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Chitinophaga soli sp. nov. and *Chitinophaga terrae* sp. nov., Isolated from Soil of a Ginseng Field in Pocheon Province, Korea

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Abstract Two novel strains of the *Cytophaga-Flexibacter-Bacteroides* (CFB) group, designated Gsoil 219^T and Gsoil 238^T, were isolated from soil of a ginseng field of Pocheon Province in Korea. Both strains were Gram-negative, aerobic, nonmotile, nonspore-forming, and rod-shaped. Phylogenetic analysis based on 16S rRNA gene sequences indicated that both isolates belong to the genus *Chitinophaga* but were clearly separated from established species of this genus. The sequence similarities between strain Gsoil 219^T and type strains of the established species and between strain Gsoil 238^T and type strains of the established species ranged from 91.4 to 94.7% and 91.6 to 94.2%, respectively. Phenotypic and chemotaxonomic data (major menaquinone, MK-7; major fatty acids, iso-$C_{15:0}$ and $C_{16:1}$ ω5c; major hydroxy fatty acid, iso-$C_{17:0}$ 3-OH; major polyamine, homospermidine) supported the affiliation of both strains Gsoil 219^T and Gsoil 238^T to the genus *Chitinophaga*. Furthermore, the results of physiological and biochemical tests allowed genotypic and phenotypic differentiation of both strains from the other validated *Chitinophaga* species. Therefore, the two isolates represent two novel species, for which the name *Chitinophaga soli* sp. nov. (type strain, Gsoil 219^T=KCTC 12650^T=DSM 18093^T) and *Chitinophaga terrae* sp. nov. (type strain, Gsoil 238^T=KCTC 12651^T=DSM 18078^T) are proposed.

Keywords: *Chitinophaga soli*, *Chitinophaga terrae*, 16S rRNA gene, polyphasic taxonomy

The genus *Chitinophaga* was originally described by Sangkhobol and Skerman [17] to include strains of filamentous, chitinolytic, gliding bacteria that transform on aging into spherical bodies. Recently, Kämpfer *et al.* [9] proposed *Chitinophaga skermanii* sp. nov. and reclassified [*Flexibacter*] *filiformis*, [*Flexibacter*] *sancti*, [*Cytophaga*] *avensicola*, and [*Flexibacter*] *japonensis* into the genus *Chitinophaga*. At the time of writing, the genus *Chitinophaga* contains 6 species with validly published names; *e.g.*, *C. pinensis*, *C. filiformis*, *C. sancti*, *C. avensicola*, *C. japonensis*, and *C. skermanii*.

During a course of study on the culturable aerobic and facultative anaerobic bacterial communities 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 two of these isolates, strains Gsoil 219^T and Gsoil 238^T. Phenotypic, chemotaxonomic, and phylogenetic analyses establish the affiliation of both isolates to the genus *Chitinophaga*. The data obtained in this study suggest that the two isolates represent two novel species of the genus *Chitinophaga*, and the names *Chitinophaga soli* sp. nov. and *Chitinophaga terrae* sp. nov. are proposed.

