Bacillus panaciterrae sp. nov., isolated from soil of a ginseng field

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A Gram-positive, non-motile, endospore-forming bacterium, designated Gsoil 1517^T, was isolated from soil of a ginseng field in Pocheon Province (South Korea) and was characterized in order to determine its taxonomic position, using a polyphasic approach. It was found to rod-shaped and aerobic or facultatively anaerobic. It grew optimally at 30 °C and at pH 6.5–7.0. Comparative 16S rRNA gene sequence analysis showed that strain Gsoil 1517^T forms a distinct phylogenetic lineage within the genus *Bacillus*, being related to *Bacillus funiculus* JCM 11201^T (96.8%). The strain showed less than 94.3% sequence similarity with other *Bacillus* species. The G + C content of the genomic DNA was found to be 47.8 mol% and the predominant respiratory quinone was MK-7. The major fatty acids were iso-C_{15:0} (42.4%), anteiso-C_{15:0} (17.4%), iso-C_{14:0} (9.7%) and C_{16:0} (6.0%). On the basis of its phenotypic properties and phylogenetic distinctiveness, strain Gsoil 1517^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus panaciterrae* sp. nov. is proposed. The type strain is Gsoil 1517^T (=KCTC 13929^T=CCUG 52470^T=LMG 23408^T).

The genus *Bacillus* was first described by Cohn in 1872 and comprises a vast diversity of physiological types (Claus & Berkeley, 1986). The G + C contents (32–69 mol%) of the known *Bacillus* species, as well as the results of DNA–DNA hybridization experiments, have revealed the heterogeneity of the genus. The introduction of molecular methods, especially the use of 16S rRNA gene sequencing, has had a major impact on *Bacillus* taxonomy and has resulted in the splitting of the genus. In fact, in the last decade, 10 new genera have been separated from this original taxon (Wisotzkey *et al.*, 1992; Ash *et al.*, 1993; Shida *et al.*, 1996; Heyndrickx *et al.*, 1998; Wainø *et al.*, 1999; Fortina *et al.*, 2001; Nazina *et al.*, 2001; Yoon *et al.*, 2001). The genus currently includes more than 100 species (Euzéby, 2006). The members of this genus are considered to be ubiquitous because they have been isolated from a wide variety of aquatic and terrestrial environments, such as dust (Venkateswaran *et al.*, 2003), river-mouth sediments (Ruiz-García *et al.*, 2005), wastewater (Ajithkumar *et al.*, 2002) and desert soils (Roberts *et al.*, 1994).

During the course of a study on the culturable aerobic/facultatively anaerobic bacterial community in soil from a ginseng field in Pocheon Province, South Korea, a large number of novel bacterial strains were isolated. In this study, we have characterized one of these isolates, namely strain Gsoil 1517^T. Phenotypic, chemotaxonomic and phylogenetic analyses have established the affiliation of this isolate with the genus *Bacillus*. The data obtained also suggest that the isolate represents a novel species of the genus *Bacillus*.

Strain Gsoil 1517^T was originally isolated from a soil sample from a ginseng field in Pocheon Province: the sample (1 g) was suspended in 10 ml 50 mM phosphate buffer (pH 7.0) and the suspension was spread on one-fifth-strength modified R2A agar plates [containing (l⁻¹) 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 sodium pyruvate, 0.3 g K₂HPO₄, 0.05 g MgSO₄, 0.05 g CaCl₂ and 15 g agar] after being serially diluted in 50 mM phosphate buffer (pH 7.0). The plates were incubated at 30 °C for 1 month. Single colonies on these plates were purified by transferring them onto new plates and subjecting them to an additional incubation on one-half-strength modified R2A for 3 days at 30 °C. The purified colonies were tentatively identified by partial sequencing of the 16S rRNA gene (Im *et al.*, 2005). One isolate, Gsoil 1517^T, was routinely cultured on R2A agar at 30 °C and maintained as a glycerol suspension (20%, w/v) at –70 °C.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Gsoil 1517^T is AB245380.

