenibacillus* was proposed by Ash *et al*. (1993) for rRNA group 3 bacilli according to comparative 16S rRNA sequence analysis. After the proposal of this genus, 105 species and 4 subspecies were reported at the time this manuscript was written (http://www.bacterio.cict.fr/) (Euzeby, 1997). Some strains of *Paenibacillus* are known to degrade the constituents in plant cell walls such as cellulose and xylan. For instance, *Paenibacillus macerans* NCDO 1764, *Paenibacillus* sp. BP-23, *Paenibacillus phyllosphaerae* PALXIL04^T, *Paenibacillus cellulosilyticus* PALXIL08^T, and *Paenibacillus curdlanolyticus* B-6 were reported as cellulolytic bacteria of this genus (Williams and Withers, 1985; Blanco and Pastor, 1993; Rivas *et al*., 2005, 2006; Pason *et al*., 2006).

Recently, a few cellulolytic bacterial strains were isolated from the rhizosphere of pine trees (*Pinus densiflora*) during a study of cultivated bacteria from the rhizosphere in the Republic of Korea. One of the cellulolytic-isolates was considered a novel species of the genus *Paenibacillus* on the basis of 16S rRNA gene sequence comparisons. Therefore, polyphasic analyses were performed to elucidate the taxonomic position of this isolate, S23^T.

Materials and Methods

Bacterial strains

Rhizosphere samples of trees were collected for the isolation of bacteria. Soil samples were diluted serially, and these dilutions were plated onto R2A agar medium (BBL, USA). The plates were then incubated at 25°C for 6 days. Selected single colonies from the R2A plates were transferred onto R2A agar plates containing carboxymethyl (CM)-cellulose, and the plates were incubated at 25°C for 6 days. CM-cellulose degrading bacteria were selected by staining the plates with a 1% Congo Red in water (Rivas *et al*. 2003). Among cellulolytic bacteria, the strain S23^T was 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). S23^T was routinely cultured on TSA agar plates (BBL) and maintained as a glycerol suspension (20%, w/v) at -70°C. This isolate was deposited into the Korean Collection for Type Cultures (KCTC) as KCTC 13695^T, the Korean Agricultural Culture Collection (KACC) as KACC 14199^T, and the Japan Collection of Microorganisms (JCM) as JCM 16419^T. *Escherichia coli* KCTC 2441^T was received from KCTC and used as a reference strain for G+C content analysis. Closely related *Paenibacillus* strains, *P. tarimensis* KACC 14087^T, *Paenibacillus humicus* KCTC 13675^T, *P. phyllosphaerae* KCTC 13018^T, *Paenibacillus castaneae* KCTC 13703^T, *Paenibacillus glycanilyticus* KCTC 3808^T, and *Paenibacillus pasadenensis* KCTC 13676^T were received from KCTC or KACC for the comparison of FAMEs and physiological characters.

Morphology and physiological characteristics

Physiological tests of the isolate were conducted under optimal growth condition, at 25~30°C and pH 7.5, if there

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was no description. The morphology of colony was observed after culturing on Nutrient Agar (NA; BBL), R2A agar, and TSA plates for 4 days at 25°C. The morphology of live cells and spores was observed using light microscopy (Nikon E600; Nikon, Japan), and the flagella of cells were observed using transmission electron microscopy (TEM). Spore formation was determined using the malachite green staining method (Schaeffer and Fulton, 1933). For TEM observation, cells were cultured for 2 days at 25°C on TSA plates, negatively stained with 1% (w/v) phosphotungstic acid, and examined on grids using a model H-7600 transmission electron microscope (Hitachi, Japan). Gram staining was performed using a Gram stain set (BBL). Anaerobic growth was evaluated by culturing the organism on a TSB agar plate under anaerobic atmosphere that was maintained ≥10% (v/v) carbon dioxide with the GasPak EZ Anaerobe Pouch System (Becton Dickinson, USA). Motility was tested by culturing the organism in TSB media that contained 0.4% agar. The oxidase activity was assessed colorimetrically using tetramethyl-p-phenylenediamine, and the catalase activity was determined by bubble production using 3% (v/v) H_2O_2. The growth at various temperatures was tested using TSA plates incubated at 4, 10, 15, 25, 30, 37, 40, and 45°C. The effects of pH on growth were tested in pH-adjusted TSB media (pH 4.0~10.0 in 0.5 unit increments). The effects of salt on growth were determined in TSB media containing 1~5% (w/v) NaCl. The growth ability on MacConkey agar was tested using standard MacConkey agar plates (BBL). The hydrolysis of casein and starch were measured using standard microbiological methods (Atlas, 1993), and the hydrolysis of Tween 80 was measured using the method of Chakrabarty *et al*. (1970). The pectinase activity was tested using R2A plates containing 0.3% citric pectin and 1% *n*-hexadecyltrimethylammonium bromide as a staining solution. Other enzyme activities of the isolate were measured with API ZYM test strips (bioMérieux, France) after 8 h incubation at 30°C. API 20NE and API 20E test strips (bioMérieux) were used to detect other biochemical and physiological traits of the isolates over a period of 2 days at 30°C. Carbon source oxidation was confirmed using an API 50CH kit and 50CHB medium (bioMérieux) over a period of 2 days at 30°C. API tests for closely related type strains were preformed for comparative study.