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Materials and Methods

Isolation of Bacterial Strain and Culture Condition

Strains Gsoil 219^T and Gsoil 238^T were originally isolated from a soil sample of a ginseng field in Pocheon Province (latitude 37°53' N, longitude 127°9' E), Korea. The soil sample was thoroughly 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. The purified colonies were tentatively identified by partial sequences of the 16S rRNA gene [8]. As they could grow well both on full-strength modified R2A agar and on commercial R2A agar (Difco), all strains were routinely cultured on R2A agar or one-half strength R2A agar (Difco) 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 [2]. Cell morphology was observed under a Nikon light microscope at ×1,000, with cells grown for three 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. Carbon-source utilization and enzyme activities were tested by using the API 20 NE, API ID 32 GN, and API ZYM test kits (bioMérieux). Degradation of DNA was determined using DNase agar from Scharlau (Scharlau Chemie S.A., Barcelona, Spain), supplemented with 1 M HCl; the hydrolysis of casein, chitin, and starch was assessed as described by Atlas [1]; degradation of lipid was performed according to Kouker and Jaeger [12]; degradation of xylan, cellulose, and collagen was determined as described by Ten *et al.* [22, 23], and reactions were read after five days. Anaerobic growth was performed in serum bottles, 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 five days of incubation. Salt tolerance was tested on R2A medium supplemented with 1–15% (w/v) NaCl after five days of incubation. Growth on nutrient agar and trypticase soy agar (TSA) 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.* [10]. The 16S rRNA gene full 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 [24]. Gaps were edited in the BioEdit program [7]. The evolutionary distances were calculated using the Kimura two-parameter model [11]. The phylogenetic trees were constructed by using the neighbor-joining method [16] and the maximum-parsimony method [6] using the MEGA3 Program [13] with bootstrap values based on 1,000 replications [5].

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 [15]. DNA base composition was determined using the HPLC method. DNA was enzymatically degraded into nucleotides as described by Mesbah *et al.* [14]. 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.* [4], 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, Isoprenoid Quinones, and Polyamines

Cellular fatty acids were analyzed in organisms grown on TSA 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 Identification software package [18]. 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.* [20]. Polyamines were extracted and analyzed according to Busse and Auling [3] and Schenkel *et al.* [19], respectively.

Nucleotide Sequence Accession Numbers

The 16S rRNA gene sequence of strain Gsoil 219^T and Gsoil 238^T determined in this study has been deposited in NCBI/EMBL/DDBJ under the accession numbers AB267723 and AB267724, respectively. The accession numbers of the reference strains, which are closely related to strains Gsoil 219^T and Gsoil 238^T, are indicated in Fig. 1.

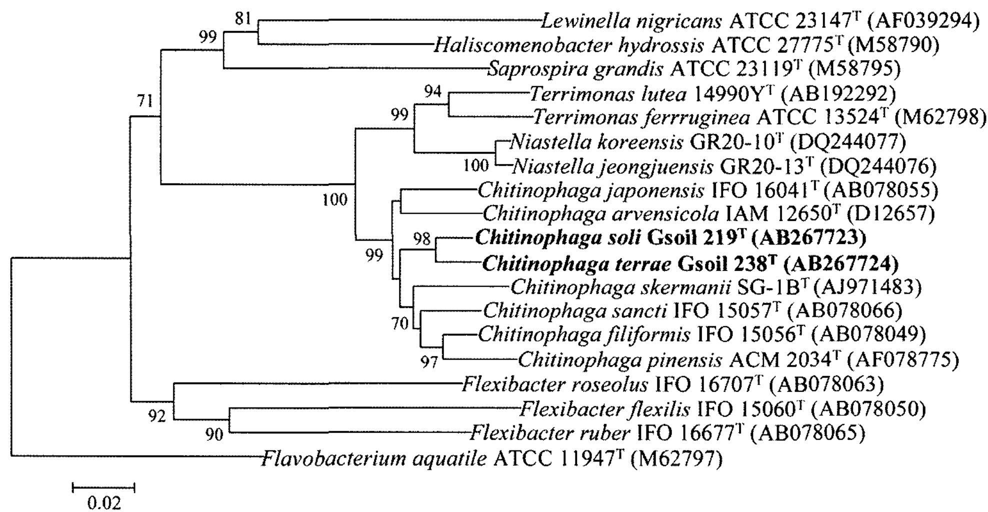

Fig. 1. Phylogenetic tree constructed from a comparative analysis of 16S rRNA gene sequences showing the relationships of *Chitinophaga soli* Gsoil 219^T and *Chitinophaga terrae* Gsoil 238^T with other related species.

