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

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A novel cellulolytic bacterium, strain S23³, 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-C₁₅₀ (51.9%), iso-C₁₀₆₀ (14.7%), and iso-C₁₆₀ (13.2%). The major isoprenoid quinone was menaquinone 7 (MK-7). Diagnostic diaminoc 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 and other Paenibacillus species were between 89.9% and 95.9%, and S23 was most closely related to Paenibacillus tarimensis SA-7-6³. On the basis of phylogenetic and phenotypic properties of strain S23³, 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 S23³ (=KCTC 13695T =KACC 14199T =JCM 16419T).

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 NCD0 1764, Paenibacillus sp. BP-23, Paenibacillus phyllosphaerae PALX104, Paenibacillus cellulolyticus PALX108³, 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, S23³.

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 S23³ 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). S23³ 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 13695T, the Korean Agricultural Culture Collection (KACC) as KACC 14199T, and the Japan Collection of Microorganisms (JCM) as JCM 16419T. Escherichia coli KCTC 2441T was received from KCTC and used as a reference strain for G+C content analysis. Closely related Paenibacillus strains, P. taminensis KACC 14087T, Paenibacillus humicus KCTC 13675T, P. phyllosphaerae KCTC 13018T, Paenibacillus castaneae KCTC 13703T, Paenibacillus glycanslyticus KCTC 3808T, and Paenibacillus pasadenensis KCTC 13676T were received from KCTC or KACC for the comparison of FAMEs and physiological characteristics.

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) H₂O₂. The growth at various temperatures was tested using TSB 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% r-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. The effects of pH on growth at various temperatures was tested using TSA plates. 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 Bigdyne terminator cycle sequencing ready reagent 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 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 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 Néi, 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 was Gram-positive, strictly aerobic, motile, catalase-negative, and oxidase-positive. Single cells of strain S23 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 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 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 TSA 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 was pH 5.5–9.0; the optimal pH was 7.5. The isolate could not grow