Paenibacillus taichungensis sp. nov., from soil in Taiwan

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Among a large collection of Taiwanese soil isolates, a novel Gram-variable, rod-shaped, motile, endospore-forming bacterial strain, strain V10537^T, was subjected to a polyphasic study including 16S rRNA and *gyrB* gene sequence analysis, DNA–DNA hybridization experiments, cell wall peptidoglycan type, cellular fatty acid composition analysis and comparative phenotypic characterization. 16S rRNA gene sequence analysis indicated that the organism belonged to the genus *Paenibacillus*. Strain V10537^T possessed meso-diaminopimelic acid as the diagnostic diamino acid of the peptidoglycan. It contained menaquinone MK-7 as the predominant isoprenoid quinone and anteiso-C_{15:0} (53.6 %) and C_{16:0} (19.0 %) as the major fatty acids. Phylogenetically, the most closely related species to strain V10537^T were *Paenibacillus pabuli*, *Paenibacillus xylanilyticus*, *Paenibacillus amylolyticus*, *Paenibacillus barcinonensis* and *Paenibacillus illinoisensis*, with 16S rRNA gene sequence similarities of 99.5, 98.8, 98.3, 98.2 and 98.1 % to the respective type strains. The *gyrB* gene sequence similarities between strain V10537^T and these strains were 76.9–85.0 %. DNA–DNA hybridization experiments showed levels of relatedness of 8.5–45.6 % between strain V10537^T and these strains. The DNA G + C content of strain V10537^T was 46.7 mol%. Strain V10537^T was clearly distinguishable from other *Paenibacillus* species and thus represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus taichungensis* sp. nov. is proposed. The type strain is V10537^T (=BCRC 17757^T =DSM 19942^T).

In a comparative analysis of 16S rRNA gene sequences of 51 species of the genus *Bacillus*, Ash *et al.* (1991) described five principal groups of strains, of which rRNA groups 3 and 4 have since been afforded generic status as *Paenibacillus* (Ash *et al.*, 1993) and *Brevibacillus* (Shida *et al.*, 1996), respectively. The description of the genus *Paenibacillus* was emended by Shida *et al.* (1997a, b). The genus *Paenibacillus* was considered to be a member of the family 'Paenibacillaceae', proposed to group several bacterial taxa previously attributed to the genus *Bacillus* (Garrity & Holt, 2001). At the time of writing, the genus

Paenibacillus comprises more than 80 recognized species, with *Paenibacillus polymyxa* as the type species.

In the present study, the taxonomic position of a Taiwanese soil isolate, strain V10537^T, was studied by using a polyphasic approach, including analysis of 16S rRNA gene sequence similarities, DNA–DNA relatedness values, cell wall peptidoglycan type, fatty acid composition, phenotypic characterization and the generally accepted standards for describing novel species (Kämpfer *et al.*, 2003; Stackebrandt & Goebel, 1994; Vandamme *et al.*, 1996; Wayne *et al.*, 1987).

Strain V10537^T was isolated on trypticase soy broth (Difco) from a sample of soil from Taiwan. *Paenibacillus amylolyticus* BCRC 14684^T, *Paenibacillus barcinonensis* BCRC 17560^T, *Paenibacillus illinoisensis* BCRC 17260^T, *Paenibacillus pabuli* BCRC 15857^T and *Paenibacillus xylanilyticus* BCRC 17561^T were used as reference strains. All strains were reactivated on nutrient agar (NA; Difco) and checked for purity on NA after cultivation at 30 °C for 24–48 h. Strains were preserved at –80 °C in trypticase soy broth or by lyophilization.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain V10537^T is EU179327; accession numbers for *gyrB* gene sequences of the six strains used in this study are EU272024–EU272029.

Maximum-parsimony and maximum-likelihood trees of *Paenibacillus* strains based on 16S rRNA and *gyrB* gene sequences are available as supplementary material with the online version of this paper.

