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

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Strain DCY37^T was isolated from a soil sample of a ginseng field in the Republic of Korea and characterized in order to determine its taxonomic position. Cells were Gram-staining-positive, heterotrophic, strictly aerobic, non-motile short rods. 16S rRNA gene sequence analysis revealed that strain DCY37^T belongs to the genus *Microbacterium*. According to 16S rRNA gene sequence analysis, it is closely related to *Microbacterium aerolatum* DSM 14217^T (98.8%), *Microbacterium hydrocarbonoxydans* DSM 16089^T (98.5%), *Microbacterium natoriense* JCM 12611^T (98.5%), *Microbacterium foliorum* (98.4%) and *Microbacterium phyllosphaerae* (98.3%). However, DNA–DNA hybridization studies showed reassociation values of less than 70% between representative strains and DCY37^T. The DNA G + C content was 64.5 mol%. Strain DCY37^T possessed chemotaxonomic markers that were consistent with classification in the genus *Microbacterium*, i.e. MK-12 and MK-13 as the major menaquinones and anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0} as the predominant cellular fatty acids. The major cell wall sugars were ribose, xylose and galactose. The diamino acid in cell-wall hydrolysates of strain DCY37^T was ornithine and major cell-wall amino acids were alanine, glycine, D-glutamic acid and serine. The major polar lipids were glycolipid, phosphatidylglycerol, diphosphatidylglycerol and unknown aminolipids. Based on these data, DCY37^T (=KCTC 19526^T =JCM 15516^T) should be classified as the type strain of a novel species of the genus *Microbacterium*, for which the name *Microbacterium ginsengiterrae* sp. nov. is proposed.

Members of the genus *Microbacterium* can be isolated from a wide range of different environmental habitats from soil to insects, human clinical specimens and marine environments. The genus *Microbacterium* (phylum *Actinobacteria*, class *Actinobacteria*, order *Actinomycetales*, family *Microbacteriaceae*) was first reported by Orla-Jensen and was emended by Collins *et al.*. More recently, the genus was emended again to unite the genera *Microbacterium* and *Aureobacterium*. At the time of writing, the genus *Microbacterium* consisted of 68 species with validly published names, including three species (*Microbacterium soli*, *Microbacterium azadirachtae* and *Microbacterium agarici*) whose descriptions were still in press. In this study, we characterized a new isolate belonging to the genus *Microbacterium*. Extensive physiological, chemotaxonomic and phylogenetic analyses were

carried out to determine the precise taxonomic position of strain DCY37^T.

Cell morphology and motility were observed with a Nikon light microscope ($\times 1000$ magnification) using the hanging drop method, with cells grown on R2A agar for 2 days at 30 °C. Transmission electron microscopy was carried out as follows. Bacteria were grown on R2A plates at 30 °C for 24 h. Resuspended cells were placed on carbon- and Formvar-coated nickel grids for 30 s. Grids were floated on one drop of 0.1% (w/v) aqueous uranyl acetate and then viewed with a Carl Zeiss LEO912AB electron microscope at 100 kV under standard operating conditions.

The Gram reaction was performed according to the non-staining method (Buck, 1982). Oxidase activity was evaluated via the oxidation of 1% *p*-aminodimethylaniline oxalate. Catalase activity was revealed by bubble production after the application of 3% (v/v) hydrogen peroxide solution. Growth on nutrient agar (NA), trypticase soy agar (TSA), R2A agar, LB agar and MacConkey agar was evaluated at 30 °C. Growth at different temperatures (4, 18, 25, 30, 37 and 42 °C) and various pH (5.0–10.0 at intervals

The GenBank/EMBL/DBBJ accession number for 16S rRNA gene sequence of strain DCY37^T is EU873314.1.

