Chryseobacterium ginsenosidimutans sp. nov., a bacterium with ginsenoside-converting activity isolated from soil of a *Rhus vernicifera*-cultivated field

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A Gram-reaction-negative, strictly aerobic, non-motile, non-spore-forming, rod-shaped bacterial strain, designated THG 15^T, was isolated from soil of a field cultivated with *Rhus vernicifera* in Okcheon province, South Korea, and its taxonomic position was investigated by using a polyphasic approach. Strain THG 15^T grew optimally at 25–30 °C and at pH 7 in the absence of NaCl on nutrient agar. Strain THG 15^T displayed β -glucosidase (aesculinase) activity that was responsible for its ability to transform ginsenoside Rb₁ (one of the dominant active components of ginseng) into compound K via Rd and F₂. On the basis of 16S rRNA gene sequence similarities, strain THG 15^T was shown to belong to the family *Flavobacteriaceae* and was most closely related to *Chryseobacterium soldanellicola* PSD1-4^T (97.7 % sequence similarity), *Chryseobacterium soli* JS6-6^T (97.5 %) and *Chryseobacterium indoltheticum* LMG 4025^T (97.3 %). The G + C content of the genomic DNA was 35.7 mol%. The major menaquinone was MK-6 and the major fatty acids were iso-C_{15:0} (50.3 %), iso-C_{17:0} 3-OH (21.9 %), summed feature 4 (comprising C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH; 9.5 %) and iso-C_{17:1} ω 9c (9.3 %). DNA sequence analysis and chemotaxonomic data supported the affiliation of strain THG 15^T to the genus *Chryseobacterium*. DNA–DNA relatedness values between strain THG 15^T and its closest phylogenetic neighbours were <15 %. Strain THG 15^T could be differentiated genotypically and phenotypically from recognized species of the genus *Chryseobacterium*. The isolate therefore represents a novel species, for which the name *Chryseobacterium ginsenosidimutans* sp. nov. is proposed. The type strain is THG 15^T (=KACC 14527^T =JCM 16719^T).

Ginseng (the root of *Panax ginseng* C. A. Meyer) is one of the most popular medicinal plants and it has been used for strengthening immunity, providing nutrition and recovering from fatigue. Ginseng saponins (ginsenosides) have been recognized as being active agents in the biological and pharmacological properties of ginseng (Choi, 2008). More than 40 different ginsenosides have been isolated from ginseng roots, six of which (ginsenosides Rb₁, Rb₂, Rc, Rd, Re and Rg₁) constitute >90 % of the total ginsenosides (Park, 2004).

Biotransformation of ginsenosides can be achieved by hydrolysing and removing a sugar moiety using bacterial and fungal strains (Kim *et al.*, 2005; Son *et al.*, 2008; Zhao *et al.*, 2009). Biotransformed ginsenoside compound K has attracted outstanding attention in recent years because of its anti-tumour (Chae *et al.*, 2009), anti-inflammatory (Choi *et al.*, 2007), anti-allergic (Shin & Kim, 2005), anti-diabetic

(Kim *et al.*, 2009) and hepato-protective effects (Lee *et al.*, 2005).

The genus *Chryseobacterium*, created by Vandamme *et al.* (1994), is a member of the family *Flavobacteriaceae*, phylum 'Bacteroidetes' (Bernardet *et al.*, 2006). At the time of writing, it consisted of 45 species with validly published names, with *Chryseobacterium gleum* as the type species (Euzéby, 1997). Many novel species of the genus *Chryseobacterium* have been described in recent years from a variety of clinical and environmental sources, such as *Chryseobacterium anthropi* (Kämpfer *et al.*, 2009), *Chryseobacterium piscicola* (Ilardi *et al.*, 2009), *Chryseobacterium bovis* (Hantsis-Zacharov *et al.*, 2008) and *Chryseobacterium hungaricum* (Szoboszlai *et al.*, 2008).

During the course of a study screening ginsenoside-converting aerobic bacterial strains in soil of a *Rhus vernicifera*-cultivated field in Okcheon province, South Korea, several novel bacterial strains were isolated. One strain, designated THG 15^T, which could convert ginsenoside

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain THG 15^T is GU138380.

Rb₁ to compound K via Rd and F₂, appeared to be a member of the genus *Chryseobacterium* and was subjected to a polyphasic taxonomy study. Here, the taxonomic characterization of strain THG 15^T is reported.