This tree was made using the neighbor-joining method with a Kimura two-parameter distance matrix and pairwise deletion. The sequence of *Flavobacterium aquatile* ATCC 11947T (M62797) was used as the outgroup. Bootstrap values (expressed as percentages of 1,000 replications) greater than 70% are shown at the branch points. Bar, 0.02 substitutions per 1 nucleotide position.

RESULTS AND DISCUSSION

Morphological and Phenotypic Characteristics

Morphological observation of strains Gsoil 219^T and Gsoil 238^T grown on R2A agar plates for three days revealed that the cells were similar to each other, *i.e.*, nonmotile rods, 0.5–0.8 µm in width, and 0.9–1.4 µm in length. Colonies grown on R2A agar were also similar to each other; smooth, circular, convex, opaque, yellowish, and 1.0–1.5 mm in diameter. Both isolates grew well on nutrient agar and trypticase soy agar (TSA). Cultivated on a R2A agar medium, strain Gsoil 219^T grew at temperatures from 4 to 30°C and at pH values between 6.0 and 8.5, whereas strain Gsoil 238^T grew at temperatures from 15 to 30°C and at pH values between 5.5 and 8.5. The optimal temperature and pH for growth of both strains were 30°C and pH 7.0. The NaCl tolerance range for growth of strain Gsoil 219^T was 0–1.0% (w/v), whereas that for growth of strain 238^T was 0–3.0% (w/v). Other physiological characteristics of strains Gsoil 219^T and Gsoil 238^T are summarized in the species description. Phenotypic and chemotaxonomic characteristics that differentiate these two strains from closest phylogenetic relatives are listed in Table 1.

Cellular Fatty Acid, Menaquinone Compositions, and Polyamines

The major respiratory quinone and polyamine of both strains were MK-7 and homospermidine, as was the case for the other species in the genus *Chitinophaga* [9]. As shown in Table 2, the major fatty acids of both strains were iso-$C_{15:0}$, $C_{16:1}$ ω5c, and the hydroxy fatty acids were iso-$C_{17:0}$ 3-OH and iso-$C_{15:0}$ 3-OH, which are consistent with other related species of the genus *Chitinophaga* [9]. However, qualitative and quantitative differences in the fatty acid compositions distinguished two strains, Gsoil 219^T and Gsoil 238^T, from the other species in the genus *Chitinophaga* (Table 2).

DNA G+C Content

The DNA G+C contents of the strains Gsoil 219^T and Gsoil 238^T were 43.2 and 46.2 mol%, respectively.

Phylogenetic Analysis

The lengths of the almost complete 16S rRNA gene sequences of strains Gsoil 219^T and Gsoil 238^T were 1,435 and 1,468 bp, respectively. Phylogenetic analysis based on 16S rRNA gene sequences indicated that both strains belong to genus *Chitinophaga* (Fig. 1). However, the 16S rRNA gene sequence similarity between strains Gsoil 219^T and Gsoil 238^T was 96.8%, suggesting that they are different at the species level [21]. Strain Gsoil 219^T showed the highest 16S rRNA gene sequence similarity to *Chitinophaga filiformis* IFO 15056^T (94.7%), followed by *Chitinophaga sancti* IFO 15057^T (93.6%), *Chitinophaga skermanii* SG-1B^T (93.5%), *Chitinophaga japonensis* IFO 16041^T (93.0%), *Chitinophaga pinensis* ACM 2034^T (92.6%), and *Chitinophaga avensicola* IAM 12650^T (91.4%). Strain Gsoil 238^T showed the highest 16S rRNA gene sequence similarity with *Chitinophaga japonensis* IFO 16041^T (94.2%), followed by *Chitinophaga sancti* IFO 15057^T (93.8%), *Chitinophaga filiformis* IFO 15056^T (93.4%), *Chitinophaga avensicola* IAM 12650^T (92.6%), *Chitinophaga skermanii* SG-1B^T (92.4%), and *Chitinophaga pinensis* ACM 2034^T (91.6%). These values (<97%) were low

Table 1. Comparison of the phenotypic characteristics of strains Gsoil 219 T and Gsoil 238 T, and related type strains of species of genus *Chitinophaga*.

