Paenibacillus tianmuensis sp. nov., isolated from soil

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Two closely related, Gram-stain-negative, rod-shaped, spore-forming strains, B27^T and F6-B70, were isolated from soil samples of Tianmu Mountain National Natural Reserve in Zhejiang, China. Phylogenetic analysis based on 16S rRNA gene and *rpoB* sequences indicated that the isolates were members of the genus *Paenibacillus*. Both isolates were closely related to *Paenibacillus ehimensis* IFO 15659^T, *Paenibacillus elgii* SD17^T and *Paenibacillus koreensis* YC300^T ($\geq 95.2\%$ 16S rRNA gene sequence similarity). DNA–DNA relatedness between strain B27^T and *P. ehimensis* DSM 11029^T, *P. elgii* NBRC 100335^T and *P. koreensis* KCTC 2393^T was 21.2, 28.6 and 16.8%, respectively. The major cellular fatty acids of strains B27^T and F6-B70 were anteiso-C_{15:0} and iso-C_{15:0}. The cell wall contained meso-diaminopimelic acid. The two isolates differed from their closest neighbours in terms of phenotypic characteristics and cellular fatty acid profiles (such as variable for oxidase, negative for methyl red test, unable to produce acid from D-fructose and glycogen and relatively higher amounts of iso-C_{15:0} and lower amounts of C_{16:0} and iso-C_{16:0}). Strains B27^T and F6-B70 represent a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus tianmuensis* sp. nov. is proposed. The type strain is B27^T (=DSM 22342^T =CGMCC 1.8946^T).

The genus *Paenibacillus* was defined by Ash *et al.* (1991, 1993) after a comparative analysis of 16S rRNA gene sequences of group 3 bacilli and then emended by Shida *et al.* (1997). While the genus included 11 species initially, it comprises more than 110 recognized species at the time of writing (<http://www.bacterio.cict.fr/p/paenibacillus.html>). Members of the genus *Paenibacillus* have been one of the hot research topics in recent years because of their ability to produce antifungal and/or antimicrobial compounds. *Paenibacillus polymyxa*, the type species of the genus *Paenibacillus*, produces a variety of antibiotics, such as polymyxin A–E, paenibacillin, jolipeptin, gavaserin, salt-avalin, fusaricidin A–D and gatavalin (He *et al.*, 2007; Ito & Koyama, 1972; Kajimura & Kaneda, 1996, 1997; Katz & Demain, 1977; Nakajima *et al.*, 1972; Pichard *et al.*, 1995). *Paenibacillus koreensis* has been reported to produce an iturin-like compound with strong antifungal activity against *Fusarium oxysporum*, *Colletotrichum lagenarium*, *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Chung *et al.*,

2000). *Paenibacillus elgii* also exhibits a broad inhibition spectrum against bacteria and pathogenic fungi (Kim *et al.*, 2004).

Two strains, B27^T and F6-B70, were isolated from soil samples collected from Tianmu Mountain National Natural Reserve (30° 20' N 119° 25' E; altitude 1200 m), Zhejiang, China. The samples were suspended in sterile distilled water and incubated on nutrient agar [0.5% (w/v) peptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 1.5% (w/v) agar; pH 7.0–7.2] at 30 °C for 3–7 days. Morphologically different colonies were purified by repeated streaking onto nutrient agar. Strain B27^T and F6-B70 were routinely cultured at 30 °C and preserved in glycerol (20%, v/v) at –80 °C. The reference strains *Paenibacillus ehimensis* DSM 11029^T, *P. elgii* NBRC 100335^T and *P. koreensis* KCTC 2393^T were routinely cultured at 30 °C on nutrient agar.

