Paenibacillus pocheonensis sp. nov., a facultative anaerobe isolated from soil of a ginseng field

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A Gram-positive, aerobic or facultatively anaerobic, rod-shaped, spore-forming bacterium, strain Gsoil 1138^T, was isolated from soil of a ginseng field in Pocheon Province, South Korea, and was characterized in order to determine its taxonomic position. On the basis of 16S rRNA gene sequence analysis, strain Gsoil 1138^T was shown to belong to the family *Paenibacillaceae* and was most closely related to the type strains of *Paenibacillus chondroitinus* (98.2 % similarity) and *Paenibacillus alginolyticus* (96.5 %). Levels of 16S rRNA gene sequence similarity between strain Gsoil 1138^T and the type strains of other recognized species of the genus *Paenibacillus* were below 96.5 %. The G + C content of the genomic DNA of strain Gsoil 1138^T was 52.1 ± 0.2 mol% (mean ± SD of three determinations). Phenotypic and chemotaxonomic data (MK-7 as the major menaquinone and anteiso-C_{15:0} and iso-C_{16:0} as the predominant fatty acids) supported the affiliation of strain Gsoil 1138^T to the genus *Paenibacillus*. The results of DNA–DNA hybridization experiments and physiological and biochemical tests allowed strain Gsoil 1138^T to be distinguished genotypically and phenotypically from recognized species of the genus *Paenibacillus*. Strain Gsoil 1138^T is therefore considered to represent a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus pocheonensis* sp. nov. is proposed. The type strain is Gsoil 1138^T (=KCTC 13941^T=LMG 23404^T).

The genus *Paenibacillus* was proposed based on the reclassification of 11 species of the genus *Bacillus* by Ash *et al.* (1993). The description of the genus *Paenibacillus* was subsequently emended by Shida *et al.* (1997). At the time of writing, the genus *Paenibacillus* comprises over 95 recognized species and two subspecies (Euzéby, 2009); during 2008 alone, nine novel species of the genus isolated from various environments were described. Members of the genus *Paenibacillus* are aerobic or facultatively anaerobic organisms that produce ellipsoidal endospores in swollen sporangia. The chemical composition of the cell wall shows typical structures of Gram-positive bacteria, although some strains are Gram-negative staining. The DNA G + C content for members of the genus ranges from 39 to 54 mol% and anteiso-C_{15:0} is the major cellular fatty acid (Shida *et al.*, 1997).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Gsoil 1138^T is AB245386.

A table giving the cellular fatty acid profile of strain Gsoil 1138^T and related type strains of species of the genus *Paenibacillus*, a transmission electron micrograph of a single negatively stained cell of strain Gsoil 1138^T, and a neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain Gsoil 1138^T among the type strains of all recognized species of the genus *Paenibacillus* are available with the online version of this paper.

During the course of a study on the culturable aerobic bacterial community of soil from a ginseng field in Pocheon Province, South Korea, a large number of novel bacterial strains were isolated (Im *et al.*, 2005). One of these isolates, strain Gsoil 1138^T, was subjected to a taxonomic investigation, including 16S rRNA gene sequence analysis and analysis of chemotaxonomic and phenotypic characteristics. On the basis of the results obtained in this study, strain Gsoil 1138^T is suggested to represent a novel species of the genus *Paenibacillus*.

Strain Gsoil 1138^T was originally isolated from soil of a ginseng field in Pocheon Province in South Korea. This soil sample was thoroughly suspended in 50 mM phosphate buffer (pH 7.0) and the suspension was spread on one-fifth-strength modified R2A agar plates (containing, per litre distilled water: 0.25 g tryptone, 0.25 g peptone, 0.25 g yeast extract, 0.125 g malt extract, 0.125 g beef extract, 0.25 g Casamino acids, 0.25 g soytone, 0.5 g glucose, 0.3 g soluble starch, 0.2 g xylan, 0.3 g C₃H₃NaO₃, 0.3 g K₂HPO₄, 0.05 g MgSO₄, 0.05 g CaCl₂ and 15 g agar) after being serially diluted with 50 mM phosphate buffer (pH 7.0). The plates were incubated for 1 month at room temperature in an anaerobic chamber (Three-Shine Inc.). The headspace was substituted with a gas mixture of N₂/CO₂/H₂ (80 : 15 : 5, by volume). Single colonies on the plates were incubated again

by using the modified R2A agar or half-strength modified R2A agar under anaerobic conditions. Purified colonies were tentatively identified from partial 16S rRNA gene sequences (Im *et al.*, 2005). Strain Gsoil 1138^T was one of the isolates that appeared on the modified R2A agar plates grown under aerobic conditions; this strain was routinely cultured on R2A agar at 30 °C and was maintained as a glycerol suspension (20 %, w/v) at -70 °C.

