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Bacillus ginsengihumi sp. nov., a Novel Species Isolated from Soil of a Ginseng Field in Pocheon Province, South Korea

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Abstract A Gram-positive, aerobic or facultative anaerobic, non motile, endospore-forming bacterial strain, designated Gsoil 114^T, was isolated from a soil sample of a ginseng field in Pocheon Province (South Korea), and was characterized taxonomically by using a polyphasic approach. It grew well on nutrient agar medium and utilized a limited number of organic substrates as sole carbon sources, including D-xylose and some other carbohydrates, but did not utilize L-amino acids and organic acids. The isolate was positive for oxidase test but negative for catalase, and negative for degradation of macromolecules such as starch, cellulose, xylan, casein, chitin, and DNA. The G+C content of the genomic DNA was 41.8 mol%. The predominant isoprenoid quinone was menaquinone 7 (MK-7). The major fatty acids were anteiso-$C_{15:0}$ (32.1%), iso-$C_{15:0}$ (30.5%), and anteiso-$C_{17:0}$ (30.2%). Comparative 16S rRNA gene sequence analysis showed that strain Gsoil 114^T fell within the radiation of the cluster comprising *Bacillus* species and joined *Bacillus shackletonii* LMG 18435^T with a bootstrap value of 95%. The highest 16S rRNA gene sequence similarities were found with *Bacillus shackletonii* LMG 18435^T (97.6%), *Bacillus acidicola* DSM 14745^T (96.9%), *Bacillus sporothermodurans* DSM 10599^T (96.5%), and *Bacillus oleronius* DSM 9356^T (96.5%). The phylogenetic distance from any other validly described species within the genus *Bacillus* was less than 96%. DNA–DNA hybridization experiments showed that the DNA-similarities between strain Gsoil 114^T and closest phylogenetic neighbors were less than 39%. On the basis of its phenotypic properties and phylogenetic distinctiveness, strain Gsoil 114^T (=KCTC 13944^T=DSMZ 18134^T) was classified in the genus *Bacillus* as the type strain of a novel species, for which the name *Bacillus ginsengihumi* sp. nov. is proposed.

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The genus *Bacillus* was first described by Cohn in 1872 and it comprises a systematically diverse assemblage of Gram-positive, aerobic or facultatively anaerobic, spore-forming organisms [6]. Members of this genus exhibit a wide range of nutritional requirements, growth conditions, DNA base compositions, and major amino acid compositions of the cell wall. In addition to phenotypic heterogeneity, they also appear to be phylogenetically diverse with five phylogenetically distinct clusters [2]. 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 splitting of the genus. In fact, over the last decade, nine new genera have been separated from this original taxon [3, 11, 14, 29, 36, 44, 46, 47]. Currently, the genus includes over 170 species [7]. Their primary habitat is the soil and associated plants, rivers, and estuarine waters, although some species are pathogenic for mammals (*e.g.*, *B. anthracis* [13]) and insects (*e.g.*, *B. sphaericus*, *B. thuringiensis* [18, 24, 30]). An important common characteristic is their ability to form endospores that allow them to survive for extended periods under adverse environmental conditions. The ability to sporulate and their metabolic diversity are significant factors that have led to their successful colonization of a wide variety of environments.

During a course of study on the culturable aerobic and facultative anaerobic bacterial community in soil of 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, strain Gsoil 114^T. Phenotypic, chemotaxonomic, and phylogenetic

analyses established the affiliation of the isolate to the genus *Bacillus*. The data obtained in this study suggest that the isolate represents a novel species of the genus *Bacillus*, and the name *Bacillus ginsengihumi* sp. nov. is proposed.

Materials and Methods

Isolation of Bacterial Strain and Culture Condition

Strain Gsoil 114^T was isolated from a soil sample of a ginseng field in Pocheon Province (South Korea). The soil sample was well suspended with 50 mM phosphate buffer (pH 7.0) and subsequently diluted serially in the same buffer. Aliquots were plated on one-fifth strength modified R2A media containing (g/l) 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 acid, 0.25 g soytone, 0.5 g dextrose, 0.3 g soluble starch, 0.2 g xylan, 0.3 g sodium pyruvate, 0.3 g K_2HPO_4, 0.05 g $MgSO_4$, 0.05 g $CaCl_2$, and 15 g agar. The plates were incubated at 30°C for one month. Single colonies on the plates were purified by transferring them onto new plates and were incubated once again under the modified R2A or one-half strength modified R2A. Strain Gsoil 114^T was one of the isolates that dominantly appeared on the modified R2A agar plates in aerobic condition. It was routinely cultured on R2A agar at 30°C and maintained as a glycerol suspension (20%, w/v) at −70°C.

