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Isolation of *Paenibacillus pinesoli* sp. nov. from Forest Soil in Gyeonggi-Do, Korea[§]

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Using a new culture method for unculturable soil bacteria, strain NB5^T was isolated from forest soil at Kyonggi University, and characterized taxonomically on the basis of 16S rRNA gene sequence as well as phenotypic and chemotaxonomic characteristics. The novel strain was a Gram- and catalase-positive, rod-shaped bacterium, which grew in the pH range 6.0–9.5 (optimum, 6.5–9.5) and at temperatures between 15°C and 45°C (optimum, 25–40°C). Growth was possible at 0–5% NaCl (optimum, 0% to 3%) in nutrient, Luria-Bertani, and trypticase soy broths (TSB), as well as R2A medium (with optimal growth in TSB). A phylogenetic analysis of the 16S rRNA gene sequence showed that the novel strain was affiliated with the genus *Paenibacillus* and had 96.8% and 96.5% similarity to *P. nanensis* MX2-3^T and *P. agaridevorans* DSM 1355^T, respectively. The predominant menaquinone in NB5^T was MK-7; the major fatty acids were ante-iso-$C_{15:0}$ and iso-$C_{16:0}$; and the DNA G+C content was 54.5 mol%. We propose this strain as a novel species of the genus *Paenibacillus*, and suggest the name *Paenibacillus pinesoli* sp. nov. (type strain, KACC 17472^T =KEMB 9005-025^T =JCM 19203^T).

Keywords: *Paenibacillus pinesoli,* forest soil, taxonomy, soil bacteria

Introduction

The genus *Paenibacillus* (Ash *et al*., 1993) was reclassified as a novel member of the family Paenibacillaceae, which belongs to the order Bacillales, in class Bacilli of the Firmicutes phylum. Species of this genus have been discovered in diverse environments, including soil, cattle feces, dead honeybees, honeybee larvae, plant roots, food, warm springs, raw and heat-treated milk, and blood cultures (Nakamura, 1996; Berge *et al*., 2002; Roux and Raoult, 2004; Scheldeman *et al*., 2004; Velázquez *et al*., 2004; Chou *et al*., 2007). These bacteria are known to produce extracellular enzymes such as polysaccharide-degrading enzymes (amylase, cellulase, and xylanase) and proteases (Alvarez *et al*., 2006; Park *et al*., 2007; Rai *et al*., 2010). Various *Paenibacillus* species also secrete antimicrobial substances that act on a wide spectrum of microorganisms such as fungi, soil, and plant pathogenic bacteria, and anaerobic pathogens such as *Clostridium botulinum* (Piuri *et al*., 1998; Girardin *et al*., 2002; von der Weid *et al*., 2003). More specifically, several *Paenibacillus* species are rhizobacteria that enhance plant growth through phosphate solubilization, nitrogen fixation, degradation of environmental pollutants, hormone production, phytopathogen control, and the stimulation of iron uptake (Kloepper *et al*., 1980; Bloemberg and Lugtenberg, 2001; Ryu *et al*., 2003; Sirota-Madi *et al*., 2010; Jin *et al*., 2011; Beneduzi *et al*., 2012; Wang *et al*., 2012). Owing to these properties, *Paenibacillus* species have played an important role in agricultural, horticultural, industrial, and medical applications (Nielsen and Sørensen, 1997; Konishi and Maruhashi, 2003; Choi *et al*., 2004; Mishra *et al*., 2012).

To date, the genus *Paenibacillus* comprises 134 described species and four subspecies that are mostly Gram-variable, facultative anaerobic or aerobic, endospore-forming bacteria (Mishra *et al*., 2012). This report describes the discovery of a novel strain, NB5^T, from pine forest surface soil during the development of a new method for isolating bacteria (Pham and Kim, 2013). NB5^T was identified as a likely member of *Paenibacillus* based on polyphasic taxonomic verification, and can potentially be useful for some of the above-described applications.

