Paenibacillus silvae sp. nov., isolated from a tropical rainforest soil

Huiqin Huang,*† Fute Zhang,† Min Liu, Ying Cui, Qianguang Sun, Jun Zhu, Xiaoxiao Zou and Shixiang Bao*

Abstract

Two Gram-stain-positive, facultatively aerobic, endospore-forming and rod-shaped bacteria, designated DB13031T and DB13311, were isolated from the soil of the Jiaxi Nature Reserve in Hainan, PR China. 16S rRNA gene analysis of strains DB13031T and DB13311 showed that they fell within the Paenibacillus cluster, with highest similarities to Paenibacillus cucumis AP-115T (98.4 and 98.3 %, respectively), Paenibacillus barcinonensis BP-23T (98.3 and 98.2 %, respectively) and Paenibacillus oceanisediminis L10T (97.7 and 97.7 %, respectively). The DNA–DNA hybridization values between strain DB13031T and the type strains of its closest related species were 48.2, 38.1 and 43.5 %. Strain DB13031T contained menaquinone-7 (MK-7) as the predominant isoprenoid quinone and anteiso-C15:0, iso-C16:0 and C16:0 as the major cellular fatty acids. The cell-wall peptidoglycan was of the A1γ type and the major polar lipid profiles were diphasatidylglycerol, phosphatidylethanolamine, four unknown aminophospholipids and four unknown phospholipids. Based on the phenotypic and genotypic data, it is proposed that the two isolates represent a novel species of the genus Paenibacillus, for which the name Paenibacillus silvae sp. nov. is proposed. The type strain is DB13031T (=CGMCC 1.12770T=DSM 28013T).

The genus Paenibacillus was originally proposed and identified as group three bacilli on the basis of 16S rRNA gene sequence analysis by Ash et al. [1]. At the time of writing, the genus Paenibacillus comprises 182 species and four subspecies with widely published names (www.bacterio.net/ paenibacillus.html). The genus Paenibacillus includes rod-shaped and endospore-forming bacteria, with anteiso-C15:0 as the major cellular fatty acid [2–4] and the G+C content of the genomic DNA ranging from 38–59 mol% [5, 6]. Phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and diphasatidylglycerol (DPG) are the major polar lipids of the type species of the genus Paenibacillus [7]. Although many cells of the genus are Gram-stain-positive rods, some Gram-stain reactions have been found to be negative or variable [1, 5]. In this study, the taxonomic status of strains DB13031T and DB13311 were analyzed according to the minimal standards for the description of aerobic, endospore-forming bacteria [8] and we propose the description of a novel species, Paenibacillus silvae sp. nov.

Strains DB13031T and DB13311 were isolated from soil collected from Jiaxi Nature Reserve in Hainan, PR China (109°10′–109°12′ E, 18°52′–18°53′ N) in July, 2012. For isolation, 10 g soil samples were suspended in 100 ml sterile distilled water and then stirred for 30 min. The liquid was incubated at 80 °C for 15 min, then spread onto trypticase soy agar (TSA, Difco) using the tenfold-dilution series method. Plates were incubated at 28 °C. After 3 days, beige colonies, designated DB13031T and DB13311, were picked out. The purified cultures were maintained on TSA slants at 4 °C for short-term storage and in a glycerol suspensions (20 %, v/v) at –70 °C for long-term preservation.

Amplification and sequencing of the almost complete 16S rRNA gene of strains DB13031T (1421 bp) and DB13311 (1407 bp) were carried out with the universal primers 27 F/C0 and 539R/C14 for 15 min, then spread onto trypticase soy agar (TSA, Difco) using the tenfold-dilution series method. Plates were incubated at 28 °C. After 3 days, beige colonies, designated DB13031T and DB13311, were picked out. The purified cultures were maintained on TSA slants at 4 °C for short-term storage and in a glycerol suspensions (20 %, v/v) at –70 °C for long-term preservation.