A neighbour-joining tree including representatives of a wider selection of *Bacillus* species is available as supplementary material in IJSEM Online.

The Gram reaction was performed by using the non-staining method, as described by Buck (1982). The cell morphology was observed under a Nikon light microscope at $\times 1000$, with cells grown for 3 days at 30 °C on R2A agar. Catalase activity was determined by bubble production in 3 % (v/v) H₂O₂, and oxidase activity was determined using 1 % (w/v) tetramethyl-*p*-phenylenediamine. To study the assimilation of sole carbon sources, a defined liquid medium containing basal salt media (containing, l⁻¹, 1.8 g K₂HPO₄, 1.08 g KH₂PO₄, 0.5 g NaNO₃, 0.5 g NH₄Cl, 0.1 g KCl, 0.1 g MgSO₄ and 0.05 g CaCl₂) was used. A vitamin solution (Widdel & Bak, 1992), a trace elements solution (SL-10; Widdel *et al.*, 1983) and a selenite/tungstate solution (Tschech & Pfennig, 1984) were added to this medium, and the pH was then adjusted to 6.8. This liquid medium was poured into 96 wells and 25 $\times$ stock solutions of carbon sources (filter-sterilized) were then added individually to each well. Growth was examined visually after incubation at 30 °C for 7 days. The negative-control well did not contain a carbon source. The positive-control culture was grown in a well containing R2A. Fermentative and oxidative carbohydrate metabolism were tested for by examining growth in O/F basal medium containing bromothymol blue (Atlas, 1993) and supplemented with 1 % carbohydrate [soft-agar stabs with (fermentative) and without (oxidative) a sterile mineral oil overlay]. The tubes containing O/F medium were incubated at 30 °C for 5 days. Some physiological characteristics were determined with API 20E galleries, according to the instructions of the manufacturer (bioMérieux). Anaerobic growth was examined in serum bottles by adding thioglycolate (1 g l⁻¹) to R2A broth, in each case, to three serum bottles (25 ml) containing 23 ml R2A broth; the rubber caps were sealed with aluminium lids and then the upper air layer was replaced with nitrogen gas. An anaerobic nitrate reduction test (to determine the final electron acceptor) was performed in serum bottles by adding thioglycolate (1 g l⁻¹) to R2A broth and substituting the air in the headspace with nitrogen gas while nitrate was added as KNO₃ at concentrations of 10 mM. Aerobic nitrate reduction was later confirmed by inoculating, in each case, three serum bottles (25 ml) containing 12 ml R2A broth, while nitrate was added as KNO₃ at a concentration of 10 mM. The reduction of nitrate was monitored by using an ion chromatograph (model 790 personal IC; Metrohm) equipped with a conductivity detector and an anion-exchange column (Metrosep Anion Supp 4; Metrohm). Tests for the degradation of DNA [DNase agar (Scharlau), with DNase activity detected 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 7 days. Growth at different temperatures (4, 15, 25, 30, 37, 42 and 45 °C) and various pH values (pH 4.0–10.0, using increments of 0.5 pH units) was assessed after 5 days incubation. Salt tolerance was tested on R2A agar supplemented with 1–10 % (w/v) NaCl after 5 days incubation. Growth at 30 °C on nutrient agar, trypticase soy agar (TSA; Difco) and MacConkey agar was also evaluated.

An almost-complete 16S rRNA gene sequence for strain Gsoil 1517^T was determined as described below. Extraction of the genomic DNA was achieved using a commercial genomic DNA-extraction kit (Core Biosystem), and PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to Kim *et al.* (2005). The full sequence of the 16S rRNA gene was compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Multiple alignments were performed by using the CLUSTAL_X program (Thompson *et al.*, 1997). Gaps were edited in the BioEdit program (Hall, 1999). The evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). The phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) and maximum parsimony (Fitch, 1971) in the MEGA3 program (Kumar *et al.*, 2004), with bootstrap values based on 1000 replications (Felsenstein, 1985).

