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Flavobacterium ginsengiterrae sp. nov., isolated from a ginseng field

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A novel strain of *Flavobacterium*, DCY55^T, a Gram-negative, yellow-pigmented, rod-shaped, non-spore-forming and gliding-motile bacterium, was isolated from the soil of a ginseng field in South Korea. Phylogenetic analysis, based on the 16S rRNA sequence, demonstrated that strain DCY55^T belongs to the genus *Flavobacterium* within the family *Flavobacteriaceae*. Strain DCY55^T showed the highest similarity with *F. johnsoniae* UW101^T (97.1%), *F. ginsenosidimutans* THG 01^T (96.8%), *F. defluvii* EMB 117^T (96.6%), *F. banpakuense* 15F3^T (96.3%) and *F. anhuiense* D3^T (95.8%). Chemotaxonomic results showed that strain DCY55^T predominantly contains menaquinone MK-6, that its DNA G+C content is 36.1 mol%, and that its major cellular fatty acids are iso-$C_{15:0}$, summed feature 3 (comprising iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$) and $C_{16:0}$. The chemotaxonomic and genotypic characteristics support the taxonomic classification of strain DCY55^T to the genus *Flavobacterium*. The results of physiological and biochemical tests confirmed that strain DCY55^T is distinct from previously validated species. We conclude that strain DCY55^T should be classified as a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium ginsengiterrae* sp. nov. is proposed, with the type strain DCY55^T (=KCTC 23319^T = JCM 17337^T).

Key Words——DCY55; *Flavobacteriaceae*; *Flavobacterium ginsengiterrae*

Introduction

The genus *Flavobacterium* was established by Bergey et al. (1923) and was subsequently amended by Bernardet et al. (1996). It belongs to the family *Flavobacteriaceae*, phylum Bacteroidetes (Bernardet et al., 2002; Ludwig and Klenk, 2001). Members of the genus *Flavobacterium* have been isolated from a variety of environments, such as the Antarctic, soil, freshwater, seawater and wastewater treatment plants (Kim et al., 2006; Liu et al., 2008; McCammon and Bowman, 2000; Park et al., 2007). Currently, the genus *Flavobacterium* consists of 70 recognized species, including the recently described species *F. dongtanense* (Xiao et al., 2011), *F. beibuense* (Fu et al., 2011), *F. ponti* (Yoon et al., 2011) and *F. sinopsychrotolerans* (Xu et al., 2011).

We isolated a novel bacterial strain from the soil of a ginseng field in South Korea. Biochemical analyses revealed this strain to be affiliated with the genus *Flavobacterium*. The strain was examined using phenotypic, physiological, and chemotaxonomic tests and by phylogenetic analysis. We report that strain DCY55^T should be classified in the genus *Flavobacterium* as a

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The NCBI GenBank accession number for the 16S rRNA gene sequence of strain DCY55^T (=KCTC 23319^T = JCM 17337^T) is HM776706.

novel species, *Flavobacterium ginsengiterrae* sp. nov.

Materials and Methods

Isolation of bacterial strain and culture conditions. We isolated the strain DCY55^T from the soil of a ginseng field in South Korea, using a standard dilution plating method on R2A agar (Difco, Franklin Lakes, NJ, USA) at 30°C, after incubation for 3 days. Purified isolates were routinely subcultured on TSA agar (Difco), identified by partial 16S rRNA gene sequences and preserved in a 30% (v/v) glycerol solution at −80°C. This isolate was deposited in the Korean Collection for Type Cultures and Japan Collection of Microorganisms (KCTC 23319^T = JCM 17337^T).

Phylogenetic analysis. The 16S rRNA gene was amplified and sequenced using the universal bacterial primers (27F, 518F and 1492R). The purified PCR products were sequenced by Genotech in Daejeon, Korea. Cycle sequencing was performed using the BigDye terminator in an Applied Biosystems 3730xl DNA analyzer with the Phred/Phrap program. After the 16S rRNA gene sequences were compiled with SeqMan software, related 16S rRNA gene sequences were collected using the EzTaxon server (http://www.eztaxon.org; Chun et al., 2007) and edited using the BioEdit program (Hall, 1999). Multiple alignments were performed by using the CLUSTAL X program (Thompson et al., 1997). A phylogenetic tree was constructed by neighbor-joining (Saitou and Nei, 1987) and