Characteristic	1	2	3	4	5	6	7	8
Pigment	Yellow	Yellow	Yellow orange	Yellow orange	Golden yellow	Golden yellow	Yellow	Yellow
Cells length (μm)	1.0–1.3	0.9–1.4	0.6–4	2–18	2–15	30–80	1–2	<40
Oxidase	+	+	+	+	ND	+	+	+
Catalase	+	+	+	+	–	–	+	+
Growth at 37°C	–	–	–	+	–	+	+	+
Highest NaCl tolerance	1.0%	3.0%	2.0%	2.0%	1.0%	0.3%	ND	ND
Urease	–	+	–	ND	ND	ND	–	+
Gelatin liquefaction	+	+	–	+	+	+	+	+
Chitin degradation	–	–	–	–	–	+	ND	+
Assimilation of								
N-Acetyl-D-glucosamine	+	+	+	+	–	–	+	+
L-Arabinose	+*	+	+*	+	+*	–	–	+
Gluconate	+*	+	–	–	+*	–	–	–
D-Mannose	+	+	+	+	+	+	+	+
D-Maltose	+	+	+	+	+	+	+	+
α-D-Melibiose	+	+	+	+	+$	+*	+*	–
L-Rhamnose	W	+$	+	+	–	–	–	+*
D-Ribose	–	–	+	–	–	–	–	–
Sucrose	+	+	+*	+	–	+	–	+*
Salicin	+	+	+	+	–	+	+	+*
Inositol	–	–	–	–	–	–	–	–
G+C content (mol%)	43.2	46.2	46.0	49.8	43.3	45.0	40.7	45.2

Strains: 1, *Chitinophaga soli* Gsoil 219T; 2, *Chitinophaga terrae* Gsoil 238T; 3, *Chitinophaga arvensicola* DSM 3695T; 4, *Chitinophaga japonensis* DSM 13484T; 5, *Chitinophaga sancti* DSM 784T; 6, *Chitinophaga filiformis* CCUG 12809T; 7, *Chitinophaga skermanii* CC-SG1BT; 8, *Chitinophaga pinensis* DSM 2588T. All the strains were isolated from soil, and acid production from glucose and H_2S production are absent. Data for taxa 3-8 from Kämpfer *et al.* [9]. The carbon assimilation tests were read after 48h of incubation; *positive after 7d of incubation, $ positive after 2 weeks of incubation. ND, not determined.

enough to place strains Gsoil 219T and Gsoil 238T as two novel species in the genus *Chitinophaga*, according to Stackebrandt and Goebel [21].

DNA-DNA Hybridization

The DNA-DNA relatedness value between strains Gsoil 219T and Gsoil 238T was 23%, which is below the threshold (70%) recommended for determining bacterial species [25]. It is clear from DNA-DNA hybridization experiments that strains Gsoil 219T and Gsoil 238T belong to two separate genomic species in the genus *Chitinophaga*.

Taxonomic Conclusions

The results obtained from the phenotypic and phylogenetic characterizations indicated that strains Gsoil 219T and Gsoil 238T belong to the genus *Chitinophaga*. The phylogenetic distinctiveness and DNA-DNA hybridization experiments confirmed that strains Gsoil 219T and Gsoil 238T represent two separate species distinct from recognized *Chitinophaga* species. There were some phenotypic differences between strains Gsoil 219T and Gsoil 238T, and phylogenetically related *Chitinophaga* species (Table 1). Therefore, on the basis of the data presented, strains Gsoil 219T and Gsoil 238T should be classified in the genus *Chitinophaga* as two novel species, for which the names *Chitinophaga soli* sp. nov. and *Chitinophaga terrae* sp. nov. are proposed.

Description of *Chitinophaga soli* sp. nov.