The soil sample was thoroughly suspended in 50 mM phosphate buffer (pH 7) and spread on modified R2A agar as described by Liu *et al.* (2006). The plates were incubated at 30 °C for 2 weeks. Single colonies were first transferred onto new modified R2A agar and then onto conventional R2A agar (BD) or nutrient agar (NA; BD). Strain THG 15^T was cultured routinely on these media at 25 °C and preserved as a suspension in nutrient broth (NB; BD) with 20 % (w/v) glycerol at –70 °C.

Genomic DNA of strain THG 15^T was extracted using a commercial genomic DNA-extraction kit (Solgent). The 16S rRNA gene was amplified from the chromosomal DNA using the universal bacterial primer pair 9F and 1512R and the purified PCR products were sequenced by Solgent, Daejeon, South Korea (Ten *et al.*, 2008). The almost complete sequence of the 16S rRNA gene was compiled using the software SeqMan (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from GenBank and from the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Multiple alignments were performed by using the program CLUSTAL_X (Thompson *et al.*, 1997) and gaps were edited using the program BioEdit (Hall, 1999). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods in the program MEGA4 (Kumar *et al.*, 2008) with bootstrap values based on 1000 replications (Felsenstein, 1985).

The 16S rRNA gene sequence of strain THG 15^T determined in this study was a continuous stretch of 1386 bp (base positions 50–1458 with respect to the *Escherichia coli* numbering system). Sequence similarity

calculations using the EzTaxon server indicated that the closest relatives of strain THG 15^T were *Chryseobacterium soldanellicola* PSD1-4^T (97.7 % sequence similarity), *Chryseobacterium soli* JS6-6^T (97.5 %), *Chryseobacterium gregarium* P 461/12^T (97.4 %), *Chryseobacterium indoltheticum* LMG 4025^T (97.3 %), *Chryseobacterium piscicola* VQ-6316s^T (97.0 %) and *Chryseobacterium taiwanense* BCRC 17412^T (97.0 %). Sequence similarities <97.0 % were found with all other recognized species of the genus *Chryseobacterium*. The relationship between strain THG 15^T and members of the genus *Chryseobacterium* was also evident in the phylogenetic tree, which was based on the comparison of 1350 nt sequences (Fig. 1). On the basis of the phylogenetic inference, *C. soldanellicola* PSD1-4^T, *C. soli* JS6-6^T, *Chryseobacterium aquaticum* 10-46^T, *Chryseobacterium scopthalmum* LMG 13028^T and *Chryseobacterium piscium* LMG 23089^T were the closest neighbours of strain THG 15^T; these strains were obtained from culture collections, grown under the same conditions and used as reference strains in most phenotypic tests and in DNA–DNA hybridization experiments.

The Gram-reaction was performed by the non-staining method as described by Buck (1982). Cell morphology and motility were observed at ×1000 magnification with a light microscope (BX50; Olympus) using cells grown for 24 h at 27 °C on NA. Catalase activity was assessed by bubble production in 3 % (v/v) hydrogen peroxide and oxidase activity was determined using 1 % (w/v) tetramethyl *p*-phenylenediamine. Carbon-source utilization and enzyme activities were tested by using API 20 NE, API ID 32 GN and API ZYM kits according to the manufacturer's instructions (bioMérieux). Tests for degradation of DNA [using DNase agar (Scharlau) flooded with 1 M HCl to reveal DNase activity], casein (skimmed milk) and starch (Atlas, 1993) were evaluated after 7 days of incubation at 27 °C. Growth was assessed after 5 days of incubation at 4, 10, 18, 27, 35, 37, 42 and 45 °C on NA and at pH 5–10 (at

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships of strain THG 15^T to related species of the genus *Chryseobacterium* and representatives of related genera in the family *Flavobacteriaceae*. Filled circles at nodes indicate generic branches that were also recovered by using the maximum-parsimony algorithm. Bootstrap values >70 % (expressed as percentages of 1000 replications) are shown at branching points. Bar, 0.01 substitutions per nucleotide position.

intervals of 0.5 pH units) in NB. Three different buffers (50 mM final concentration) were used to adjust the pH of NB. 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 NB supplemented with 1–10 % (w/v) NaCl (at intervals of 1 %) after 5 days of incubation. Growth was determined by monitoring OD₆₀₀. Anaerobic growth was tested in serum bottles containing NB plus thioglycolate (1 g l⁻¹) and substituting the air atmosphere of the bottle with nitrogen gas. Growth was also evaluated on trypticase soy agar (TSA; BD), MacConkey agar (BD) and cetrimide agar (BD) at 27 °C. The presence of flexirubin-type pigments was tested as described previously (Fautz & Reichenbach, 1980) using 20 % (w/v) KOH. Conversion of ginsenoside Rb₁ was assessed using TLC and HPLC analysis as described by Kim *et al.* (2005).