Cell morphology and mobility were examined using light microscopy (E600; Nikon), transmission electron microscopy (JEM-1230; JEOL) and scanning electron microscopy (S3000N; Hitachi). Gram staining was carried out by the standard Gram reaction in parallel with the KOH lysis test (Gregersen, 1978). Growth was tested in basal nutrient broth at 4, 10, 20, 30, 37, 40, 45 and 50 °C, at pH 5, 5.6, 6, 7, 8, 9 and 10 and with 1–5% NaCl (at intervals of 1% NaCl). Anaerobic growth was tested in nutrient broth using Hungate anaerobic culture tubes with nitrogen gas at

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains B27^T and F6-B70 are FJ719490–FJ719492 and GU647099, GQ240305 and GU647100, respectively, and for the *rpoB* sequences of strains F6-B70, B27^T, *Paenibacillus ehimensis* DSM 11029^T, *Paenibacillus elgii* NBRC 100335^T and *Paenibacillus koreensis* KCTC 2393^T are GU979421 and GQ499850–GQ499853, respectively.

Two supplementary figures are available with the online version of this paper.

30 °C. Oxidase and catalase were determined using Kovács' reagent (Kovács, 1956) and 3 % (v/v) hydrogen peroxide, respectively. Acid production from 49 carbon sources was tested using the API 50 CHB system (bioMérieux). Other physiological and biochemical characteristics were determined according to the standard methods described elsewhere (Barrow & Feltham, 1993; Smibert & Krieg, 1994). The diamino acid of the peptidoglycan was determined as outlined by Hasegawa *et al.* (1983). Cellular fatty acids were analysed following the instructions of the Sherlock Microbial Identification System (MIDI) after cultivation on trypticase soy agar (5459; Merck) for 24 h at 30 °C.

Genomic DNA was extracted and purified according to standard methods (Moore & Dowhan, 1995). Fragments of the 16S rRNA gene were amplified, cloned into pMD19-T (TaKaRa) and sequenced as described previously (Wu *et al.*, 2008). For amplification of almost-complete *rpoB* sequences from the isolates and the reference strains, three degenerate PCR primer pairs were designed using an alignment of *rpoB* sequences from *Bacillus subtilis* 168^T, *Geobacillus kaustophilus* HTA 426, *Anoxybacillus flavithermus* WK1, *Escherichia coli* K12 and *Streptomyces coelicolor* A3(2) (GenBank accession numbers AL009126, BA000043, CP000922, CP000948 and AL939121, respectively). The primer pairs were 73F (5'-GAGYTGCCGAACYTGATYGYRATYCA-3') and 1584R (5'-RATCAAGCCGATRTTVG-GDCCYT-3'), 1429F (5'-TATGCTGARCTBACSCAYAAR-CG-3') and 2177R (5'-TGGTTRTAGCANGTGCCYTGR-TT-3'), and 1876F (5'-CGCCCGTTCTTWGAAAAYGAY-GAC-3') and 3306R (5'-GTGGTCGGAYTTRACCGTCA-ARAT-3'), which correspond to positions (*B. subtilis* *rpoB* numbering) 73–99, 1584–1561, 1429–1451, 2177–2155, 1876–1899 and 3306–3283, respectively. The reaction mixture (50 µl final volume) contained 5 µl 10 × PCR buffer, 100 µM each dNTP, 500 nM each primer, 1.25 U DNA polymerase (TaKaRa) and 10 ng genomic DNA. Amplification conditions were 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 60 °C (56 °C for primers 1429F and 2177R) for 1 min and 72 °C for 2 min, and a final extension step at 72 °C for 7 min. Amplified sequences were cloned into pMD19-T (TaKaRa) and sequenced (Wu *et al.*, 2008). The partially overlapping sequences were combined into a single consensus sequence using sequence assembler software (Applied Biosystems).