Materials and Methods

Sampling and cultivation

A soil sample was collected from the forest at Kyonggi University, Suwon, Kyonggi-do, South Korea (37° 17.990′ N and 127° 2.325′ E) and passed through a mesh sieve to preserve microorganisms. Bacteria were isolated from the soil using a polycarbonate transwell plate as previously described (Pham and Kim, 2013). Briefly, 3 g of soil was added to each well and overlaid with the transwell insert. Nutrient broth (NB) (3 ml) and a soil suspension (100 µl) as an inoculum were added to the insert before incubation for 2 weeks at 28°C with shaking. The resultant culture in each well was serially diluted, and 100 µl of each diluted inoculum was spread on nutrient agar (NA) plates, which were incubated at 28°C for 1 week under aerobic conditions until colonies appeared. *Paenibacillus nanensis* KCTC 13044^T and *Paenibacillus agaridevorans* KACC 13412^T were used as reference species.

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Cell morphology

Sporulation was induced in Schaeffer's medium [0.1% KCl, 0.01% $MgCl_2$, 1.0 mM $Ca(NO_3)_2$, 0.01 mM $MnCl_2$, 0.001 mM $FeSO_4$, and 0.8% (w/v) NB] (Kempf *et al*., 2005). The size of cells grown on trypticase soy agar (TSA) for 2 days and morphology of spores incubated for 1 week were determined by phase contrast microscopy (BX50 light microscope; Olympus, Japan) at 1,000× magnification. Motility was assayed on 0.4% agar TSA. Gram staining was carried out as previously described (Doetsch, 1981), and the flagellum type was determined by staining (Forbes, 1981). The shape of isolated colonies grown on TSA at 30°C for 1 day was recorded.

Physiological tests

The thermal tolerance of *Paenibacillus* strains was tested at different temperatures (4, 10, 15, 20, 25, 30, 37, 45, 50, and 55°C) for 5 days. Salt tolerance was tested by incubating cells for 5 days at 30°C in trypticase soy broth (TSB) supplemented with 0–10% NaCl (w/v). Growth at a range of pH values from 4.0 to 12.0 (in 0.5 increments) was tested using acetate, phosphate, or Tris buffer for pH 5.0, 6.0–8.0, and 9.0–12.0, respectively. Growth on various media was tested by incubating for 5 days at 30°C on NA, Luria-Bertani agar (LBA), R2A agar, and TSA. To examine the growth pattern as a function of O_2 level, a traditional deep tube culture method with 0.7% agar medium was employed (Johnson and Case, 2007). Oxidase activity was determined using 1% (v/v) *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride. Catalase activity was assessed by the production of bubbles in 3% H_2O_2. Carbon source utilization was determined using the API 50CH system (bioMérieux, France) according to the manufacturer's instructions.

Phylogenetic analysis

Genomic DNA was extracted using an InstaGene Matrix kit (Bio-Rad, USA), and the 16S rRNA gene was amplified by PCR using the universal primer set for bacteria: 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′). The PCR product was purified with a multiscreen filter plate (Millipore Corp., USA) and the sequencing reaction was prepared with the PRISM BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). The reaction was incubated at 95°C for 5 min, cooled on ice for 5 min, and then analyzed with an ABI Prism 3730XL DNA Analyzer automated DNA sequencing system (Applied Biosystems). The near-complete sequence of the 16S rRNA gene was compiled using the SeqMan software (DNASTAR Inc., USA). Phylogenetic neighbors were identified, and pairwise 16S rRNA gene sequence similarities were calculated using the EzTaxon server (Kim *et al*., 2012). The related 16S rRNA gene sequences (GenBank) were edited using the BioEdit program (Hall, 1999). Multiple alignments were performed with the CLUSTAL_X program (Thompson *et al*., 1997). The phylogenetic tree was reconstructed using the neighbor-joining (Saitou and Nei, 1987), maximum likelihood, and maximum parsimony methods with the MEGA5.03 program (Tamura *et al*., 2011) and a bootstrap value of 1,000 replications (Felsenstein, 1985).