A test for the Gram reaction was performed by the non-staining method as described by Buck (1982). Cell morphology was observed under a Nikon light microscope ($\times 1000$) and with transmission electron microscopy, by using cells grown for 3 days at 30 °C on R2A agar. For the latter, cells were negatively stained with 1 % (w/v) phosphotungstic acid and, after air-drying, grids were examined with a transmission electron microscope (model CM-20; Philips). Catalase activity was determined by means of bubble production in 3 % (v/v) H₂O₂. Oxidase activity was determined by using 1 % (w/v) tetramethyl *p*-phenylenediamine. Anaerobic growth was tested in serum bottles containing R2A broth supplemented with thioglycolate (1 g l⁻¹) and in which the headspace was substituted with nitrogen gas. Tests for the degradation of DNA (DNase agar Scharlau by flooding plates with 1 M HCl), casein, chitin, starch (Atlas, 1993), lipid (Kouker & Jaeger, 1987), xylan and cellulose (Ten *et al.*, 2004) were performed and evaluated after 5 days. Other physiological characteristics were determined by using API 20NE, API 50CH and API ZYM galleries according to the manufacturer's instructions (bioMérieux). Growth at different temperatures (4, 15, 25, 30, 37 and 42 °C) and different pH (5.0–10.0, at increments of 0.5 pH units) was assessed after 5 days incubation. Salt tolerance was tested on R2A medium supplemented with 1–10 % (w/v) NaCl after 5 days incubation. Growth on nutrient agar, trypticase soy agar and MacConkey agar was evaluated at 30 °C.

Extraction of genomic DNA was performed with a commercial genomic DNA extraction kit (Core Biosystems) and PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to Ten *et al.* (2008). Complete 16S rRNA gene sequences were compiled by using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from GenBank. Multiple alignments were performed with CLUSTAL_X (Thompson *et al.*, 1997). Gaps at the 5' and 3' ends and ambiguous bases were removed from the alignment by using BIOEDIT (Hall, 1999). Evolutionary distances were calculated with the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were constructed by using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods via MEGA, version 3 (Kumar *et al.*, 2004) with bootstrap values based on 1000 replications (Felsenstein, 1985).

To determine the G+C content of the chromosomal DNA, genomic DNA of strain Gsoil 1138^T was extracted and purified as described by Moore & Dowhan (1995) and

was enzymically degraded into nucleosides. The G+C content was determined as described by Mesbah *et al.* (1989) by using reversed-phase HPLC. Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under a vacuum and re-extracted in *n*-hexane/water (1:1, v/v). The crude quinone in the *n*-hexane solution was purified by using a Sep-Pak (Waters) cartridge and was subsequently analysed by HPLC as described by Hiraishi *et al.* (1996). For determination of cellular fatty acids, strain Gsoil 1138^T and reference strains were grown on R2A agar (Difco) for 2 days since one of the reference strains, *Paenibacillus alginolyticus* DSM 5050^T, did not grow on trypticase soy agar (Difco). Cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI Inc.). Fatty acids were analysed by GC (6890; Hewlett Packard) and were identified by using the Microbial Identification software package (Sasser, 1990).

Cells of strain Gsoil 1138^T were Gram-positive, aerobic or facultatively anaerobic, motile, rod-shaped and had peritrichous flagella (see Supplementary Fig. S1 in IJSEM Online). Colonies grown on R2A agar plates (Difco) for 2 days were irregular, concentric, undulate, convex, light yellow and 1–2 mm in diameter. The strain grew well on nutrient agar, trypticase soy agar and R2A agar. On R2A agar, strain Gsoil 1138^T was able to grow at 15–30 °C, but not at 4 or 37 °C. The physiological characteristics of strain Gsoil 1138^T are summarized in the species description below and comparisons of selective characteristics with those of the type strains of closely related species are shown in Table 1.

