Brachybacterium ginsengisoli sp. nov., isolated from soil of a ginseng field

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A novel Gram-staining-positive, aerobic bacterium, designed DCY80T, was isolated from soil of a ginseng field in the Republic of Korea. 16S rRNA gene sequence analysis revealed that strain DCY80T belonged to the genus Brachybacterium (95.8–98.2 % similarity) and was most closely related to Brachybacterium faecium DSM 4810T (98.2 %). Colonies were circular, entire, low-convex, opaque and 0.5–1.0 mm in diameter after growth for 2 days on TSA at 30 °C. Growth occurred at 4–34 °C (optimum, 25 °C), at pH 5.0–10.0 (optimum, pH 6.5–7.0) and in the presence of 0–7 % NaCl. Strain DCY80T produced siderophores and was sensitive to penicillin G, erythromycin, cefazolin, oleandomycin, ceftazidine, vancomycin, tetracycline, novobiocin, carbamycin, rifampicin and neomycin. The DNA G+C content was 71.0 mol%. Levels of DNA–DNA relatedness between strain DCY80T and B. faecium DSM 4810T, B. paraconglomeratum KCTC 9916T, B. saurushtrense DSM 23186T and B. conglomeratum KCTC 9915T were 46.9±0.5, 28.9±0.6, 20.4±0.9 and 17.3±0.4 %, respectively. The cell-wall peptidoglycan of strain DCY80T contained meso-diaminopimelic acid as the diagnostic diamino acid. The menaquinones were MK-7 (85.8 %) and MK-8 (14.2 %). The major cellular fatty acids were anteiso-C15 : 0 (69.1 %) and anteiso-C17 : 0 (12.2 %). Phosphatidylglycerol, diphosphatidylglycerol, an unidentified glycolipid, two unidentified phospholipids and five unidentified polar lipids were found. On the basis of our phenotypic and genotypic analyses, strain DCY80T represents a novel species of the genus Brachybacterium, for which the name Brachybacterium ginsengisoli sp. nov. is proposed (type strain DCY80T=KCTC 29226T=JCM 19356T).

The genus Brachybacterium was established by Collins et al. (1988) with Brachybacterium faecium as the type species. At the time of writing, the genus Brachybacterium encompassed 14 species: B. faecium (Collins et al., 1988), B. nesterenkovi (Gvozdyak et al., 1992), B. conglomeratum, B. paraconglomeratum, B. rhammosum (Takeuchi et al., 1995), B. alimentarium, B. tyrofermentans (Schubert et al., 1996), B. fresonis, B. sacelli (Heyrman et al., 2002), B. muris (Buczolits et al., 2003), B. zhongsanense (Zhang et al., 2007), B. phenoliresistens (Chou et al., 2007), B. saurushtrense (Gontia et al., 2011) and B. squillarum (Park et al., 2011). Members of the genus Brachybacterium are Gram-staining-positive, have coccoid-shaped cells and contain A4γ-type peptidoglycan with meso-diaminopimelic acid, N-glycolyl muramic acid in the cell wall, menaquinone 7 (MK-7) as the principal menaquinone, cellular fatty acids with anteiso-C15 : 0 and anteiso-C17 : 0 predominant and diphosphatidylglycerol and phosphatidylglycerol as the major polar lipids. Strains of the genus Brachybacterium have been isolated from a variety of environments, such as the surfaces of cheeses, oil brine, poultry deep litter, a mural painting, mouse liver, milk, oil-contaminated coastal sand and corn-steep liquor. During the course of an investigation of the bacterial community in soil from a ginseng field in Yeoncheon province, Republic of Korea, a large number of bacterial strains was isolated. In this study, we have characterized one of these isolates, strain DCY80T, by phenotypic, chemotaxonomic and phylogenetic analyses and found that the strain is affiliated to the genus Brachybacterium. The results obtained suggest that strain DCY80T represents a novel species of the genus.

