**Paenibacillus panaciterrae** sp. nov., isolated from ginseng-cultivated soil

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A novel bacterium, designated DCY95<sup>T</sup>, was isolated from ginseng-cultivated soil in Quang Nam province, Vietnam. On the basis of 16S rRNA and gyrB gene sequence analysis, this isolate was assigned to the genus *Paenibacillus* and found to be closely related to *Paenibacillus sacheonensis* SY01<sup>T</sup> (97.1 % 16S rRNA gene sequence similarity) and *Paenibacillus taihuensis* THMBG22<sup>T</sup> (96.4 %). The partial gyrB gene of DCY95<sup>T</sup> possessed 69.6–83.9 % sequence identity to those of other members of the genus *Paenibacillus*. Strain DCY95<sup>T</sup> was Gram-reaction-negative, catalase-negative, oxidase-positive, strictly aerobic, rod-shaped and motile by means of peritrichous flagella. Ellipsoidal free spores or subterminal endospores were produced in sporangia. MK-7 was the diagnostic menaquinone. The cell-wall peptidoglycan contained meso-diaminopimelic acid as the diamino acid. Whole-cell sugars comprised ribose, mannose and glucose. The major cellular fatty acids were anteiso-C<sub>15</sub>:0, iso-C<sub>16</sub>:0 and C<sub>16</sub>:0. The major polar lipids were phosphatidylethanolamine, phosphatidyglycerol, three unidentified aminophospholipids, and two unidentified phospholipids. The genomic DNA G+C content was 60.7 ± 0.9 mol%. Phenotypic and chemotaxonomic results placed strain DCY95<sup>T</sup> within the genus *Paenibacillus*. However, DNA–DNA relatedness values between strain DCY95<sup>T</sup> and *P. sacheonensis* KACC 14895<sup>T</sup> or *P. taihuensis* NBRC 108766<sup>T</sup> were lower than 36 %. The low DNA relatedness data in combination with phylogenetic and (GTG)<sub>9</sub>–PCR analyses, as well as biochemical tests, indicated that strain DCY95<sup>T</sup> could not be assigned to any recognized species. In conclusion, the results in this study support the classification of strain DCY95<sup>T</sup> as a representative of a novel species within the genus *Paenibacillus*, for which the name *Paenibacillus panaciterrae* is proposed. The type strain is DCY95<sup>T</sup> (=KCTC 33581<sup>T</sup>=DSM 29477<sup>T</sup>).

The genus *Paenibacillus* was proposed by Ash *et al.* (1993, 1994), and then emended by Shida *et al.* (1997) and Behrendt *et al.* (2010). At the time of writing, 166 species and subspecies with validly published names exist (http://www.bacterio.net/paenibacillus.html). The genus *Paenibacillus* consists a large group of versatile species isolated from a variety of sources. Many species of the genus *Paenibacillus* have been isolated from soil material, for instance *Paenibacillus graminis* (Berge *et al.*, 2002), *P. cineris* (Logan *et al.*, 2004), *P. gansuensis* (Lim *et al.*, 2006), *P. sabinae* (Ma *et al.*, 2007), *P. forsythiae* (Ma & Chen, 2008), *P. souci* (Hong *et al.*, 2009), *P. pinihumi* (Kim *et al.*, 2009), *P. sophorae* (Jin *et al.*, 2011), *P. telluris* (Lee *et al.*, 2011), *P. catalpae* (Zhang *et al.*, 2013), *P. darwinianus* (Dsouza *et al.*, 2014) and *P. doosanensis* (Kim *et al.*, 2014). In particular, several species of the genus *Paenibacillus* have been isolated from soil used for ginseng cultivation in Korea, including *Paenibacillus panacisoli* (Ten *et al.*, 2006), *P. ginsengarvi* (Yoon *et al.*, 2007), *P. ginsengihumi* (Kim *et al.*, 2008) and *P. pocheonensis* (Baek *et al.*, 2010). To the best of our knowledge, no detection of novel species of the genus *Paenibacillus* in Vietnamese ginseng-cultivated soil has been described, even though many studies have focused on extracting compounds from Vietnamese ginseng and their applications (Tran *et al.*, 2001; Le *et al.*, 2014). During investigation of the bacterial population in Vietnamese ginseng-cultivated soil, one supplementary table and five supplementary figures are available with the online Supplementary Material.
ginseng-cultivated soils, we isolated strain DCY94T and identified it as the type strain of a novel species in the genus Paenibacillus.