The 16S rRNA gene sequence of strain Gsoil 1138^T determined in this study was 1488 bp in length. 16S rRNA gene sequence similarity calculations (via the EzTaxon server, <http://www.eztaxon.org>; Chun *et al.*, 2007) after simple alignment analysis indicated that the closest relatives of strain Gsoil 1138^T were *Paenibacillus chondroitinus* (98.2 %) and *P. alginolyticus* (96.5 %). Lower levels of 16S rRNA gene sequence similarity (<96.5 %) were found with the type strains of other recognized species of the genus *Paenibacillus*. This relationship between strain Gsoil 1138^T and other members of the genus *Paenibacillus* was also evident in the phylogenetic tree obtained with the neighbour-joining method (Fig. 1; based on 1455 bp). The results thus showed that strain Gsoil 1138^T is closely related to *P. chondroitinus* and *P. alginolyticus*, with which it forms a monophyletic clade with high bootstrap support. The same result was evident in the maximum-parsimony tree. Furthermore, strain Gsoil 1138^T occurred as a separate cluster with *P. chondroitinus* and *P. alginolyticus* in the neighbour-joining phylogenetic tree encompassing the type strains of all recognized species of the genus *Paenibacillus* (see Supplementary Fig. S2; based on 1299 bp). Differential characteristics between strain Gsoil 1138^T and its closest phylogenetic neighbours are presented in Table 1.

Table 1. Differential physiological characteristics between strain Gsoil 1138^T and related type strains of the genus *Paenibacillus*

Strains: 1, Gsoil 1138^T; 2, *P. chondroitinus* DSM 5051^T; 3, *P. alginolyticus* DSM 5050^T; 4, *P. polymyxa* DSM 36^T. All data were obtained in the present study. All strains were positive in tests for the hydrolysis of aesculin and 4-nitrophenyl β -D-galactopyranoside, and for the assimilation of D-glucose, D-mannitol, maltose, glycerol, L-arabinose, D-ribose, D-xylose, methyl β -D-xylopyranoside, D-galactose, methyl α -D-glucopyranoside, amygdalin, D-cellobiose, D-lactose, melibiose, sucrose, trehalose, raffinose, gentiobiose, turanose and potassium gluconate. All strains were negative for indole production, D-glucose fermentation, hydrolysis of L-arginine, urea, 2-naphthyl phosphate, 2-naphthyl myristate, L-leucyl-2-naphthylamide, L-valyl-2-naphthylamide, L-cystyl-2-naphthylamide, N-benzoyl-DL-arginine-2-naphthylamide, N-glutaryl-phenylalanine-2-naphthylamide, 6-Br-2-naphthyl- α -D-galactopyranoside, naphthol-AS-BI- β -D-glucuronide, 2-naphthyl- α -D-glucopyranoside, 1-naphthyl-N-acetyl- β -D-glucosaminide, 6-Br-2-naphthyl- α -D-mannopyranoside, 2-naphthyl- α -L-fucopyranoside, and for assimilation of capric acid, adipic acid, trisodium citrate, phenylacetic acid, erythritol, D-arabinose, L-xylose, D-adonitol, L-sorbose, L-rhamnose, dulcitol, D-sorbitol, methyl α -D-mannopyranoside, inulin, melezitose, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol and potassium 5-ketogluconate. +, Positive; w, weakly positive; -, negative.

Characteristic	1	2	3	4
Reduction of nitrate to nitrite	-	-	-	+
Hydrolysis of:				
2-Naphthyl butyrate	+	+	w	-
Naphthol-AS-BI-phosphate	+	w	-	w
2-Naphthyl caprylate	w	w	+	-
2-Naphthyl- β -D-galactopyranoside	-	w	w	-
6-Br-2-naphthyl- β -D-glucopyranoside	-	-	+	-
Gelatin	-	-	-	+
Assimilation of:				
N-Acetylglucosamine	+	+	+	-
Arbutin	-	-	-	+
D-Fructose	-	-	+	+
Glycogen	-	-	-	+
Inositol	-	-	+	-
Malic acid	-	-	+	-
D-Mannose	-	+	w	+
Potassium 2-ketogluconate	+	-	w	+
Salicin	+	+	+	-
Starch	-	-	-	+

DNA–DNA hybridization experiments were performed between strain Gsoil 1138^T and *P. chondroitinus* DSM 5051^T according to the method described by Ezaki *et al.* (1989) by using photobiotin-labelled DNA probes and micro-dilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values in each sample were excluded and the remaining three values were used for calculation of the mean level of DNA–DNA relatedness. The level of DNA–DNA relatedness between strain Gsoil 1138^T and *P. chondroitinus* DSM

5051^T was 35 %, whereas reciprocal hybridization resulted in a lower value of 31 %. Therefore, based on the recommended DNA–DNA relatedness threshold value of 70 % for species delineation (Wayne *et al.*, 1987), strain Gsoil 1138^T represents a separate species from *P. chondroitinus*.