Genomic DNA was extracted and purified using the Qiagen Blood and Cell Culture DNA kit. The 16S rRNA gene was PCR-amplified and sequenced by using the MicroSeq Full Gene 16S rRNA gene Bacterial Identification kit (Applied Biosystems). Sequencing was performed with a 3730 DNA sequencer (Applied Biosystems and Hitachi). Sequence assembly was performed by using the ABI PRISM DNA Sequencing Analysis software (PE Applied Biosystems). Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). The 16S rRNA gene sequence of strain V10537^T was aligned with those of *Paenibacillus* species retrieved from GenBank; the sequence of *Bacillus subtilis* subsp. *subtilis* DSM 10^T was also included in the phylogenetic analysis for comparative purposes. Phylogenetic analysis was performed using the software CLUSTAL_X (Thompson *et al.*, 1997) for alignment and phylogenetic trees were inferred by using the PHYLIP package (Felsenstein, 2007). Phylogenetic distances were calculated according to Kimura's two-parameter model (Kimura, 1980) and trees were constructed on the basis of the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods. Bootstrap analyses were made with 1000 replicates, except in the maximum-likelihood method, where only 100 replicates were generated. Tree figures were drawn by MEGA4 (Tamura *et al.*, 2007). The *gyrB* gene encoding the subunit B protein of DNA gyrase was amplified using the universal primers UP-1 and UP-2r as described previously (Yamamoto & Harayama, 1995). PCR products were purified with the PCR-M clean up system (Viogene) and sequenced using the ABI BigDye Terminator v3.1 cycle sequencing kit. DNA sequencing was determined using *gyrB* degenerate primers for strain V10537^T [PB-1s (5'-GGGGYGYTCCGCAGATATGA-3')

and PB-3sr (5'-CCCHCCTTCGACGATGTACA-3')] and for five type strains of the most closely related *Paenibacillus* species [PB-2s (5'-CCGGATCCGGARATTTTCAC-3') and PB-3sr]. Other sequences obtained from GenBank and the ICB database were also aligned and phylogenetic trees were constructed. The sequence of *B. subtilis* subsp. *subtilis* BCRC 10255^T was included in the phylogenetic analysis for comparative purposes.

DNA G + C content and levels of DNA–DNA relatedness were determined with genomic DNA prepared using a commercial kit (Genomic-tips; Qiagen). The G + C content was determined by reversed-phase HPLC according to Tamaoka & Komagata (1984) with slight modifications. Nucleotides were separated using a Cosmosil 5C₁₈ column (4.6 × 150 mm) (Waters) in a mobile phase composed of 0.2 M NH₄H₂PO₄/acetonitrile (20:1, v/v) at a flow rate of 1 ml min⁻¹ at room temperature. Nucleotides were detected and quantified by absorption at 260 nm. DNA–DNA relatedness values between the novel strain and the type strain of the phylogenetically most closely related species of *Paenibacillus* were determined by using the fluorometric hybridization method in microdilution wells (Ezaki *et al.*, 1989; Tai *et al.*, 2006). The fluorescence intensity of each well was measured with a Fluoroskan II microplate fluorometer (Labsystems) at wavelengths of 360 nm for excitation and 450 nm for emission. Hybridization temperature was 42 °C.

Phylogenetic analysis by the neighbour-joining (Figs 1 and 2), maximum-parsimony and maximum-likelihood methods (see Supplementary Figs S1–S4, available in IJSEM Online) produced similar trees with the exception of minor differences in the tree topology of the basal branches. The phylogenetic tree constructed by using the neighbour-joining method based on 1369 bp of the 16S rRNA gene sequence (positions 71–1446; *Escherichia coli* K-12

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships among *Paenibacillus* species. Database accession numbers for the sequences used in the analysis are given in parentheses. Only bootstrap percentages above 50% are shown (based on 1000 replications). *Bacillus subtilis* subsp. *subtilis* DSM 10^T was used as outgroup to root the tree. Bar, 1% nucleotide substitution rate.

