Brevibacillus ginsengisoli sp. nov., a denitrifying bacterium isolated from soil of a ginseng field

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A Gram-positive, rod-shaped, spore-forming bacterium, Gsoil 3088^T, was isolated from soil from a ginseng field in Pocheon Province in South Korea and characterized in order to determine its taxonomic position. On the basis of 16S rRNA gene sequence similarity, strain Gsoil 3088^T was shown to belong to the family *Paenibacillaceae*, being related to *Brevibacillus centrosporus* (96.6%), *Brevibacillus borstelensis* (96.3%), *Brevibacillus parabrevis* (96.1%), *Brevibacillus formosus* (96.1%), *Brevibacillus brevis* (96.1%) and *Brevibacillus laterosporus* (96.0%). The phylogenetic distances from other validly described species within the genus *Brevibacillus* were greater than 4.0% (i.e. there was less than 96.0% similarity). The G + C content of the genomic DNA was 52.1 mol%. Phenotypic and chemotaxonomic data (major menaquinone, MK-7; fatty acid profile, iso-C_{15:0}, iso-C_{14:0} and anteiso-C_{15:0}) supported the affiliation of strain Gsoil 3088^T to the genus *Brevibacillus*. The results of physiological and biochemical tests allowed strain Gsoil 3088^T to be distinguished genotypically and phenotypically from *Brevibacillus* species with validly published names. Strain Gsoil 3088^T, therefore, represents a novel species of the genus *Brevibacillus*, for which the name *Brevibacillus ginsengisoli* sp. nov. is proposed. The type strain is Gsoil 3088^T (= KCTC 13938^T = LMG 23403^T).

Bacillus brevis was first described in 1900 (Migula, 1900) and was reclassified as a novel species (*Brevibacillus brevis*) of the novel genus *Brevibacillus*, along with nine other species of the *Bacillus brevis* group (Shida *et al.*, 1996). Later, *Brevibacillus invocatus* (Logan *et al.*, 2002) was isolated from contaminants of an industrial fermentation process and a strain of *Brevibacillus brevis* was reclassified as a novel *Brevibacillus* species, *Brevibacillus limnophilus* (Goto *et al.*, 2004). Recently, *Brevibacillus levickii*, isolated from the slope of Mount Melbourne, was proposed as a novel species (Allan *et al.*, 2005). Currently, there are 13 *Brevibacillus* species with validly published names (<http://www.bacterio.cict.fr/b/brevibacillus.html>). Members of this genus contain MK-7 as the major respiratory quinone, produce oval endospores in swollen sporangia, possess anteiso-C_{15:0} and iso-C_{15:0} or

just iso-C_{15:0} as the major cellular fatty acids and have DNA G + C contents in the range 42.8–57.4 mol% (Shida *et al.*, 1996).

During a course of 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 3088^T, was subjected to a taxonomic investigation.

In the present study, we conducted a phylogenetic analysis based on 16S rRNA gene sequences, chemotaxonomic characteristics and some important phenotypic characteristics in order to determine the precise taxonomic position of this strain. On the basis of the results obtained in this study, strain Gsoil 3088^T is proposed as a novel species of the genus *Brevibacillus*.

Strain Gsoil 3088^T was originally isolated from soil from a ginseng field in Pocheon Province in South Korea. The 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 the following,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Gsoil 3088^T is AB245376.

A transmission electron micrograph of a cell of strain Gsoil 3088^T and a table comparing the cellular fatty acid profiles of this strain and members of the genus *Brevibacillus* are available as supplementary material in IJSEM Online.

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 $C_3H_3NaO_3$, 0.3 g K_2HPO_4 , 0.05 g $MgSO_4$, 0.05 g $CaCl_2$, 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 (Labplus). The headspace was substituted with a gas mixture comprising $N_2/CO_2/H_2$ (80:15:5, by vol.). Single colonies on the plates were purified by transferring them onto new plates which were incubated using the modified R2A agar or half-strength modified R2A agar under anaerobic conditions. The purified colonies were tentatively identified from partial sequences of the 16S rRNA gene (Im *et al.*, 2005). Strain Gsoil 3088^T was one of the isolates that appeared on the modified R2A agar plates grown under anaerobic conditions; this strain was routinely cultured on R2A agar at 30 °C and maintained as a glycerol suspension (20 %, w/v) at -70 °C.

A Gram reaction was performed by using 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, using cells grown for 3 days at 30 °C on R2A agar. For the latter, the 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) H_2O_2 . Oxidase activity was determined using 1 % (w/v) tetramethyl-*p*-phenylenediamine. For studies on the assimilation of single carbon sources, a defined liquid medium containing a basal salt mixture, comprising (g l^{-1}) 1.8 g K_2HPO_4 , 1.08 g KH_2PO_4 , 0.5 g $NaNO_3$, 0.5 g NH_4Cl , 0.1 g KCl , 0.1 g $MgSO_4$ and 0.05 g $CaCl_2$. A vitamin solution (Widdel & Bak, 1992), a trace element solution (SL-10; Widdel *et al.*, 1983) and a selenite/tungstate solution (Tschech & Pfennig, 1984) were added to this medium and the pH was adjusted to 6.8 by the addition of 1.0 M HCl. This liquid medium was poured into 96-well plates and filter-sterilized 25 $\times$ carbon sources were added to each well. The plates were incubated at 30 °C for up to 7 days and growth was examined visually. The negative control well did not contain any carbon source solution and the positive control culture was grown in one well containing R2A broth.

