Microbacterium ginsengisoli sp. nov., a β -glucosidase-producing bacterium isolated from soil of a ginseng field

Min-Ju Park,¹ Myung Kyum Kim,¹ Ho-Bin Kim,¹ Wan-Taek Im,² Tae-Hoo Yi,¹ Se-Young Kim,¹ Nak-Kyun Soung³ and Deok-Chun Yang¹

Correspondence
Deok-Chun Yang
dcyang@khu.ac.kr

¹Department of Oriental Medicinal Material and Processing College of Life Science, Kyung Hee University, 1 Seocheon-dong, Giheung-gu, Yongin-si, Gyeonggi-do 446-701, Republic of Korea

²Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1, Guseong-dong, Yuseong-gu, Daejeon 305-701, Republic of Korea

³Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD 20892, USA

Strain Gsoil 259^T, a β -glucosidase-producing bacterium, was isolated from a soil sample from a ginseng field in the Republic of Korea and characterized in order to determine its taxonomic position. Cells were Gram-positive, heterotrophic, strictly aerobic, non-motile short rods. 16S rRNA gene sequence analysis revealed that strain Gsoil 259^T belonged to the genus *Microbacterium* and was closely related to *Microbacterium arborescens* IFO 3750^T (98.5 %) and *Microbacterium imperiale* IFO 12610^T (97.9 %). However, it has low values for DNA–DNA relatedness with the above strains (20.7 and 17.5 %, respectively). Strain Gsoil 259^T possessed chemotaxonomic markers that were consistent with classification in the genus *Microbacterium*, i.e. MK-11 and MK-12 were the major menaquinones and anteiso-C_{17:0}, anteiso-C_{15:0} and iso-C_{16:0} were the predominant cellular fatty acids. The DNA G + C content was 69.4 mol%. The cell-wall sugar was rhamnose and the diamino acid in the cell-wall peptidoglycan was ornithine. On the basis of data from this polyphasic study, strain Gsoil 259^T represents a novel species of the genus *Microbacterium*, for which the name *Microbacterium ginsengisoli* sp. nov. is proposed. The type strain is Gsoil 259^T (=KCTC 19189^T =DSM 18659^T).

The genus *Microbacterium* was first described by Orla-Jensen (1919), and its description was emended by Collins *et al.* (1983) and again more recently by Takeuchi & Hatano (1998) to unite the genera *Microbacterium* and *Aureobacterium*. At the time of writing, the genus *Microbacterium* consists of 45 species with validly published names (type species, *Microbacterium lacticum*). The members of the genus were isolated from various environments (Collins & Bradbury, 1992; Takeuchi & Hatano 1998; Rivas *et al.*, 2004; Park *et al.*, 2006).

During a study on the culturable aerobic bacterial community in soil from a ginseng field of Pocheon in the Republic of Korea, a large number of novel bacterial strains were isolated (Im *et al.*, 2005). On the basis of 16S rRNA gene sequencing data, one of these strains, Gsoil

259^T, which appeared on 10-fold-diluted R2A agar, was found to be a member of the genus *Microbacterium* and was subjected to taxonomic investigation.

The Gram reaction was performed by the non-staining method, as described by Buck (1982). Cell morphology and motility were observed under a Nikon light microscope ($\times 1000$ magnification) using the hanging drop method, with cells grown on R2A agar (Difco) for 2 days at 30 °C. Catalase activity was determined by assessing bubble production with 3 % (v/v) H₂O₂, and oxidase activity was determined using 1 % (w/v) tetramethyl-*p*-phenylenediamine. Growth at different temperatures (4, 18, 25, 30, 37 and 42 °C) and various pH values (pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0) was assessed after 5 days incubation in R2A broth. For the pH experiments, three different buffers were used (final concentration, 50 mM): acetate buffer was used for pH 5.0–5.5; phosphate buffer was used for pH 6.0–8.0; Tris buffer was used for pH 8.5–10.0.) Salt tolerance was tested in R2A broth supplemented with 1–10 % (w/v) NaCl after 5 days incubation. Growth on nutrient agar, trypticase soy agar (Difco) and MacConkey agar was also evaluated at 30 °C. Anaerobic

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Gsoil 259^T is AB271048.