Considering that complete *rpoB* sequences are not yet available for all of the species of the genus *Paenibacillus* with validly published names, the phylogenetic analysis used partial *rpoB* sequences (corresponding to *B. subtilis* *rpoB* positions 1571–1934). The sequences obtained in this study and sequences extracted from GenBank were aligned using CLUSTAL W (Thompson *et al.*, 1994) and edited manually. Distances were calculated using distance options according to Kimura's two-parameter model (Kimura, 1980). Phylogenetic trees were reconstructed using the maximum-likelihood (Felsenstein, 1981) algorithm contained in PHYLIP version 3.63 (Felsenstein, 2005) and the

neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Kluge & Farris, 1969) algorithms available in MEGA version 4.0 (Tamura *et al.*, 2007). The resultant tree topologies were evaluated by bootstrap analysis based on 100, 1000 and 1000 resamplings for the maximum-likelihood, neighbour-joining and maximum-parsimony algorithms, respectively. Determination of DNA G+C content and DNA–DNA hybridization were performed by the thermal denaturation method described by De Ley *et al.* (1970).

Strains B27^T and F6-B70 had similar phenotypic characteristics. Cells were strictly aerobic, stained Gram-negative and also gave a Gram-negative result with the KOH lysis test. Cells were non-motile rods (0.75–1.5 × 3.0–7.5 µm) with no flagella (Supplementary Fig. S1, available in IJSEM Online) and formed ellipsoidal spores that lay terminally or subterminally in swollen sporangia (Supplementary Fig. S2). The isolates were positive for catalase and negative for indole and H₂S production. Aesculin, casein, DNA and gelatin were hydrolysed. Oxidase was variable and depended on the growth stage. Other phenotypic characteristics are given in the species description and Table 1. The isolates could be clearly distinguished from the three reference strains by the phenotypic characteristics listed in Table 1, such as oxidase, methyl red test and acid production from different carbon sources. Moreover, the isolates were different from *P. ehimensis* DSM 11029^T and *P. koreensis* KCTC 2393^T with respect to their inability to grow at 50 °C and different from *P. elgii* NBRC 100335^T by having no urease activity. Strains B27^T and F6-B70 both contained *meso*-diaminopimelic acid in the cell wall and their DNA G+C contents were 55.4 and 55.5 mol%, respectively. The isolates also possessed identical cellular fatty acid profiles, with anteiso-C_{15:0} and iso-C_{15:0} as the major components (Table 2). Distinct quantitative differences were found in the fatty acid profiles of the isolates in comparison with their closest neighbours: for instance, strains B27^T and F6-B70 contained relatively higher amounts of iso-C_{15:0} (22.1 and 18.0 %, respectively) and lower amounts of C_{16:0} (1.4 and 1.8 %) and iso-C_{16:0} (1.2 and 0.9 %) than the reference strains (Table 2).

The cloned 16S rRNA genes revealed three types of sequences in strains B27^T and F6-B70, indicating intra-genomic heterogeneity, which has been found in other species of the genus *Paenibacillus* (Bosshard *et al.*, 2002; Hamasaki *et al.*, 2005; Nübel *et al.*, 1996; Roux & Raoult, 2004). Comparative 16S rRNA gene sequence analyses showed that strains B27^T and F6-B70 were phylogenetically affiliated to the genus *Paenibacillus*. Sequences of the same type from strains B27^T and F6-B70 exhibited >99.5 % 16S rRNA gene sequence similarity, which suggested that the isolates are members of the same species. Moreover, the isolates exhibited 99.9 % *rpoB* sequence similarity (3177 bp) and 70 % DNA–DNA relatedness, which verified that they should be assigned to the same species (Adékambi *et al.*, 2008; Wayne *et al.*, 1987). The maximum-likelihood tree based on 16S rRNA gene sequences (Fig. 1) showed

Table 1. Differential phenotypic characteristics of strains B27^T and F6-B70 and closely related type strains of species of the genus *Paenibacillus*

Strains: 1, *Paenibacillus tianmuensis* sp. nov. B27^T and F6-B70; 2, *P. ehimensis* DSM 11029^T; 3, *P. elgii* NBRC 100335^T; 4, *P. koreensis* KCTC 2393^T. All data were taken from this study. All species are positive for catalase, nitrate reduction, β -galactosidase and hydrolysis of casein, gelatin, aesculin and DNA. All strains are negative for H₂S and indole production and citrate utilization. +, Positive; v, variable; w, weakly positive; –, negative.