Chemotaxonomy

The cellular fatty acid compositions of S23^T and closely related *Paenibacillus* strains were determined using cells that were grown on TSA agar plates for 2 days at 30°C. Fatty acids were extracted according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990) and analyzed using a gas chromatograph (model 6890N and autosampler 7683; Agilent, USA) and the Microbial Identification Sherlock software package (MIDI, USA). Isoprenoid quinone was determined using freeze-dried cells previously grown in TSA broth at 25°C for 2 days. Isoprenoid quinone was extracted according to the method of Collins and Jones (1981) and then purified by preparative TLC (silica gel F254; Merck). The identity of the quinone was determined by HPLC (Hitachi L-5000) using a reversed-phase column (YMC pack ODS-AM; YMC Co.) as described by Shin *et al*. (1996). The diamino acid of the peptidoglycan was determined by TLC (DC-Alufoline cellulose; Merck) as described by Komagata and Suzuki (1987).

Determination of G+C content, 16S rRNA gene sequence, and phylogenetic analysis

The G+C content of the isolate was determined by the HPLC method described by Meshbah *et al*. (1989). Genomic DNAs of strain S23^T and *E. coli* KCTC 2441^T were extracted according to the method described by Sambrook and Russell (2001), hydrolyzed with nuclease P1, and then dephosphorylated with alkaline phosphatase. The mixture of nucleosides was analyzed by HPLC using a reversed-phase column (Supelcosil LC-18-S; Supelco, Germany). The 16S rRNA gene sequence was amplified by PCR using universal primers fD1 and rD1 as previously described by Weisburg *et al*. (1991). The sequencing reaction and analysis were performed at SolGent Co. Republic of Korea, using an ABI prism Bigdye terminator cycle sequencing ready reaction kit V.3.1 and ABI 3730XL capillary DNA Sequencer (Applied Biosystems, USA). The nearly-full sequence of the 16S rRNA gene was assembled with Vector NTI software (Invitrogen, USA).

The 16S rRNA gene sequence of strain S23^T was compared with available 16S rRNA gene sequences from GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/) and the Eztaxon server [http://www.eztaxon.org/; Chun *et al*. (2007)]. The 16S rRNA gene sequences of strain S23^T and closely related type strains were aligned using CLUSTAL X software (Thompson *et al*., 1997). The evolutionary distances were computed by Kimura's two-parameter method (Kimura, 1980), and the phylogenetic trees were constructed with the PHYLIP package (Felsenstein, 1993) using the neighbor-joining (Saitou and Nei, 1987), maximum parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods. The topologies of tree were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings.

Results and Discussion

Morphology and physiological characteristics

Strain S23^T was Gram-positive, strictly aerobic, motile, catalase-negative, and oxidase-positive. Single cells of strain S23^T were observed as rods that measured 1.6~3.5 μm in length and 0.6~0.8 μm in width and formed peritrichous flagella on TSA medium (Fig. 1). Ellipsoidal spores were observed in terminal or subterminal positions. Colonies of S23^T are circular, rough, convex in elevation, and entire in margin on NA, R2A, and TSA plates. The colony color on NA, R2A, and TSA plates are cream, white, and cream, respectively. The diameter of colonies on NA, R2A, and TSA was 1.0, 1.5, and 2.0 mm, respectively, after 4 days at 25°C. Strain S23^T grew at 15~37°C, optimally at 25~30°C, on TSA plates but not under 10°C or over 40°C. Growth was observed in TSB media that contained 0~3% (w/v) NaCl but not in media containing ≥4% (w/v) NaCl. The initial media pH range that allowed growth of strain S23^T was pH 5.5~9.0; the optimal pH was 7.5. The isolate could not grow