Cells of strain THG 15^T were Gram-reaction-negative, strictly aerobic, non-spore-forming, non-motile, oxidase-positive and catalase-positive rods that produced flexirubin-type

pigments. Activities of α - and β -glucosidases were detected in strain THG 15^T; these enzymes are responsible for the gradual conversion of ginsenoside Rb₁ to compound K (data not shown). The phenotypic characteristics of strain THG 15^T and five reference type strains are summarized in the species description and given in Table 1.

The genomic DNA of strain THG 15^T was extracted and purified as described by Moore & Dowhan (1995) and its G + C content was determined as described by Mesbah *et al.* (1989) using reversed-phase HPLC. The cellular fatty acid profiles of strain THG 15^T and five reference type strains were determined using cells grown on NA for 2 days at 27 °C. Cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock microbial identification system (MIDI) and analysed by capillary GLC (Hewlett Packard 6890) using the Microbial Identification software package with the Sherlock system MIDI 6.0 and the Sherlock Aerobic Bacterial Database (TSBA60) (Sasser, 1990). For isoprenoid quinone analysis,

Table 1. Differential characteristics of strain THG 15^T and type strains of related species of the genus *Chryseobacterium*

Strains: 1, THG 15^T; 2, *C. soldanellicola* PSD1-4^T; 3, *C. soli* JS6-6^T; 4, *C. aquaticum* 10-46^T; 5, *C. scopthalmum* LMG 13028^T; 6, *C. piscium* LMG 23089^T. All data are from this study, except for the DNA G + C contents of the reference strains (taken from Park *et al.*, 2006; Weon *et al.*, 2008; Kim *et al.*, 2008; Vandamme *et al.*, 1994; de Beer *et al.*, 2006). All strains were Gram-reaction-negative rods, catalase- and oxidase-positive and tolerate 1 % NaCl. In API 20 NE and API ID 32 GN kits, all strains were positive for arginine dihydrolase and urease activities, gelatin and casein hydrolysis, production of flexirubin-type pigments and assimilation of D-glucose, maltose and D-mannose. All strains were negative for nitrate reduction, acid production from glucose and assimilation of the following substrates: 2-ketogluconate, 3-hydroxybenzoate, 3-hydroxybutyrate, 4-hydroxybenzoate, 5-ketogluconate, adipate, caprate, citrate, D-mannitol, melibiose, D-ribose, D-sorbitol, gluconate, *myo*-inositol, itaconate, lactate, L-fucose, L-histidine, L-serine, malate, malonate, N-acetylglucosamine and suberate. In API ZYM kits, all strains were positive for alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-B1-phosphohydrolase, α - and β -glucosidase activities but negative for esterase (C4), lipase (C14), cystine arylamidase, α -chymotrypsin, α - and β -galactosidase, β -glucuronidase, α -mannosidase and α -fucosidase. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2	3	4	5	6
Indole production	–	+	+	–	+	+
Hydrolysis of:						
DNA	+	–	+	+	+	–
Starch	+	+	+	+	–	–
Assimilation of (API 20NE/API ID 32 GN):						
Acetate	+	+	+	+	–	–
Sucrose	–	+	–	+	+	–
Glycogen	+	+	–	+	+	+
L-Alanine	+	+	–	–	–	+
L-Arabinose	+	+	–	–	+	–
L-Proline	+	–	–	–	+	+
L-Rhamnose	–	+	+	–	+	–
Phenylacetate	–	–	–	–	–	+
Propionate	+	–	+	+	–	+
Salicin	–	–	–	–	+	–
Valerate	+	–	–	–	–	+
Enzyme activity (API ZYM)						
Esterase lipase (C8)	w	+	w	w	w	w
Trypsin	–	–	+	+	+	+
N-Acetyl- β -glucosaminidase	+	–	–	–	+	–
DNA G + C content (mol%)	35.7	29	39.9	38.5	34.1	34

strain THG 15^T was grown in modified NB for 2 days at 27 °C. Cells were lyophilized for 24 h and isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under vacuum 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 15^T, *C. soli* JS6-6^T and *C. soldanellicola* PSD1-4^T according to the method described by Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and micro-dilution wells. Hybridization was performed at 39 °C 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 (%).

