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Mesorhizobium hankyongi sp. nov. Isolated from Soil of Ginseng Cultivating Field

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Abstract

A Gram-negative, non-spore-forming and rod-shaped, bacterium (designated Gsoil 531^T) was isolated from soil of a ginseng field. On the basis of 16S rRNA gene sequence, strain Gsoil 531^T clustered with species of the genus *Mesorhizobium* and was closely related to *M. camelthorni* CCNWXJ 40-4^T (98.9%) and *M. alhagi* CCNWXJ12-2^T (98.7%). The DNA G + C content was 62.9 mol% and the predominant quinone was ubiquinone-10 (Q-10). The major cellular fatty acids were $C_{16:0}$, $C_{19:0}$ cyclo *ω*8*c* and summed feature 8 ($C_{18:1}$ *ω*7*c*/$C_{18:1}$ *ω*6*c*). The DNA–DNA hybridization values were less than 35.0% between novel isolate and its closest reference strains *M. camelthorni* HAMBI 3020^T, *M. alhagi* HAMBI 3019^T and *M tamadayense* LMG 26736^T. Physiological, biochemical and low values of DNA–DNA hybridization results enabled strain Gsoil 531^T to be differentiated genotypically and phenotypically from all known species of the genus *Mesorhizobium*. Therefore, strain Gsoil 531^T signifies a novel species of the genus *Mesorhizobium*, for which the name *Mesorhizobium hankyongi* sp. nov. is proposed. The type strain Gsoil 531^T (= KACC 19443^T = LMG 30463^T).

Introduction

The soil of ginseng field is good habitat for soil-borne microbial communities. Ginseng plants are able to grow in the shade. To study the microbial community of ginseng soil, a novel bacterium, designated Gsoil 531^T, was isolated. Phylogenetic analysis allocated strain Gsoil 531^T to the genus *Mesorhizobium* of the family *Phyllobacteriaceae*, order *Rhizobiales*, class *Alphaproteobacteria* and phylum *Proteobacteria*. The genus *Mesorhizobium* was first proposed by Jarvis et al. [13], by transferring the five *Rhizobium* species to *Mesorhizobium* gen. nov. Members of this genus are Gram-stain-negative, and are rod-shaped. At the time of writing the genus contained more than 40 species (http://www.bacterio.net), including, *Mesorhizobium calcicola* [6]; *Mesorhizobium japonicum* [17], *Mesorhizobium sediminum* [33] and *Mesorhizobium kowhaii* [6].

The NCBI GenBank accession number for the 16S rRNA gene sequence of strain Gsoil 531^T is KY078835.

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Materials and Methods

Isolation of Bacterial Strain

To study the culturable aerobic, facultative bacterial and anaerobic strains living in the soil of a ginseng field Pocheon province [(37°91′96″N, 127°22.4′59.1″E) South Korea], a number of novel bacterial strains including novel genus (*Pseudobacter, Panacibacter, Anseongella*) and novel species (*Lysobacter pocheonensis, Arachidicoccus ginsenosidivorans,, Mucilaginibacter ginsenosidivorans* and *Aeromicrobium panacisoli*) were isolated on

R2A and 1/2 R2A agar plates [14, 24–29]. Here, in this study, we report another novel bacterial strain, designated Gsoil 531^T, which appeared to be a member of the genus *Mesorhizobium*.

Strain Gsoil 531^T was routinely cultured on R2A agar at 30 °C and maintained as a glycerol suspension (R2A broth with 20%, v/v), at − 80 °C. The reference strains (*M. camelthorni* HAMBI 3020^T, *M. alhagi* HAMBI 3019^T, *M. tamadayense* LMG 26736^T) were obtained from two different culture collections and were used in most of the comparative analysis.

Strain Gsoil 531^T was deposited to the Korean Agricultural Culture Collection under the accession number (=KACC 19443^T) and Belgian Coordinated Collections of Microorganisms (BCCM/LMG) under the accession number (=LMG 30463^T).