Chemotaxonomic and genotypic characteristics

Isoprenoid quinone was extracted with chloroform:methanol (2:1), filtered through Whatman paper (No. 2), evaporated under vacuum at 50°C, and extracted again with *n*-hexane. The crude *n*-hexane-quinone mixture was purified with a Sep-Pak Vac silica cartridge (Waters, Ireland). The purified quinone in *n*-hexane was evaporated, eluted with hexane: diethyl ether (98:2) to produce menaquinone, and re-extracted with acetone before analyzing by high performance liquid chromatography (HPLC) (Collins and Jones, 1981). Cells grown for 3 days on TSA at 30°C were used for fatty acid methyl ester analysis, in which esters were saponified, methylated, and extracted as per the instructions of the Sherlock Microbial Identification System (TSBA6, Version 6.0; MIDI, Inc., USA) (Sasser, 1990). Polar lipids were extracted using a previously published procedure (Minnikin *et al*., 1984) and separated by two-dimensional thin layer chromatography followed by spraying with appropriate detection reagents (Minnikin *et al*., 1984; Komagata and Suzuki, 1987).

The DNA G+C content of NB5^T and reference strains was determined by reversed phase HPLC as described (Mesbah *et al*., 1989). DNA from the NB5^T strain was labeled with photobiotin and used as the hybridization probe in reference species. Fluorometry was used to determine levels of relatedness of DNA-DNA hybridizations (Mehlen *et al*., 2004). The 16S rRNA gene sequence for the strain was deposited in the European Molecular Biology Laboratory database under the accession number KC415175.

Table 1. Differential phenotypic characteristics of strain NB5^T and the two closest *Paenibacillus* species

Characteristics	1	2	3
Maximal growth temp. (°C)	45	(45)	(35)
Anaerobic growth	–	(facultative)	ND
Growth at pH 6.0	+	(–)	(+)
Growth in 5% NaCl	+	(–)	(+)
Endospore type	unswollen	swollen	(unswollen)
Acid production from:			
Glycerol	–	w	w
L-Arabinose	–	w	w
D-Ribose	–	+	+
Methyl-β-D-xylopyranoside	–	w	w
L-Rhamnose	–	w	w
D-Mannitol	+	+	–
Methyl-α-D-mannopyranoside	–	w	w
N-Acetylglucosamine	w	w	–
Amygdaline	+	–	w
D-Lactose	–	+	+
Inulin	–	w	–
L-Fucose	–	w	w
Potassium gluconate	w	w	–
Major fatty acids (> 10%)	anteiso-$C_{15:0}$, iso-$C_{16:0}$	anteiso-$C_{15:0}$, iso-$C_{16:0}$, $C_{16:0}$, anteiso-$C_{17:0}$	anteiso-$C_{15:0}$, iso-$C_{16:0}$
DNA G+C content (mol%)	54.5	(52.9)	(51.0)

Strains: 1, strain NB5^T; 2, *P. nanensis* MX2-3^T; 3, *P. agaridevorans* DSM 1355^T. Data for strain NB5^T and related taxa were obtained in this study; data for other strains (in parentheses) were taken from Khianngam *et al.* (2009) and Uetanabaro *et al.* (2003). +, positive; –, negative; w, weakly positive, ND, not determined.

Table 2. Cellular fatty acid composition of strain NB5^T and the two closest *Paenibacillus* species

Fatty acid	1	2	3
Saturated			
$C_{16:0}$	8.4	15.2	9.1
Branched saturated			
iso-$C_{14:0}$	2.9	1.6	2.6
iso-$C_{15:0}$	7.8	4.8	9.3
iso-$C_{16:0}$	18.2	22.2	17.3
iso-$C_{17:0}$	5.1	7.0	6.0
anteiso-$C_{15:0}$	49.8	35.7	46.8
anteiso-$C_{17:0}$	5.1	10.1	5.7

All data are expressed as percentages and were obtained in this study (shown for >1.0% only). Strains: 1, *P. moonii* NB5^T; 2, *P. nanensis* MX2-3^T; 3, *P. agaridevorans* DSM 1355^T.

