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Paenibacillus camelliae sp. nov., Isolated from Fermented Leaves of *Camellia sinensis*

Hyun-Woo Oh[1], Byung-Chun Kim[1], Kang Hyun Lee[1], Do Young Kim[1], Doo-Sang Park[1], Hee-Moon Park[2], and Kyung Sook Bae[1]*

[1]*Biological Resources Center, KRIBB, Daejeon 305-806, Republic of Korea*
[2]*School of Bioscience and Biotechnology, Chungnam National University, Daejeon 305-764, Republic of Korea*

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A novel bacterium, strain b11s-2^T was isolated from Pu'er tea. The isolate was Gram-positive, endospore-forming motile rod that grew at 15~42°C and pH 6.0~10.2. The DNA G+C content was 48.3 mol%, the predominant isoprenoid quinone was MK-7, and the predominant cellular fatty acid was anteiso-C15:0 (54.2%) followed by C16:0 (15.5%) and iso-C16:0 (8.2%). The polar lipid pattern of b11s-2^T was characterized by the presence of diphosphatidylglycerol, phosphatidylethanolamine, and phosphatidylglycerol. Phylogenetic analysis based on 16S rRNA gene sequence showed that the strain was affiliated within the *Paenibacillaceae*. The strain was most closely related to *Paenibacillus granivorans* A30^T, with a similarity of 97.1%. Based on the phylogenetic and phenotypic characteristics of strain b11s-2^T, the isolate is thought to represent a novel taxon in the genus *Paenibacillus*. The name *Paenibacillus camelliae* sp. nov. is proposed for the fermented tea isolate; the type strain is b11s-2^T (= KCTC 13220^T= CECT 7361^T).

***Keywords*:** Pu'er tea, novel bacterium, *Paenibacillus camelliae*

The genus *Paenibacillus* was separated from the genus *Bacillus* for the rRNA group 3 bacilli on the basis of phylogenetic analysis of 16S rRNA gene sequences (Ash *et al*., 1993). The ability to form spores and the production of diverse degrading enzymes make it possible for *Paenibacillus* to persist in diverse environments. Therefore, many *Paenibacillus* species have been isolated from a wide variety of sources, including antarctic sediments (Montes *et al*., 2004), air (Rivas *et al*., 2005), alkaline soils (Yoon *et al*., 2005), cow feces (Velázquez *et al*., 2004), garden peas (Šmerda *et al*., 2005), rice fields (Sánchez *et al*., 2005), soils (Kanzawa *et al*., 1995), and warm springs (Saha *et al*., 2005).

In a study that analyzed the cultivated bacterial community present in Pu'er tea, which is a fermented tea made from leaves of the tea plant, *Camellia sinensis*, a number of novel bacterial strains were isolated. The isolate, designated strain b11s-2^T, was related with *Paenibacillus* on the basis of a 16S rRNA gene sequence comparison. Therefore, this study was conducted to elucidate the taxonomic position of strain b11s-2^T based on phenotypic, genetic, and chemotaxonomic analyses.

Materials and Methods

Bacterial strains

Strain b11s-2^T was isolated from Pu'er tea collected in China by plating serial dilutions onto R2A agar medium (Difco, USA) that was then incubated at 30°C for 3 days. The isolated colony was streaked for three times to obtain pure culture. Strain b11s-2^T was deposited into the KCTC (Korean Collection for Type Cultures) as KCTC 13220^T, and also deposited into the CECT (Spanish Type Culture Collection) as CECT 7361^T. *Paenibacillus polymyxa* KCTC 3858^T, a reference strain for polar lipid analysis, was obtained from KCTC.