A soil sample was collected from a ginseng-cultivated area in Nam Tra My district, Quang Nam province, Vietnam (15° 01’ 54” N 107° 58’ 45” E). Sterilized saline (0.85 %, w/v, NaCl) was used for suspending the soil sample and for serial dilutions. Subsequently, 100 µl of soil suspension was spread onto a modified Reasoner’s 2A (R2A; MB cell) agar medium (1/5-strength R2A). After 3 days of culturing at 30 °C, colonies with different morphologies were picked up and purified. Among the bacteria, strain DCY95T was isolated and identified. For long-term storage, cells were mixed with R2A broth containing 30 % (v/v) glycerol and maintained at −70 °C. Three-time-R2A broth (3-R2A) and five-time R2A (5-R2A) agar were used to maintain strain DCY95T.

After isolation and purification, we started identification by sequencing the 16S rRNA gene of strain DCY95T. The 16S rRNA gene was amplified from the chromosomal DNA of strain DCY95T using the universal bacterial primers 27F, 518F, 800R and 1492R, and the purified PCR products were sequenced by Genotech, Daejeon, Korea (Weisburg et al., 1991; Kim et al., 2005). The gyrB gene, encoding the B subunit of the DNA gyrase, was amplified using primers UP-1 and UP-2r (Yamamoto & Harayama, 1995). The partial sequences of the 16S rRNA gene and gyrB gene were compiled with SeqMan software. The resulting sequences were aligned with those of the representative species of the genus Paenibacillus using the software CLUSTAL X 2.0.10 (Larkin et al., 2007). Alignment gaps were trimmed manually using the BioEdit program (Hall, 1999). The phylogenetic affiliation of the sequences retrieved was generated by using the MEGA 6.0 program (Tamura et al., 2013). The Tamura–Nei model (Tamura & Nei, 1993) was used to model nucleotide substitution in maximum-likelihood (Felsenstein, 1981), neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) analyses. Bootstrap analysis with 1000 replicates was also conducted in order to estimate the confidence levels of the tree topologies (Felsenstein, 1985). The nearly full-length sequence of the 16S rRNA gene (1465 bp) of strain DCY95T was obtained and uploaded to the EzTaxon-e server (Kim et al., 2012) to determine the pairwise similarity with other sequences. Pairwise alignment of the gyrB gene sequences was computed by using the BioEdit program (Hall, 1999) with the optimal global alignment method. Sequence similarity values were then calculated after trimming gaps.

According to an EzTaxon-e search, strain DCY95T represents a member of the genus Paenibacillus. The closest relatives were Paenibacillus sacheonensis SY01T (97.1 % 16S rRNA gene sequence similarity; Moon et al., 2011) and Paenibacillus taihuensis THMBG22T (96.4 %; Wu et al., 2013). Phylogenetic analyses showed that strain DCY95T formed a discrete cluster with P. sacheonensis SY01T and P. taihuensis THMBG22T (Fig. 1) with high bootstrap support (99 %). This topology was conserved in all trees reconstructed by maximum-likelihood, neighbour joining and maximum-parsimony methods. However, strain DCY95T also formed a separated branch within this group. The phylogenetic trees of gyrB gene sequences (see Fig. S1 available in the online Supplementary Material) also showed the segregation of the cluster containing strain DCY95T with P. sacheonensis KACC 14895T and P. taihuensis NBRC 108766T. Pairwise alignment showed that partial gyrB gene of DCY95T possessed 83.9 % sequence identity to that of P. sacheonensis KACC 14895T, 82.4 % sequence identity to that of P. taihuensis NBRC 108766T, and 69.6–77.4 % sequence identities to those of other members of the genus Paenibacillus. These interspecies gyrB gene sequence similarity values were low enough to distinguish strain DCY95T from other species of the genus Paenibacillus as Hu et al. (2010) suggested a threshold value of 98 % gyrB gene sequence similarity for species delineation.