Phenotypic and Biochemical Characteristics

Gram reaction was performed by the nonstaining method as described by Buck [5]. Cell morphology was observed under a Nikon light microscope at ×1,000, with cells grown for 3 days at 30°C on R2A agar. Catalase activity was determined by bubble production in 3% (v/v) H_2O_2, and oxidase activity was determined using 1% (w/v) tetramethyl *p*-phenylenediamine. Single-carbon source assimilation tests were performed using API 50 CH strips and ID 32 GN test kits (bioMérieux) according to the manufacturer's instructions. Degradation of DNA was determined using DNase agar from Scharlau, supplemented with 1 M HCl; the hydrolysis of casein, chitin, and starch was assessed as described by Atlas [4]; degradation of lipid was performed according to Kouker and Jaeger [21]; degradation of xylan, cellulose, and collagen was determined as described by Ten *et al.* [40, 41], and reactions were read after 5 days. Anaerobic growth was performed in serum bottles by adding the thioglycolate (1 g/l) to R2A broth and substituting the upper air layer with nitrogen gas. Nitrate reduction, acid production from carbohydrates, and some other physiological characteristics were determined with API 20 E and API 20 NE galleries according to the instructions of the manufacturer (bioMérieux). Growth at different temperatures (4, 15, 20, 25, 30, 37, 42, and 45°C) and various pH values (pH 4.5–10.0 at intervals of 0.5 pH units) was assessed after 5 days of incubation. Salt tolerance was tested on R2A medium supplemented with 1–15% (w/v) NaCl after 5 days of incubation. Growth on nutrient agar, trypticase soy agar (TSA), and MacConkey agar was also evaluated at 30°C.

PCR Amplification, 16S rRNA Gene Sequencing, and Phylogenetic Analysis

DNA was extracted using a commercial genomic DNA extraction kit (Solgent Co., Korea) and PCR-mediated amplification of the 16S rRNA gene and sequencing of purified PCR product were carried out according to Kim *et al.* [19]. The 16S rRNA full gene sequences were compiled using SeqMan software (DNASTAR, Madison, WI, U.S.A.). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database. The multiple alignments were performed by the Clustal_X program [42]. Gaps were edited in the BioEdit program [12]. The evolutionary distances were calculated using the Kimura two-parameter model [20]. The phylogenetic trees were constructed by using the neighbor-joining method [34] and the maximum-parsimony method [10] using the MEGA3 Program [22] with bootstrap values based on 1,000 replications [9].

DNA Extraction and Determination of DNA G+C Content

Chromosomal DNA for determination of G+C content was extracted from cells and purified as described by Moore [28]. DNA base composition was determined using the HPLC method. DNA was enzymatically degraded into nucleotides as described by Mesbah *et al.* [26]. The nucleotide mixture obtained was then separated by HPLC using a Waters Nova-Pak C_{18} column (3.9×300 mm) and eluted by a mixture of 0.2 M $(NH_4)H_2PO_4$ and acetonitrile (20:1, v/v) at a flow rate of 1.0 ml/min and detected by UV absorbance at 270 nm. DNA of *E. coli* (Sigma) was used as the calibration reference.

DNA-DNA hybridization

DNA-DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* [8], with photobiotin-labeled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded, and the means of the remaining three values were quoted as the DNA relatedness value.

Cellular Fatty Acids and Isoprenoid Quinones

Cellular fatty acids were analyzed in organisms grown on R2A agar for two days. The cellular fatty acids were saponified, methylated, and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI, 1999). The fatty acids analyzed by a gas chromatograph (Hewlett Packard 6890) were identified by the Microbial