The DNA G + C content of strain THG 15^T was 35.7 mol%, a value within the range reported for species of the genus *Chryseobacterium* (Bernardet *et al.*, 2006). The major respiratory quinone of strain THG 15^T was MK-6, which is in line with all other known members of the family *Flavobacteriaceae* (Bernardet & Nakagawa, 2006). The fatty acid profiles of strain THG 15^T and the five reference strains are shown in Table 2. The fatty acid profile of strain THG 15^T was mainly composed of iso-C_{15:0} (50.3 %), iso-C_{17:0} 3-OH (21.9 %), summed feature 4 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 9.5 %) and iso-C_{17:1}ω9c (9.3 %), which is similar to the profiles of its five closest relatives. DNA–DNA relatedness values of strain THG 15^T with *C. soli* JS6-6^T and *C. soldanellicola* PSD1-4^T were 12 and 15 %, respectively.

In summary, the characteristics of strain THG 15^T were consistent with those of members of the genus

Chryseobacterium with regard to morphological, biochemical and chemotaxonomic properties. However, the phylogenetic distance and the low DNA–DNA relatedness values (Wayne *et al.*, 1987) between strain THG 15^T and recognized species of the genus *Chryseobacterium* along with its unique phenotypic characteristics (Table 1) suggests strain THG 15^T represents a novel species of the genus *Chryseobacterium*, for which the name *Chryseobacterium ginsenosidimutans* sp. nov. is proposed.

Description of *Chryseobacterium ginsenosidimutans* sp. nov.

Chryseobacterium 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-reaction-negative, strictly aerobic, non-motile and non-spore-forming rods, 0.3–0.5 × 1.5–2.0 µm after culture on NA for 2 days. Colonies are smooth, transparent, deep-orange, convex, circular with regular margins and 1.0–1.5 mm in diameter after 2 days of growth on NA. Growth also occurs on TSA and R2A agar but not on MacConkey or cetrimide agars. Growth occurs on NA at 10–37 °C and pH 5.5–10.0 but not at 4 °C 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 with 2.0 % (w/v) NaCl. Catalase- and oxidase-positive. Starch, gelatin, casein and DNA are hydrolysed. Flexirubin-type pigments are produced. Arginine dihydrolase and urease activities are present. Nitrate is not reduced. Acid is not produced from glucose. The substrates utilized as sole carbon sources (API ID 32 GN, API 20 NE) and the enzyme activities (API

Table 2. Cellular fatty acid profiles (%) of strain THG 15^T and phylogenetically related species of the genus *Chryseobacterium*

Strains: 1, THG 15^T; 2, *C. soldanellicola* PSD1-4^T; 3, *C. soli* JS6-6^T; 4, *C. aquaticum* 10-46^T; 5, *C. scopthalmum* LMG 13028^T; 6, *C. piscium* LMG 23089^T. All data are from this study. All strains were cultured on NA for 2 days at 27 °C. Fatty acids amounting to <1 % of the total fatty acids in all strains are not listed. –, Not detected.

Fatty acid	1	2	3	4	5	6
Saturated						
C _{16:0}	—	—	—	6.5	—	—
Branched-chain						
iso-C _{13:0}	—	—	—	2.7	—	—
iso-C _{15:0}	50.3	47.8	42.2	48.3	44.4	47.3
iso-C _{17:1} ω9c	9.3	13.6	14.3	—	20.1	17.0
anteiso-C _{15:0}	3.8	5.0	8.1	5.4	3.2	4.5
Hydroxy						
iso-C _{15:0} 3-OH	5.2	4.1	4.3	3.9	4.7	5.4
iso-C _{16:0} 3-OH	—	—	—	4.7	—	—
iso-C _{17:0} 3-OH	21.9	19.9	20.8	16.9	17.6	20.0
Summed feature 4*	9.5	9.7	10.4	11.6	10.1	5.9

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 4 comprises C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

ZYM) are listed in Table 1. MK-6 is the predominant respiratory quinone. Major cellular fatty acids (>9%) are iso-C_{15:0}, iso-C_{17:0} 3-OH, summed feature 4 (comprising C_{16:1ω7c} and/or iso-C_{15:0} 2-OH) and iso-C_{17:1ω9c}.

The type strain is THG 15^T (=KACC 14527^T =JCM 16719^T), isolated from soil of a *Rhus vernicifera*-cultivated field in Okcheon province, South Korea. The genomic DNA G+C content of the type strain is 35.7 mol%.

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