A transmission electron micrograph of cells of strain DCY37^T, and polar lipid and fatty acid profiles of strain DCY37^T, are available with the online version of this paper.

of 0.5 pH units) was assessed after 5 days of incubation on R2A plates. Salt tolerance was tested in R2 broth supplemented with 0–10 % (w/v) NaCl after 5 days of incubation. Strain DCY37^T and the type strains *Microbacterium hydrocarbonoxydans* DSM 16089^T, *Microbacterium natoriensense*

JCM 12611^T, *Microbacterium aerolatum* DSM 14217^T and *Microbacterium foliorum* DSM 12966^T were grown on TSA for 2 days at 30 °C for fatty acid analysis. The API 20NE, API ZYM and API ID32 GN microtest systems were employed following the recommendations of the manufacturer

Table 1. Phenotypic characteristics differentiating strain DCY37^T and related type strains of species of the genus *Microbacterium*

Taxa: 1, *Microbacterium ginsengiterrae* sp. nov. DCY37^T; 2, *M. hydrocarbonoxydans* DSM 16089^T; 3, *Microbacterium natoriensense* JCM 12611^T; 4, *Microbacterium aerolatum* DSM 14217^T; 5, *Microbacterium foliorum* DSM 12966^T. Data from this study, except where indicated. All five strains were positive for activities of esterase (C4), esterase lipase (C8), arginine dihydrolase, urease, α -glucosidase, β -glucosidase and assimilation of sucrose. All five strains were negative for the reduction of nitrates to nitrites, indole production, alkaline phosphatase, valine arylamidase, cystine arylamidase, trypsin and α -fucosidase activities, assimilation of suberic acid, sodium malonate, potassium 5-ketogluconate, L-fucose, D-sorbitol, capric acid, adipate and phenylacetate, and for gelatin hydrolysis. +, Positive; –, negative; w, weakly positive; ND, no data available; PY, pale yellow; Y, yellow; DY, deep yellow.

Characteristic	1	2	3	4	5
Colour of colonies	PY	Y	PY	Y	DY
Growth at 6 % NaCl	+	+	–	+	–
Enzyme activity (API ZYM)					
Lipase (C14)	–	–	w	w	–
Leucine arylamidase	–	–	+	+	–
α -Chymotrypsin	–	–	+	–	–
Acid phosphatase	–	–	+	–	–
Naphthol-AS-BI-phosphohydrolase	+	–	–	–	–
β -Galactosidase	–	w	+	–	+
β -Glucuronidase	–	–	–	–	w
α -Mannosidase	+	+	+	–	–
Assimilation of (API 20 NE):					
Glucose	–	+	+	–	–
Arabinose	–	+	+	–	–
Mannose	–	+	+	–	–
Mannitol	–	–	+	–	w
N-Acetylglucosamine	–	+	+	–	w
Maltose	–	+	+	–	w
Gluconate	–	+	+	–	w
Malate	–	+	+	–	–
Trisodium citrate	–	+	+	w	w
Assimilation of (API 32 GN):					
L-Rhamnose	w	+	–	–	–
D-Ribose	+	–	–	–	–
Inositol	–	–	+	–	–
Itaconic acid	–	–	–	w	–
Sodium acetate	–	w	+	–	–
Lactic acid	–	w	–	–	–
L-Alanine	–	w	w	–	–
Glycogen	–	+	+	w	+
3-Hydroxybenzoic acid	+	–	w	+	+
L-Serine	–	w	w	–	–
Salicin	–	+	+	w	+
Melibiose	–	–	+	–	–
L-Histidine	–	+	+	w	w
Potassium 2-ketogluconate	+	w	–	w	w
Major menaquinones (MK)	12, 13	11, 12 ^{a*}	9, 10, 11, 12 ^b	11, 12, 13 ^c	10, 11, 12 ^d
DNA G + C content (mol%)	64.5	ND	69.1 ^b	68.1 ^c	67 ^d

*Data from: a, Schippers *et al.* (2005); b, Liu *et al.* (2005); c, Zlamala *et al.* (2002); d, Behrendt *et al.* (2001).

(bioMérieux). The API kits were incubated at 30 °C and results were checked after 24 h and 48 h, except for API ZYM where results were recorded after 10 h. Cells grown in LB for 6 days at 26 °C were used for determination of the respiratory quinone system. Differential phenotypic characteristics between strain DCY37^T and the most closely related type strains of species of the genus *Microbacterium* are summarized in Table 1.

Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), purified by TLC and subsequently analysed by HPLC, as described by Collins & Jones (1981) and Shin *et al.* (1996). For fatty acid analysis, strain DCY37^T and the other type strains (*M. hydrocarbonoxydans* DSM 16089^T, *M. natoriense* JCM 12611^T, *M. aerolatum* DSM 14217^T and *M. foliorum* DSM 12966^T) were grown on TSA for 48 h at 30 °C, and 50 mg of the biomass was harvested. Fatty acid methyl esters were prepared, separated and identified with the Sherlock Microbial Identification System (MIDI; Sasser, 1990).

Polar lipids were extracted and examined by two-dimensional TLC (Minnikin *et al.*, 1977; Bligh & Dyer, 1959). Amino-acid composition in the cell wall peptidoglycan was determined by using TLC after hydrolysis with 6 M HCl at 100 °C for 18 h (Busse *et al.*, 1996; Schenkel *et al.*, 1995; Komagata & Suzuki, 1987). Cell wall sugars were analysed as described by Stanek & Roberts (1974).

Genomic DNA of the novel strain was extracted and purified, degraded by P1 nuclease into nucleosides, and the G + C content was determined as described by Mesbah *et al.* (1989) using reversed-phase HPLC.

DNA–DNA hybridization was carried out with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989), using a BioAssay reader for fluorescence measurement. The hybridization temperature was 37 °C. Five replications were performed for each sample; the highest and lowest values were excluded and the remaining three values were used for calculation of hybridization values. Reciprocal experiments were performed.

Genomic DNA of strain DCY37^T was extracted and purified by using a Genomic DNA Isolation kit (Core Bio System). The 16S rRNA gene was amplified and sequenced by using the universal bacterial primer sets 9F and 1512R, and 27F and 1492R, respectively (Kim *et al.*, 2005). The purified PCR products were sequenced by Genotec, Daejeon, Korea. 16S rRNA gene sequences were compiled by using SeqMan software, and edited using the BioEdit program (Hall, 1999). Multiple alignments were performed with the CLUSTAL X program (Thompson *et al.*, 1997), and the Kimura two-parameter model (Kimura, 1983) was used to calculate evolutionary distances. Neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony trees were reconstructed using the MEGA 3 (Felsenstein, 1985) and PHYLIP programs (Choi *et al.*, 2000), respectively.

Strain DCY37^T was an aerobic, Gram-positive, rod-shaped (0.5–0.7 µm in diameter and 1.5–2.0 µm in length;

Supplementary Fig. S1, available in IJSEM online) and non-motile bacterium. Colonies on R2A agar were pale yellow, circular and slightly convex after 3 days of cultivation at 30 °C. Growth occurred between 15 and 45 °C and at pH 5 to 9; optimal growth occurred at 30 °C and pH 7.0. Poor growth was observed in the absence of NaCl and in the presence of >8 % (w/v) NaCl. The strain was slightly halophilic. The physiological characteristics of strain DCY37^T are summarized in the species description and a comparison of selected characteristics with those of related type strains is shown in Table 1.

The main fatty acids detected in strain DCY37^T were anteiso-C_{15:0} (44.9 %), anteiso-C_{17:0} (30.6 %) and iso-C_{16:0} (11.2 %) (Supplementary Table S1). The major menaquinones detected for this strain were MK-12 and MK-13. This pattern has been reported for other species of the genus *Microbacterium* (Schippers *et al.*, 2005). The major polar lipids were glycolipid, phosphatidylglycerol, diphosphatidylglycerol and unknown aminolipids. The genus *Microbacterium* (and *Aureobacterium*) is characterized by diphosphatidylglycerol, phosphatidylglycerol and an unusual glycolipid, and these have been detected as the major polar lipids in numerous species of the genus (Young *et al.*, 2010). The major cell-wall sugars were ribose, xylose and galactose. The diamino acid in cell-wall hydrolysates of strain DCY37^T was ornithine. The major cell-wall amino acids were alanine, glycine, D-glutamic acid and serine. Analytical data suggested the presence of peptidoglycan type B2β in strain DCY37^T.