Strain DCY80T was isolated from soil by plating with the serial dilution method on TSA (Difco). One gram of the soil sample was suspended in 10 ml sterile normal saline (0.85 % NaCl) containing 0.8 % (w/v) peptone. Serial dilutions were prepared up to 10−4 using the same solution. Subsequently,
100 μl of each diluted sample was plated five times onto TSA. The plates were incubated at 30 °C for 7 days. Single colonies were purified by transfer to new TSA plates. One isolate, designated DCY80T, was selected for further study. The isolate was routinely cultivated on TSA at 25 °C and stored at −80 °C as a suspension in 30% (v/v) glycerol. The following reference strains were obtained from the DSMZ and the Korean Collection for Type Cultures: B. faecium DSM 4810T, B. saurushtrense DSM 23186T, B. paraconglomeraturn KCTC 9916T and B. conglomeraturn KCTC 9915T.

Colony morphology of strain DCY80T was observed by using cells grown on TSA (Difco) at 25 °C for 2 days. Motility was determined by the hanging-drop technique (Bernardet et al., 2002). Cell size, shape, morphology, flagellation and motility of strain DCY80T were observed by phase-contrast microscopy (×1000 magnification; Nikon Optiphot-2) and by transmission electron microscopy after growth on TSA and in TS (Difco) for 1 day at 25 °C. Suspended cells were placed on carbon- and Formvar-coated nickel grids for 30 s. Grids were floated on a drop of 0.1% (w/v) aqueous uranyl acetate, blotted dry and then viewed with a Carl Zeiss electron microscope (LOE912AB) at 100 kV under standard operating conditions. Gram staining was performed using a bioMérieux Gram stain kit according to the manufacturer’s instructions. Growth was checked on nutrient agar (NA; Difco), TSA (Difco), R2A agar (Difco), MacConkey agar (Difco) and Luria–Bertani agar (LB agar; to the manufacturer’s instructions. Growth was checked on TSA at 25 °C. Suspended cells were placed on carbon- and Formvar-coated nickel grids for 30 s. Grids were floated on a drop of 0.1% (w/v) aqueous uranyl acetate, blotted dry and then viewed with a Carl Zeiss electron microscope (LOE912AB) at 100 kV under standard operating conditions. Gram staining was performed using a bioMérieux Gram stain kit according to the manufacturer’s instructions. Growth was checked on nutrient agar (NA; Difco), TSA (Difco), R2A agar (Difco), MacConkey agar (Difco) and Luria–Bertani agar (LB agar; Difco) and in potato dextrose broth (PDB; Difco) at 25 °C. The temperature range for growth was tested by checking growth in TSA and on TSA at 4, 10, 20, 28, 30, 34, 35 and 37 °C. Tolerance of NaCl was evaluated in TSA supplemented with 0–15.0% (w/v) NaCl (at 1.0% intervals) at 25 °C. The pH range for growth was examined at pH 4.0–10.0 in 0.5 pH unit intervals in R2A broth adjusted with 10 mM phosphate/citrate buffer (pH 4.0–5.0), MES/NaOH (pH 5.5–6.5), HEPES/NaOH (pH 7.0–8.0), Tris/HCl (pH 8.5–9.0) and NaHCO3/Na2CO3 (pH 9.5–10.0). Catalase activity was determined by the formation of bubbles in 3% (v/v) H2O2 solution. Oxidase activity was determined by using 1% (w/v) N,N,N’,N’-tetramethyl 1,4-phenylenediamine reagent (bioMérieux) according to the manufacturer’s instructions. H2S production was evaluated on triple-sugar-iron agar. Hydrolysis of the following substrates was tested: gelatin (on TSA containing 1% starch (Difco) at 25 °C), aesculin (on TSA containing aesculin (0.3%; Sigma) and ferric citrate (0.02%; Fluka)) and Tweens 20 and 80 (on TSA containing 1% Tween 20 or 80 and 0.02% CaCl2). Indole production was analysed using Kovács’ reagent in 1% tryptone broth. Nitrate reduction was tested in nitrate broth containing 0.2% KNO3 (Skerman, 1967). Urease activity was evaluated in Christensen’s medium (Christensen, 1946). Production of siderophores was tested by using the blue agar chrome azurol sulfonate assay method according to Schwyn & Neilands (1987). Growth under anaerobic conditions was assessed after 10 days of incubation on R2A agar at 30 °C in the GasPak EZ anaerobic container system (BD). Carbon utilization and enzyme production were examined using the API 20NE, API ID 32 GN and API ZYM strips according to the manufacturer’s instructions (bioMérieux). The results of the API ID 32 GN and API 20NE strips were recorded after 24 h and those of the API ZYM strips were recorded after 4 h of incubation. Antibiotic susceptibility was evaluated using Oxoid antibiotic discs on Müller–Hinton agar plates incubated at 30 °C for 48 h as described by Bauer et al. (1966). Inhibition zones were assessed according to the manufacturer’s manual. The antibiotic discs used were: penicillin G (10 U), erythromycin (15 μg), cefazolin (30 μg), oleandomycin (15 μg), ceftazidime (30 μg), vancomycin (30 μg), tetracycline (30 μg), novobiocin (30 μg), carbamycin (100 μg), rifampicin (5 μg) and neomycin (30 μg).