Characteristic	1	2	3	4
Gram-staining	–	v	v	v
Oxidase	v	+	–	+
Urease	–	–	+	–
Methyl red test	–	+	+	+
Growth at/with:				
50 °C	–	+	–	+
pH 5.6	–	–	–*	+
Acid production from:				
L-Arabinose	–	+	–	+
D-Fructose	–	+	w	+
D-Mannose	–	+	–*	+
Inositol	–	+	–	+
Mannitol	–	+	–	+
Sorbitol	–	+	–	+
Methyl α -D-mannoside	–	w	–	+
Methyl α -D-glucoside	+	+	–	+
Amygdalin	–	+	–	†
Arbutin	–	+	–	+
Salicin	–	+	–	†
Cellobiose	w	+	–	+
Lactose	–	+	–	+
Melibiose	–	+	–	+
Melezitose	–	+	–	–
Raffinose	–	+	–	+
Starch	w	+	+	+
Glycogen	–	+	+	+
Xylitol	+	–	–	–
D-Gentiobiose	w	+	–	+
D-Arabitol	–	+	–	+

*Data differ from those obtained by Kim *et al.* (2004).

†Data differ from those obtained by Chung *et al.* (2000).

that strains B27^T and F6-B70 were closely related to *P. ehimensis* IFO 15659^T, *P. elgii* SD17^T and *P. koreensis* YC300^T, with which strain B27^T shared 96.0–97.2, 95.8–97.1 and 95.2–96.3 % 16S rRNA gene sequence similarity, respectively. The phylogenetic trees inferred with the neighbour-joining and maximum-parsimony algorithms showed relationships similar to those inferred with the maximum-likelihood algorithm.

To further validate the phylogenetic affinity of the isolates, this study analysed sequences of the *rpoB* gene, which has been shown to be more discriminative than the 16S rRNA gene in distinguishing members of the genus *Paenibacillus*

Table 2. Cellular fatty acid profiles of strains B27^T and F6-B70 and closely related type strains of species of the genus *Paenibacillus*

Strains: 1, *Paenibacillus tianmuensis* sp. nov. B27^T; 2, *P. tianmuensis* sp. nov. F6-B70; 3, *P. ehimensis* DSM 11029^T; 4, *P. elgii* NBRC 100335^T; 5, *P. koreensis* KCTC 2393^T. All data were taken from this study. –, Not detected.

Fatty acid (%)	1	2	3	4	5
Saturated					
C _{14:0}	1.0	0.9	0.8	2.3	1.2
C _{16:0}	1.4	1.8	3.2	7.6	9.5
Branched					
iso-C _{14:0}	0.9	0.6	2.5	2.3	2.7
iso-C _{15:0}	22.1	18.0	10.6	10.7	7.0
anteiso-C _{15:0}	58.1	58.2	53.9	51.8	44.5
iso-C _{16:0}	1.2	0.9	9.5	6.0	14.4
iso-C _{17:0}	2.8	3.3	3.1	2.8	3.9
anteiso-C _{17:0}	4.8	7.6	6.8	5.5	8.5
Unsaturated					
C _{16:1} ω 7c alcohol	0.9	0.7	2.6	1.6	1.5
C _{16:1} ω 11c	3.2	3.5	4.5	5.4	5.1
iso-C _{17:1} ω 10c	2.2	2.3	1.3	1.1	0.6
Summed feature 4*	1.4	2.3	0.8	–	0.5

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 4 consisted of iso-C_{17:1} I and/or anteiso-C_{17:1} B.

(da Mota *et al.*, 2004). The maximum-likelihood tree based on partial *rpoB* gene sequences (Fig. 2) showed that strains B27^T and F6-B70 fell within the cluster comprising the genus *Paenibacillus* and formed a separate lineage within the *P. ehimensis*–*P. koreensis*–*P. elgii* cluster. The overall partial *rpoB* gene sequence similarity between strain B27^T and members of the genus *Paenibacillus* was 71.3–94.8 %. The almost-complete *rpoB* gene sequence similarities between strain B27^T and *P. ehimensis* DSM 11029^T, *P. koreensis* KCTC 2393^T and *P. elgii* NBRC 100335^T were 95.3, 95.2 and 94.4 %, respectively, which were quite similar to the partial *rpoB* gene sequence similarities (94.8, 94.2 and 90.1 %, respectively). All *rpoB* gene sequence similarities were below the 97.7 % cut-off value suggested for the genomic distinction of species (Adékambi *et al.*, 2008) and indicated that strain B27^T represented a novel species of the genus *Paenibacillus*.