The 16S rRNA gene sequence of strain DCY37^T, a continuous stretch of 1427 nt, was sequenced. The phylogenetic tree shows that strain DCY37^T was clustered within the genus *Microbacterium* of the class *Actinobacteria*. It is closely related to *M. aerolatum* DSM 14217^T (98.8 %), *M. hydrocarbonoxydans* DSM 16089^T (98.5 %), *M. natoriense* JCM 12611^T (98.5 %), *M. foliorum* DSM 12966^T (98.4 %) and *Microbacterium phyllosphaerae* DSM 13468^T (98.3 %) (Fig. 1). The DNA G + C content of strain DCY37^T was 64.5 mol%, a value within the range reported for members of the genus *Microbacterium* (Table 1). However, the DNA–DNA relatedness values between strain DCY37^T and strains *M. aerolatum* DSM 14217^T, *M. hydrocarbonoxydans* DSM 16089^T, *M. natoriense* JCM 12611^T and *M. foliorum* DSM 12966^T were 51.8 ± 0.7, 41.8 ± 0.4, 35.4 ± 0.8 and 29.8 ± 0.3 %, respectively. Reciprocal hybridization tests resulted in DNA–DNA relatedness values of 37.7 % between *M. hydrocarbonoxydans* DSM 16089^T and strain DCY37^T. These results show that strain DCY37^T is an independent species at the genotype level according to the limits devised by Wayne *et al.* (1987).

Based on the phylogenetic analysis and phenotypic characteristics, strain DCY37^T is described as a novel species in the genus *Microbacterium*, for which the name *Microbacterium ginsengiterrae* sp. nov. (type strain DCY37^T = KCTC 19526^T = JCM 15516^T) is proposed.

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic relationships of strain DCY37^T and representative members of the genus *Microbacterium*. *Rarobacter faecitabidus* DSM 4813^T was used as an outgroup. Bootstrap values >50% based on 1000 replications are shown at branching points. Filled circles indicate that the corresponding nodes were also recovered in the tree generated using the maximum-parsimony algorithm. Bar, 0.01 substitutions per nucleotide position.

Description of *Microbacterium ginsengiterrae* sp. nov.

Microbacterium ginsengiterrae (gin.seng.i.ter'rae. N.L. n. *ginsengum* ginseng; L. n. *terra* soil; N.L. gen. n. *ginsengiterrae* of soil of a ginseng field, the source of the type strain).

Cells are Gram-positive, heterotrophic, strictly aerobic, non-motile and rod-shaped (0.5–0.7 µm diameter and 1.5–2.0 µm length). Colonies on R2A agar are circular with regular edges, yellow and translucent with a diameter of 2–3 mm after 2 days of incubation on R2A agar. Grows between 15 and 45 °C and at pH 5 to 9. Optimal growth at 30 °C and pH 7.0. Poor growth in the absence of NaCl and in the presence of >8% (w/v) NaCl. Slightly halophilic. Catalase, oxidase and DNase activities are present. In the API ZYM kit, α-glucosidase, β-glucosidase, esterase (C4), esterase lipase (C8), naphthol-AS-BI-phosphohydrolase and α-mannosidase activities are present; alkaline phosphatase, lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase and α-fucosidase activities are absent. In the API 20NE kit, arginine dihydrolase and urease reactions are positive; the following reactions

are negative: reduction of nitrate to nitrite, reduction of nitrate to nitrogen, indole production, protease, β-galactosidase, glucose acidification, and assimilation of L-arabinose, D-mannitol, N-acetylglucosamine, maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. In the API 32GN kit, the following substrates are assimilated: D-ribose, L-rhamnose, sucrose, 3-hydroxybenzoic acid and 4-hydroxybenzoic acid; the following substrates are not assimilated: glucose, inositol, itaconic acid, suberic acid, sodium malonate, sodium acetate, L-alanine, glycogen, L-serine, salicin, melibiose, L-fucose, D-sorbitol, propionic acid, capric acid, valeric acid, L-histidine, 3-hydroxybutyric acid and L-proline. The predominant menaquinones are MK-12 and MK-13. The major fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. The major cell-wall sugars are ribose, xylose and galactose. The cell-wall peptidoglycan is of the B2β type, (L-Hsr)–D-Glu→Gly→D-Orn. The major polar lipids are glycolipid, phosphatidylglycerol, diphosphatidylglycerol and unknown aminolipids. The DNA G+C content of the type strain is 64.5 mol%.

The type strain, DCY37^T (=KCTC 19526^T =JCM 15516^T), was isolated from soil of a ginseng field in the Republic of Korea.

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