Physiological characteristics were determined by using API 20E galleries according to the manufacturer's instructions (bioMérieux). 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. The anaerobic nitrate reduction test to determine the final electron acceptor 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; nitrate was added as KNO_3 at a concentration of 10 mM. Aerobic nitrate reduction was later confirmed by inoculation into 12 ml R2A broth

supplemented with 10 mM KNO_3 in three 25 ml serum bottles. 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 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. Growth at different temperatures (4, 15, 25, 30, 37 and 42 °C) and various pH values (pH 5.0–10.0, using 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 also evaluated at 30 °C.

Extraction of genomic DNA was performed with 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). Complete 16S rRNA gene sequences were compiled 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 were edited using BIOEDIT (Hall, 1999). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). A phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) using the MEGA3 program (Kumar *et al.*, 2004) with bootstrap values based on 1000 replications (Felsenstein, 1985).

To determine the G + C content of chromosomal DNA, genomic DNA of the novel strain was extracted and purified as described by Moore & Dowhan (1995) and was enzymically degraded into nucleosides. DNA G + C content was determined as described by Mesbah *et al.* (1989) using a reverse-phase HPLC. Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under a vacuum and reextracted in *n*-hexane/water (1:1, v/v). The crude quinone in the *n*-hexane solution was purified using Sep-Pak cartridges (Waters) and subsequently analysed by HPLC as described previously (Hiraishi *et al.*, 1996). Cellular fatty acids were determined for strains grown on trypticase soy agar (Difco) for 2 days. Cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acids analysed by GC (6890; Hewlett Packard), were identified by the Microbial Identification software package (Sasser, 1990).

Cells of strain Gsoil 3088^T were found to be Gram-positive, aerobic or facultatively anaerobic, motile, rod-shaped and 0.3–0.5 μm in diameter and 3.5–5.0 μm in length. They possessed peritrichous flagella (see Supplementary Fig. S1 available in IJSEM Online). The novel strain could grow

anaerobically in the presence of nitrate, but not in the absence of nitrate. Thus the novel strain was able to grow anaerobically using nitrate as a terminal electron acceptor. Colonies grown on R2A agar plates (Difco) for 2 days were smooth, circular, glossy, white and 2–4 mm in diameter. On R2A agar, strain Gsoil 3088^T was able to grow at 20–42 °C, but not at 4 or 45 °C. The physiological characteristics of strain Gsoil 3088^T are summarized in the species description and comparisons of selective characteristics with closely related type strains are shown in Table 1.

The 16S rRNA gene sequence of strain Gsoil 3088^T determined in this study was 1494 bp in length. Sequence similarity calculations after a simple alignment analysis indicated that the closest relative of strain Gsoil 3088^T was *Brevibacillus centrosporus* (96.6 %). Lower levels of sequence similarity (< 96.5 %) were found with the other recognized *Brevibacillus* species. The relationship between strain Gsoil 3088^T and other members of the genus *Brevibacillus* was also evident in the phylogenetic tree obtained using the neighbour-joining method (Fig. 1, using 1465 bases). The results obtained showed that strain Gsoil 3088^T is closely related to *Brevibacillus laterosporus*, with which it forms a monophyletic clade with a high bootstrap value.

DNA–DNA hybridization between strain Gsoil 3088^T and its nearest phylogenetic neighbours was not attempted since strains differing by > 3.0 % at the 16S rRNA gene level are unlikely to exhibit > 70 % similarity at whole-genome level (Stackebrandt & Goebel, 1994).

The DNA G+C content of strain Gsoil 3088^T was 52.1 mol%. The respiratory quinone system present in strain Gsoil 3088^T supports its affiliation to the class *Bacilli*; indeed, the majority of species in the genus *Brevibacillus* have MK-7 as the major quinone (Shida *et al.*, 1996). The fatty acid profile of strain Gsoil 3088^T (see Supplementary Table S1 available in IJSEM Online) mainly comprised iso-C_{15:0} (36.3 %), iso-C_{14:0} (15.0 %) and anteiso-C_{15:0} (14.8 %). These chemotaxonomic data support the affiliation of strain Gsoil 3088^T to the genus *Brevibacillus*.