Phylogenetic trees were reconstructed with the MEGA 5 program [12], using three tree-making algorithms: the neighbour-joining [13], maximum-likelihood [14] and maximum-
parsimony [15] methods. The confidence values of branches of the trees were determined using bootstrap analysis based on 1000 resamplings [16]. *Bacillus subtilis* DSM106 was used as the outgroup in the phylogenetic trees. Strains DB13031T and DB13311 shared 99.9% 16S rRNA gene sequence similarities, and were clustered into the same phylogenetic branch. The closest relatives were *Paenibacillus cucumis* AP-115T (98.4 and 98.3%, respectively) [17], *Paenibacillus barcinonensis* BP-23T (98.3 and 98.2%, respectively) [18] and *Paenibacillus oceaniseminis* L10T (97.7 and 97.7%, respectively) [19]. Other 16S rRNA gene sequence similarities were all below 97.5%. The neighbour-joining tree indicated that the two strains were most closely related phylogenetically to *P. cucumis* AP-115T, followed by *P. barcinonensis* BP-23T and *P. oceaniseminis* (Fig. 1). The topologies of the phylogenetic trees reconstructed using the maximum likelihood method (Fig. S1, available in the online Supplementary Material), the maximum parsimony method (Fig. S2) and the tree including all species of the genus *Paenibacillus* (Fig. S3) supported this result and confirmed that the isolates always grouped with *P. cucumis* AP-115T. Hence, type strains in the present study of the three closest related species (*P. cucumis* AP-115T, *P. barcinonensis* BP-23T and *P. oceaniseminis* L10T) were analysed together as reference strains.

Morphological, physiological and biochemical analyses were performed with strains cultivated for 2 days at 28 °C in TSA medium, unless mentioned otherwise. Gram-staining was performed by using a Gram stain kit (Beijing Land Bridge). Endospore formation was examined using a spore-staining kit (Guangzhou Huankai) using cells cultivated on endospore-forming agar for 3 days. The endospore-forming agar contained: 0.7 g yeast extract, 1 g peptone, 1 g glucose, 0.2 g (NH₄)₂SO₄, 0.2 g MgSO₄·7H₂O, 1 g K₂HPO₄, 20 g agar, 1000 ml distilled water and was adjusted to pH 7.2. The morphological characteristics of cells were checked by light and scanning electron microscopy. Motility was tested in TSB (tryptic soy broth) containing 0.3% (w/v) agar [20]. Anaerobic growth was tested as described by Valverde et al. [21]. Growth at different temperatures (4, 10, 16, 20, 28, 30, 32, 37, 42, 50, 53 and 55 °C) and different NaCl concentrations [0–5% (w/v) with 1.0% increments] was tested. The pH range for growth was adjusted to various pH values (4.0–12.0, with increments of 1.0) by the addition of 1 M HCl or NaOH. Antibiotic sensitivity was tested on TSA medium by Sensi-Discs (6 mm; BBL) with the following antibiotics (µg per disc): achoemycin (30), ampicillin (10), chloromycetin (30), erythromycin (15), kanamycin (30), gentamicin (10), nalidixic acid (30), neomycin (30), novobiocin (30), and rifampicin (5). Other physiological and biochemical properties were tested as described previously by Logan and De Vos [22].

Cell biomass of the novel strains was collected for chemotaxonomic and molecular systematic studies and was obtained by cultivation using TSB medium at 28 °C for 2 days in a rotary shaker at 150 r.p.m., harvested by centrifugation and washed twice with sterile distilled water. Fatty acid methyl esters were prepared and determined by using gas chromatography/mass spectroscopy (model GC-2010; Shimadzu) [23]. Polar lipids were extracted and examined using 10×10 cm TLC Silica gel 60 F₂₅₄ plates (Merck) [24, 25]. Isoprenoid quinones were purified [26] by TLC and analyzed by HPLC [27]. The cell-wall peptidoglycan was analyzed using the method described by Schumann [28]. Genomic DNA was extracted with a Bacterial Genomic DNA Isolation Kit (Foregene Biosciences), according to the manufacturer’s protocol. DNA–DNA hybridization was carried out using the method of Ezaki et al. [29]. Homology values were calculated according to Christensen et al. [30]. The G+C content was analyzed by the hydrolysis of DNA to nucleosides and quantified by HPLC using the method of Mesbah et al. [31].

Strains DB13031T and DB13311 were Gram-stain-positive, facultatively anaerobic, rod-shaped and non-motile. Cells were 0.4–1.0 µm wide and 1.3–3.5 µm long (Fig. S4). Colones were slightly irregular, smooth, beige and 0.4–0.8 mm

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**Fig. 1.** Neighbour-joining tree, based on the 16S rRNA gene sequences of strains DB13031T and DB13311 and related type strains of species of the genus *Paenibacillus*. *Bacillus subtilis* DSM106 was used as the outgroup. Values indicate the percentage occurrence in 1000 bootstrapped trees with values >50% shown. Bar, 0.01 substitutions per nucleotide position.
in diameter after growth for 48 h at 28 °C on TSA medium. Ellipsoidal endospores were formed in a terminal position in swollen sporangia (Fig. S5). Other physiological properties are given in Table 1 and in the species description.