DNA G + C content was determined based on three replications and is expressed as the mean ($\pm$ SD) of these three values. The DNA G + C content of strain Gsoil 1138^T was 52.1 ± 0.2 mol%. The respiratory quinone system present in strain Gsoil 1138^T (MK-7 as the major menaquinone) supported its affiliation to the class *Bacilli*; indeed, the majority of species in the genus *Paenibacillus* have MK-7 as the major quinone (Shida *et al.*, 1997). The fatty acid profile of strain Gsoil 1138^T (see Supplementary Table S1 in IJSEM Online) mainly comprised anteiso-C_{15:0} (64.0 %) and iso-C_{16:0} (12.8 %). These chemotaxonomic data support the affiliation of strain Gsoil 1138^T to the genus *Paenibacillus*.

The suggestion that strain Gsoil 1138^T represents a novel species of the genus *Paenibacillus* is strengthened on the basis of differences with respect to recognized species of this genus (Table 1). Distinct differences were also observed in the fatty acid composition of strain Gsoil 1138^T and recognized species of the genus *Paenibacillus*.

All of the characteristics determined for strain Gsoil 1138^T are in accordance with those previously described for the genus *Paenibacillus*. However, on the basis of phylogenetic distance from recognized species of the genus *Paenibacillus*, as indicated by relatively low levels of 16S rRNA gene sequence similarity (<98.2%; see Fig. 1 and Supplementary Fig. S2), and the combination of phenotypic characteristics (Table 1), it is apparent that strain Gsoil 1138^T is not affiliated to any recognized species of the genus *Paenibacillus*. Therefore, on the basis of the data presented, strain Gsoil 1138^T is considered to represent a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus pocheonensis* sp. nov. is proposed.

Description of *Paenibacillus pocheonensis* sp. nov.

Paenibacillus pocheonensis (po.che.on.en'sis. N.L. masc. adj. *pocheonensis* pertaining to Pocheon Province in South Korea, the source of the soil sample from which the type strain was isolated).

Cells are Gram-positive, motile rods, 0.5–1.0 μ m in diameter and 3.0–6.0 μ m in length after 2 days culture on R2A agar. Colonies grown on R2A agar for 2 days are irregular, concentric, undulate, light yellow and convex. Subterminal oval spores are formed in swollen sporangia. Aerobic or facultatively anaerobic, and catalase- and oxidase-positive. Grows at 15–30 °C, at pH 5.0–8.5 and in the presence of 2.0 % (w/v) NaCl. Growth occurs on nutrient agar and trypticase soy agar, but not on MacConkey agar. Does not reduce nitrate to nitrite. MK-7 is the predominant menaquinone, and anteiso-C_{15:0} and

Fig. 1. Neighbour-joining phylogenetic tree, constructed based on a comparative analysis of 16S rRNA gene sequences, showing the relationships between strain Gsoil 1138^T and the type strains of related species of the genus *Paenibacillus*. Filled circles indicate generic branches that were also recovered by using the maximum-parsimony algorithm. Bootstrap values (expressed as percentages of 1000 replications) of >65% are shown at branch points. Bar, 0.01 substitutions per nucleotide position. An expanded version of this tree, including the type strains of all recognized species of the genus *Paenibacillus*, is available as Supplementary Fig. S2 in IJSEM Online.

iso-C_{16:0} are the major cellular fatty acids (Supplementary Table S1). Does not degrade xylan, cellulose, chitin, casein or DNA; other physiological characteristics are given in Table 1.

The type strain, Gsoil 1138^T (=KCTC 13941^T=LMG 23404^T), was isolated from soil of a ginseng field in Pocheon Province, South Korea. The G+C content of the genomic DNA of the type strain is 52.1±0.2 mol% (mean±SD of three determinations; HPLC).

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