To measure the G + C content of the chromosomal DNA, the genomic DNA was extracted and purified as described by Moore (1995), enzymically degraded into nucleosides. The DNA G + C content was then determined as described by Mesbah *et al.* (1989), using reversed-phase HPLC. Isoprenoid quinones were extracted with chloroform/methanol (2 : 1, v/v), evaporated under vacuum conditions and re-extracted in *n*-hexane/water (1 : 1, v/v). The crude quinone in *n*-hexane was purified using silica Sep-Pak Vac cartridges (Waters) and subsequently analysed by HPLC, as described previously (Hiraishi *et al.*, 1996). Cellular fatty acids were analysed in organisms grown on TSA for 2 days. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acids were then analysed by gas chromatography (model 6890; Hewlett Packard) using the Microbial Identification software package (Sasser, 1990).

Strain Gsoil 1517^T was Gram-positive, spore-forming, non-motile and rod-shaped. Central ellipsoidal endospores were observed in swollen sporangia. Colonies grown on R2A agar plates (Difco) for 2 days were smooth, shiny, circular, entire, light-yellowish white in colour and 1.5–2.0 mm in diameter. The oxidase and catalase reactions were positive. On R2A agar, the optimum temperature for growth was 30 °C and the optimum pH range was 6.5–7.0. Other physiological characteristics of strain Gsoil 1517^T are summarized in the species description. The phenotypic and chemotaxonomic characteristics that differentiate strain Gsoil 1517^T from related *Bacillus* species are listed in Table 1.

The 16S rRNA gene sequence of strain Gsoil 1517^T was a continuous stretch of 1476 bp. Comparative 16S rRNA gene sequence analyses showed that the strain was phylogenetically affiliated with *Bacillus* species. The phylogenetic tree based on the neighbour-joining method placed strain Gsoil 1517^T within the radiation of the cluster comprising *Bacillus*

Table 1. Phenotypic characteristics of strain Gsoil 1517^T and some related *Bacillus* species

Taxa: 1, Gsoil 1517^T (data from this study); 2, *B. funiculus* (Ajithkumar *et al.*, 2001, 2002); 3, *Bacillus cereus* (Claus & Berkeley, 1986; Priest *et al.*, 1988); 4, *Bacillus pseudomyoides* (Nakamura, 1998); 5, *Bacillus mycoides* (Ajithkumar *et al.*, 2001, 2002; Venkateswaran *et al.*, 2003). Results are scored as: +, positive; V, variable among strains; –, negative; ND, no data available. All species produce centrally located ellipsoidal or oval spores.

Characteristic	1	2	3	4	5
Gram staining	+	–	+	+	+
Budding cells	–	+	–	–	–
Anaerobic growth	+	–	+	+	+
Growth at/in:					
1 % (w/v) NaCl	+	ND	+	+	+
5 % (w/v) NaCl	–	–	+	+	–
10 °C	–	–	V	–	ND
42 °C	+	–	V	–	ND
Oxidase	+	–	V	–	ND
Catalase	+	+	ND	+	ND
Citrate utilization	+	–	+	V	–
Indole production	–	–	ND	–	–
Voges–Proskauer test	–	+	+	+	+
Hydrolysis of:					
Casein	–	–	+	+	+
Gelatin	+	–	+	ND	+
Starch	–	+	V	+	ND
Acid production from:					
D-Glucose	–	ND	+	+	+
D-Mannitol	–	ND	–	–	+
DNA G+C content (mol%)	47.8	37.2	31.7–40.1	34–36	35.5–36.2

species; the strain joined *Bacillus funiculus* JCM 11201^T with a bootstrap resampling value of 96.0 % (Fig. 1). Strain Gsoil 1517^T exhibited 16S rRNA gene sequence similarity of 96.8 % with respect to the type strain of *B. funiculus* and

showed sequence similarities below 94.3 % with respect to the type strains of other *Bacillus* species with validly published names (see an extended tree, available as Supplementary Fig. S1 in IJSEM Online).

