Flavobacterium ginsenosidimutans sp. nov., a bacterium with ginsenoside converting activity isolated from soil of a ginseng field

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A Gram-negative, aerobic, non-motile, non-spore-forming, yellow-pigmented, rod-shaped bacterium, designated strain THG 01^T, was isolated from the soil of a ginseng field in Pocheon province, South Korea, and its taxonomic position was investigated by using a polyphasic approach. Strain THG 01^T grew well at 25–37 °C and pH 6.0–7.5 in the absence of NaCl on nutrient agar. On the basis of 16S rRNA gene sequence similarity data, strain THG 01^T was shown to belong to the family *Flavobacteriaceae* and was related to *Flavobacterium anhuiense* D3^T (97.5 % similarity), *Flavobacterium johnsoniae* UW101^T (96.8 %) and *Flavobacterium denitrificans* ED5^T (96.7 %). 16S rRNA gene sequence similarities between the novel strain and members of other recognized species within the family *Flavobacteriaceae* were less than 96.7 %. The G + C content of the genomic DNA of strain THG 01^T was 32.1 mol%. Phenotypic and chemotaxonomic data (major menaquinone was MK-6 and major fatty acids were iso-C_{15:0}, iso-C_{15:0} 3-OH and C_{16:1ω7c} and/or C_{16:1ω6c}) supported the affiliation of strain THG 01^T to the genus *Flavobacterium*. DNA–DNA hybridization experiments showed that DNA–DNA relatedness values between strain THG 01^T and its closest phylogenetic neighbours were below 11 %. The results of physiological and biochemical tests enabled strain THG 01^T to be differentiated genotypically and phenotypically from recognized species of the genus *Flavobacterium*. The isolate therefore represents a novel species, for which the name *Flavobacterium ginsenosidimutans* sp. nov. is proposed, with THG 01^T (=KACC 14525^T=JCM 16720^T) as the type strain.

The genus *Flavobacterium*, created by Bergey *et al.* (1923), has a rapidly growing membership, as demonstrated by the multitude of novel species that have been proposed in recent years, e.g. *Flavobacterium caeni* (Liu *et al.*, 2010), *Flavobacterium reichenbachii* (Ali *et al.*, 2009), *Flavobacterium chungangense* (Kim *et al.*, 2009), *Flavobacterium cheniae* (Qu *et al.*, 2008) and *Flavobacterium aquidurens* (Cousin *et al.*, 2007). The genus belongs to the family *Flavobacteriaceae*, phylum ‘*Bacteroidetes*’, and, at the time of writing, comprised 89 species, with *Flavobacterium aquatile* as the type species (Euzéby, 1997). Species of the genus *Flavobacterium* have been isolated from diverse habitats such as fresh and salt water, soil, sediment, sea ice, diseased fish and microbial mats (Bernardet & Bowman, 2006). Furthermore, the description of the genus has been emended several times (Bernardet *et al.*,

1996). *Flavobacterium* species are generally Gram-negative, aerobic, non-spore-forming, yellow-pigmented, rod-shaped bacteria that are usually motile by gliding and have menaquinone-6 (MK-6) as the major respiratory quinone. Most members of the genus *Flavobacterium* have DNA G + C contents in the range 30–37 mol%. In this paper, a polyphasic taxonomic approach was used to characterize a novel *Flavobacterium*-like strain, THG 01^T, isolated from the soil of a ginseng field.

Strain THG 01^T was isolated from soil samples from a ginseng field of Pocheon province, South Korea. Soil samples were thoroughly suspended in 50 mM phosphate buffer (pH 7.0) and spread on modified R2A agar (Liu *et al.*, 2006). The plates were incubated at 28 °C for 2 weeks. Single colonies on the plates were purified by transferring them onto new plates of R2A agar (BD) followed by further incubation. One isolate, THG 01^T, was cultured routinely on R2A agar or nutrient agar (BD) at 28 °C and preserved in a glycerol solution (20 %, w/v) at –70 °C.

Gram-reaction testing was performed by the non-staining method as described by Buck (1982). Cell morphology was

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain THG 01^T is GU138377.

A supplementary table is available with the online version of this paper.

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observed at $\times 1000$ magnification with a light microscope (BX50; Olympus) using cells grown for 48 h at 28 °C on nutrient agar. Catalase activity was determined by bubble production in 3 % (v/v) H₂O₂ and oxidase activity was determined using 1 % (w/v) tetramethyl *p*-phenylenediamine. Carbon-source utilization and some enzyme activities were tested by using API 20NE, ID 32GN and API ZYM kits according to the instructions of the manufacturer (bioMérieux). Tests for hydrolysis of DNA (DNase agar, Scharlau; DNase activity revealed by flooding the plates with 1 M HCl), casein and starch (Atlas, 1993) were evaluated after 5 days of incubation at 28 °C. Conversion of ginsenoside Rb₁ was performed using TLC analysis as described by Kim *et al.* (2005). Growth at different temperatures (4, 10, 18, 25, 28, 35, 37 and 42 °C) and pH (pH 5.0–10.0 at intervals of 0.5) was assessed after 5 days of incubation. 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; and Tris buffer was used for pH 8.5–10.0. Salt tolerance was tested in nutrient broth (BD) supplemented with 1–10 % (w/v) NaCl after 5 days of incubation. Growth was estimated by monitoring the OD₆₀₀. Growth on nutrient agar, tryptic soy agar (TSA) and MacConkey agar was also evaluated at 28 °C.