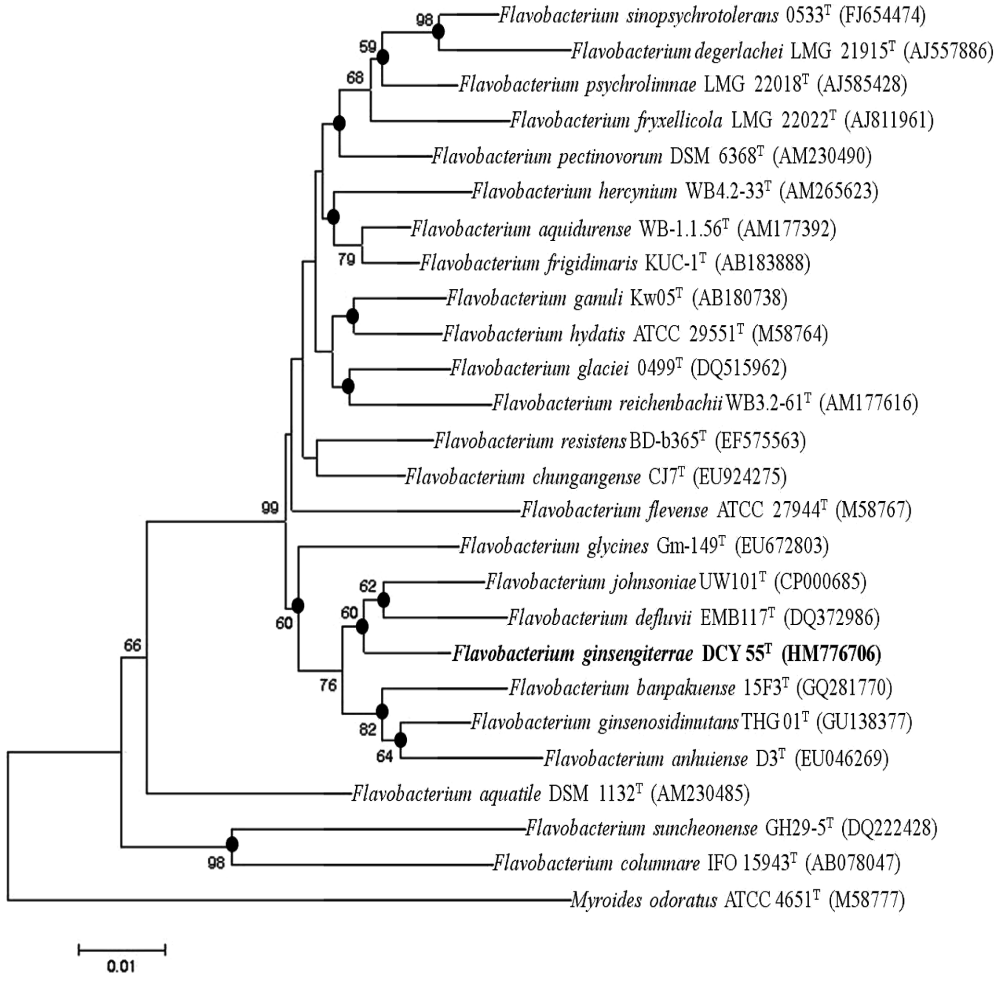

Fig. 1. A neighbor-joining tree showing the phylogenetic relationships of strain DCY55^T and members of the genus *Flavobacterium*.

Bootstrap values > 60% based on 1,000 replications are shown at branching points. Filled circles indicate that the corresponding nodes were also recovered in the tree constructed with the maximum-parsimony algorithm. *Myroidesodoratus odoratus* ATCC 4651^T (M58777) was used as an outgroup. Scale bar, 0.01 substitutions per nucleotide position.

maximum parsimony (Fitch, 1971) algorithms using MEGA 4 (Tamura et al., 2007) with bootstrap analyses based on 1,000 replications (Felsenstein, 1985). The evolutionary distances of the neighbor-joining method were calculated using the Kimura two-parameter model (Kimura, 1983). The resulting neighbor-joining tree showed that strain DCY55^T was affiliated with the genus *Flavobacterium* (Fig. 1).