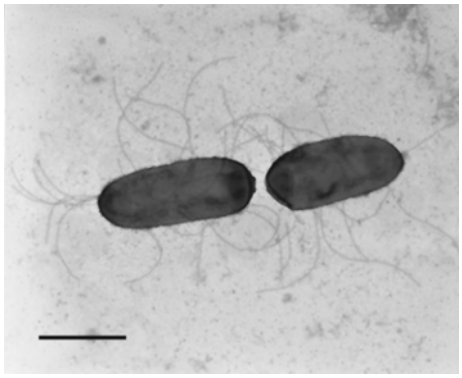

Fig. 1. Transmission electron micrograph of isolate S23^T grown on TSA medium. Bar=1 μm.

on MacConkey agar. Differentiating characteristics of strain S23^T and other related type strains are shown in Table 1. The physiological and biochemical properties of strain S23^T are given in the species description.

Chemotaxonomy

Shida *et al*. (1997) reported that *Paenibacillus* strains contain MK-7 as the major respiratory quinone and anteiso-$C_{15:0}$ as the major fatty acid. The predominant isoprenoid quinone of strain S23^T was unsaturated menaquinone with seven isoprene units. The major cellular fatty acids of strain S23^T were anteiso-$C_{15:0}$ (51.9%), iso-$C_{16:0}$ (14.7%), and iso-$C_{15:0}$ (13.2%). The fatty acid content of strain S23^T is clearly discriminated with that of closely related type strains of *Paenibacillus* (Table 2). Strain S23^T contained *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan.

G+C content and phylogenetic analysis

The G+C content of strain S23^T was 49.5 mol%. The nearly complete 1,474 bp 16S rRNA gene sequence of strain S23^T was determined and subjected to comparative analysis with the 16S rRNA genes of closely related reference strains. The phylogenetic tree based on the nucleotide sequences of 16S rDNA is shown in Fig. 2. In an NJ tree, strain S23^T is closely related to *Paenibacillus phyllosphaerae* PALXIL04^T, *Paenibacillus tarimensis* SA-7-6^T, *Paenibacillus humicus* PC-

Table 1. Phenotypic characteristics of strain S23^T and some closely related strains
Species: 1, strain S23^T (n=2); 2, *P. tarimensis* KACC 14087^T; 3, *P. humicus* KCTC 13675^T; 4, *P. phyllosphaerae* KCTC 13018^T; 5, *P. castaneae* KCTC 13703^T; 6, *P. glycanilyticus* KCTC 3808^T; and 7, *P. pasadenensis* KCTC 13676^T. Data are from this study and from Wang *et al*. (2008), Vaz-moreira *et al*. (2007), Rivas *et al*. (2005), Valverde *et al*. (2008), Dasman *et al*. (2002), and Osman *et al*. (2006). All strains are motile and hydrolyze aesculin. +, positive; -, negative.

Characteristic	1	2	3	4	5	6	7
Oxidase	+	-	+	+	+	-	+
Catalase	-	+	+	+	+	+	+
Hydrolysis of[a] :							
Cellulose	+	+	+	+	+	-	+
Pectin	-	+	-	+	-	-	-
Starch	+	+	+	+	-	+	+
Tween 80	-	-	-	+	-	-	-
API test[a] :							
Gelatinase	-	+	+	-	-	-	+
Assimilation of :							
Potassium gluconate	-	+	+	+	-	-	-
Acid production from :							
N-Acetyl-glucosamine	-	+	-	-	-	-	+
d-Arabinose	-	+	-	-	-	+	-
l-Arabinose	-	-	-	-	+	+	+
Arbutin	-	+	-	-	-	+	+
Cellobiose	-	+	+	+	+	+	+
Fructose	-	-	+	-	+	+	+
Glucose	-	+	+	-	+	+	+
Maltose	-	+	+	+	+	+	+
Mannitol	-	+	+	+	+	+	+
Mannose	-	-	-	-	+	+	+
Melezitose	-	+	+	-	-	+	+
Melibiose	-	+	+	+	+	+	+
Methyl-α, d-Glucoside	-	-	-	+	-	+	+
Sucrose	-	+	+	+	+	+	+
d-Turanose	-	+	+	+	+	+	+
G+C content (mol%)	49.5	53.7	58	50.7	46	50.5	64