An extended neighbour-joining phylogenetic tree based on 16S rRNA gene sequences and a table showing fatty acid compositions for Gsoil 259^T and members of the genus *Microbacterium* are available as supplementary material with the online version of this paper.

growth was performed in serum bottles by adding thioglycolate (1 g l^{-1}) to the R2A broth and replacing the upper air layer with nitrogen gas. Tests for the degradation of DNA [using DNase agar (Scharlau); degradation by DNase was assessed by flooding plates with 1 M HCl], chitin, starch, skimmed milk (Atlas, 1993), lipid (Kouker & Jaeger, 1987), xylan and cellulose (Ten *et al.*, 2004) were performed and evaluated after 2 weeks. The utilization of substrates as sole carbon sources and the activities of some enzymes were tested by using the API ZYM, API 50 CH, API 20NE and API 32GN galleries according to the instructions of the manufacturer (bioMérieux).

To measure the G+C content of the chromosomal DNA, genomic DNA from the novel strain was extracted and purified as described by Moore & Dowhan (1995), degraded enzymically into nucleosides and then the G+C content was 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 n-hexane-quinone solution was purified using Sep-Pak Vac silica 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 for 3 days. The fatty acid methyl esters of cells grown on trypticase soy agar for 3 days at 30°C were prepared according to the protocol of the Sherlock Microbial Identification System (MIDI system; <http://www.midi-inc.com/>) and analysed by GC (6890; Hewlett Packard) using the Microbial Identification software package (Sasser, 1990). The amino acid composition of the cell-wall peptidoglycan was determined using TLC after hydrolysis with 6 M HCl at 100°C for 18 h, as described by Komagata & Suzuki (1987). The cell-wall sugars were analysed as described by Stanek & Roberts (1974). DNA–DNA hybridization was performed fluorometrically using the method of Ezaki *et al.* (1989), with photobiotin-labelled DNA probes and microdilution wells.

Extraction of genomic DNA was performed with a commercial genomic DNA-extraction kit (from Solgent) 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). Full sequences of the 16S rRNA gene were compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from GenBank. Multiple alignments were performed using the CLUSTAL_X program (Thompson *et al.*, 1997), gaps were edited in the BioEdit program (Hall, 1999) and evolutionary distances were calculated using Kimura's two-parameter model (Kimura, 1983). Phylogenetic trees were constructed with neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods, using the MEGA3 program (Kumar *et al.*, 2004) and bootstrap values based on 1000 replications (Felsenstein, 1985).

The physiological characteristics of strain Gsoil 259^T are summarized in the species description and selective characteristics are compared with those of closely related type strains in Table 1.

The 16S rRNA gene sequence of strain Gsoil 259^T determined in this study was a continuous stretch of 1458 bp (base positions 20–1499 with respect to the *Escherichia coli* numbering system). In the phylogenetic tree (Fig. 1; an extended version of this tree is available as Supplementary Fig. S1 in IJSEM Online), strain Gsoil 259^T clustered within the genus *Microbacterium* of the class *Actinobacteria*. The highest levels of 16S rRNA gene sequence similarity (98.5 and 97.9 %) were found with respect to *Microbacterium arborescens* IFO 3750^T and *Microbacterium imperiale* IFO 12610^T, but the corresponding levels of DNA–DNA relatedness were 20.7 and 17.5 %, respectively. This level is sufficiently low to permit the classification of strain Gsoil 259^T within a distinct species with respect to previously described type strains (Wayne *et al.*, 1987).

The chemotaxonomic characteristics of strain Gsoil 259^T were in agreement with the results of the phylogenetic inference, confirming that the novel bacterium belongs to the genus *Microbacterium*. The major menaquinones of Gsoil 259^T were MK-11 (37 %) and MK-12 (63 %), matching the profile reported for the phylogenetically closest species, *M. arborescens* and *M. imperiale* (Takeuchi & Hatano, 1998). The cellular fatty acids (representing >1 % of the total) comprised branched fatty acids anteiso- $\text{C}_{15:0}$ (32.80 %), iso- $\text{C}_{15:0}$ (3.30 %), iso- $\text{C}_{16:0}$ (19.47 %), anteiso- $\text{C}_{17:0}$ (40.37 %) and iso- $\text{C}_{17:0}$ (1.34 %) and straight-chain fatty acid $\text{C}_{16:0}$ (1.84 %) (Supplementary Table S1). However, the novel bacterium clearly differed from related species in terms of cell-wall composition (Table 1). The cell-wall diamino acid of Gsoil 259^T was ornithine, while that of *M. arborescens* and *M. imperiale* was lysine; only rhamnose was found in the cell wall of Gsoil 259^T, whereas the phylogenetically related species contained several sugars. The DNA G+C content of strain Gsoil 259^T was 69.4 mol%.