numbering) with gaps showed that strain V10537^T belonged to the genus *Paenibacillus* (Fig. 1). The level of 16S rRNA gene sequence similarity between strain V10537^T and the type strains of *P. pabuli*, *P. xylanilyticus*, *P. amylolyticus*, *P. barcinonensis* and *P. illinoisensis*, the phylogenetically most closely related species, were 99.5, 98.8, 98.3, 98.2 and 98.1 %, respectively. The values do not seem to exclude a possible relationship at the species level with any one of these taxa (Rivas *et al.*, 2005; Sánchez *et al.*, 2005). Nevertheless, strain V10537^T shared 76.9–85.0 % *gyrB* gene sequence similarity with other *Paenibacillus* species. A good correlation between *gyrB* sequence dendrograms and DNA–DNA association data has been reported for the *B. subtilis* group and the genera *Acinetobacter* and *Pseudomonas* (Wang *et al.*, 2007; Yamamoto *et al.*, 1999; Cámara *et al.*, 2007).

The tree based on 770 bp *gyrB* gene sequences clearly placed strain V10537^T on a separate branch with 100 % bootstrap support (Fig. 2). Strain V10537^T shared 76.9–85.0 % *gyrB* gene sequence similarity with the phylogenetically most closely related species. The correlation between *gyrB* gene sequence similarity and levels of DNA–DNA relatedness in species of the *B. subtilis* group has been reported previously (Wang *et al.*, 2007); a linear correlation was observed between them. Strains with 95 % or higher *gyrB* gene sequence similarity in the cluster exhibited DNA–DNA relatedness of >70 %, an acceptable value for proposal of a single species.

In the present study, DNA–DNA relatedness values between strain V10537^T and the type strains of *P. pabuli*, *P. illinoisensis*, *P. barcinonensis*, *P. xylanilyticus* and *P. amylolyticus* were 45.6, 28.7, 25.0, 22.7 and 8.5 %, respectively. Among the species compared, none gave a value for DNA–DNA relatedness higher than the 70 % guideline (Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002). Strain V10537^T showed low *gyrB* gene sequence similarities (76.9–85.0 %) and low DNA–DNA relatedness of 8.5–45.6 % with the strains compared. The results demonstrated that the

resolution of *gyrB* gene sequences also enabled strain V10537^T to be differentiated from the above-mentioned species. Therefore, the isolate is considered to represent a novel species of the genus *Paenibacillus*.

Phenotypic data are included in Table 1 and the species description. All phenotypic tests were performed on bacteria grown on NA at 30 °C unless otherwise specified. General cell morphology was studied by using phase-contrast microscopy (Eclipse E600; Nikon). The Gram reaction was performed with a four-step Gram stain kit (Becton Dickinson) according to the manufacturer's instructions. Catalase activity, oxidase activity and oxidation–fermentation reactions were determined by using standard methods (Barrow & Feltham, 1993). Additional physiological and biochemical tests were performed with the API 50CHB, API ZYM and API 20E systems (bioMérieux) and Biolog GP (Biolog) according to the manufacturers' instructions.

The fatty acid composition was determined using the Sherlock Microbial Identification system (MIDI) as described by Chern *et al.* (2004). Extracts of methylated fatty acids were prepared according to the protocol provided by the manufacturer and analysed with an Agilent 6890N GC equipped with a flame-ionization detector and 7683 Automatic Liquid Sampler; results obtained were compared with data in the built-in TSBA 50 database (MIDI).

Analysis of whole-cell diaminopimelic acid isomers and sugars was done using the method of Hasegawa *et al.* (1983). Respiratory quinones of the novel bacterium were identified according to Collins & Jones (1981) and Komagata & Suzuki (1987). The TLC-purified quinones were analysed with a Nova-Pak C₁₈ (3.9 × 150 mm) column (Waters). Peaks were detected at 270 nm after elution with methanol/2-propanol (2:1) at flow rate of 1 ml min⁻¹. The fatty acid profiles of strain V10537^T and related taxa are presented in Table 2. Only minor differences were noted between strain V10537^T and other *Paenibacillus* species. The dominant fatty acids for all strains investigated (mean level above 10 %) were anteiso-C_{15:0} and C_{16:0} (53.6 and 19.0 % of the total fatty acids, respectively, in strain V10537^T) (Table 2). The cell-wall peptidoglycan contained *meso*-diaminopimelic acid. Strain V10537^T contained menaquinone MK-7 as the predominant isoprenoid quinone (98.3 %) and MK-6 (1.7 %) as a minor component; this is in accordance with all other members of the genus *Paenibacillus* (Shida *et al.*, 1997a; Takeda *et al.*, 2005).