Morphological and Biochemical Characteristics

The Gram reaction was determined using the non-staining method [2]. Cell morphology was examined by transmission electron microscope (Hitachi SU-3500), after cells grown on R2A agar medium for 2 days at 30 °C. Catalase and oxidase tests were performed as described previously [3]. Biochemical and phenotypic tests were carried out using API ID 32GN, API 20NE and API ZYM test kits according to the manufacturer (bioMérieux, France) instructions. Tests for degradation of DNA, casein, Tween-20 and starch were evaluated after 5 days of incubation at 30 °C [1]. Growth at different temperatures (4, 10, 15, 18, 20, 25, 30, 37, 42 and 45 °C) and various pH values (pH 3.5–10.0 at intervals of 1.0 and 0.5 pH units) was assessed after 5 days of incubation at 30 °C on R2A agar medium. The following buffers (final concentration, 20 mM) were used to adjust the pH of R2A broth: acetate buffer (pH 3.5–5.5), phosphate buffer (pH 6.0–8.0) and Tris buffer (pH 8.5–10.0). Salt tolerance was tested on R2A agar medium supplemented with 1–10% (w/v at intervals of 1% unit) NaCl after 5 days of incubation at 30 °C. Growth on different media [nutrient agar (NA, Difco), R2A agar (Difco), Luria–Bertani (Difco), DNAse agar (Difco), MacConkey and TSA agar (Difco)] were also evaluated after 5 days of incubation at 30 °C.

Phylogenetic Analysis and DNA G + C Content (mol%) Analysis

Genomic DNA was extracted using a genomic DNA extraction kit (Solgent Co. Ltd, Korea) and the 16S rRNA gene was amplified from the chromosomal DNA using the universal bacterial primer set (800R, 1492R, 27F and 518F) [16]. Then, the purified PCR products were sequenced by Solgent Co. Ltd. (Daejeon, South Korea) as described previously [12]. Almost full-length 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 and EzTaxon-e server [http://www.ezbiocloud.net/eztaxon]. Multiple sequence alignments were performed by Clustal X program [31] and the gaps were edited in the BioEdit program [10]. Evolutionary distances were calculated using the Kimura two-parameter model [15] and the phylogenic trees were constructed with neighbor-joining [22], maximum-likelihood and maximum-parsimony [9] algorithms by using MEGA 6 Program [30] with bootstrap values of 1000 replications [8].

For the measurement of DNA G + C content, genomic DNA of the novel strain was extracted and purified as described by Moore and Dowhan [20] and was enzymatically degraded into nucleosides, and was determined as described before [18] using a reverse-phase HPLC.

DNA–DNA Hybridization

DNA–DNA hybridization experiment was carried out in triplicate by using the fluorometric microplate method [7].

Chemotaxonomic Analysis

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). The crude *n*-hexane-quinone solution was purified using Sep-Pak Vac cartridges silica (Waters) and subsequently analyzed by HPLC as previously described [11]. Cellular fatty acids profiles were determined for strains grown on R2A agar for 48 h. The cellular fatty acids were saponified, methylated, and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acid methyl esters were then analyzed by gas chromatography (model 6890; Hewlett Packard) using the Microbial Identification software package [23]. Strain Gsoil 531^T was examined for their polar lipid contents as described by Minnikin et al. [19].

Results and Discussion

Morphological and Phenotypic Characteristics

Colonies of strain Gsoil 531^T grown on R2A agar plates for 2 days at 30 °C were convex, light yellow colored and rod-shaped, (0.6–1.2 μm in diameter and 0.8–1.6 μm in length) as shown in Fig. S1. Strain Gsoil 531^T was able to grow at

Table 1 Physiological and biochemical characteristics between strain Gsoil 531^T and closely related species of the genus *Mesorhizobium*