Morphology and physiological characteristics

The morphology of live cells and spores was determined using light microscopy (Nikon E600; Nikon, Japan) and transmission electron microscopy (TEM). For TEM observation, cells from an exponentially grown culture were negatively stained with 1% (w/v) phosphotungstic acid and allowed to air dry. Next, the cell was examined by using a model H-7600 transmission electron microscope (Hitachi, Japan). Thin sections of cells were prepared using the method described by Paster and Canale-Parola (1982). The physiological characteristics of the isolate were determined using cells cultivated aerobically in Tryptic Soy Broth (TSB) or on TSB agar (Difco, USA) for 2 days at 30°C. Gram-staining was performed using a Gram stain set (Difco, USA). The ability to grow under anaerobic conditions was evaluated by culturing the organisms on a TSB agar plate supplemented with nitrate in a sealed container that contained a BBL GasPak Pouch. Motility was tested by culturing the organism in TSB medium that contained 0.4% agar. Nitrate reduction was confirmed by culturing the organisms on a TSB plate according to the methods described by Lanyi (1987). The presence of oxidase activity was determined using an Oxy-swab (bioMérieux, France), and catalase activity was detected by placing drops of 3% (v/v) H_2O_2 on cultures grow-

* To whom correspondence should be addressed.
(Tel) 82-42-860-4610; (Fax) 82-42-861-2675
(E-mail) ksbae@kribb.re.kr

ing on TSB and observing the production of oxygen bubbles. To determine growth at various pHs, cells were inoculated in pH-adjusted media (pH 5.0~10.5 in 0.5 unit increments) at 30°C for 48 h, and the growth was then evaluated by measuring the optical density at 595 nm using a microplate reader (BIO-RAD, USA). To determine the optimum growth temperature and tolerance to NaCl in TSB medium, cells were cultured at temperatures ranging from 4 to 65°C and in the presence of NaCl concentrations ranging from, 0~10% (w/v), for 2 days. Xylanase activity was determined by Congo red staining using the following method (Skipper *et al*., 1985): Briefly, cells were grown in M9 minimal media (Difco, USA) that contained 0.5% yeast extract and 0.5% birchwood xylan (Sigma, USA). The colonies were then washed with water, after which the plate was stained with Congo red (2 mg/ml) for a few minutes, and then rinsed with 1 M NaCl. Unstained areas were assumed to indicate the hydrolysis of xylan. GP Biolog microplates (BIOLOG, USA), containing 95 different carbon compounds, were used to confirm the substrate oxidation. Biochemical and physiological traits of isolate were analyzed using API 20 NE test strip and an API 50CH kit (bioMérieux, France) over a period of 48 h.

Chemotaxonomy

The diamino acid of the peptidoglycan was determined by TLC (DC-Alufoline cellulose; Merck, USA) as described by Komagata and Suzuki (1987). Isoprenoid quinone and polar lipid of strain b11s-2^T were obtained from 100 mg freeze-dried cell grown in TSB at 30°C. Isoprenoid quinone was extracted according to the method of Collins and Jones (1981) and then purified by preparative TLC (silica gel F254; Merck, USA). The identity of the quinone was determined by HPLC (Hitachi L-5000; Hitachi, Japan) equipped with a reversed-phase column (YMC pack ODS-AM; YMC Co., Japan) as described by Shin *et al*. (1996). Polar lipids were extracted according to the procedures described by Minnikin *et al*. (1984). Extracted lipids were separated by two-dimensional TLC and identified by spraying with appropriate detection reagent (Minnikin *et al*., 1984; Komagata and Suzuki, 1987). Cellular fatty acid composition was determined using isolate that was grown on TSB agar for 2 days. Saponification, methylation, and extraction were performed according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The fatty acids were then analyzed using a gas chromatograph (model 6890N and autosampler 7683; Agilent, USA) and identified using the Microbial Identification Sherlock software package (MIDI, USA).

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

The DNA G+C content was determined using the method described by Tamaoka and Komagata (1984). Briefly, chromosomal DNA was extracted and purified according to the method described by Sambrook and Russell (2001), and then

Table 1. Physiological properties of strain b11s-2^T and other related *Paenibacillus* species
Species: 1, *Paenibacillus camelliae* b11s-2^T; 2, *P. granivorans* A30^T (Data from Van Der Maarel *et al*., 2000); 3, *P. agaridevorans* DSM 1355^T (Data from Uetanabaro *et al*., 2003); 4, *P. alkaliterrae* KSL-134 ^{T} (Data from Yoon *et al*., 2005). +, positive; -, negative; NA, data not available. All strains are positive for catalase.