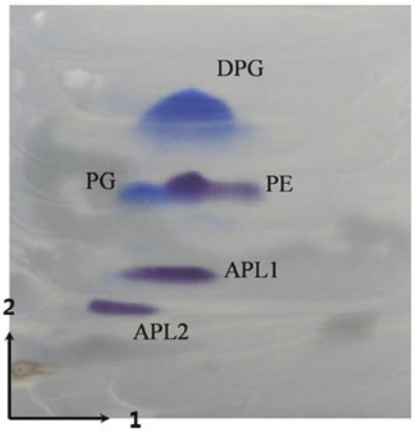

Fig. 1. Polar lipid profile of *M. pinesoli* strain NB5^T as determined by two-dimensional thin layer chromatography. Arrows with numbers indicate 1st and 2nd dimensions. APL1 and APL2, unknown aminophospholipids; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

Results and Discussion

Morphological and physiological characteristics

Bacteria in strain NB5^T were rod-shaped cells that were aerobic, Gram-positive, and motile by means of peritrichous flagella with a cell length of 1.5–4.0 μm and a diameter of 0.9–1.2 μm. Central or subterminal ellipsoidal endospores were observed in unswollen sporangia (Supplementary data Fig. S1). Colonies were 1–3 mm in diameter, yellowish, butyrous, circular, raised, and had entire margins after 2 days of growth on TSA at 30°C. Growth occurred within a wide range of temperatures (15–45°C, with an optimum range of 25–40°C) and pH (6.0–9.5, with optimum range of 6.5–9.5). Its salt tolerance ranged from 0% to 5% NaCl with an optimal salinity of 0–3%. Cells were positive for oxidase and catalase activities. Other phenotypic features are described in Table 1.

Chemotaxonomic characteristics

The predominant isoprenoid quinone found in strain NB5^T was the menaquinone MK-7, as for the reference species *P.*

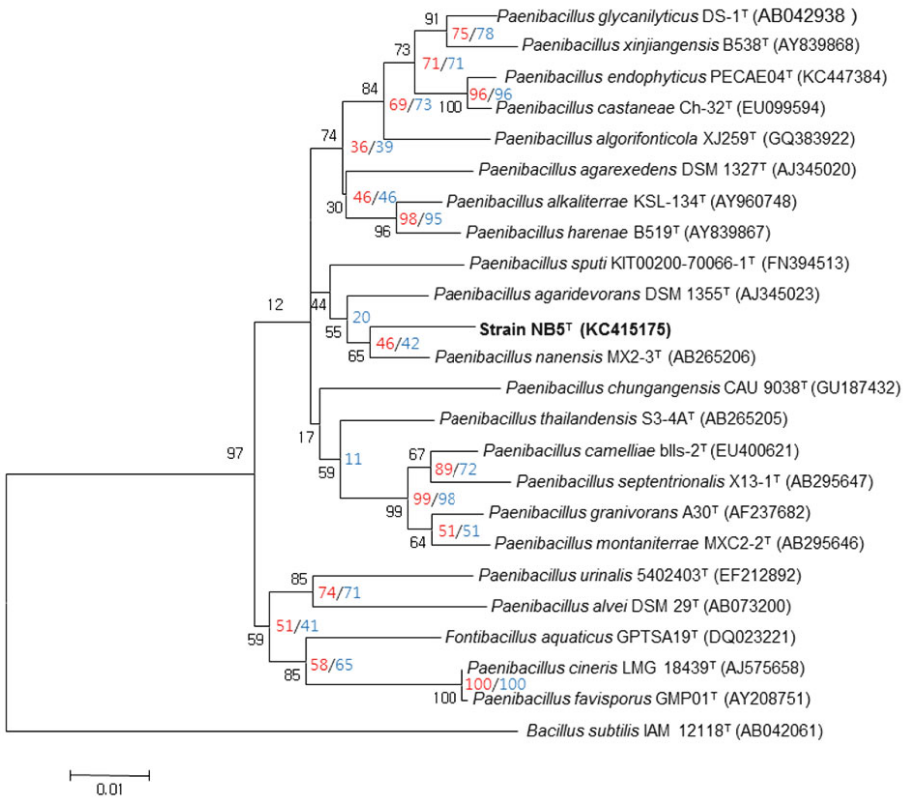

Fig. 2. Neighbor-joining phylogenetic tree of *M. pinesoli* strain NB5^T and related taxa based on an analysis of the 16S rRNA gene sequence. Numbers indicate bootstrap values from 1,000 resamplings (black, neighbor-joining; red, maximum likelihood; blue, maximum parsimony). Scale bar = 0.01 nucleotide substitution per sequence position.