Characteristic	1	2	3	4
Isolation sources	Soil	*Alhagi sparsifolia*[a]	*Alhagi sparsifolia*[b]	Root nodules[c]
Colony colour	Light yellow	White[a]	White[b]	White[c]
Growth at/with				
pH 5	−	−	−	+
pH 10	−	−	−	+
2% NaCl	+	+	−	−
Enzyme activity				
Alkaline phosphatase	−	+	+	+
N-acetyl-β-glucosaminidase	+	−	−	−
Cystine arylamidase	−	+	+	+
α-Chymotrypsin	−	−	−	+
Esculin hydrolysis	+	−	−	+
Esterase (C4)	−	+	−	+
Esterase lipase (C8)	−	+	−	+
β-Galactosidase (PNPG)	−	−	+	−
β-Galactosidase	+	−	−	−
β-Glucuronidase	+	−	−	+
β-Glucosidase	+	+	−	+
Lipase (C14)	−	−	−	+
Nitrate reduction	−	+	+	+
Trypsin	−	+	+	+
Urease	−	+	+	+
Assimilation of				
Acetate	−	−	−	+
L-Arbinose	−	+	+	+
N-Acetyl-D-glucosamine	+	−	−	+
Adipate	−	−	+	+
Citrate	−	+	−	−
Caprate	−	−	−	+
L-Fucose	−	−	+	+
D-Glucose	−	−	−	+
Gluconate	+	−	−	+
L-Histidine	−	−	−	+
3-Hydroxy-butyrate	+	+	−	+
Inositol	+	+	+	−
2-Ketogluconate	−	−	+	−
5-Ketogluconate	−	+	+	−
Malate	−	+	−	+
Malonate	−	+	+	+
D-Mannose	+	−	−	+
D-Mannitol	+	−	−	+
D-Maltose	−	+	+	+
D-Melibiose	+	−	+	+
Propionate	−	+	+	+
L-Proline	+	−	+	+
D-Ribose	+	−	−	−
D-Sorbitol	−	+	+	+
D-Sucrose	+	−	−	+
Valerate	+	+	−	−
DNA G+C (mol%)	62.9	59.5–63.7[a]	59.5–63.3[b]	60.3[c]

Table 1 (continued)

All strains are positive for L-rhamnose, *N*-acetyl-D-glucosamine, inositol, D-maltose, glycogen, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and negative for indole production, glucose acidification, gelatin hydrolysis, D-glucose, caprate, phenyl-acetate, salicin, caprate, L-histidine, 4-hydroxy-benzoate, itaconate, suberate, acetate, lactate, 3-hydroxy-benzoate, L-serine, lipase (C14), α-chymotrypsin, α-mannosidase and α-fucosidase. '+' positive; '−' negative

1, Gsoil 531^T; 2, *M. camelthorni* HAMBI 3020^T; 3, *M. alhagi* HAMBI 3019^T; 4, *M. tamadayense* LMG 26736^T. All tests were obtained in this study

[a,b,c]Data taken from: Chen et al. [5], Chen et al. [4], Ramírez-Bahena et al. [21], respectively.

18–42 °C, but not grow bellow 15 and above 42 °C. Furthermore, the physiological characteristics of strain Gsoil 531^T are summarized in the species description and Table 1. Similarly, list of all negative traits are mentioned in Table S1.

Phylogenetic and DNA G + C Content Analysis

Almost complete 16S rRNA gene sequence of strain Gsoil 531^T (1406 nt, accession number KY078835) was assembled using SeqMan software (DNASTAR) program and compared with the 16S rRNA gene sequences of related taxa, which were obtained from the EzBiocloud server [http://www.ezbiocloud.net/eztaxon] and GenBank data base, it was noticed that novel isolate belong to the genus *Mesorhizobium* and show highest sequence similarity to *M. camelthorni* HAMBI 3020^T (98.9%), and *M. alhagi* HAMBI 3019^T (98.7%). The phylogenetic study (based on the neighbor-joining, maximum-likelihood and maximum-parsimony methods) also approve that strain Gsoil 531^T clustered within the genus *Mesorhizobium* and forming a clade with *M. camelthorni* HAMBI 3020^T and *M. alhagi* HAMBI 3019^T (Fig. 1). DNA of strain Gsoil 531^T was 62.9 mol%.

DNA–DNA Hybridization

The DNA–DNA hybridization relatedness between strain Gsoil 531^T and *M. camelthorni* HAMBI 3020^T, *M. alhagi* HAMBI 3019^T and *M. tamadayense* LMG 26736^T were 32.2 ± 1.46% (30.1 ± 0.9%, reciprocal), 30.9 ± 0.9% (31.1 ± 1.2%, reciprocal) and 27.6 ± 1.01% (25.2 ± 1.8%, reciprocal), respectively; this hybridization value is below the 70% threshold proposed for species delineation, Wayne et al. [32].

Chemotaxonomic Characteristics

The quinone detected in strain Gsoil 531^T was ubiquinone-10 (Q-10), which was similar to the other species of genus *Mesorhizobium*. The major cellular fatty acids of strain Gsoil 531^T were mainly composed of $C_{16:0}$ (18.8%), $C_{19:0}$ Cyclo $\omega 8c$ (25.9%) and summed feature 8 [comprising $C_{18:1}$ $\omega 7c$/$C_{18:1}$ $\omega 6c$ (35.6%)], which were similar to those of described species of genus *Mesorhizobium* (Table 2). The major polar lipids were diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and one unidentified polar lipid (L1). Minor lipids were phosphatidylcholine (PC), eight unidentified polar lipids (L2–L8), and two unidentified phospholipids (PL1–PL2) (Fig. S2). From the polar lipids analysis, the novel isolate share same major polar lipids DPG, PG and PE to the described species of the genus *Mesorhizobium* [33].