To explore similar as well as different characteristics of strain DCY95T and related type strains, phenotypic assays were carried out. Colony morphology was observed after three days of incubation in 5-R2A agar at 30 °C. Cell shape and size, and flagella were checked by transmission electron microscopy. Young cells (24 h old) were negatively stained with 2 % (w/v) phosphotungstic acid (PTA; pH 7.2) on Formvar-carbon-coated nickel grids, and examined with transmission electron microscopy (LEO912AB, Carl Zeiss) at 80 kV under standard operating conditions. The occurrence of spores was checked using cells cultured on YEP medium supplemented 5 mg MnCl2 l−1 for 4 days. Spores were stained with malachite green (Prescott & Harley, 2001) and observed with an Imager. Z1 fluorescence microscope (Carl Zeiss) equipped with an MRC5 digital CCD camera (AxioCam). Gram staining was tested using a Gram staining kit (Sigma-Aldrich). Oxidase activity was determined by adding oxidase reagent (bioMérieux) to young cells in 5-R2A agar; appearance of a blue–purple colour within 15–30 s indicates a positive test. Catalase activity was determined by placing cells onto a glass slide and adding 3 % (v/v) H2O2 solution; bubbles observed within 10 s indicates a positive reaction. Ability to grow under anaerobic conditions was determined by incubating bacteria on R2A and 5-R2A plates in a BD GasPak EZ anaerobe pouch system with indicator (Becton Dickinson) and AnaeroPack Rectangular Jar (Mitsubishi Gas Chemical) that contained an AnaeroPack-anaero (Mitsubishi gas chemical) for 14 days at 30 °C. The temperature for growth was tested at 4, 10, 15, 20, 25, 28, 30, 37 and 40 °C in 3-R2A broth over 5 days. The pH range for growth was examined between pH 4 and 10 at intervals of 0.5 using pH-adjusted R2A broth and incubation for 5 days at 30 °C; pH values were adjusted by using the following buffers: citric acid/sodium citrate (pH 4.0–6.0), Na2HPO4/NaH2PO4 (pH 6.0–8.0), Tris/HCl (pH 8.5–9.0) and glycine/NaOH (pH 9.5–10.0) (Gomori, 1955). A saline tolerance test was performed at concentrations of 0–3 % (w/v) NaCl in increments of 0.2 % in R2A broth at 30 °C for 7 days. Hydrolysis of casein,
DNA, 1-tyrosine, starch, Tween 20 and Tween 80 was evaluated as described by Barrow & Feltham (1993). Phenylalanine deaminase activity, methyl red test, nitrate reduction and nitrite reduction were assessed according to the method of Lányi (1987). Production of H2S using thiosulfate iron was also checked (Levine et al., 1934). Biochemical characteristics were further determined with API ZYM, API 50CH and API 20E kits; API tests were performed according to the instructions of the manufacturer (bioMérieux). Antibiotic susceptibility was assayed by the disc diffusion method according to Nokhal & Schlegel (1983). Inocula were prepared using culture in 3-R2A broth at an optical density adjusted 0.2 at 600 nm. The bacterial inocula were then spread on Mueller–Hinton agar (Difco) plates. The following antibiotics (μg per disc, except penicillin G; Oxoid) were tested: carbenicillin (100), cefazolin (30), ceftazidime (30), erythromycin (15), lincomycin (15), neomycin (30), novobiocin (30), oleandomycin (15), penicillin G (10 units), rifampicin (5), tetracycline (30) and vancomycin (30).