nanensis (Khianngam *et al*., 2009). Fatty acids present at >1% abundance were $C_{15:0}$ anteiso (49.8%), $C_{16:0}$ iso (18.2%), $C_{16:0}$ (8.4%), $C_{15:0}$ iso (7.8%), $C_{17:0}$ iso (5.1%), $C_{17:0}$ anteiso (5.1%), and $C_{14:0}$ iso (2.9%). Differences in the fatty acid profiles of strain NB5^T and the two reference species are shown in Table 2. The two most abundant fatty acids were the same in all three bacterial strains. The predominant polar lipid component of strain NB^T was diphosphatidylglycerol (DPG), while phosphatidylethanolamine, phosphatidylglycerol, and two unknown aminophospholipids APL1 and APL2 were also present in moderate amounts (Fig. 1).

DNA-DNA hybridization and DNA G+C content

The DNA G+C content of strain NB5^T was 54.5%, which was higher than the two reference species (51.0% and 52.9%, respectively) (Uetanabaro *et al*., 2003; Khianngam *et al*., 2009). Hybridization experiments showed that the DNA of NB5^T had 48.5% and 22.3% similarity to *P. nanensis* and *P. agaridevorans*, respectively. These data demonstrate that strain NB5^T belongs to a distinct taxon.

Phylogenetic analysis

A phylogenetic tree was constructed using the neighbor-joining method based on the similarities in the 16S rRNA genes of strain NB5^T, closely related species, and neighboring taxa (Fig. 2). Strain NB5^T grouped with the genus *Paenibacillus* and had the highest similarity with *P. nanensis* (96.8%) and *P. agaridevorans* (96.5%). The tree was verified using two other methods (maximum likelihood and maximum parsimony), with bootstrap values indicated at many common nodes (Fig. 2).

Taxonomic conclusion

The phenotypic and chemotaxonomic properties, as well as 16S rRNA gene sequence and DNA-DNA hybridization similarities, indicate that strain NB5^T represents a member of a novel species, for which the name *Paenibacillus pinesoli* sp. nov. is proposed.

Description of *Paenibacillus pinesoli* sp. nov.

Paenibacillus pinesoli (pínésóli. L. gen. n. *pinesoli* of pine soil, referring to the surface soil in a pine tree forest).

Cells are aerobic, Gram-positive, motile, central or subterminal endospore-forming in unswollen sporangia, 1.5–4.0 μm long and 0.9–1.2 μm in diameter. Colonies on TSA at 30°C after 2 days are yellowish, butyrous, circular, raised, have entire margins, and are 1–3 mm in diameter. Growth occurs at 15–45°C (optimum, 25–40°C), pH 6.0–9.5 (optimum, pH 6.5–9.5), and 0–5% NaCl (w/v) (optimum, 0–3%). Cells have catalase and oxidase activities, and produce acid from D-xylose, D-glucose, D-mannitol, methyl-α-D-glucopyranoside, amygdaline, D-maltose, D-trehalose, amidon, glycogen, and gentiobiose; and weakly from D-galactose, D-fructose, D-mannose, *N*-acetyl-glucosamine, arbutin, salicin, D-cellobiose, D-melibiose, D-saccharose, D-melezitose, D-raffinose, D-turanose, potassium gluconate, and potassium-5-cetogluconate; but not from glycerol, L-arabinose, D-ribose, methyl-β-D-xylopyranoside, L-rhamnose, methyl-α-D-mannopyranoside, D-lactose, inulin, or L-fucose. The predominant isoprenoid quinone is MK-7. The major cellular fatty acids (> 10%) are $C_{15:0}$ anteiso and $C_{16:0}$. The predominant polar lipid is DPG. The DNA G+C content was 54.5% by HPLC. The type strain NB5^T (=KACC 17472^T =KEMB 9005-025^T =JCM 19203^T) was isolated from the surface soil in a pine tree forest at Kyonggi University in Suwon, South Korea.

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