Morphological and physiological characteristics. Cell morphology was observed with a Nikon light microscope (1,000×magnification). Transmission electron microscopy (TEM) was performed using the following procedure: bacteria were grown on TSA plates at 30°C for 24 h; resuspended cells were placed on carbon- and formvar-coated nickel grids for 30s; and grids were floated on 1 drop of 0.1% (w/v) aqueous uranyl acetate, blotted dry, and then viewed with a Carl Zeiss LEO912AB electron microscope at 100 kV under standard operating conditions. Gliding motility was observed using the hanging drop technique (Bernardet et al., 2002).

Gram reactions were tested according to the non-staining method as described by Buck (1982). Catalase activity was determined by bubble production in the presence of 3% (v/v) hydrogen peroxide solution, and oxidase activity was evaluated via the oxidation of 1% (w/v) tetramethyl-*p*-phenylenediamine (Merck, Whitehouse Station, NJ, USA). Growth on nutrient agar (NA), trypticase soy agar (TSA), and R2A agar was evaluated at 30°C. Growth at different temperatures was assessed at 4, 10, 20, 25, 30, 35, 37 and 42°C on TSA agar. Various pH values (pH 5.0−10.0 at intervals of 1.0 pH units) were assessed in trypticase soy broth (TSB), and salt tolerance was also tested in trypticase soy broth supplemented with 0, 1, 2, 3 and 5% (w/v) NaCl after 3 days' incubation. The production of flexirubin-type pigments was investigated by the method of Bernardet et al. (2002) as described previously. Hydrolysis of Tween 20 and Tween 80 was investigated according to Gerhardt et al. (1994). DNase activity and hydrolysis of casein, gelatin, tyrosine, starch and urea were also investigated as described by Cowan and Steel (1965). Hydrolysis tests of esculin and nitrate reduction were done as presented by Lanyi (1987).

Table 1. Physiological characteristics of strain DCY55^T and related type strains of species of the genus *Flavobacterium*.

Characteristic	1	2	3	4	5	6
Tolerance of 2% NaCl	+	−	−	−	−	+
Oxidase activity	+	+	+	−	−	−
Gliding motility	+	+	−	+	+	+
Flexirubin-type pigments	+	+	NR	+	+	+
Nitrate reduction ($NO_3 \rightarrow NO_2$)	−	+	−	−	+	−
Degradation of:						
Gelatin	−	+	NR	+	+	−
Starch	+	+	+	−	+	+
DNA	+	+	−	+	NR	−
Assimilation of:						
L-Arabinose	+	+	+	(+)	+	+
N-acetyl-D-glucosamine	+	+	−	+	+	+
Enzymatic activities (API ZYM)						
α-Galactosidase	−	−	−	(+)	−	−
α-Glucosidase	+	+	NR	+	+	(+)
β-Galactosidase	(+)	+	+	(+)	+	+
β-Glucuronidase	−	+	−	+	(+)	−
β-Glucosidase	+	+	+	+	+	−
DNA G+C content (mol%)	36.1	35.2	32.1	33.5	31.1	31.4

Strains: 1, *Flavobacterium ginsengiterrae* DCY 55^T; 2, *Flavobacterium johnsoniae* DSM 2064^T; 3, *Flavobacterium ginsenosidimutans* THG 01^T (data from Yang et al., 2011); 4, *Flavobacterium defluvii* DSM 17963^T; 5, *Flavobacterium banpakuense* 15F3^T (data from Kim et al., 2011); 6, *Flavobacterium anhuiense* KCTC 22128^T. +, Positive result; (+), weakly positive; −, negative result; NR, not reported.

Hydrolysis of CM-cellulose was tested as described by Ramesh et al. (2008). Enzyme activities and additional biochemical tests were tested by determining API 20NE and API ZYM systems (bioMérieux, Marcy l'Etoile, France) according to the instructions of the manufacturer. The phenotypic characteristics of strain DCY55^T are given in the species description and in Table 1.

Chemotaxonomic characteristics. Isoprenoidquinones were extracted and analyzed by HPLC according to Komagata and Suzuki (1987). For analysis of fatty acid composition, type strains were grown on TSA plates for 48 h at 28°C, and the cells were harvested. Fatty acids were extracted, methylated, and separated by gas chromatography, and the identification and quantification of methyl esters were conducted using a TSBA library (version 4.5) from Sherlock Microbial Identification Systems (MIDI, Newark, DE, USA) as described by Sasser (1990). The fatty acid profiles of strain DCY55^T and related type strains are represented in Table 2.

Table 2. Fatty acid profiles of strain DCY55^T and related species of the genus *Flavobacterium*.