All of the characteristics determined for strain Gsoil 3088^T are in accordance with those for the genus *Brevibacillus*. However, on the basis of the phylogenetic distance from recognized *Brevibacillus* species, indicated by relatively low 16S rRNA gene sequence similarities (< 97 %; see Fig. 1), and the combination of unique phenotypic characteristics (Table 1), it is apparent that strain Gsoil 3088^T is not affiliated to any recognized species of the genus *Brevibacillus*. Therefore, on the basis of the data presented above, strain Gsoil 3088^T represents a novel species of the genus *Brevibacillus*, for which the name *Brevibacillus ginsengisoli* sp. nov. is proposed.

Description of *Brevibacillus ginsengisoli* sp. nov.

Brevibacillus ginsengisoli (gin.sen.gi.so'li. N.L. n. *ginsengum* ginseng; L. n. *solum* soil; N.L. gen. n. *ginsengisoli* of the soil of a ginseng field, the source of the organism).

Table 1. Physiological characteristics of strain Gsoil 3088^T and related type strains of *Brevibacillus* species

Strains: 1, Gsoil 3088^T (data from this study); 2, *Brevibacillus laterosporus*; 3, *Brevibacillus borstelensis*; 4, *Brevibacillus centrosporus*; 5, *Brevibacillus parabrevis*; 6, *Brevibacillus formosus*; 7, *Brevibacillus brevis*. Data for reference taxa are from Logan *et al.* (2002). +, Positive; –, negative; v, variable.

Characteristic	1	2	3	4	5	6	7
Growth temperature (°C):							
Range	20–42	15–50	20–50	10–40	20–45	20–45	20–45
Optimum	30	30	30	28	35–40	35–40	35–40
Nitrate reduction	+	+	+	v	+	+	+
Hydrolysis of:							
Casein	+	+	+	–	+	+	+
Gelatin	+	+	+	–	+	+	+
Starch	–	–	–	–	–	–	+
Utilization of sole carbon sources:							
L-Alanine	+	+	+	–	+	+	+
D-Gluconate	–	–	+	+	+	+	+
2-Ketogluconate	–	v	–	+	+	+	v
DL-Lactate	–	–	–	+	–	+	–
Maltose	–	+	–	–	+	+	+
D-Mannitol	–	+	–	+	+	+	+
N-Acetyl-D-glucosamine	+	+	–	+	+	+	+
Phenylacetate	–	–	+	–	+	+	+
Sucrose	–	–	–	–	+	+	+

Fig. 1. Neighbour-joining phylogenetic tree, constructed from a comparative analysis of 16S rRNA gene sequences, showing the relationships between strain Gsoil 3088^T and related species. Filled circles indicate generic branches that were also recovered by using maximum-parsimony algorithms. Bootstrap values (expressed as percentages of 1000 replications) greater than 65% are shown at the branch points. Bar, 0.01 substitutions per 1 nucleotide position.

Cells are Gram-positive, aerobic or facultatively anaerobic, motile, slightly curved rods, 0.3–0.5 µm in diameter and 3.5–5.0 µm in length after 2 days culture on R2A agar. Colonies grown on R2A agar for 2 days are smooth, circular, glossy, white and convex. Central and subterminal oval spores are formed in swollen sporangia. Grows well at 20–42 °C and pH 5.0–8.5, but does not grow at 4 or 45 °C. Growth occurs in the absence of NaCl and in the presence of 2.0% (w/v) NaCl, but not 4% (w/v) NaCl. Able to grow anaerobically in denitrifying conditions. Xylan, chitin, starch, cellulose and DNA are not degraded. Substrate utilization, enzyme production, acid production and other physiological characteristics are indicated in Table 1. MK-7 is the predominant respiratory quinone. The major cellular fatty acids are iso-C_{15:0}, iso-C_{14:0} and anteiso-C_{15:0} (see Supplementary Table S1). Urease, β-glucosidase, protease and malic acid assimilation are positive in tests using API 20E and API 20NE strips. Reactions for ONPG hydrolysis, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, hydrogen sulfide production, tryptophan deaminase, indole production, acetoin production and adipic acid assimilation are negative. The following carbon sources are utilized in the API 50 CH and ID 32GN tests: glycerol, D-glucose, salicin, D-celiobiose, sodium acetate, L-serine, valeric acid, L-histidine, 3-hydroxybutyric acid and L-proline. Tests are negative for the utilization of the following substrates: erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-galactose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, arbutin, D-maltose, D-lactose, D-melibiose, D-trehalose, inulin, D-melezitose, D-raffinose, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, itaconic acid, suberic acid, sodium malonate,

3-hydroxybenzoic acid, propionic acid, capric acid, trisodium citrate and 4-hydroxybenzoic acid. The G + C content of the genomic DNA is 52.1 mol% (as determined by HPLC).

The type strain, Gsoil 3088^T (=KCTC 13938^T=LMG 23403^T), was isolated from soil from a ginseng field in Pocheon Province, South Korea.

Acknowledgements

This work was supported by the 21C Frontier Microbial Genomics and Application Center Program, Ministry of Science and Technology (grant MG05-0101-4-0), Republic of Korea.

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