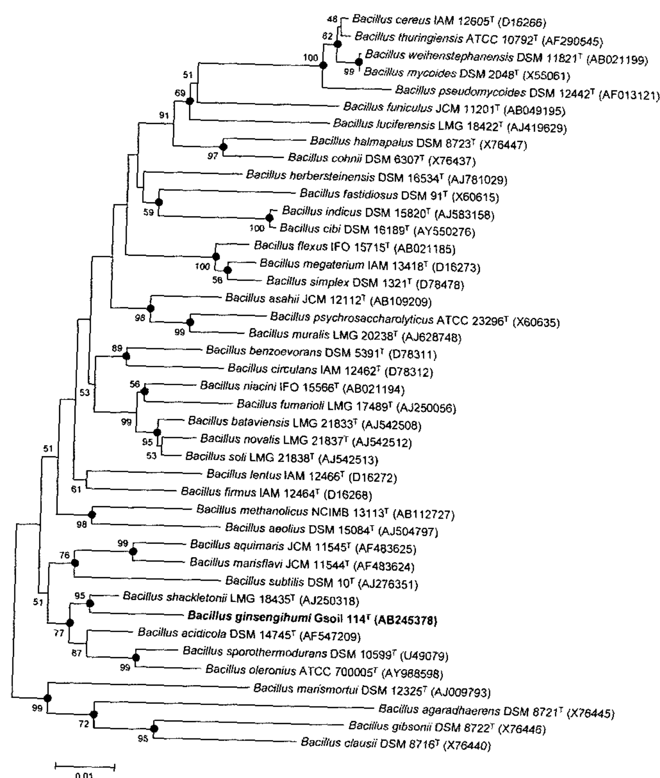

Fig. 1. Neighbor-joining tree (based on 16S rRNA gene sequences) showing the phylogenetic positions of strain Gsoil 114^T among phylogenetic neighbors.

Numbers on branch nodes are bootstrap values (1,000 resamplings; only values over 50% are given). Dots indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. Bar, 1% sequence divergence.

Identification software package [35]. Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under vacuum conditions, and reextracted in *n*-hexane-water (1:1, v/v). Then, the crude quinone in purified *n*-hexane was purified using Sep-Pak Vac Cartridges Silica (Waters) and subsequently analyzed, as previously described by Shin *et al.* [38].

Nucleotide Sequence Accession Numbers

The 16S rRNA gene sequence of strain Gsoil 114^T determined in this study has been deposited in NCBI/EMBL/DDBJ under the accession number AB245378. The accession numbers of the reference strains, which are closely related to strain Gsoil 114^T, are indicated in Fig. 1.

Results and Discussion

Morphological and Phenotypic Characteristics

Strain Gsoil 114^T was a Gram-positive, aerobic or facultatively anaerobic, and rod-shaped organism that was about 3.0–4.5 μm long and 0.8–1.0 μm wide. Colonies of Gsoil 114^T were flat, smooth, irregular, undulate, and opaque, with a slight yellow tint and 1.0–2.0 mm in diameter after incubation for one day on R2A at 30°C. The isolate was non motile in contrast to its closest phylogenetic relatives presented in Table 1. The optimal temperature for growth was 30°C and growth occurred within the range 20–45°C. The pH range for growth was 5.0–10.0 and the optimum growth pH was 6.5–7.0. The strain Gsoil 114^T has been isolated from a nonsaline environment and does not require NaCl to grow, but in contrast to *B. acidicola*, *B. sporothermodurans*, and *B. oleronius*, it can tolerate 10% (w/v) NaCl. Other physiological characteristics of strain Gsoil 114^T are summarized in the species description. Phenotypic and chemotaxonomic characteristics that differentiate strain Gsoil 114^T from its closest phylogenetic relatives are listed in Table 1.

Cellular Fatty Acid and Menaquinone Compositions

The predominant menaquinone was MK-7. The major fatty acids found in strain Gsoil 114^T are shown in Table 2 and are compared with values available for phylogenetically related *Bacillus* strains. Strain Gsoil 114^T contained a large amount of iso- and anteiso-branched fatty acids; the major components were 12-methyl tetradecanoic acid (anteiso-$C_{15:0}$), 13-methyl tetradecanoic acid (iso-$C_{15:0}$), and 14-methyl hexadecanoic acid (anteiso-$C_{17:0}$), typical for members of the genus *Bacillus* [16]. Some qualitative and quantitative differences in fatty acid content could be observed between the strain Gsoil 114^T and the phylogenetically closest relatives. One of the major fatty acids found in the strain Gsoil 114^T was anteiso-$C_{17:0}$, comprising 30.2%. This value is generally much higher than that reported for *B. shackletonii*, *B. acidicola*, *B. marisflavi*, *B. aquimaris*, *B. oleronius*, and *B. subtilis*. Strain Gsoil 114^T could also be differentiated from these phylogenetically closest relatives (partially, except *B. oleronius*) by the absentce of saturated straight-chain fatty acids and any monounsaturated fatty acids, except Summed feature 5.