An almost-complete 16S rRNA gene sequence for strain THG 01^T was determined as described below. Extraction of the genomic DNA was achieved using a commercial genomic DNA-extraction kit (Solgent). The 16S rRNA gene was amplified from the chromosomal DNA using the universal bacterial primer set, comprising 9F and 1512R, and the purified PCR products were sequenced by Solgent, Daejeon, South Korea (Im *et al.*, 2010). The full sequence of the 16S rRNA gene was compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from GenBank. Multiple alignments were performed by using the CLUSTAL_X program (Thompson *et al.*, 1997). Gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). The phylogenetic tree was constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods in the MEGA4 program (Kumar *et al.*, 2008), with bootstrap values based on 1000 replications (Felsenstein, 1985).

For measurement of the G + C content of the chromosomal DNA, genomic DNA of the novel strain was extracted and purified as described by Moore & Dowhan (1995) and G + C content was determined as described by Mesbah *et al.* (1989) using reverse-phase HPLC. Cellular fatty acid profiles were determined for strains grown on nutrient agar (BD) at 28 °C for 2 days. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). Fatty acids analysed by GC (6890; Hewlett Packard) were identified by the Microbial Identification software package (Sasser, 1990). For isoprenoid quinone

analysis, strain THG 01^T was grown on modified nutrient broth for 2 days at 28 °C. Collected cells were lyophilized for 24 h. 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). DNA–DNA hybridization experiments were performed between strain THG 01^T and closely related type strains of species of the genus *Flavobacterium* according to Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and micro-dilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values were converted to DNA–DNA relatedness values (%).

Table 1. Physiological characteristics of strain THG 01^T and related type strains of species of genus *Flavobacterium*

Strains: 1, THG 01^T; 2, *F. anhuiense* D3^T; 3, *F. defluvii* EMB117^T; 4, *F. denitrificans* ED5^T; 5, *F. daejeonense* GH1-10^T; 6, *F. johnsoniae* UW101^T (all data are from this study). All strains were Gram-negative, rod-shaped, catalase- and oxidase-positive, and tolerated 1 % NaCl. In API 20NE and ID 32GN tests, all strains were positive for hydrolysis of casein and starch, and assimilation of D-glucose, L-arabinose, D-mannose, maltose and glycogen, but negative for assimilation of the following substrates: 2-ketogluconate, 3-hydroxybenzoate, 3-hydroxybutyrate, 4-hydroxybenzoate, 5-ketogluconate, acetate, adipate, caprate, citrate, D-mannitol, D-ribose, D-sorbitol, myo-inositol, itaconate, lactate, L-histidine, L-proline, L-serine, malonate, phenylacetate, propionate, suberate and valerate. In API ZYM tests, all strains were positive for β -glucosidase and β -galactosidase, but negative for esterase (C4), esterase lipase (C8), lipase (C14), cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -glucuronidase, α -mannosidase and α -fucosidase.

Characteristic	1	2	3	4	5	6
Indole production	–	–	–	–	+	–
Hydrolysis of DNA	–	+	+	+	+	+
API 20NE/ID 32GN tests						
Nitrate reduction to nitrite	–	–	–	+	–	+
Assimilation of carbon sources:						
L-Fucose	+	–	–	–	–	–
Malate	–	+	–	–	+	–
Melibiose	+	+	–	+	–	–
Salicin	+	–	+	–	–	–
Sucrose	+	–	–	+	–	+
API ZYM results						
N-Acetyl- β -glucosaminidase	–	–	–	–	–	+
Acid phosphatase	+	–	+	+	–	+
Alkaline phosphatase	+	+	+	+	+	–
Leucine arylamidase	+	–	–	–	+	+
Naphthol-AS-BI-phosphohydrolase	–	–	+	–	+	+
Valine arylamidase	+	–	+	+	–	–

Fig. 1. Neighbour-joining phylogenetic tree constructed from a comparative analysis of 16S rRNA gene sequences showing the relationship between strain THG 01^T and related strains. Filled circles at nodes indicate generic branches that were also recovered by using maximum-parsimony algorithms. Bootstrap values (expressed as percentages of 1000 replications) >65% are shown at the branch points. Bar, 0.02 substitutions per nucleotide position.