Fatty acid	1	2	3	4	5	6
Saturated						
$C_{14:0}$	1.6	1.5	tr	1.9	1.2	1.2
$C_{16:0}$	14.4	13.4	6.1	10.8	4.7	5.3
Unsaturated						
$C_{15:1}$ ω6*c*	tr	tr	tr	1.5	3.0	2.8
$C_{16:1}$ ω5*c*	2.1	1.5	tr	1.0	tr	tr
$C_{17:1}$ ω6*c*	tr	tr	tr	tr	1.4	1.2
$C_{17:1}$ ω8*c*	tr	tr	tr	tr	1.2	tr
Branched						
iso-$C_{15:0}$	24.1	23.6	31.2	25.7	24.7	31.4
iso-$C_{15:0}$ 3-OH	5.1	4.8	8.9	5.4	8.3	7.3
iso-$C_{15:1}$G	2.2	1.7	1.2	2.7	5.2	3.5
anteiso-$C_{15:0}$	3.0	2.6	1.5	1.7	1.5	1.6
iso-$C_{16:0}$	tr	tr	1.2	tr	tr	tr
iso-$C_{16:0}$ 3-OH	tr	tr	tr	tr	1.0	tr
iso-$C_{17:0}$	tr	tr	1.5	tr	tr	tr
iso-$C_{17:0}$ 3-OH	5.2	5.5	10.3	4.3	8.2	6.2
iso-$C_{17:1}$ ω9*c*	2.0	2.6	6.5	2.0	tr	2.3
Hydroxy						
$C_{15:0}$ 3-OH	—	—	—	1.18	1.7	1.3
$C_{16:0}$ 3-OH	5.4	5.4	2.6	6.9	5.7	3.8
Summed feature 3[a]	19.9	22.7	12.1	22.9	14.6	14.0

[a]Summed feature 3 comprises iso-$C_{15:0}$ 2-OH and/or $C_{16:1}$ ω7*c* that could not be separated by GLC with the MIDI system.

Strains: 1, *Flavobacterium ginsengiterrae* DCY55^T; 2, *Flavobacterium johnsoniae* DSM 2064^T; 3, *Flavobacterium ginsenosidimutans* THG 01^T (nutrient agar at 28°C for 2 days, data from Yang et al., 2011); 4, *Flavobacterium defluvii* DSM 17963^T; 5, *Flavobacterium banpakuense* 15F3^T (tryptic soy agar at 30°C for 3 days, data from Kim et al., 2011); 6, *Flavobacterium anhuiense* KCTC 22128^T. All data are from the present study except for column 3 and 5. Fatty acids amounting to <1% in all strains tested are not listed. tr, Trace amount (<1.0%); −, not detected.

DNA base composition. For G+C content analysis, the genomic DNA was extracted and purified using the Genomic DNA Isolation Kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. The DNA was degraded into nucleosides using P1 nuclease, and the G+C content mol% was measured using reverse-phase HPLC as described by Mesbah et al. (1989). Rather than 97.0%, a 16S rRNA gene sequence similarity threshold range of 98.7−99% is the point at which DNA−DNA reassociation experiments should be mandatory for testing the genomic uniqueness of a novel isolate (Stackerbrandt and Ebers, 2006).

Results and Discussion

Phylogenetic analysis

The sequence of strain DCY55^T was compared with 16S rRNA sequences obtained from EzTaxonserver (http://www.eztaxon.org) using its BLAST program. 16S rRNA gene sequence analyses determined that strain DCY55^T belongs to the genus *Flavobacterium* within the family *Flavobacteriaceae*. The highest similarity of sequence was with *F. johnsoniae* UW101^T (97.1%), followed by *F. ginsenosidimutans* THG 01^T (96.8%), *F. defluvii* EMB 117^T (96.6%), *F. banpakuense* 15F3^T (96.3%) and *F. anhuiense* D3^T (95.8%), in diminishing order (Fig. 1).

Morphological and physiological characteristics

Cells are Gram-negative, non-spore-forming, nonflagellated, aerobic rods (0.6−0.9 μm wide and 3.2−4.3 μm long) with gliding motility. Colonies grown on TSA agar are yellow, irregular, and slightly convex, with a diameter of 1−1.5 mm after 2 days of incubation on TSA agar. Growth occurs at 4−35°C (optimum, 30°C) and at pH 5−9 (optimum, pH 7.0). Growth occurs in the presence of 0−2% NaCl (optimum, 0−1%). Growth also occurs on NA and R2A. Flexirubin-type pigments are produced. Catalase and oxidase are positive. Nitrate is not reduced to nitrite. The bacteria

degrade esculin, casein, CM-cellulose, DNA, starch and Tween 20, but not gelatin, tyrosine, urea or Tween 80. Acid production from carbohydrates is not present. The physiological characteristics of strain DCY55^T and related type strains are summarized in Table 1.