DNA–DNA hybridization between strain Gsoil 1517^T and the nearest phylogenetic neighbours was not attempted since strains differing by >3.0 % at the 16S rRNA gene sequence level are unlikely to exhibit >70 % relatedness at the whole-genome level (Stackebrandt & Goebel, 1994).

The G + C content of the genomic DNA was 47.8 mol% and the predominant respiratory quinone was MK-7. The fatty acid profile of strain Gsoil 1517^T is shown in Table 2 and was compared with those available for phylogenetically related *Bacillus* strains. Strain Gsoil 1517^T contained large amounts of iso- and anteiso-branched fatty acids; the major components were 13-methyltetradecanoic acid (iso-C_{15:0}), 12-methyltetradecanoic acid (anteiso-C_{15:0}) and 12-methyltridecanoic acid (iso-C_{14:0}), which is typical of members of the genus *Bacillus* (Kämpfer, 1994). Some qualitative and quantitative differences in fatty acid content could be observed between strain Gsoil 1517^T and the phylogenetically closest relatives (Table 1), perhaps because of differences in cultivation conditions (Kämpfer, 1994; Ajithkumar *et al.*, 2002; Venkateswaran *et al.*, 2003).

All of the characteristics determined for strain Gsoil 1517^T were in accordance with those of the genus *Bacillus*. However, on the basis of phylogenetic distances from *Bacillus* species with validly published names, indicated by the relatively low 16S rRNA gene sequence similarities (<97 %) and the combination of unique phenotypic characteristics (Table 1), it is demonstrable that Gsoil 1517^T is not affiliated with any known species of the genus *Bacillus*. Therefore, on the basis of the data presented above, strain Gsoil 1517^T represents a novel species within the genus *Bacillus*, for which the name *Bacillus panaciterrae* sp. nov. is proposed.

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic positions of Gsoil 1517^T and selected *Bacillus* species. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. The tree was rooted using *Paenibacillus polymyxa* DSM 36^T as an outgroup. Bar, 0.01 substitutions per nucleotide position. An extended tree including representatives of a wider selection of *Bacillus* species is available as Supplementary Fig. S1 in IJSEM Online.

Table 2. Cellular fatty acid profiles of strain Gsoil 1517^T and related *Bacillus* species

Strains: 1, Gsoil 1517^T (data determined after growth on trypticase soy agar for 2 days in this study); 2, *B. funiculus* JCM 11201^T (nutrient broth/glucose agar for 4 days at 30 °C; data from Ajithkumar *et al.*, 2002); 3, *B. cereus* DSM 31^T (tryptone soy agar for 24 h at 30 °C; Kämpfer, 1994); 4, *B. mycoides* ATCC 6462^T (trypticase soy broth, overnight at 32 °C; Venkateswaran *et al.*, 2003). Fatty acid data have not been reported for *B. pseudomycoloides*. —, Not detected/reported.

Fatty acid	1	2	3	4
Straight-chain saturated				
C _{14:0}	—	—	3.5	3.0
C _{16:0}	6.0	8.8	3.6	6.2
C _{17:0}	—	1.2	—	—
C _{18:0}	1.1	1.9	—	—
Branched saturated				
iso-C _{12:0}	—	—	—	1.4
iso-C _{13:0}	3.6	—	9.5	7.6
iso-C _{14:0}	9.7	—	4.0	5.0
iso-C _{15:0}	42.4	18.0	35.9	20.9
iso-C _{16:0}	2.5	3.8	1.3	10.2
iso-C _{17:0}	1.9	—	8.9	6.9
anteiso-C _{13:0}	—	—	0.6	1.9
anteiso-C _{15:0}	17.4	44.3	4.7	4.5
anteiso-C _{17:0}	1.1	—	0.8	2.2
Unsaturated				
C _{11:1}	—	4.3	—	—
Summed feature 2*	2.4	—	—	—
C _{16:1} ω11c	1.6	—	1.8	4.0
C _{16:1} (branched)	—	—	3.3	—
C _{16:1} ω7c alcohol	1.8	—	—	4.6
Summed feature 4*	1.4	—	—	7.1
anteiso-C _{16:1}	—	—	8.4	—
iso-C _{17:1} ω10c	0.8	—	0.8	11.8
Summed feature 5*	—	—	—	2.7
anteiso-C _{16:1}	—	—	8.4	—
anteiso-C _{17:1}	—	—	3.4	—
C _{17:1} (branched)	—	—	6.3	—
C _{20:2} ω16,9c	0.9	—	—	—
Unknown†				
ECL 13-961	5.4	—	—	—
C _{15:4} hydroxylated, branched	—	13.8	—	—