DNA G+C Content

The G+C content for strain Gsoil 114^T was 41.8 mol%, which lies within the range of 39–54% reported for the genus *Bacillus* [37].

Phylogenetic Analysis

The lengths of the almost complete 16S rRNA gene sequence of strain Gsoil 114^T was 1,495 bp. Preliminary comparison of the sequence against the GenBank database indicated that members of the genus *Bacillus* were the closest phylogenetic neighbors. The phylogenetic tree (Fig. 1) based on the neighbor-joining algorithm showed that strain Gsoil 114^T appeared within the genus *Bacillus* and occupied a distinct phylogenetic position within the genus. The closest phylogenetic neighbors of strain Gsoil

Table 1. Comparison of the phenotypic characteristics of *Bacillus ginsengihumi* sp. nov. and phylogenetically related *Bacillus* species.

Characteristic	1	2	3	4	5	6	7	8
Gram staining	+	v	+	+ or v	v	+	+	−
Anaerobic growth	+	−	−	−	−	−	−	−
Motility	−	+	+	+	+	+	+	+
Nitrate reduction	−	−	−	ND	ND	+	−	−
Indole production	−	−	−	ND	ND	ND	−[a]	−
Voges-Proskauer test	+	−[b]	−	ND	ND	+[c]	−	+
Growth at/in:								
5% (w/v) NaCl	+	ND	−	+	+	+	−	+
10% (w/v) NaCl	(+)	ND	−	+	+	+	−	−
10°C	−	−	−	+	+	+	+	−
45°C	+	+	+	+	−	+	+	ND
pH 5.0	+	+	+	+	+	ND	ND	ND
pH 10.0	(+)	−	−	ND	−	ND	ND	ND
Enzymes								
Oxidase	+	ND	−	−	−	+	+	ND
Catalase	−	+	+	+	+	+	+	+
Lysine decarboxylase	−	−	+	ND	ND	ND	ND	−
Arginine dihydrolase	+	−	ND	ND	ND	−[c]	ND	ND
Utilization of								
Citrate	−	−[b]	+	ND	ND	+	−	−
Propionate	−	ND	-	ND	ND	−	−[a]	−
D-Mannitol	+	ND	ND	ND	ND	−[c]	ND	+
Hydrolysis of								
Casein	−	(+)	−	+	+	+	−	−
Gelatin	−	−[b]	−	ND	ND	−[c]	−	+
Starch	−	−	ND	−	+	+	−	+
Aesculin	−	+	ND	+	−	ND	+	+
Acid production from								
D-Glucose	−	+	+	+	+	+	−	+
D-Mannitol	−	+	−	+	−	+	−	−
Inositol	−	−	NK	−	−	−[c]	−[d]	−
D-Sorbitol	−	−	−	−	−	−[c]	ND	−
L-Rhamnose	−	−	−	−	−	ND	ND	−
D-Sucrose	−	−	+	+	+	+[c]	ND	−
D-Melibiose	−	−	NK	+	−	−[c]	−[d]	−
Amygdalin	−	+	NK	+	+	+[c]	v	−
L-Arabinose	(+)	−	−	−	−	+	−[d]	−
DNA G+C (mol%)	41.8	35.4	43.2	49.0	38.0	43.0	36.0	35.0

Strains: 1, *Bacillus ginsengihumi* Gsoil 114[T] (present study); 2, *Bacillus shackletonii* LMG 18435[T] [25]; 3, *Bacillus acidicola* DSM 14745[T] [1]; 4, *Bacillus marisflavi* JCM 11544[T] [48]; 5, *Bacillus aquimaris* JCM 11545[T] [48]; 6, *Bacillus subtilis* NRRL NRS-744[T] [33]; 7, *Bacillus sporothermodurans* DSM 10599[T] [31]; 8, *Bacillus oleronius* DSM 9356[T] [22]. Results are scored as +, positive; (+), weakly positive; v, variable among strains; −, negative; ND, not determined.

[a] Data from Montanari *et al.* [27]

[b] Citrate utilization, gelatin hydrolysis, and Voges-Proskauer reactions may become positive slowly at 40°C.