Characteristic	1	2	3	4
Anaerobic growth	+[a]	-	-	-
Oxidase	+	-	+	+
Nitrate reduction	+	+	-	-
Growth at pH 5.7	-	-	-	-
Hydrolysis of				
Starch (Lugol)	+	+	-	+
Agar	-	NA	+	-
Casein	+	-	-	-
Gelatin	+	-	-	-
Tween 80	+	-	-	+
Utilization of				
D-Fructose	+	NA	-	+
D-Mannose	+	NA	-	+
D-Xylose	+	+	-	+
Citrate	+	-	-	-
L-Malate	+	-	-	-
Succinate	+	-	-	+
Major fatty acid	anteiso-$C_{15:0}$	anteiso-$C_{15:0}$	NA	anteiso-$C_{15:0}$
DNA G+C mol (%)	48.3	47.8	51	49.4

[a] Culture medium supplemented with nitrate.

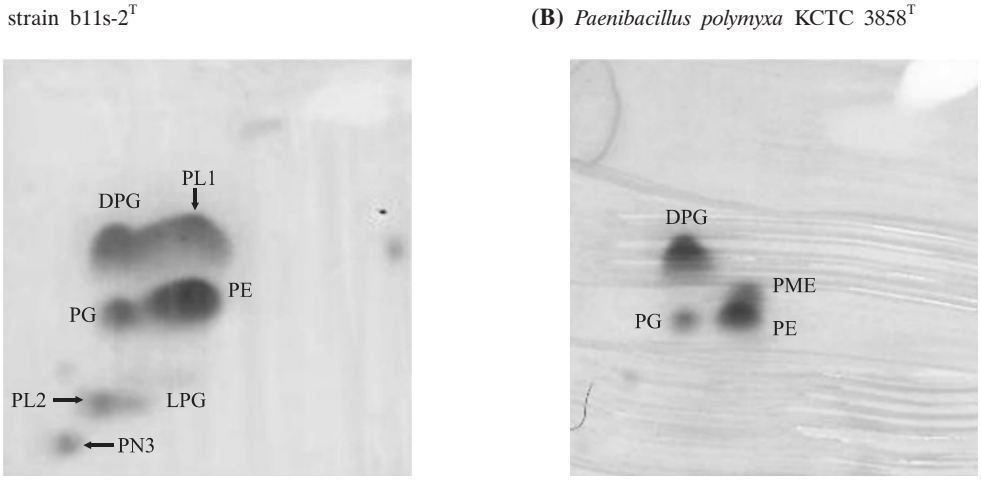

Fig. 1. Two-dimensional thin-layer chromatograms after staining with molybdatophosphoric acid (ZInzadze reagent) showing the total polar lipid profiles of *Paenibacillus* sp. b11s-2^T (left) and *Paenibacillus polymyxa* KCTC 3858^T (reference; right).
Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PME, phosphatidyl-*N*-methylethanolamine; LPG, lysyl-phosphatidylglycerol; PL1~2, unknown phospholipids; PN3, unkown aminophospholipid

treated with nuclease P1 and alkaline phosphatase. The resultant nucleotides were then analysed by HPLC using a reversed-phase column (Supelcosil LC-18-S; Supelco, Germany). The 16S rDNA of the isolate was amplified by PCR using universal primers fD1 and rD1, as previously described by Weiburg *et al*. (1991). The full sequence of the 16S rRNA gene was compiled with SeqMan software (DNASTAR). The 16S rRNA gene sequence of the isolate was compared with available 16S rRNA gene sequences from GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/) and the Ribosomal Database Project (Maidak *et al.*, 2001). The 16S rRNA gene sequence of the isolated strain was aligned with the 16S rRNA gene sequences of representatives of the genus *Paenibacillus* and related taxa, using CLUSTAL_X software (Thompson *et al*., 1997). Phylogenetic trees were constructed with MEGA (Molecular Evolutionary Genetics Analysis) program (Kumar *et al.*, 2004) using the neighbour-joining (Saitou and Nei, 1987) and maximum likelihood (Felsenstein, 1981) method based on distance-matrix data. The topology of the phylogenetic tree was evaluated with bootstrap analysis (Felsenstein, 1985) of the neighbour-joining data, based on 1,000 replications.