Digital Protologue Number

The digital protologue number of strain Gsoil 531^T is TA00389.

Taxonomic Conclusions

In summary, the characteristics of strain Gsoil 531^T are consistent with descriptions of the genus *Mesorhizobium* with regard to morphological, biochemical and chemotaxonomic properties. However, on the basis of phylogenetic distance from known *Mesorhizobium* species indicated by 16S rRNA gene sequence similarities and the combination of unique phenotypic characteristics (Table 1), strain Gsoil 531^T represents a novel species with in the genus *Mesorhizobium*, for which the name *Mesorhizobium hankyongi* sp. nov is proposed.

Description of *Mesorhizobium hankyongi* sp. nov

Mesorhizobium hankyongi (hank.yong'i. N.L. gen. n. *hankyongi* of Hankyong National University where taxonomy study was performed).

Cells are gram-stain-negative, aerobic, non-spore-forming and non-motile. Colonies grown on R2A agar are opaque, circular and light yellow coloured. Growth occurs at 18–42 °C in the presence of 0–2% NaCl (w/v) and at pH 6–8. Optimum growth occurs at 30 °C and pH 7.0 in the absence of NaCl. Oxidase positive and catalase negative. Positive for casein hydrolysis but negative for DNase, starch and Tween-20 hydrolysis. The strain grow well on R2A agar medium, whereas weakly grow on TSA, NA and LB agar media, but