[a] data from this study

Table 2. Fatty acid methyl ester compositions of strain S23^T and some closely related strains
Species: 1, strain S23^T; 2, *P. tarimensis* KACC 14087^T; 3, *P. humicus* KCTC 13675^T; 4, *P. phyllosphaerae* KCTC 13018^T; 5, *P. castaneae* KCTC 13703^T; 6, *P. glycanilyticus* KCTC 3808^T; and 7, *P. pasadenensis* KCTC 13676^T. 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. All strains were cultured on TSA for 2 days at 30°C.

Fatty acid	1	2	3	4	5	6	7
Saturated							
$C_{10:0}$	0.6	-	-	-	-	-	-
$C_{14:0}$	1.1	1.3	1.5	1.6	1.7	0.8	0.5
$C_{16:0}$	6.7	8.7	5.3	16.2	8.2	9.1	3.8
$C_{17:0}$	0.5	-	0.1	0.7	-	0.5	0.2
Unsaturated							
$C_{16:1}\omega7c$ OH	-	-	0.1	0.9	-	0.1	0.8
$C_{16:1}\omega11c$	0.2	1.7	-	0.8	-	-	0.4
iso-$C_{17:1}\omega10c$	-	0.8	-	-	-	-	-
Branched							
iso-$C_{14:0}$	3.8	0.5	4.0	1.7	2.8	2.0	2.3
iso-$C_{15:0}$	13.2	13.0	12.4	9.2	7.3	5.2	7.6
iso-$C_{16:0}$	14.7	2.6	15.0	10.9	6.3	21.2	19.3
iso-$C_{17:0}$	3.3	5.4	2.1	3.6	1.5	3.6	2.9
anteiso-$C_{13:0}$	0.2	-	0.1	-	0.9	-	-
anteiso-$C_{15:0}$	51.9	48.3	54.2	45.1	67.2	48.8	51.6
anteiso-$C_{17:0}$	3.2	16.3	5.3	8.4	3.6	6.6	10.2
Summed features[a]							
4	-	1.1	-	0.7	-	-	-

[a] Summed features represent groups of two or three fatty acids that could not be separated using GLC with the Microbial Identification System (MIDI). Summed feature 4 contains one or more of the following fatty acids: $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2OH.

147^T, and *Paenibacillus pasadenensis* SAFN-007^T. The topology is similar to those of MP and ML trees. The 16S rRNA gene sequence similarity values between strain S23^T and the other *Paenibacillus* species ranged from 89.9~95.9%; and strain S23^T showed the highest similarity to *P. tarimensis* SA-7-6^T (95.9%), with the next highest similarities being *P.*