Results and Discussion

Morphology and physiological characteristics

Cells of strain b11s-2^T was observed as rods that measured 2.3~3.3 μm in length and 0.5~0.7 μm in diameter. Subterminal ellipsoidal spore was observed in swollen sporangium. Strain b11s-2^T was motile, Gram-positive, oxidase-positive, catalase-positive, and capable of growth under both aerobic and anaerobic conditions. The strain was also capable of growth on TSB medium that contained 0% to 2% (w/v) NaCl; however, it was not capable of growth in the presence of ≥3% (w/v) NaCl. Growth of strain b11s-2^T occurred between 15 and 42°C and at pH 6.0~10.2. Physiological properties of strain b11s-2^T and some other related type strains are shown in Table 1.

Chemotaxonomy

Strain b11s-2^T did not contain diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. The polar lipid content of strain b11s-2^T after separation by two-dimensional TLC is shown in Fig. 1. The polar lipid patterns were characterized by the presence of diphosphatidylglycerol, phosphatidylethanolamine, and phosphatidylglycerol. The predominant isoprenoid quinone of the strain was unsaturated menaquinone with seven isoprene units (MK-7). The principal cellular fatty acids of strain b11s-2^T grown on TSB agar were anteiso-C15:0 (46.9~54.2%) followed by C16:0 (5.0~15.5%) and iso-C16:0 (2.7~8.2%). Like other species of *Paenibacillus* spp., the predominant cellular fatty acid of the isolate was anteiso-C15:0. Shida *et al*. (1997) reported that *Paenibacillus* strains have MK-7 as the major respiratory quinone and anteiso-C15:0 as the major fatty acid.

G+C content and phylogenetic analysis

DNA G+C content of strain b11s-2^T was 48.3 mol%. The sequence of the isolate was manually aligned with representatives of the genus *Paenibacillus* and related taxa. The 16S rRNA gene sequence of strain b11s-2^T showed the highest similarity to that of *Paenibacillus granivorans* A30^T (97.1%), and *Paenibacillus agaridevorans* DSM 1355^T (95.6%). A rooted phylogenetic tree showing the relationship between the isolates and representatives of the genus *Paenibacillus* is shown in Fig. 1. The isolate b11s-2^T was clearly discriminated from the type species of *Paenibacillus*. The result of phylogenetic analyses demonstrated that strain b11s-2^T was not related to any of the recognized members of the genus *Paenibacillus* at the species level.

Based on the characteristics of strain b11s-2^T, we propose that this organism represents a novel species with the name of *Paenibacillus camelliae,* which is phylogenetically distinct from closely related members belonging to the genus *Paeni-*