The major polar lipids of DB13031T were found to be DPG, PE, four unknown aminophospholipids (AL1–AL4) and four unknown phospholipids (PL1–PL4) (Fig. S6). The diagnostic diamino acid was meso-DAP and the cell wall type was A1γ. The predominant isoprenoid quinone was identified as MK-7. The major fatty acids (>5 %) were anteiso-C15:0 (54.9 %), C16:0 (13.7 %) and iso-C16:0 (12.1 %). The complete fatty acid compositions of strains DB13031T, DB13311 and the reference species are shown in Table 2.

The DNA G+C contents of strains DB13031T and DB13311 were 49.7 and 49.6 mol%, respectively. The DNA hybridization values between strain DB13031T, which was selected as the representative strain, with DB13311, P. cucumis AP-115T, P. barcinonensis BP-23T and P. oceanisediminis L10T were 81.9, 48.2, 38.1 and 43.5 %, respectively. These data clearly support the conclusion that strains DB13031T and DB13311 are members of a single species [32].

Table 1. Phenotypic properties distinguishing strains DB13031T and DB13311 from the closest phylogenetic neighbours

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<td>49.6</td>
<td>51.4*</td>
<td>45.0†</td>
<td>44.0‡</td>
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</table>

*Data from: Kämpfer et al. [17].
†Data from: Sánchez et al. [18].
‡Data from: Lee et al. [19].

The DNA–DNA hybridization values between strain DB13031T and the type strains of P. cucumis AP-115T, P. barcinonensis BP-23T and P. oceanisediminis L10T were significantly lower than the threshold value (70 %) recommended for species delineation [33]. Several physiological features (NaCl concentration for growth, temperature and pH ranges for growth; hydrolysis of starch, D-mannose, inositol and Tween 80; nitrate reduction; oxidase activity and acid production among others) and fatty acid profiles also clearly differentiate the isolates from their closest phylogenetic neighbours. In conclusion, the results of this polyphasic study suggest that strains DB13031T and DB13311 represent a novel species of the genus Paenibacillus, and the name Paenibacillus silvae sp. nov. is proposed.

**DESCRIPTION OF PAENIBACILLUS SILVAE SP. NOV.**

*Penibacillus silvae* (sil’vae. L. gen. n. silvae of a forest). The organism was isolated from the soil of an original forest in Jiaxi Nature Reserve in Hainan, PR China).

Cells are Gram-stain-positive, facultatively anaerobic, rod-shaped, and 0.4–1.0 µm wide and 1.3–3.5 µm long. Ellipsoidal endospores are formed in a terminal position and cells are motile. After 48 h growth on TSA medium, colonies are round, raised, beige and 1–4 mm in diameter. Growth occurs between pH 5.0 and 11.0 (optimum pH 7.0–9.0), between 10 and 53 °C (optimum 30–37 °C) and in 0–4 % (w/v) NaCl (optimum 0–2 %). Catalase-positive and oxidase-negative. Tweens 20, 40, 60 and 80 are hydrolysed, but starch, carabamide, gelatin and cellulose not. Nitrate reduction and the methyl red test are positive. The Voges-Proskauer reaction is negative and the pH in Voges-Proskauer broth is 3.93. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are produced, but not H₂S, indole, L-tyrosine and phenylalanine.
deaminase. Among the carbon sources, cellobiose, D-trehalose, D-mannose, D-raffinose, D-xylose, glucose, L-arabinose, maltose and starch are utilized, but citrate, D-galactose, D-fructose, D-ribose, D-sorbitol, glycine, inositol, L-arginine, L-ornithine, propionate, sorbic acid and α-lactate are not.

Acid is produced from cellobiose, D-raffinose, D-fructose, D-ribose, D-trehalose, glucose, inositol, maltose, mannotol, sorbitol, starch, sucrose, xylose, α-arabinose, α-galactose and α-lactate, but not from D-mannose. Susceptible to achoemycin, ampicillin, chloromycetin, erythromycin, kanamycin, gentamicin, nalidixic acid, neomycin, novobiocin and rifampicin. The major polar lipids present are diphosphatidylglycerol, phosphatidylethanolamine, four unknown amiphibolipids and four unknown phospholipids. The diagnostic diamino acid is meso-DAP, the cell wall type was A1γ and the predominant isoprenoid quinone is MK-7. The major cellular fatty acids are anteiso-C15:0, C16:0 and iso-C16:0.

The type strain, DB13031^T (=CGMCC 1.12770^T=DSM 28013^T), was isolated from a soil sample collected from the Jiaxi Nature Reserve in Hainan, PR China. The DNA G+C content of the type strain is 49.7 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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