Chitinophaga soli (so'li. L. n. *solum* soil; L. gen. n. *soli* of the soil).

Cells are Gram-negative, aerobic, nonmotile, and rod shaped, 0.5–0.7 μm in diameter, and 1.0–1.3 μm in length for three days culture on R2A agar. Colonies grown on a R2A agar (Difco) for three days are smooth, circular, convex, opaque, and yellowish. Optimal growth is observed at 30°C and pH 7.0. Temperature, pH, and NaCl tolerance ranges are 4–30°C, pH 6.0–8.5, and 0–1% (w/v), respectively. It cannot reduce nitrate. Anaerobic growth does not occur. It cannot degrade DNA, chitin, xylan, cellulose, or collagen. Substrate utilization, enzyme production, and other physiological characteristics are indicated in Table 1. In addition to the sources in Table 1, it utilizes L-alanine, L-fucose, gluconate, glucose, histidine, mannitol, L-lactate, L-proline, L-serine, and D-sorbitol. It does not utilize acetate, adipate, caprate, citrate, glycogen, 3-hydroxybenxoate,

Table 2. Cellular fatty acid profiles of stains Gsoil 219^T and Gsoil 238^T, and related type strains of species of genus *Chitinophaga*.

	1	2	3	4	5	6	7	8
Unknown 13.565	0.5		3.6	3.1	3.2	2.5	4.4	2.6
$C_{14:0}$	1.6	1.4	1.4	0.5	0.7	0.9	1.8	0.7
$C_{15:0}$ 2-OH			0.3					
Iso-$C_{15:0}$ 3-OH			3.0	2.4	3.4	3.3	2.6	3.1
Iso-$C_{15:0}$	**53.2**	**52.9**	**35.3**	**40.0**	**44.0**	**37.3**	**47.3**	**30.4**
Anteiso-$C_{15:0}$	0.9		0.6	0.5				
Unknown 11.543			0.5				0.8	
$C_{15:0}$	1.0		0.4				0.4	0.4
Unknown 14.959	0.9		0.3				0.4	0.3
Summed feature 2			0.4	0.5			0.4	
Iso-$C_{17:1}$ ω9c			0.3		1.1			
Summed feature 3			3.7	3.3	16.0	11.2	3.5	7.7
Summed feature 4	2.4	4.5		1.3	0.8		0.4	
$C_{16:0}$ 10 methyl			0.4	0.9				
$C_{16:1}$ ω11c			0.5	1.0	0.9		0.4	1.9
$C_{16:1}$ ω5c	**18.2**	**20.3**	**33.6**	**22.2**	**13.5**	**25.5**	**24.4**	**33.2**
$C_{16:0}$ 2-OH				3.0	0.7	1.1		0.7
$C_{16:0}$	4.6	5.4	5.1	3.0	4.2	4.3	3.5	4.2
Iso-$C_{16:0}$			0.3					
Iso-$C_{16:0}$ 3-OH	3.5	4.0	0.5	0.4			0.4	0.4
$C_{16:0}$ 3-OH	2.5	1.6	1.7	0.8	0.5	1.2	1.6	1.2
Anteiso-$C_{17:0}$				0.4				
$C_{17:0}$ 2-OH			0.4	0.4				
Iso-$C_{17:0}$			0.4	1.8	0.6		0.6	0.4
Unknown 16.582			0.8	1.1	1.3	0.9	0.7	1.1
Iso-$C_{17:0}$ 3-OH	10.0	9.8	5.9	13.0	9.1	11.8	5.0	11.5
$C_{18:0}$	0.6							

Strains: 1, *Chitinophaga soli* Gsoil 219^T; 2, *Chitinophaga terrae* Gsoil 238^T; 3, *Chitinophaga arvensicola* DSM 3695^T; 4, *Chitinophaga japonensis* DSM 13484^T; 5, *Chitinophaga sancti* DSM 784^T; 6, *Chitinophaga filiformis* CCUG 12809^T; 7, *Chitinophaga skermanii* CC-SG1B^T; 8, *Chitinophaga pinensis* DSM 2588^T. Data for taxa 3-8 from Kämpfer *et al.* [9]. Summed feature 2 comprises any combination of $C_{12:0}$ aldehyde, $C_{14:0}$ 3-OH, and/or iso-$C_{16:1}$. Summed feature 3 comprises any combination of iso-$C_{15:0}$ 2-OH and/or $C_{16:1}$ ω7c. Summed feature 4 comprises any combination of iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B.