On the basis of the phenotypic, chemotaxonomic and phylogenetic data, strain Gsoil 259^T represents a novel species of the genus *Microbacterium*, for which the name *Microbacterium ginsengisoli* sp. nov. is proposed.

Description of *Microbacterium ginsengisoli* sp. nov.

Microbacterium ginsengisoli (gin.seng.i.so'li. N.L. n. *ginsengum* ginseng; L. n. *solum* soil; N.L. gen. n. *ginsengisoli* of soil of a ginseng field, the source of the type strain).

Cells are Gram-positive, heterotrophic, strictly aerobic, non-motile, short rods, about $0.3\text{--}0.4 \mu\text{m}$ in width and $0.6\text{--}0.8 \mu\text{m}$ in length. Colonies grown on R2A agar for 2 days are smooth, circular, convex, transparent and yellow. Growth occurs at $18\text{--}30^\circ\text{C}$, pH 5–9 and in the presence of up to 4 % (w/v) NaCl. No growth occurs on MacConkey agar. Catalase- and oxidase-positive. Arginine

Table 1. Selective phenotypic characteristics of strain Gsoil 259^T and related type strains of species of the genus *Microbacterium*

Strains: 1, Gsoil 259^T; 2, *M. arborescens* IFO 3750^T; 3, *M. imperiale* IFO 12610^T; 4, *M. ulmi* LMG 20991^T. Data for reference strains are from Takeuchi & Hatano (1998), Rivas *et al.* (2004) and Park *et al.* (2006). All four strains are positive for acid production from cellobiose, fructose, maltose, mannose, rhamnose, trehalose, D-xylose, mannitol and aesculin, for utilization of D-glucose, maltose and mannitol and for hydrolysis of 2-naphthyl β -D-galactopyranoside and 1-naphthyl *N*-acetyl- β -D-glucosaminide. All four strains are negative for the production of arginine dihydrolase, acid production from 2-ketogluconate, D- and L-fucose, D-lyxose, ribose, sorbose, D-tagatose, L-xylose, adonitol, D- and L-arabitol, dulcitol, sorbitol, xylitol, inulin and glycogen, for utilization of 3-hydroxybenzoate, caprate, itaconate, malonate, suberate, L-fucose, D-ribose, D-sorbitol and glycogen and for hydrolysis of 2-naphthyl myristate. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2	3	4
Motility	–	+	+	–
Colony colour	Yellow	Dirty orange	Red–orange	White
Growth at 37 °C	–	–	+	+
Catalase	+	+	+	–
Urease	–	+	–	–
Acid production from:				
Gluconate	–	+	+	–
Galactose	–	+	+	+
Lactose	–	+	+	+
Melezitose	–	+	–	–
Methyl β -D-xyloside	–	+	–	+
Glycerol	+	+	–	–
<i>N</i> -Acetylglucosamine	–	+	+	–
Salicin	–	+	+	+
Utilization of:				
3-Hydroxybutyrate	+	–	–	–
Acetate	+	+	+	–
Adipate	–	–	+	–
Citrate	–	w	–	–
Malate	+	+	+	–
Phenylacetate	–	–	–	+
Propionate	+	–	–	+
Valerate	+	–	+	–
L-Arabinose	–	+	+	–
Mannose	+	–	–	+
Inositol	+	–	–	–
L-Alanine	+	–	–	–
L-Proline	+	–	–	–
L-Serine	+	–	–	–
<i>N</i> -Acetylglucosamine	–	+	w	–
Hydrolysis of:				
Gelatin	–	+	–	+
2-Naphthyl butyrate*	+	+	–	–
2-Naphthyl caprylate*	+	+	–	–
6-Bromo-2-naphthyl α -D-galactopyranoside*	–	+	+	–
2-Naphthyl α -D-glucopyranoside*	+	+	–	–
6-Bromo-2-naphthyl β -D-glucopyranoside*	+	+	+	–
DNA G + C content (mol%)	69.4	70.9	71.3	69.0
Cell-wall sugar(s)†	Rha	6dTal, Man, Gal	Rha, Man, Gal	Gal, Fuc, Xyl, Rha
Cell-wall diamino acid	Orn	Lys	Lys	Orn
Major menaquinones	11, 12	11, 12	11, 12	12, 13

*Data from API ZYM tests. The intensity of the colour was measured on a scale from 0 to 5. Values of 0 and 1 were interpreted as negative and values between 2 and 5 were interpreted as positive (Mudarris *et al.*, 1994).