On the basis of the phylogenetic, phenotypic, genotypic and chemotaxonomic results, it is proposed that strain V10537^T represents a novel species of the genus *Paenibacillus*, *Paenibacillus taichungensis* sp. nov. The phenotypic features that enable differentiation of this novel species from closely related *Paenibacillus* species are shown in Table 1.

Fig. 2. Neighbour-joining tree based on *gyrB* gene sequences of *Paenibacillus* strains. Database accession numbers for the sequences used in the analysis are given in parentheses. Only bootstrap percentages above 50 % are shown. *Bacillus subtilis* subsp. *subtilis* BCRC 10255^T was used as an outgroup. Bar, 10 % nucleotide substitution rate.

Table 1. Differential phenotypic characteristics of strain V10537^T and type strains of phylogenetically related *Paenibacillus* species

Strains: 1, strain V10537^T; 2, *P. pabuli* BCRC 15857^T; 3, *P. amylolyticus* BCRC 14684^T; 4, *P. barcinonensis* BCRC 17560^T; 5, *P. illinoisensis* BCRC 17260^T; 6, *P. xylanilyticus* BCRC 17561^T. Data were obtained in this study. +, Positive; w, weak; –, negative.

Characteristic	1	2	3	4	5	6
Growth at:						
5 °C	+	+	+	+	–	+
45 °C	–	–	–	–	+	+
2 % NaCl	+	+	+	+	–	+
7 % NaCl	+	+	–	–	+	+
pH 4	–	–	w	–	+	+
pH 10	+	+	–	+	+	+
pH 11	+	+	–	+	–	+
pH 12	–	–	–	–	–	+
Hydrolysis of:						
Casein	–	–	+	–	–	–
Starch	+	+	+	–	+	+
Acid production from (API 50CHB):						
Glycerol	+	+	–	+	+	+
D-Arabinose	–	–	–	+	–	–
Inulin	+	+	–	–	+	+
Melezitose	+	+	–	+	–	–
Starch	+	+	+	–	+	+
Glycogen	+	+	+	–	+	+
Gluconate	–	–	–	+	+	+
Enzyme activity (API 20E)						
β-Galactosidase	+	+	+	–	+	+
Urease	–	–	+	–	–	–
Gelatinase	–	–	–	+	–	–
Rhamnose	–	w	w	+	w	w
Nitrate reduction	–	–	+	–	+	–
Enzyme activity (API ZYM)						
Trypsin	–	–	–	+	–	–
α-Chymotrypsin	+	+	–	+	+	+
Acid phosphatase	+	–	+	+	+	+
N-Acetyl-β-glucosaminidase	+	+	+	+	+	–
α-Fucosidase	–	–	+	–	–	–
Utilization of (Biolog GP2):						
L-Fucose	+	–	–	–	–	–
D-Galacturonic acid	+	–	–	w	–	–
myo-Inositol	+	–	–	–	–	–
D-Lactose	–	+	+	+	+	+
Melezitose	+	w	–	+	w	–
Methyl α-D-glucoside	–	+	–	+	+	+
Methyl α-D-mannoside	+	–	–	–	–	–
D-Sorbitol	+	+	–	+	+	w

Description of *Paenibacillus taichungensis* sp. nov.

Paenibacillus taichungensis (tai.chung.en'sis. N.L. masc. adj. *taichungensis* of Taichung, a city in Taiwan, where the type strain was isolated).