Strain DCY80T was Gram-staining-positive and aerobic and its cells were non-motile, coccoïd to ovoid and 0.6–0.8 μm in diameter (Fig. S1, available in the online Supplementary Material). Cells were catalase-positive and oxidase-negative. Colonies were circular, entire, low-convex, opaque and 0.5–1.0 mm in diameter after 2 days of growth on TSA at 30 °C. Growth occurred at 4–34 °C (optimum, 25 °C), at pH 5.0–10.0 (optimum, pH 6.5–7.0) and in the presence of 0–7.0% NaCl. Strain DCY80T grew on LB agar, R2A agar, TSA and NA but not in PDB or on MacConkey agar. Siderophores were produced but urease and indole were not. Nitrate was reduced to nitrite. Hydrolysis of casein, gelatin, starch, cellulose and DNase was positive, but Tweens 20 and 80 were not hydrolysed. Cells were sensitive to penicillin G, erythromycin, cefazolin, oleandomycin, ceftazidime, vancomycin, tetracycline, novobiocin, carbamycin, rifampicin and neomycin. The results of the other physiological and biochemical tests are given in the species description and in Table 1.

Genomic DNA of strain DCY80T was extracted and purification by using the Genomic DNA isolation kit (Core Bio System) according to the manufacturer’s instructions. The 16S rRNA gene was amplified from chromosomal DNA of strain DCY80T using bacterial universal primers 27F, 518F and 1492R (Lane, 1991; Anzai et al., 2005). The full sequence (1416 bp) of the 16S rRNA gene was compiled by using SeqMan software version 4.1 (DNASTAR INC.). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Multiple alignments were performed with the CLUSTAL_X program (Thompson et al., 1997). Gaps were edited by using the BioEdit program (Hall, 1999). Evolutionary distances were calculated using Kimura’s two-parameter model (Kimura, 1983). The phylogenetic tree was reconstructed by using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) algorithms within the MEGA4 program (Tamura et al., 2007) with bootstrap values determined based on 1000 replications (Felsenstein, 1985). Comparison of the 16S rRNA gene
sequence of strain DCY80\textsuperscript{T} with existing sequences from the
databases was carried out using EzTaxon-e server (Kim et al.,
2012). The DNA G+C content was determined by the
method of Mesbah et al. (1989). Genomic DNA of strain
DCY80\textsuperscript{T} was extracted and purified using an Exgene Cell
SV mini-kit (Gene All Biotechnology). An aliquot (10 \textmu l) of
genomic DNA was mixed with 10 \textmu l of glycine buffer (pH 10.0) and
then heated in a boiling water bath for 5 min and then cooled in an
ice-water bath. The denatured DNA solution was mixed with 10 \textmu l
nuclease P1 solution (20 U \textmu l\textsuperscript{-1}) and incubated at 37 °C for
1 h. An aliquot (10 \textmu l) of glycine buffer (pH 10.0) and 10 \textmu l of
alkaline phosphatase (40 U \textmu l\textsuperscript{-1}) were added to the sample and
the mixture was incubated for 1 h at 37 °C. Subsequently, the
nucleoside mixture was separated by HPLC [Futecs NS-6000A;
reversed-phase column YMC-Triart C18 (4.6 × 250 mm,
5 \textmu m)]. The mobile phase used was 25 mM NH\textsubscript{4}H\textsubscript{2}PO\textsubscript{4}/
acetonitrile (20:1, v/v). Genomic DNA of Escherichia coli
strain B (D4889; Sigma-Aldrich) was used as a standard.
DNA–DNA hybridization was performed fluorometrically
according to the method of Ezaki et al. (1989), using
photobiotin-labelled DNA probes and microdilution wells.
Hybridization was conducted in five replications for each
sample; the highest and lowest values obtained for each
sample were excluded, and the means of the remaining
three values are quoted as DNA–DNA relatedness values.