4-hydroxybenzoate, 3-hydroxybutyrate, itaconate, 2-ketogluconate, 5-ketogluconate, malate, malonate, phenylacetate, propionate, suberate, and valerate. According to the API ZYM gallery, it is positive for *N*-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, cystine arylamidase, esterase (C4), esterase lipase (C8), α-fucosidase, α-glucosidase, β-glucosidase, leucine arylamidase, trypsin, and valine arylamidase. It is negative for chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, lipase (C14), α-mannosidase, and naphthol-AS-BI-phosphohydrolase. According to the API 20NE gallery, it is positive for β-glucosidase, but negative for arginine dihydrolase, β-galactosidase, tryptophan degradation, and glucose fermentation. MK-7 is the strain's predominant menaquinone and iso-$C_{15:0}$ and $C_{16:1}$ ω5c are the predominant cellular fatty acids. The G+C content of genomic DNA is 43.2 mol%.

The type strain, Gsoil 219^T (=KCTC 12650^T=DSM 18093^T), was isolated from soil of a ginseng field of Pocheon Province, Korea.

Description of *Chitinophaga terrae* sp. nov.

Chitinophaga terrae (ter'ra.e. L. gen. n. *terrae* of the earth)

Cells are Gram-negative, aerobic, nonmotile, and rod-shaped, 0.5–0.8 μm in diameter, and 1.0–1.3 μm in length for three day's culture on R2A agar. Colonies grown on a R2A agar (Difco) for three days are smooth, circular, convex, opaque, and yellowish. Optimal growth is observed at 30°C and pH 7.0. Temperature, pH, and NaCl tolerance ranges are 15–30°C, pH 5.5–8.5, and 0–3.0% (w/v), respectively. It can reduce nitrate to nitrite. Anaerobic growth does not occur. It cannot degrade DNA, chitin, xylan, cellulose, or collagen. Substrate utilization, enzyme production, and other physiological characteristics are indicated in Table 1. In addition to the sources in Table 1, it utilizes acetate, citrate, L-fucose, glucose, gluconate, histidine, 5-ketogluconate, L-proline, and valerate. It does not utilize adipate, L-alanine, caprate, citrate, glycogen, 3-hydroxybenxoate, 4-hydroxybenzoate, 3-hydroxybutyrate, itaconate, 2-ketogluconate, L-lactate, mannitol, malate, malonate, phenylacetate, propionate, L-serine, D-sorbitol and

suberate. According to the API ZYM gallery, it is positive for *N*-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, chymotrypsin, cystine arylamidase, esterase (C4), esterase lipase (C8), α-fucosidase, β-galactosidase, α-glucosidase, β-glucosidase, leucine arylamidase, trypsin, and valine arylamidase. It is negative for β-glucuronidase, lipase (C14), α-mannosidase, and naphthol-AS-BI-phosphohydrolase. According to the API 20NE gallery, it is positive for β-galactosidase, β-glucosidase, arginine dihydrolase, and glucose fermentation, but negative for tryptophan degradation. MK-7 is the strain's predominant menaquinone and iso-$C_{15:0}$ and $C_{16:1}$ ω5c are the predominant cellular fatty acids. The G+C content of the genomic DNA is 46.2 mol%.

The type strain, Gsoil 238^T (=KCTC 12651^T=DSM 18078^T), was isolated from soil of a ginseng field of Pocheon Province, Korea.

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