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Mucilaginibacter composti sp. nov., with Ginsenoside Converting Activity, Isolated from Compost

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The Gram-negative, strictly aerobic, non-motile, non-spore-forming, rod shaped bacterial strain designated TR6-03^T was isolated from compost, and its taxonomic position was investigated by using a polyphasic approach. Strain TR6-03^T grew at 4-42°C and at pH 6.0-8.0 on R2A and nutrient agar without NaCl supplement. Strain TR6-03^T had *β*-glucosidase activity, which was responsible for its ability to transform ginsenoside Re (one of the dominant active components of ginseng) to Rg_2. On the basis of 16S rRNA gene sequence similarity, strain TR6-03^T was shown to belong to the family *Sphingobacteriaceae* and to be related to *Mucilaginibacter lappiensis* ANJLI2^T (96.3% sequence similarity), *M. dorajii* FR-f4^T (96.1%), and *M. rigui* WPCB133^T (94.1%). The G+C content of the genomic DNA was 45.6%. The predominant respiratory quinone was MK-7 and the major fatty acids were summed feature 3 (comprising $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2OH), iso-$C_{15:0}$ and iso-$C_{17:0}$ 3OH. DNA and chemotaxonomic data supported the affiliation of strain TR6-03^T to the genus *Mucilaginibacter*. Strain TR6-03^T could be differentiated genotypically and phenotypically from the recognized species of the genus *Mucilaginibacter*. The isolate therefore represents a novel species, for which the name *Mucilaginibacter composti* sp. nov. is proposed, with the type strain TR6-03^T (=KACC 14956^T =KCTC 12642^T =LMG 23497^T).

Keywords: 16S rRNA gene, polyphasic taxonomy, *Mucilaginibacter composti*, ginsenoside

During the course of a study to screen ginsenoside converting aerobic bacterial strains in soil and compost samples, several novel bacterial strains showing ginsenoside-converting activity were screened. Among them, a strain designated TR6-03^T isolated from a compost sample, which could convert ginsenoside Re to Rg_2, became the subject of a taxonomic investigation.

Biotransformation of ginsenoside (deglycosylation) can be achieved by hydrolyzing and removing a sugar moiety from the major ginsenosides using bacterial and fungal strains (Kim *et al*., 2005; Zhao *et al*., 2009; Park *et al*., 2010). This is desirable because the efficacy of ginsenoside increases with the extent of deglycosylation, which enhances its hydrophobicity and cell wall permeability.

The genus *Mucilaginibacter* was recently proposed by Pankratov *et al*. (2007) and emended by Urai *et al*. (2008) and Baik *et al*. (2010) in order to accommodate Gram-negative, strictly aerobic or facultatively anaerobic, chemoheterotrophic, rod shaped bacteria. It contains menaquinone-7 (MK-7) as the predominant respiratory quinone and unsaturated and branched saturated fatty acids as the major fatty acids. The G+C content of the genomic DNA of this genus ranges from 42.4 to 47.0 mol% (Pankratov *et al*., 2007; Urai *et al*., 2008; Baik *et al*., 2010). At the time of writing, the genus consisted of 13 validly named species, with *M. paludis* as the type species (Euzéby, 1997).

In the present study, we describe the taxonomic position of strain TR6-03^T based on the results of polyphasic analyses, and we propose the name *Mucilaginibacter composti*.

Materials and Methods

Isolation of bacterial strain and culture conditions

Strain TR6-03^T was originally isolated from compost in Chungwon province, South Korea. This compost sample was thoroughly suspended in 50 mM phosphate buffer (pH 7.0) and spread on nutrient agar (NA, Difco, USA) plates. The plates were incubated at 30°C for 2 weeks. The TR6-03^T was one of the single colonies on the plates and was purified by transferring it onto a new nutrient agar plate. It was cultured routinely on R2A agar (Difco) or NA at 25°C and preserved as a suspension in nutrient broth with glycerol (20%, w/v) at -70°C. Strain TR6-03^T was deposited in the Korean Agricultural Culture Collection (=KACC 14956^T), the Korean Collection for Type Cultures (=KCTC 12642^T) and the Belgian Co-ordinated Collections of Micro-organisms/Laboratorium voor Microbiologie (=LMG 23497^T).