†6dTal, 6-Deoxytalose; Fuc, fucose; Gal, galactose; Man, mannose; Rha, rhamnose; Xyl, xylose.

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the phylogenetic relationships of strain Gsoil 259^T and members of the genus *Microbacterium*. Filled circles indicate branches that were also recovered by using the maximum-parsimony algorithm. Bootstrap percentages (based on 1000 replications) greater than 60% are shown at branch points. Bar, 0.02 substitutions per nucleotide position.

dihydrolase and urease activities are absent. Negative for aerobic and anaerobic nitrate reduction. Aesculin and DNA are hydrolysed, but starch, xylan, gelatin, chitin, cellulose and skimmed milk are not hydrolysed. Acid is produced from the following carbon sources in the API 50 CH test: fructose, cellobiose, maltose, mannose, rhamnose, sucrose, trehalose, turanose, D-xylose, glycerol and mannitol. Acid is not produced from gluconate, 2-ketogluconate, 5-ketogluconate, D- and L-arabinose, D- and L-fucose, galactose, gentiobiose, lactose, D-lyxose, melezitose, melibiose, raffinose, ribose, sorbose, D-tagatose, L-xylose, methyl α -D-glucoside, methyl α -D-mannoside, methyl β -D-xyloside, adonitol, D- and L-arabitol, dulcitol, erythritol, inositol, sorbitol, xylitol, N-acetylglucosamine, starch, amygdalin, arbutin, inulin, salicin and glycogen. The following carbon sources are utilized in the API 20NE and API 32GN tests: D-glucose, maltose, mannose, L-rhamnose, sucrose, inositol, mannitol, L-alanine, L-proline, L-serine, 3-hydroxybutyrate, acetate, gluconate, malate, propionate and valerate. Negative for the utilization of the following substrates: L-arabinose, L-fucose, melibiose, D-ribose, D-sorbitol, L-histidine, N-acetylglucosamine, salicin, glycogen, 2-ketogluconate, 5-ketogluconate, 3-hydroxybenzoate, 4-hydroxybenzoate, adipate, caprate, citrate, itaconate, D- and L-lactate, malonate, phenylacetate and suberate. In API ZYM assays, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, β -galactosidase, α -glucosidase, β -glucosidase and N-acetyl- β -glucosaminidase activities are present, but alkaline phosphatase, lipase (C14), cystine arylamidase, α -chymotrypsin, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -glucuronidase, α -mannosidase and α -fucosidase are absent. Cell-wall peptidoglycan contains ornithine. Cell-wall sugar is rhamnose. The predominant menaquinones are MK-11 and MK-12. The major fatty acids are anteiso-C_{17:0}, anteiso-C_{15:0} and iso-C_{16:0}. The DNA G + C content is 69.4 mol%.

The type strain, Gsoil 259^T (=KCTC 19189^T =DSM 18659^T), was isolated from a soil sample from a ginseng field in Pocheon in the Republic of Korea.

Acknowledgements

This work was supported by a Korea Science and Engineering Foundation (KOSEF) grant (no. R01-2006-000-11178-0) funded by

the Korean Government (MOST) and by a BK21 research fellowship from the Ministry of Education and Human Resource Development, Republic of Korea.