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 2 contained C_{13:0} 3-OH and/or iso-C_{15:1} H. Summed feature 4 contained iso-C_{15:0} 2-OH and/or C_{16:1}ω7c. Summed feature 5 contained iso-C_{17:1} I and/or anteiso-C_{17:1} B.

†Unknown fatty acids have no names listed in the peak library file of the MIDI system and therefore cannot be identified. ECL, Equivalent chain length.

Description of *Bacillus panaciterrae* sp. nov.

Bacillus panaciterrae (pa.na.ci.ter'rae. N.L. n. *Panax* -acis scientific name for ginseng; L. n. *terra* soil; N.L. gen. n. *panaciterrae* of soil of a ginseng field).

Cells are Gram-positive, rod-shaped, 0.5–0.8 µm in diameter and 3.0–5.0 µm in length, non-motile and occur singly or in chains. Central ellipsoidal endospores are observed in swollen sporangia. The optimum temperature for growth is 30 °C and the growth temperature range is between 20 and 45 °C. The minimum pH for growth lies between 5.0 and 5.5, the optimum pH is 6.5–7.0 and the maximum pH lies between 8.0 and 8.5. Tolerates 1 % (w/v) NaCl, but not 2 %. Growth occurs on TSA but not on MacConkey agar. Able to hydrolyse chitin, but not starch, cellulose, DNA, olive oil or xylan. The following substrates are utilized for growth: D-glucose, D-fructose, L-xylose, N-acetylglucosamine, pyruvate, acetate, 3-hydroxybutyrate, valerate, fumarate, salicin, citrate, lactate, malate, succinate, tartrate, sucrose, D-trehalose, gluconate, inositol (weakly), D-mannitol, xylitol, glycerol, inulin, L-alanine, L-arginine, L-asparagine, L-aspartate, L-glutamate, L-glutamine, L-histidine and L-proline. The following substrates are not utilized for growth: D-galactose, D-mannose, D-fucose, ethanol, L-rhamnose, L-sorbose, D- and L-arabinose, D-lyxose, D-ribose, D-xylose, formate, propionate, caprate, maleate, phenylacetate, benzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, malonate, glutarate, itaconate, adipate, suberate, oxalate, D-cellobiose, D-lactose, D-maltose, D-melibiose, D-raffinose, D-adonitol, dulcitol, D-sorbitol, amygdalin, methanol, glycogen, dextran, L-cysteine, glycine, L-isoleucine, L-leucine, L-lysine, L-threonine, L-methionine, L-phenylalanine, L-serine, L-tryptophan, L-tyrosine and L-valine. In API 20E tests, positive for gelatin hydrolysis and negative for β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, indole and H₂S production, the Voges–Proskauer reaction and urease production. Acid is not produced from the following carbohydrates: L-arabinose, D-mannitol, inositol, D-sorbitol, L-rhamnose, sucrose, D-melibiose, D-glucose and amygdalin. The major fatty acids are iso-C_{15:0} (42.4 %), anteiso-C_{15:0} (17.4) and iso-C_{14:0} (9.7 %). The G + C content of genomic DNA is 47.8 mol%. MK-7 is the predominant respiratory quinone.

The type strain, Gsoil 1517^T (=KCTC 13929^T=CCUG 52470^T=LMG 23408^T), was isolated from soil from a ginseng field in Pocheon Province, South Korea.

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