Physiological and biochemical characteristics of strain DCY95T are summarized in the species description and the comparison of selective characteristics with those of related type strains is shown in Table 1. Cells were rod-shaped, 0.6–0.8 μm in width, 2.1–2.5 μm in length, and motile by means of peritrichous flagella (see Fig. S2). Ellipsoidal free spores or subterminal endospores were formed in sporangia (see Fig. S3). Cells could not grow under anaerobic conditions. Gram reaction and catalase activity were negative, but oxidase activity was positive. These results obtained satisfy the physiological characteristics of members of the genus Paenibacillus (Shida et al., 1997). The negative result in the catalase reaction of strain DCY95T is similar to that seen in P. sacheonensis KACC 14895T, but opposite of those found in other species of the genus Paenibacillus in this study. Strain DCY95T could be distinguished from its closest phylogenetic neighbours by its ability to produce acid from erythritol, D-adenitol, D-sorbitol and D-xylitol, but not from N-acetylglucosamine. Strain DCY95T, P. sacheonensis KACC 14895T and P. taihuensis NBRC 108766T were sensitive to carbenicillin,
cefazolin, ceftazidime, erythromycin, lincomycin, neomycin, novobiocin, oleandomycin, penicillin G, rifampicin, tetracycline and vancomycin, which is a common characteristic of members of the genus *Paenibacillus*.

In order to determine whether strain DCY95\(^T\) represented a novel species, DNA–DNA relatedness examination was performed between strain DCY95\(^T\) and its closest relatives, as per the method of Ezaki *et al.* (1989). The genomic DNA of strains for molecular analysis was extracted and purified with an Exgene Cell SV mini-kit (Gene All Biotechnology). The hybridization temperature was 49 °C. The experiment was carried out with five replications for each sample. The highest and lowest values obtained from each sample were excluded, and the mean of the remaining three values was calculated for DNA–DNA relatedness. Mean (±SEM) levels of DNA–DNA relatedness of strain DCY95\(^T\) and *P. sacheonensis* KACC 14895\(^T\) and *P. taihuensis* NBRC 108766\(^T\) were 27% (±9%) and 18% (±6%), respectively. Based on the criterion of Wayne *et al.* (1987), strain DCY95\(^T\) represents a member of a single novel species of the genus *Paenibacillus*.

Five rep-PCR (repetitive extragenic palindromic-PCR) (GTG)\(_5\)-PCR genomic fingerprinting method was performed as described by Versalovic *et al.* (1994). The fingerprint of strain DCY95\(^T\) was visually different from those of *P. sacheonensis* KACC 14895\(^T\) and *P. taihuensis* NBRC 108766\(^T\) (see Fig. S4). This result confirmed that strain DCY95\(^T\) could be discriminated from members of related species.

### Table 1. Different characteristics of strain DCY95\(^T\) and related type strains of species of the genus *Paenibacillus*