References

- Atlas, R. M. (1993). *Handbook of Microbiological Media*. pp. 196, 843. Edited by L. C. Parks. Boca Raton, FL: CRC Press.
- Buck, J. D. (1982). Nonstaining (KOH) method for determination of Gram reactions of marine bacteria. *Appl Environ Microbiol* **44**, 992–993.
- Collins, M. D. & Bradbury, J. F. (1992). The genera *Agromyces*, *Aureobacterium*, *Clavibacter*, *Curtobacterium*, and *Microbacterium*. In *The Prokaryotes*, 2nd edn, pp. 1355–1368. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. Berlin: Springer.
- Collins, M. D., Jones, D. & Kroppenstedt, R. M. (1983). Reclassification of *Brevibacterium imperiale* (Steinhaus) and “*Corynebacterium laevaniformans*” (Dias and Bhat) in a redefined genus *Microbacterium* (Orla-Jensen), as *Microbacterium imperiale* comb. nov. and *Microbacterium laevaniformans* nom. rev., comb. nov. *Syst Appl Microbiol* **4**, 65–78.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Felsenstein, J. (1985). Confidence limit on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.
- Hiraishi, A., Ueda, Y., Ishihara, J. & Mori, T. (1996). Comparative lipoquinone analysis of influent sewage and activated sludge by high-performance liquid chromatography and photodiode array detection. *J Gen Appl Microbiol* **42**, 457–469.
- Im, W.-T., Jung, H.-M., Cui, Y.-S., Liu, Q.-M., Zhang, S.-L. & Lee, S.-T. (2005). Cultivation of the three hundreds of bacterial species from soil of a ginseng field and mining the novel lineage bacteria. In *Proceedings of the International Meeting of the Federation of Korean Microbiological Societies*, abstract A035, pp. 169. Seoul: Federation of Korean Microbiological Societies.
- Kim, M. K., Im, W.-T., Ohta, H., Lee, M. & Lee, S.-T. (2005). *Sphingopyxis granuli* sp. nov., a β -glucosidase producing bacterium in the family *Sphingomonadaceae* in α -4 subclass of the *Proteobacteria*. *J Microbiol* **43**, 152–157.
- Kimura, M. (1983). *The Neutral Theory of Molecular Evolution*. Cambridge: Cambridge University Press.

- Komagata, K. & Suzuki, K. (1987).** Lipid and cell-wall analysis in bacterial systematics. *Methods Microbiol* **19**, 161–207.
- Kouker, G. & Jaeger, K.-E. (1987).** Specific and sensitive plate assay for bacterial lipase. *Appl Environ Microbiol* **53**, 211–213.
- Kumar, S., Tamura, K. & Nei, M. (2004).** MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989).** Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Moore, D. D. & Dowhan, D. (1995).** Preparation and analysis of DNA. In *Current Protocols in Molecular Biology*, pp. 2–11. Edited by F. W. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. New York: Wiley.
- Mudarris, M., Austin, B., Segers, P., Vancanneyt, M., Hoste, B. & Bernardet, J. F. (1994).** *Flavobacterium scopthalmum* sp. nov., a pathogen of turbot (*Scophthalmus maximus* L.). *Int J Syst Bacteriol* **44**, 447–453.
- Orla-Jensen, S. (1919).** *The Lactic Acid Bacteria*. Copenhagen: Host & Sons.
- Park, H. Y., Kim, K. K., Jin, L. & Lee, S.-T. (2006).** *Microbacterium paludicola* sp. nov., a novel xylanolytic bacterium isolated from swamp forest. *Int J Syst Evol Microbiol* **56**, 535–539.
- Rivas, R., Trujillo, M. E., Sánchez, M., Mateos, P. F., Martínez-Molina, E. & Velázquez, E. (2004).** *Microbacterium ulmi* sp. nov., a xylanolytic, phosphate-solubilizing bacterium isolated from sawdust of *Ulmus nigra*. *Int J Syst Evol Microbiol* **54**, 513–517.
- Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sasser, M. (1990).** *Identification of bacteria by gas chromatography of cellular fatty acids*, MIDI Technical Note 101. Newark, DE: MIDI Inc.
- Staneck, J. L. & Roberts, G. D. (1974).** Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. *Appl Microbiol* **28**, 226–231.
- Takeuchi, M. & Hatano, K. (1998).** Union of the genera *Microbacterium* Orla-Jensen and *Aureobacterium* Collins *et al.* in a redefined genus *Microbacterium*. *Int J Syst Bacteriol* **48**, 739–747.
- Ten, L. N., Im, W.-T., Kim, M.-K., Kang, M.-S. & Lee, S.-T. (2004).** Development of a plate technique for screening of polysaccharide-degrading microorganisms by using a mixture of insoluble chromogenic substrates. *J Microbiol Methods* **56**, 375–382.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997).** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987).** International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.