Chemotaxonomic characteristics

The predominant quinone was MK-6; other members of the family *Flavobacteriaceae*, including the genus *Flavobacterium*, contain MK-6 as the predominant quinone (Bernardet et al., 2002). The cellular fatty acid profiles of strain DCY55^T and other species are shown in Table 2. Strain DCY55^T mainly contains fatty acids (>10% of the total composition) of iso-$C_{15:0}$ (24.1%), summed feature 3 (comprising iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$, 19.9%) and $C_{16:0}$ (14.4%). The major fatty acid profiles of strain DCY55^T are highly similar to its closest type strain, *F. johnsoniae* DSM 2046^T.

DNA base composition

The DNA G+C content of strain DCY55^T was 36.1%. The DNA G+C content is in the range of 30–52 mol% (Liu et al., 2008), supporting that strain DCY55^T is within the genus *Flavobacterium*.

Based on the results of polyphasic analysis, strain DCY55^T should be classified as a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium ginsengiterrae* sp. nov. has been proposed.

Description of *Flavobacterium ginsengiterrae* sp. nov.

Flavobacterium ginsengiterrae (gin.sen. gi. ter'rae. N. L. n. *ginsengum* ginseng; L. n. *terrae* soil; N.L. gen. n. *ginsengiterrae* of soil of a ginseng field, the source of the type strain). Cells are Gram-negative, non-spore-forming, non-flagellated, aerobic rods (0.6–0.9 μm wide and 3.2–4.3 μm long) with gliding motility. Colonies on TSA agar are yellow, circular, and slightly convex with a diameter of 1–1.5 mm after 2 days of incubation on TSA agar. Growth occurs at 4–35°C (optimum, 28–30°C), at pH 5–9 (optimum, pH 6.0–8.0) and in the presence of 0–2% NaCl (optimum, 0–1%). Growth also occurs on NA and R2A. Flexirubin-type pigments are produced. Catalase and oxidase are positive. Indole and H_2S production are negative. The bacteria degrade esculin, casein, CM-cellulose, DNA, Starch and Tween 20, but not gelatin, tyrosine, urea or Tween 80. Acid production from carbohydrates is not present. In the API 20NE kit, esculin hydrolysis and β-galactosidase were positive, but reduction of nitrate to nitrite, indole production, glucose acidification, and arginine dihydrolase were negative. The species displayed assimilation of D-glucose, L-arabinose, D-mannose, *N*-acetyl-D-glucosamine and D-maltose but not D-mannitol, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate or phenylacetic acid. In the API ZYM kit, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucinearylamidase, valinearylamidase, cystinearylamidase, acid phosphatase, naphtol-AS-BI-phosphohydrolase, β-galactosidase (weak), α-glucosidase, β-glucosidase and *N*-acetyl-β-glucosaminidase activities were detected. But lipase (C14), trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase activities were not detected. The major cellular fatty acids are iso-$C_{15:0}$, summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$) and $C_{16:0}$. The major isoprenoid quinone is menaquinone-6 (MK-6), and the DNA G+C content is 36.1 mol%.

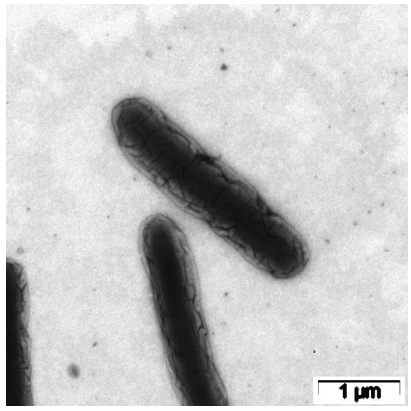

Fig. 2. Transmission electron micrograph of *Flavobacterium ginsengiterrae* DCY55^T.
Bar, 1 μm.

The type strain, DCY55^T (=KCTC 23319^T = JCM 17337^T), was isolated from a soil sample from a ginseng field in South Korea.

Acknowledgments

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