<table>
<thead>
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<tr>
<td>DNA G + C content (mol%)</td>
<td>60.7 ± 0.9</td>
<td>56.1(^a*)</td>
<td>55.2(^b)</td>
<td>50.7(^c)</td>
<td>51(^d)</td>
<td>50-52(^e)</td>
<td>43-46(^e)</td>
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\(^a\)Data cited from: *a*, Moon *et al.* (2011); *b*, Wu *et al.* (2013); *c*, Rivas *et al.* (2005); *d*, Rivas *et al.* (2006); *e*, Shida *et al.* (1997).
The genomic DNA G+C content was analysed according to Mesbah et al. (1989) with modification as described by Mesbah et al. (2011). After DNA denaturation in boiling water for 5 min and immediate cooling in ice, P1 nuclease was added to cleave ssDNA to nucleotides and was kept at 37 °C for 2 h. Phosphate residues were removed by using alkaline phosphatase incubated at 37 °C, overnight. The nucleosides collected were detected by HPLC (NS-4000, Futecs) using an YMC-Triart C18 column (250 × 4.6 mm, 5 μm). Elution was achieved with a mixture of 25 mM (NH₄)₂HPO₄/acetonitrile (20 : 1, v/v) at the flow rate of 0.6 ml min⁻¹. Detection was performed by UV absorption at a wavelength of 270 nm. The genomic DNA of Escherichia coli strain B (D4889, Sigma-Aldrich) was used as a standard. This experiment was carried out in triplicate. The DNA G+C content of strain DCY95T was 60.7 ± 0.9 mol%, indicating that strain DCY95T belongs to the high G+C group within the genus Paenibacillus (Vaz-Moreira et al., 2007).

Fatty acids were analysed as described by Sassar (1990) from cells grown in 3-R2A agar at 30 °C for 24 h. Fatty acids were separated by gas chromatography (GC 6890, Agilent). Identification of the methyl esters was conducted by using the TSBA library (version 6.1). Polar lipids of strain DCY95T and the most closely related type strain, P. sacheonensis KACC 14895T, were extracted and analysed by two-dimensional TLC (Minnikin et al., 1984). Polar lipid extracts were spotted onto the lower left-hand corner of thin layer plates (silica gel 60 pre-prepared plates, 10 × 10 cm, Merck). The plates were developed with chloroform/methanol/water (65 : 25 : 4, by vol.) in the first direction and chloroform/acetic acid/methanol/water (80 : 15 : 12 : 4, by vol.) in the second direction. Isoprenoid quinones were determined as described by Collins et al. (1977). Quinones were extracted from 100 mg freeze-dried cells and analysed using HPLC. The extracted quinone was detected in an Agilent Technologies 1260 infinity HPLC system with an Agilent Poroshell 120 EC-C18 column (3.0 × 50 mm, 2.7 μm) at 270 nm. The mobile phase was a mixture of methanol/2-propanol (7 : 5, v/v) and the flow rate was 0.4 ml min⁻¹. The quinones extracted from P. sacheonensis KACC 14895T containing MK-7 (Moon et al., 2011) was used as a reference. The amino acid composition in the peptidoglycan and whole-cell sugars were analysed as described by Stanec & Roberts (1974) and Komagata & Suzuki (1987), respectively. However, solvent systems for running TLC cellulose Merck KgaA (20 × 20 cm) were modified according to Schumann (2011), including the solvent 1-butanol/pyridine/water (5 : 3 : 2, by vol.) for whole-cell sugars, and methanol/pyridine/HCl (12 M)/water (32 : 4 : 1 : 7, by vol.) for peptidoglycan analyses.