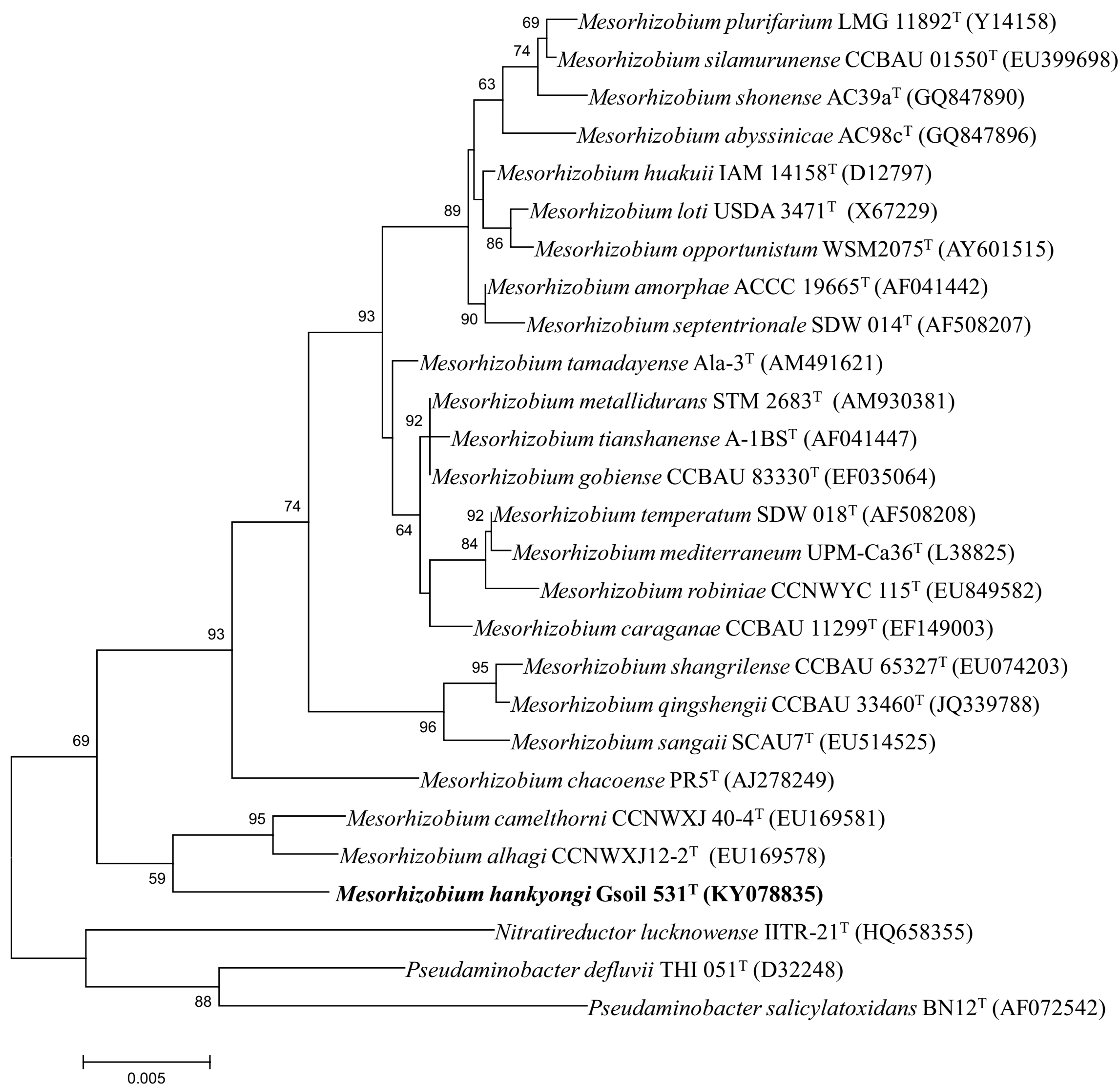

Fig. 1 Phylogenetic tree showing the relationships of strain Gsoil 531^T with other related species of the genus *Mesorhizobium*. The tree was made using the neighbor-joining method. Circle dots indicate generic branches that were also recovered by using maximum-parsimony and maximum-likelihood algorithms. Bootstrap values (expressed as percentages of 1000 replications) greater than 60% are shown at the branch points. Bar 0.005 substitutions per single nucleotide position

did not grow on DNAse agar and MacConkey agar. In API kit system positive for esculin hydrolysis, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, glycogen, D-maltose, L-proline, L-rhamnose, *N*-acetyl-D-glucosamine, D-ribose, Inositol, D-sucrose, 3-hydroxy-butyrate, D-glucose, D-melibiose, D-mannose, D-mannitol, *N*-acetyl-D-glucosamine and gluconate. The predominant quinone is Q-10. The major cellular fatty acids are $C_{16:0}$, $C_{19:0}$ cyclo $\omega 8c$ and summed feature 8. The polar lipids are DPG, PG, PE, PC, nine unidentified polar lipids and two unidentified phospholipids. The DNA G+C content of genomic DNA is 62.9 mol%.

The type strain, Gsoil 531^T (=KACC 19443^T=LMG 30463^T) was isolated from soil of a ginseng field of Pocheon province, South Korea.

Table 2 Fatty acid profiles of strain Gsoil 531^T and related species of the genus *Mesorhizobium*

Fatty acid	1	2	3	4
Saturated				
$C_{16:0}$	18.8	21.6	19.27	11.8
$C_{17:0}$	0.7	–	–	1.0
$C_{18:0}$	5.5	6.3	5.21	6.0
Unsaturated			–	–
$C_{18:1}$ *ω*9*c*	1.1	1.5	–	–
$C_{20:2}$ *ω*9*c*	0.7	–	–	–
Branched-chain fatty acid				
iso-$C_{13:0}$ 3-OH	–	0.6	–	0.8
iso-$C_{15:0}$	0.7	0.5	–	0.5
iso-$C_{17:0}$	4.8	3.1	4.3	3.3
iso-$C_{19:0}$	–	–	0.6	–
Anteiso-$C_{17:1}$ A	1.2	–	–	–
$C_{18:1}$ *ω*7*c* 11-methyl	–	–	3.6	–
$C_{17:0}$ cyclo	0.7	–	–	1.3
$C_{19:0}$ cyclo *ω*8*c*	25.9	26.0	24.3	22.5
Summed feature				
Sum 8; $C_{18:1}$ *ω*7*c*/$C_{18:1}$ *ω*6*c*	35.6	36.8	40.0	46.3

All strains were cultured on R2A agar medium for 48 h at 30 °C. Fatty acids amounting to $<0.5\%$ of the total fatty acids in all strains are not listed. tr, trace amounting (tr $>0.5\%$); '–' not detected

1. Gsoil 531^T; 2. *M. camelthorni* HAMBI 3020^T; 3. *M. alhagi* HAMBI 3019^T; 4. *M. tamadayense* LMG 26736^T

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