DNA–DNA relatedness between strain B27^T and *P. ehimensis* DSM 11029^T, *P. elgii* NBRC 100335^T and *P. koreensis* KCTC 2393^T was 21.2, 28.6 and 16.8 %, respectively, which further verified that strains B27^T and F6-B70 belong to a novel species of the genus *Paenibacillus* (Wayne *et al.*, 1987).

Therefore, on the basis of phenotypic, genotypic and chemotaxonomic data, strains B27^T and F6-B70 represent a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus tianmuensis* sp. nov. is proposed.

Fig. 1. Maximum-likelihood tree based on 16S rRNA gene sequences showing the phylogenetic position of *Paenibacillus tianmuensis* sp. nov. within the genus *Paenibacillus*. Bootstrap values (>50%) based on 100 resamplings are shown at branch nodes. Filled circles indicate that corresponding nodes were also recovered in trees generated with the neighbour-joining and maximum-parsimony algorithms. *Brevibacillus brevis* DSM 30^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Description of *Paenibacillus tianmuensis* sp. nov.

Paenibacillus tianmuensis (ti.an.mu.en'sis. N.L. masc. adj. *Tianmuensis* pertaining to Tianmu Mountain National Natural Reserve in Zhejiang Province, China, where the type strain was isolated).

The species description is based on two strains. Cells are strictly aerobic, Gram-stain-negative, non-motile rods (0.75–1.5 × 3.0–7.5 µm). No flagella are observed. Ellipsoidal spores are formed at terminal or subterminal positions in swollen sporangia. After 48 h on nutrient agar at 30 °C,

colonies are 1–2 mm in diameter, circular, flat, glossy, translucent and pale yellow. Growth occurs at 20–37 °C, at pH 6–8 and with 0–2% (w/v) NaCl. Optimum growth occurs at 30 °C and pH 7. Oxidase is variable, depending on the growth stage. Positive for catalase, β-galactosidase and nitrate reduction. Negative for urease, indole and H₂S production, Voges–Proskauer test, methyl red test and citrate utilization. Aesculin, casein, DNA and gelatin are hydrolysed and starch is weakly hydrolysed. Acid is produced from D-ribose, D-galactose, D-glucose, methyl α-D-glucoside, N-acetylglucosamine, aesculin, maltose, sucrose, trehalose, xylitol and turanose. Acid is weakly produced from glycerol, cellobiose, starch and gentiobiose. Acid is not produced from erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, D-adonitol, methyl β-D-xyloside, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannoside, amygdalin, arbutin, salicin, lactose, melibiose, inulin, melezitose, raffinose, glycogen, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. The major fatty acids are anteiso-C_{15:0} and iso-C_{15:0}. The diamino acid in the cell wall is meso-diaminopimelic acid. The DNA G + C content of the type strain is 55.4–55.5 mol%.

The type strain, B27^T (=DSM 22342^T =CGMCC 1.8946^T), was isolated from soil of Tianmu Mountain National Natural Reserve in Zhejiang, China.

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Fig. 2. Maximum-likelihood tree based on partial *rpoB* sequences showing the phylogenetic position of *Paenibacillus tianmuensis* sp. nov. within the genus *Paenibacillus*. Bootstrap values (>50%) based on 100 resamplings are shown at branch nodes. Filled circles indicate that corresponding nodes were also recovered in trees generated with the neighbour-joining and maximum-parsimony algorithms. *Bacillus subtilis* 168^T was used as an outgroup. Bar, 0.05 substitutions per nucleotide position.

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