The fatty acids profile of strain DCY95T was composed of a predominant amount of anteiso-C₁₅:₀ (49.2%), major amounts of iso-C₁₆:₀ (17.6%) and C₁₆:₀ (12.1%), moderate amounts of anteiso-C₁₇:₀ (6.7%) and iso-C₁₅:₀ (5.1%), and small to trace amounts of iso-C₁₄:₀ (2.2%), iso-C₁₇:₀ (1.6%), C₁₄:₀ (1.6%) and C₁₈:₀ (<1 %), which is in good agreement with those of species of the genus Paenibacillus (Shida et al., 1997; see Table S1). This fatty acids profile was compared with those of P. sacheonensis KACC 14895T and P. taihuensis NBRC 108766T. strain DCY95T contained higher amounts of C₁₆:₀ and C₁₇:₀; strain DCY95T was different from P. sacheonensis KACC 14895T by a lesser amount of C₁₈:₀ and did not contain summed feature 5 (anteiso-C₁₈:₀ and/or C₁₈:₀); the polar lipids profile of strain DCY95T consisted of major amounts of diphosphatidylglycerol (DPG), phosphatidylethanolamine, three unidentified aminophospholipids (APL1–3) and two unidentified phospholipids (PL1–2); minor amounts of phosphatidylglycerol (PG), one unidentified glycolipid, one unidentified aminolipid (AL1), one unidentified aminophosphoglycolipid, five unidentified phospholipids (PL3–7), four unidentified aminophospholipids (APL4–7) and four unidentified polar lipids (L1–4) (see Fig. S5a). The major polar lipids of P. sacheonensis KACC 14895T were PG and DPG (see Fig. S5b). Moreover, the presence of APL7, PL5, PL7 and L3–4, as well as the absence of PL8–10, APL8, and AL2–3 in the polar lipids profile could distinguish strain DCY95T from P. sacheonensis KACC 14895T. The quinone system of strain DCY95T contained MK-7, which is in accordance with other members of the genus Paenibacillus. The peptidoglycan possessed meso-diaminopimelic acid, glutamic acid and alanine, which is consistent with the previous study reported by Behrendt et al. (2010). Whole-cell sugars were composed of ribose, mannose and glucose. Collectively, the chemotaxonomic characteristics support the inclusion of strain DCY95T in the genus Paenibacillus.

Taken together, the results obtained in this study support the proposal of a novel species represented by strain DCY95T, for which the name Paenibacillus panaciterrae sp. nov. is proposed.

Description of Paenibacillus panaciterrae sp. nov.

Paenibacillus panaciterrae (pa.na.ci.ter’rae. N.L. n. Panax–acis scientific name of ginseng; L. n. terra soil; N.L. gen. n. panaciterrae soil from a ginseng field, the source of the organism).

Cells are Gram-reaction-negative, catalase-negative, oxidase-positive, strict aerobic rods approximately 0.6–0.8 μm in width, 2.1–2.5 μm in length, and motile by means of peritrichous flagella. Ellipsoidal free spores or subterminal endospores are formed in sporangia. Colonies on 5-R2A agar are creamy-coloured, circular, flat and 1–3 mm in diameter. The presence of PL8–10, APL8, and AL2–3 in the polar lipids profile could distinguish strain DCY95T from P. sacheonensis KACC 14895T. The quinone system of strain DCY95T contained MK-7, which is in accordance with other members of the genus Paenibacillus. The peptidoglycan possessed meso-diaminopimelic acid, glutamic acid and alanine, which is consistent with the previous study reported by Behrendt et al. (2010). Whole-cell sugars were composed of ribose, mannose and glucose. Collectively, the chemotaxonomic characteristics support the inclusion of strain DCY95T in the genus Paenibacillus.
arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, tryptophan deaminase and gelatinase are absent. In the API ZYM strip, activities of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-galactosidase are detected; but activities of lipase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are not. Acid is produced from glycerol, erythritol, L-arabinose, D-ribose, D-xylene, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, mellezitose, raffinose, starch, glycogen, D-xyitol, turanose and D-lyxose; but not from D-arabinose, L-xylene, L-sorbos, dulcitol, inositol, N-acetyl-glucosamine, inulin, gentiobiose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. The major cellular fatty acids are anteiso-C15 : 0, iso-C16 : 0 and C16 : 0. The major polar lipids are phosphatidylethanolamine, diphasphatidylglycerol, three unidentified aminophospholipids and two unidentified phospholipids. Menaquinone is MK-7. Whole-cell sugars are composed of ribose, mannose and glucose. Peptidoglycan contains meso-diaminopimelic acid, glutamic acid and alanine.

The type strain, DCY95T (=KCTC 33581T=DSM 29477T), was isolated from a soil sample cultivated with ginseng in Quang Nam province, Vietnam. The genomic DNA G+C content of the type strain is 60.7 ± 0.9 mol%.

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References