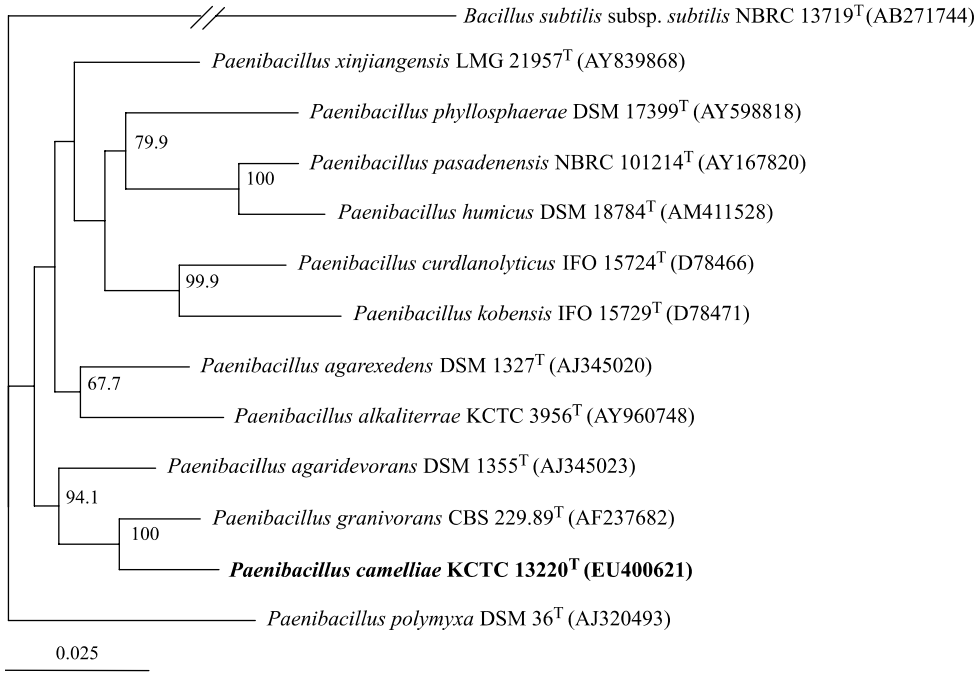

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of strain b11s-2^T within closely related species of the genus *Paenibacillus.*
Bootstrap values (1000 replications) are shown as percentages at each node only if they are 50% or greater. Bar, 0.025 substitutions per nucleotide position. *Bacillus subtilis* NBRC 13719^T was used as the outgroup.

bacillus.

Description of *Paenibacillus camelliae* sp. nov.

Paenibacillus camelliae (ca.mel.li'ae: N.L. gen. n. camelliae, of *Camellia*, referring to the isolation of the type strain from fermented green tea, *Camellia sinensis*).

Cells are Gram-positive, catalase-positive, oxidase-positive, motile, and have subterminal ellipsoidal spores in swollen sporangia. Colonies grown on TSB agar are round, cream-white, and approximately 2 mm in diameter. Single cells are slightly curved rods, measuring 2.3~3.3×0.5~0.7 μm. Growth occurs at temperatures between 15 and 42°C in TSB medium, with the optimum growth occurring at 30°C. Strains grow in the presence of 0~2% NaCl and at pH ranging from 6.0~10.2. The strains grow well on the surface of TSB agar when incubated under aerobic or anaerobic conditions. Casein, cellulose, gelatin, pectin, starch, Tween 80, and xylan are hydrolyzed. Positive for nitrate reduction, acetoin production, lysine decarboxylase, orinithine decarboxylase, and citrate utilization, and negative for β-galactosidase, arginine dihydrolase, urease, tryptophane deaminase, H_2S production, and indole production. Acid is produced from L-arabinose, amygdalin, arbutin, cellobiose, fructose, galactose, gentiobiose, glucose, glycogen, inositol, inulin, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, sorbitol, sucrose, starch, melibiose, ribose, salicin, trehalose, and D-xylose; but not from adonitol, D-arabitol, L-arabitol, D-arabinose, dulcitol, erythritol, D-fucose, D-lyxose, β-methyl-D-xylose, α-methyl-D-mannose, α-methyl-D-glucose, rhamnose, sorbose, D-tagatose, xylitol, and L-xylose. Cells cultured in TSB medium contain anteiso-C15:0 as the predominant cellular fatty acid. The predominant isoprenoid quinone is MK-7. Diphosphatidylglycerol, phosphatidylethanolamine, and phosphatidylglycerol are the main polar lipids. The G+C content of the DNA of the type strain is 48.3 mol%. The type species is *Paenibacillus camelliae* b11s-2^T (= KCTC 13220^T= CECT 7361^T).

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