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

Byung-Chun Kim¹, Kang Hyun Lee¹, Mi Na Kim¹, Eun-Mi Kim¹, Sung Ran Min², Hyun Soon Kim³, and Kee-Sun Shin*¹

¹Biological Resources Center, KRIBB, Daejeon 305-806, Republic of Korea
²Plant Genomics Research Center, KRIBB, Daejeon 305-806, Republic of Korea

(Received November 2, 2009 / Accepted December 15, 2009)

Strain S22², 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 ellipsoid spores. S22² 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-C₁₅:₀ (52.9%), iso-C₁₆:₀ (11.3%), and iso-C₁₆:₁ (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. S22² 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 anareicusana MH21⁷ and Paenibacillus ginsengsoli Gsoil 1638⁴, with equal similarities of 95.8%. This polyphasic evidence suggested that strain S22² should be considered a novel species in the genus Paenibacillus, for which the name, Paenibacillus pini sp. nov., is proposed. The type strain is S22² (=KCTC 13694⁷ =KACC 14198⁷ =JCM 16148⁷).

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 N₂ fixation (Höffich 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 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 rhizophaeae from Cicer arietinum (Rivas et al., 2005), Paenibacillus nograndensis from Triticum aestivum (Beneduzi et al., 2009), Paenibacillus sonchii 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 S22².

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% 2,3,5-triphenyltetrazolium chloride (TTC) and 0.1% Coomassie Brilliant Blue R-250. Strain S22², 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. S22² 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 13694⁷, the Korean Agricultural Culture Collection (KACC) as KACC 14198⁷, and the Japan Collection of Microorganisms (JCM) as JCM 16148⁷. Closely related Paenibacillus strains, Paenibacillus anareicusana MH21⁷ (KACC 11533⁷), Paenibacillus barengoltzii SAFN-016⁷ (KCTC 13674⁷), Paenibacillus chibensis NRRL B-142⁷ (KCTC 3758⁷), Paenibacillus cookii LMG 18419⁷ (KCTC 3999⁷), Paenibacillus ginsengsoli Gsoil 1638⁷ (KCTC 13931⁷), and Paenibacillus
motobuensis MC107 (KCTC 3992T), 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. Growth was measured by OD600 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 20E 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 S22T 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 2441T was obtained from KCTC. Strain S22T and *E. coli* KCTC 2441T 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 S22T 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 S22T 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 S22T is GQ423056 (http://www.ncbi.nlm.nih.gov/)

![Fig. 1. Light photo micrograph showing strain S22T spores stained with malachite green. Bar, 10 µm.](image)