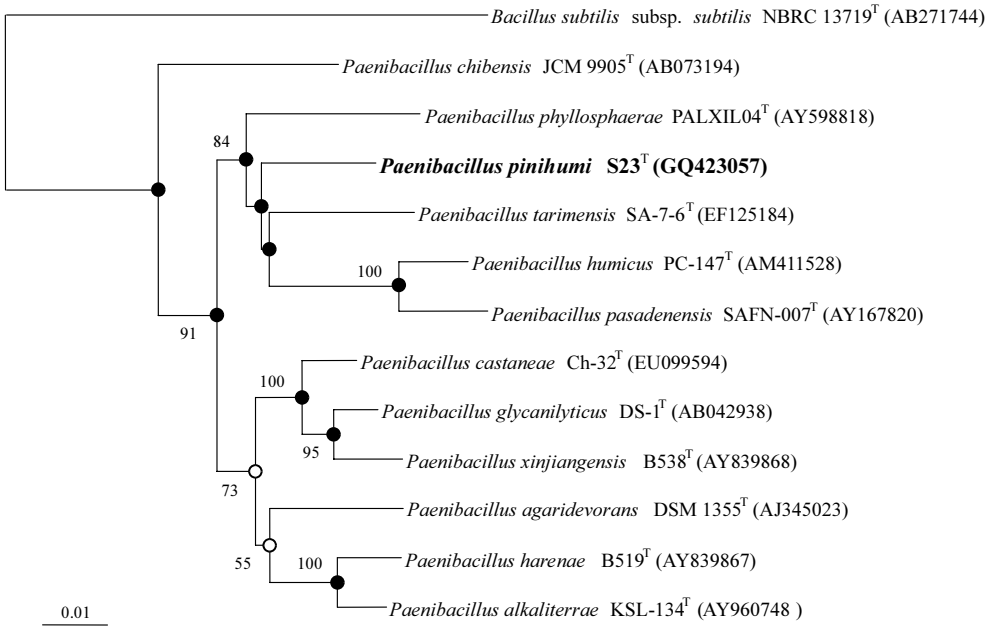

Fig. 2. Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain S23^T within closely related strains in the genus *Paenibacillus*. Closed circles represent that the corresponding branches are also recovered in maximum-likelihood and maximum-parsimony methods. Open circles represent that the corresponding branches are recovered in maximum-parsimony method. GenBank accession no. of the 16S rRNA gene sequences are given in parentheses. Bootstrap values (based on 1,000 replications) greater than or equal to 500 are shown as percentages at each nodes. Bar, 0.01 substitutions per nucleotide position. *Bacillus subtilis* subsp. *subtilis* NBRC 13719^T was used as the outgroup.

humicus PC-147^T (95.3%) and *P. phyllosphaerae* PALXIL04^T (95.2%). The 16S rRNA gene sequence of strain S23^T has been deposited in the GenBank nucleotide database under accession number GQ423057 at the NCBI website (http://www.ncbi.nlm.nih.gov). Strains that have less than 97.0% 16S rRNA gene sequence similarity will not reassociate to more than 60%, no matter which hybridization method is applied (Stackebrandt and Goebel, 1994), and strains with approximately 70% or greater DNA-DNA relatedness were considered as a species (Wayne *et al*., 1987). The 16S rRNA gene sequence similarity demonstrates that strain S23^T is a distinct species.

On the basis of phenotypic, chemotaxonomic and phylogenetic analyses, it is evident that S23^T represents a novel species within *Paenibacillus* for which the name *Paenibacillus filicis* sp. nov. is proposed.

Description of *Paenibacillus pinihumi* sp. nov.

Paenibacillus pinihumi (pi.ni.hu'mi. L. n. *pinus* a pine, L. n. *humus* soil, N. L. gen. n. *pinihumi* of soil from a pine tree) Cells are Gram-positive, strictly aerobic, catalase-negative, oxidase-positive, and motile by means of peritrichous flagella. Ellipsoidal spores are formed in terminal or subterminal positions. Colonies grown on NA and TSA agar are circular, rough, cream, convex, and have entire margins. Single cells are rod-shaped, measuring 1.6~3.5×0.6~0.8 μm. Growth occurs in the presence of 0~3% NaCl, at pHs ranging from 5.5~9.0 (optimally at pH 7.5), and at temperatures between 15°C and 37°C (optimally at 25~30°C). Starch and CM-cellulose are hydrolyzed, but not casein, gelatin, pectin, or Tween 80. In API ZYM tests, positive reactions for alkaline phosphatase, esterase lipase, leucine arylamidase, α-galactosidase, and β-galactosidase, and negative reactions for esterase, lipase, valine arylamidase, crystine arylamidase, trypsin, α-chymotrypsin, acid phospatase, naphtol-AS-BI-phosphohydrolase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase were observed. In API 50CH, acid is produced from esculin and trehalose but not from other carbon sources. In API 20NE, esculin hydrolysis, para-nitrophenyl-β-D-galactopyranosidase, assimilation of d-mannitol and d-maltose are positive, but all the other tests is negative. In API 20E, ortho-nitrophenyl-β-D-galactopyranosidase and acetoin production is positive, but all the other tests are negative. The major isoprenoid quinone is MK-7. Cell wall peptidoglycan contains *meso*-diaminopimelic acid. The predominant fatty acid is anteiso-$C_{15:0}$. The genomic DNA G+C content is 49.5 mol%. The type strain is S23^T (=KCTC 13695^T =KACC 14199^T =JCM 16419^T).

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