[c] Data from Venkateswaran *et al.* [43] for *B. subtilis* IAM 1026[T].

[d] Data from Heyrman *et al.* [15].

Table 2. Fatty acid composition of *Bacillus ginsengihumi* sp. nov. and phylogenetically related *Bacillus* species[a].

Fatty acids	1	2	3	4	5	6	7
Straight-chain saturated							
$C_{14:0}$	−[b]	1.4	0.9	1.7	0.9	−	1.0
$C_{15:0}$	−		−	1.1	0.5	−	−
$C_{16:0}$	−	−	0.8	1.0	0.5	3.1	2.0
$C_{17:0}$ 2-OH	−	−	−	1.2	−	−	−
Branched saturated							
iso-$C_{13:0}$	−		−	−	0.4	−	−
iso-$C_{14:0}$	−	1.2	−	9.1	6.5	1.1	−
iso-$C_{15:0}$	30.5	30.9	57.9	22.9	46.6	29.3	47.0
iso-$C_{16:0}$	1.2	6.0	−	7.7	4.2	2.4	3.0
iso-$C_{17:0}$	3.1	−	3.3	1.2	0.8	9.6	5.0
iso-$C_{14:0}$ 3-OH	−	−	−	2.0	−	−	−
iso-$C_{15:0}$ 2-OH	−	−	−	5.5	−	−	−
iso-$C_{15:0}$ 3-OH	−	−	−	6.1	−	−	−
iso-$C_{16:0}$ 3-OH	−	−	−	1.5	−	−	−
anteiso-$C_{15:0}$	32.1	36.6	24.3	27.6	22.2	40.2	25.0
anteiso-$C_{17:0}$	30.2	18.1	8.5	7.1	2.3	9.4	16.0
Monounsaturated							
$C_{16:1}$ ω5c	−	−	−	−	−	1.5	−
$C_{16:1}$ ω7c alcohol	−	−	−	2.5	8.4	−	−
$C_{16:1}$ ω11c	−	1.6	0.7	−	1.3	−	−
iso-$C_{17:1}$ ω7c	−	−	−	−	−	1.7	
iso-$C_{17:1}$ ω10c	−	1.5	1.7	−	1.8	−	−
Summed feature 5[c]	2.9	2.7	1.9	1.8	2.9	−	−
Others	−	−	−	−	0.7	1.7	1.0

Strains: 1, *Bacillus ginsengihumi* Gsoil 114[T] (present study); 2, *Bacillus shackletonii* LMG 18435[T] [25]; 3, *Bacillus acidicola* DSM 14745[T] [1]; 4, *Bacillus marisflavi* JCM 11544[T] [48]; 5, *Bacillus aquimaris* JCM 11545[T] [48]; 6, *Bacillus subtilis* NRRL NRS-744[T] [32]; 7, *Bacillus oleronius* DSM 9356[T] [22]. Data were not found for *Bacillus sporothermodurans* DSM 10599[T].

[a] Values are shown as a percentage of the total fatty acid content for each strain.

[b] Not detected.

[c] Summed feature represents a group of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 5 contained iso-$C_{17:1}$I and/or anteiso-$C_{17:1}$B.

114[T] were *Bacillus shackletonii* LMG 18435[T] (97.6%), *Bacillus acidicola* DSM 14745[T] (96.9%), *Bacillus sporothermodurans* DSM 10599[T] (96.5%), and *Bacillus oleronius* DSM 9356[T] (96.5%). The phylogenetic distance from any other validly described species within the genus *Bacillus*, including *Bacillus marisflavi* JCM 11544[T], *Bacillus aquimaris* JCM 11545[T], and *Bacillus subtilis* DSM 10[T], was less than 96%. These data indicate that strain Gsoil 114[T] is a species that is clearly separate from other *Bacillus* spp., with the exceptions of the above-mentioned four species [39]. To differentiate strain Gsoil 114[T] from these closely related species, a DNA-DNA hybridization test was performed.

DNA-DNA Hybridization

The DNA-DNA relatedness value of strain Gsoil 114^T to above-mentioned the closest four species of genus *Bacillus* was 17–38%; *i.e.*, to *Bacillus shackletonii* KCTC 13003^T (38%), *Bacillus acidicola* DSM 14745^T (31%), *Bacillus sporothermodurans* DSM 10599^T (23%), and *Bacillus oleronius* DSM 9356^T (17%). The DNA-DNA hybridization level was less than 70%, which defines a genomic species [39, 45]. These results strongly suggested that strain Gsoil 114^T represents a distinct species of the genus *Bacillus*.