Phylogenetic analysis based on 16S rRNA gene sequences
revealed that strain DCY80\textsuperscript{T} formed a monophyletic cluster
with the members of the genus Brachybacterium (Fig. 1).
This cluster was also recovered with high bootstrap values
in the tree generated by the maximum-parsimony algo-


Table 1. Differential characteristics of strain DCY80\textsuperscript{T} and type strains of related species of the genus Brachybacterium

<table>
<thead>
<tr>
<th>Characteristic</th>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>W</td>
<td>+</td>
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<td>7\textsuperscript{b}</td>
<td>7\textsuperscript{a}</td>
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<td>DNA G+C content (mol%)</td>
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<td>71.5\textsuperscript{a}</td>
<td>68.6\textsuperscript{b}</td>
<td>73.0\textsuperscript{c}</td>
<td>76.6\textsuperscript{b}</td>
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</table>

\*Data taken from: a, Collins et al. (1988); b, Takeuchi et al. (1995); c, Gontia et al. (2011).
(97.8%) and B. conglomeratum KCTC 9915T (97.7%).
Levels of DNA–DNA relatedness (mean ± SD, n = 3) between
strain DCY80T and B. faecium DSM 4810T, B. paraconglomerat
um KCTC 9916T, B. saurashtrense DSM 23186T, B. conglomera
tum KCTC 9915T were 46.9 ± 0.5, 28.9 ± 0.6, 20.4 ± 0.9 and 17.3 ± 0.4 %, respectively. All DNA–DNA
relatedness values were significantly lower than the threshold
value of 70% recommended for recognition of separate
species (Wayne et al., 1987). The DNA G+C content of
strain DCY80T was 71.0 mol%, which is consistent with
previously reported values for members of the genus
Brachybacterium.

For isoprenoid quinone analysis, cell biomass was grown in
TSB (Difco) at 25 °C for 48 h and then freeze-dried.
Menaquinones were extracted from dry cells (50 mg) with
chloroform/methanol (2:1, v/v) and then concentrated at
40 °C using a vacuum rotary evaporator; the residue was
subsequently extracted with 10 ml hexane. After using a
Sep-Pak Vac 6cc silica cartridge for purification, samples
were analysed by HPLC [Futecs NS-6000A; reversed-phase
column YMC-Triart C18 (4.6 × 250 mm, 5 μm); meth-
anol/2-propanol (7:3, v/v) as mobile phase] according to
Collins (1985). Cellular fatty acid profiles were determined
for strain DCY80T and the four reference type strains
grown on TSA (Difco) for 2 days at 25 °C. Cellular fatty
acids were saponified, methylated and extracted according
to the protocol of the Sherlock Microbial Identification
System (MIDI). Fatty acid methyl esters were analysed by GC
(Hewlett Packard 6890) and identified according to the
Microbial Identification software package (Sasser, 1990). The
polar lipids of strain DCY80T and three of the refer-
cence strains (B. faecium DSM 4810T, B. paraconglomerat
um KCTC 9916T and B. conglomeratum KCTC 9915T) were
extracted and analysed by two-dimensional TLC followed by
spraying with molybdatophosphoric acid, molybdenum
blue, α-naphthol and ninhydrin reagents as described by
Minnikin et al. (1977). The peptidoglycan was analysed as
described by Schleifer & Kandler (1972) by using TLC on
cellulose sheets. The menaquinones of strain DCY80T were
MK-7 (85.8 %) and MK-8 (14.2 %). The cellular fatty acid
profiles of strain DCY80T and the four references strains
are shown in Table 2. The major cellular fatty acids
of strain DCY80T were anteiso-C15:0 (69.1 %) and anteiso-
C17:0 (12.2 %), followed by iso-C16:0 (7.3 %). Strain
DCY80T contained phosphatidylglycerol and diphosphati-
dylglycerol as major polar lipids, while an unidentified
glycolipid, two unidentified phospholipids and five un-
identified polar lipids were also detected as minor
components (Fig. S2). The peptidoglycan contained the

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain DCY80T with
other members of the genus Brachybacterium. Bootstrap values >70% based on 1000 replications are shown as percentages
at branching points. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with
the maximum-parsimony algorithm. Dermabacter hominis DSM 7083T was used as an outgroup. Bar, 0.005 substitutions per
nucleotide position.
amino acids *meso*-diaminopimelic acid, alanine, glutamic acid and glycine.