Table 2. Cellular fatty acid compositions of strain V10537^T and type strains of selected *Paenibacillus* species

Strains: 1, strain V10537^T; 2, *P. pabuli* BCRC 15857^T; 3, *P. amylolyticus* BCRC 14684^T; 4, *P. barcinonensis* BCRC 17560^T; 5, *P. illinoisensis* BCRC 17260^T; 6, *P. xylanilyticus* BCRC 17561^T. Data were obtained in this study. All strains were grown in TSA medium (BBL) at 28 °C for 24 h. Values are percentages of total fatty acids. ND, Not detected.

Fatty acid	1	2	3	4	5	6
iso-C _{14:0}	2.8	1.6	1.0	3.9	1.7	1.8
C _{14:0}	4.2	2.6	1.4	8.1	2.9	2.4
iso-C _{15:0}	5.8	6.7	10.2	7.6	4.8	5.2
anteiso-C _{15:0}	53.6	57.1	45.6	34.8	50.2	49.2
iso-C _{16:0}	5.5	4.4	9.7	3.7	4.5	5.1
C _{16:1ω11c}	1.6	3.3	ND	5.6	2.3	1.6
C _{16:0}	19.0	13.3	11.1	31.8	21.1	19.2
iso-C _{17:0}	3.1	4.7	8.0	2.3	4.7	6.3
anteiso-C _{17:0}	4.4	6.4	12.9	1.6	7.7	9.2

Cells are rods (1.8–6.8 × 0.9–1.4 μm), Gram-variable, facultatively anaerobic, mesophilic, capable of forming ellipsoidal endospores and motile. Colonies are flat with undulate margins and greyish-white on NA. Grows at 5–40 °C (optimum 30 °C) on NA. Unable to grow above 40 °C. The pH range for growth is 5–11 (optimum pH 6–8). Grows on NA containing 7 % NaCl (w/v). Positive for catalase, oxidase and β-galactosidase, but negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, tryptophan deaminase and gelatinase. Nitrate is not reduced to nitrite or nitrogen. Acetoin is produced, but indole and H₂S are not. Acid is produced from sucrose, melibiose and amygdalin (API 20E). Hydrolyses starch and casein. Positive for alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase and N-acetyl-β-glucosaminidase, but negative for lipase, cystine arylamidase, trypsin, β-glucuronidase, α-mannosidase and α-fucosidase (API ZYM). Acid is produced from glycerol, L-arabinose, ribose, D-xylose, methyl β-D-xyloside, galactose, glucose, fructose, mannose, mannitol, methyl α-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, gentiobiose and turanose; no acid is produced from any other substrate in the API 50CHB system. Positive for α-cyclodextrin, β-cyclodextrin, dextrin, glycogen, N-acetyl-D-glucosamine, amygdalin, L-arabinose, arbutin, cellobiose, D-fructose, L-fucose, D-galacturonic acid, D-gluconic acid, myo-inositol, lactulose, maltose, maltotriose, D-mannose, melezitose, methyl α-D-galactoside, methyl β-D-galactoside, 3-methyl glucose, methyl α-D-glucoside, methyl α-D-mannoside, palatinose, D-psicose, raffinose, D-ribose, salicin, D-sorbitol, stachyose, sucrose, trehalose, D-xylose, acetic acid, pyruvic

acid methyl ester, succinic acid monomethyl ester, pyruvic acid, 2,3-butanediol, glycerol, adenosine, 2'-deoxyadenosine, inosine, thymidine and uridine, but negative or borderline for other substrates in the Biolog GP2 MicroPlate system. Cell wall peptidoglycan contains meso-diaminopimelic acid. The major fatty acids are anteiso-C_{15:0} and C_{16:0}. The predominant menaquinone is MK-7; MK-6 is a minor component.

The type strain is V10537^T (=BCRC 17757^T =DSM 19942^T), isolated in 2000 from soil in Taichung City, Taiwan. The DNA G+C content of the type strain is 46.7 mol%.

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