Taxonomic Conclusions

The results obtained from the phenotypic and phylogenetic characterizations indicated that strain Gsoil 114^T belongs to the genus *Bacillus*. The phylogenetic distinctiveness and DNA-DNA hybridization experiments confirmed that strain Gsoil 114^T represents a species distinct from recognized *Bacillus* species. There were some phenotypic differences between strain Gsoil 114^T and phylogenetically related *Bacillus* species (Table 1). Therefore, on the basis of the data presented, strain Gsoil 114^T should be classified in the genus *Bacillus* as a novel species, for which the name *Bacillus ginsengihumi* sp. nov. is proposed.

Description of *Bacillus ginsengihumi* sp. nov.

Bacillus ginsengihumi (gin.sen.gi.humi. N.L. n. ginsengum, ginseng; L. n. humus, soil; N.L. gen. n. ginsengihumi, of soil of a ginseng field).

Cells are Gram-positive, aerobic or facultative anaerobic, nonmotile, and rod-shaped, 3.0–4.5 μm in length and 0.8–1.0 μm in width. Terminal oval spores are observed in swollen sporangia. After one day on R2A, colonies are 1.0–2.0 mm in diameter, flat, smooth, irregular, undulate, and opaque with a slight yellow tint. The oxidase reaction is positive, and catalase and lipase are negative. The bacterium grows within a temperature range of 20 to 45°C; the optimum temperature for growth is 30°C. It grows within pH values of between 5.0 and 10.0; the optimum pH is 6.5–7.0. The strain tolerates 10% (w/v) NaCl, but not 12%. Growth occurs on TSA and NA, but not on MacConkey agar. The strain hydrolyzes aesculin, but not starch, chitin, casein, collagen, CM-cellulose, DNA, and xylan. The following substrates are utilized for growth: D-glucose, L-arabinose, D-mannose, D-maltose, D-fructose, D-xylose, D-cellobiose, D-melezitose, methyl-α-D-glucopyranoside (w), D-mannitol, D-sorbitol, and glycerol. The following substrates are not utilized for growth: *N*-acetyl-glucosamine, L-rhamnose, D-galactose, L-xylose, D-sucrose, D-melibiose, L-fucose, D-fucose, L-rhamnose, D-arabinose, L-sorbose, D-tagatose, D-lactose, D-trehalose, D-ribose, methyl-β-D-xylopyranoside, methyl-α-D-mannopyranoside, D-raffinose, D-turanose, D-sucrose, gentiobiose, salicin, glycogen, amygdalin, arbutin, esculin, inulin, starch, glycogen, D-adonitol, xylitol, dulcitol, inositol, D-arabitol, L-arabitol, erythriol, acetate, 3-hydroxybutylate, 3-hydroxybenzoate, 4-hydroxybenzoate, malonate, valerate, citrate, lactate, 5-ketogluconate, 2-ketogluconate, propionate, caprate, malate, phenylacetate, itaconate, adipate, suberate, gluconate, L-alanine, L-histidine, L-proline, and L-serine. In API 20E and API 20NE tests, the ONPG reaction, Voges-Proskauer test, arginine dihydrolase, and urease production are positive; nitrate reduction, gelatin and aesculin hydrolysis, hydrogen sulfide production, lysine decarboxylase, ornithine decarboxylase, tryptophane deaminase, β-galactosidase, and indole production are all negative. Acid is weakly produced from L-arabinose, but not from D-mannitol, inositol, D-sorbitol, L-rhamnose, D-sucrose, D-melibiose, D-glucose, and amygdalin. The major fatty acids are anteiso-$C_{15:0}$ (32.0%), iso-$C_{15:0}$ (30.5%), and anteiso-$C_{17:0}$ (30.2%). The G+C content of genomic DNA is 41.8 mol%.

The type strain Gsoil 114^T was isolated from a soil sample of a ginseng field in Pocheon Province (South Korea). The strain has been deposited in the Korean Collection for Type Cultures and Deutsche Sammlung von Mikroorganismen und Zellkulturen as KCTC 13944^T and DSMZ 18134^T, respectively.

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