Cells of strain THG 01^T were Gram-negative, aerobic, non-spore-forming, non-motile, rod-shaped, and oxidase- and catalase-positive. Colonies grown on nutrient agar plates for 2 days were smooth, transparent and deep orange in colour. On nutrient agar, THG 01^T was able to grow at 10–37 °C, but not at 4 or 42 °C. The isolate grew on TSA (BD), but not on MacConkey agar. The isolate could hydrolyse starch, casein and DNA. It had β -glucosidase activity linked with ginsenoside-converting activity. It could convert ginsenoside Rb₁ {3-O-[β -D-glucopyranosyl-(1-2)- β -D-glucopyranosyl]-20-O-[β -D-glucopyranosyl-(1-6)- β -D-glucopyranosyl]-20(S)-protopanaxadiol} to C-K [20-O-(β -D-glucopyranosyl)-20(S)-protopanaxadiol]. Physiological characteristics of strain THG 01^T are summarized in the species description and a comparison of selective characteristics of strain THG 01^T and related type strains is given in Table 1.

The 16S rRNA gene sequence of strain THG 01^T determined in this study was a continuous stretch of 1386 bp (positions 50–1458 with respect to the *Escherichia coli* numbering system). Sequence similarity calculations using the EzTaxon server [http://www.eztaxon.org; Chun *et al.* (2007)] indicated that the closest relatives of strain THG 01^T were *Flavobacterium anhuiense* D3^T (97.5%

sequence similarity), *Flavobacterium johnsoniae* UW101^T (96.8%) and *Flavobacterium denitrificans* ED5^T (96.7%). Lower sequence similarities (<96.7%) were found with strains of all other recognized species of the family *Flavobacteriaceae*. This relationship between strain THG 01^T and other members of the genus *Flavobacterium* was also evident in the phylogenetic tree, which used over 1350 nt (Fig. 1). The DNA–DNA relatedness value between strain THG 01^T and *Flavobacterium anhuiense* D3^T was 11%. This low value, together with 16S rRNA gene sequence similarities of <97% with other strains, suggested that strain THG 01^T represented a novel *Flavobacterium* species according to Wayne *et al.* (1987) and Stackebrandt & Goebel (1994).

The DNA G+C content of strain THG 01^T was 32.1 mol%. The respiratory quinone system of strain THG 01^T was MK-6, which was consistent with the quinone systems of members of the genus *Flavobacterium*. The fatty acid profiles of isolate THG 01^T and five recognized species of the genus *Flavobacterium* are shown in Supplementary Table S1 (available in IJSEM Online). The fatty acid profile of strain THG 01^T was composed mainly of iso-C_{15:0} (24.7%), C_{16:1} ω 7c and/or C_{16:1} ω 6c

(14.6%), iso-C_{15:0} 3-OH (8.5%) and iso-C_{17:0} 3-OH (8.2%), fatty acids that are typical of those of members of the genus *Flavobacterium*.

In summary, the characteristics of strain THG 01^T were consistent with those of members of the genus *Flavobacterium* with regard to morphological, biochemical and chemotaxonomic properties. However, on the basis of phylogenetic distances between strain THG 01^T and recognized species of the genus *Flavobacterium*, as indicated by 16S rRNA gene sequence similarity values and the combination of unique phenotypic characteristics (Table 1) and low DNA–DNA relatedness values, strain THG 01^T should be assigned to the genus *Flavobacterium* as a representative of a novel species, for which the name *Flavobacterium ginsenosidimutans* sp. nov. is proposed.

Description of *Flavobacterium ginsenosidimutans* sp. nov.

Flavobacterium ginsenosidimutans (gin.se.no.si.di.mu'tans. N.L. n. *ginsenosidum* ginsenoside; L. part. adj. *mutans* transforming, converting; N.L. part. adj. *ginsenosidimutans* ginsenoside-converting).

Cells are Gram-negative, aerobic, yellow-pigmented, non-motile, non-spore-forming and rod-shaped, 0.2–0.4 µm in diameter and 1.0–1.5 µm in length, after cultivation on R2A agar for 2 days. Colonies grown on nutrient agar or R2A agar plates for 2 days are smooth, transparent and deep orange in colour. Grows at 10–37 °C and pH 5.5–10.0, but not at 4 or 42 °C. Optimum growth occurs at 25–30 °C and pH 7.0. Growth occurs in the absence of NaCl and in the presence of 1.0% (w/v) NaCl, but not in 2.0% (w/v) NaCl. Catalase- and oxidase-positive. Data for carbon assimilation tests (API ID 32GN, API 20NE) and enzyme activities (API ZYM) are listed in Table 1. MK-6 is the predominant menaquinone; iso-C_{15:0}, iso-C_{15:0} 3-OH and C_{16:1}ω7c and/or C_{16:1}ω6c are the major cellular fatty acid components.

The type strain is THG 01^T (=KACC 14525^T=JCM 16720^T), isolated from the soil of a ginseng field in Pocheon province, South Korea. The genomic DNA G+C content of the type strain is 32.1 mol%.

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