The results of our phylogenetic and chemotaxonomic analyses suggested that strain DCY80T belongs to the genus *Brachybacterium*. The phylogenetic, biochemical and physiological characteristics of strain DCY80T indicated that the strain represents a novel species of the genus *Brachybacterium* clearly differentiated from related species. Therefore, on the basis of the data presented, a novel species, *Brachybacterium ginsengisoli* sp. nov., is proposed for strain DCY80T.

**Description of Brachybacterium ginsengisoli** sp. nov.

*Brachybacterium ginsengisoli* (gin.sen.gi.so’li. N.L. n. ginsengum ginseng; L. n. solum soil; N.L. gen. n. ginsengisoli of soil of a ginseng field).

Cells are Gram-staining-positive, coccoid to ovoid, with a diameter of 0.6–0.8 μm (Fig. S1). Catalase-positive, oxidase-negative, aerobic and non-motile. Colonies are circular, entire, low-convex, opaque and 0.5–1.0 mm in diameter after 2 days of growth on TSA at 30 °C. Growth occurs at 4–34 °C (optimum, 25 °C), at pH 5.0–10.0 (optimum, pH 6.5–7.0) and in the presence of 0–7.0 % NaCl. Cells are capable of producing siderophores. Sensitive to penicillin G, erythromycin, cefazolin, oleandomycin, ceftazidine, vancomycin, tetracycline, novobiocin, carbenicillin, rifampicin and neomycin. According to the API ZYM test, positive for alkaline phosphatase, esterase (C4), lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *x*-galactosidase, *β*-galactosidase, *β*-glucuronidase, *x*-glucosidase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase and *z*-mannosidase, but negative for lipase (C14), trypsin, *x*-chymotrypsin and *x*-fucosidase. According to the API 20NE test, positive for nitrate reduction, aesculin hydrolysis, glucose fermentation, *β*-galactosidase, gelatinase and assimilation of D-glucose, *N*-acetylglucosamine, maltose, gluconic acid, capric acid, adipic acid, L-arabinose, maltale, trisodium citrate, D-mannose and phenylacetic acid, but negative for arginine dihydrolase, urease, indole production and assimilation of D-mannitol. According to the API ID 32 GN test, positive for assimilation of *N*-acetylglucosamine, *myo*-inositol, sucrose, maltose, suberic acid, sodium acetate, sodium malonate, lactic acid, L-alanine, L-serine, D-glucose, salicin, melibiose, L-arabinose, L-fucose, capric acid, valeric acid, trisodium citrate, L-histidine, 2-ketogluconate, 3-hydroxybutyric acid, 4-hydroxybenzoic acid and L-proline, but negative for assimilation of L-ribonose, D-ribose, itaconic acid, 5-ketogluconate, glycerogen, 3-hydroxybenzoic acid, D-mannitol, D-sorbitol and propionic acid. The predominant menaquinones are MK-7 and MK-8. *meso*-Diaminopimelic acid is the diagnostic diamino acid of the cell-wall peptidoglycan. The major cellular fatty acids are anteiso-C15:0 and anteiso-C17:0. Phosphatidylglycerol, diphosphatidylglycerol, an unidentified glycolipid, two unidentified phospholipids and five unidentified polar lipids are found.

The type strain is DCY80T (=KCTC 29226T=JCM 19356T), isolated from soil of a ginseng field in Yeoncheon province, Republic of Korea (38° 04' 00" N 126° 57' 00" E). The DNA G+C content of the type strain is 71.0 mol%.

**Acknowledgements**

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Bernardet, J. F., Nakagawa, Y., Holmes, B. & Subcommittee on the taxonomy of Flavobacterium and Cytophaga-like bacteria of the