Phenotypic and biochemical characteristics

The Gram reaction was determined using the non-staining method, as described by Buck (1982). Cell morphology and motility were observed under a Nikon light microscope at 1000× using the hanging drop technique (Perry, 1973), with cells grown on R2A agar for 2 days at 30°C. Catalase and oxidase tests, and spore observation were performed as outlined by Cappuccino and Sherman (2002). Cells

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grown on R2A agar for 1 day were used as inocula for the physiological and biochemical tests. Anaerobic growth was determined in serum bottles containing R2A broth supplemented with thioglycolate (1 g/L), in which the upper air layer had been replaced with nitrogen. In addition, biochemical phenotypic tests were carried out using API 20NE, API ID 32GN, and API ZYM test kits according to the instructions of the manufacturer (bioMérieux, France). Tests for hydrolysis of DNA (using DNase agar from Scharlau, with DNase activity determined by flooding plates with 1 M HCl), casein, starch, tween 80, pullulan, laminarin (Atlas, 1993), xylan and carboxymethyl-cellulose (Ten *et al*., 2004) were performed and evaluated after 7 days. Growth at different temperatures (4, 10, 18, 30, 37, 42, and 45°C) and various pH values (pH 4.5-10.0 at intervals of 0.5 pH units) was assessed after 7 days of incubation. Salt tolerance was tested on R2A medium supplemented with 1-10% NaCl (w/v, at intervals of 1%) after 7 days of incubation. Growth on trypticase soy agar (TSA, Difco), and MacConkey agar (Difco) was also evaluated at 30°C.

Biotransformation of ginsenosides

Ginsenosides Rb_1, Rc, Rd, Re, Rg_1, Rg_2(S), Rh_1(S), F_2, and compound K were purchased from Dalian Green Bio Ltd (China). The reaction mixture, containing 200 μl of 1 mM ginsenosides (Re and Rg_1, respectively) and 200 μl of a bacterial suspension inoculated in a nutrient broth, was incubated for 4 days, at 150 rpm and 30°C. During the reaction, a 50 μl aliquot was taken daily, extracted with an equal volume of water-saturated *n*-butanol, and subjected to TLC analysis. TLC was performed using $60F_{254}$ silica gel plates (Merck, Germany) with $CHCl_3$-CH_3OH-H_2O (65:35:10, v/v, lower phase) as the solvent. The spots on the TLC plates were detected by spraying with 10% (v/v) H_2SO_4 followed by heating at 110°C for 5 min.

PCR amplification, 16S rRNA gene sequencing, and phylogenetic analysis

The genomic DNA of strain TR6-03^T was extracted using a commercial genomic DNA-extraction kit (Solgent, Korea). 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 Co. Ltd (Im *et al.*, 2010). Full sequences of the 16S rRNA gene were compiled using SeqMan software (DNASTAR, USA). The 16S rRNA gene sequences of related taxa were obtained from the GenBank and EzTaxon servers (Chun *et al.*, 2007). Multiple alignments were performed using the CLUSTAL X program (Thompson *et al*., 1997) and gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). The phylogenetic trees were constructed by using the neighbor-joining (Saitou and Nei, 1987) and the maximum-parsimony (Fitch, 1971) methods with the MEGA4 Program (Kumar *et al.*, 2008), with bootstrap values based on 1,000 replications (Felsenstein, 1985).

Isoprenoid quinones and cellular fatty acids

Cell biomass of strain TR6-03^T needed for isoprenoid quinones was obtained from cultures grown on R2A agar for 2 days at 30°C. Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under a vacuum and re-extracted in *n*-hexane-water (1:1, v/v). The crude quinone in *n*-hexane solution was purified using Sep-Pak Vac Cartridges Silica (Waters) and subsequently analyzed using a reverse-phase HPLC system (Younglin, Korea), as described by Hiraishi *et al*. (1996). Cellular fatty acid profiles were determined for strains grown on R2A agar for 48 h at 30°C. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acid methyl esters were then analyzed by gas chromatography (model 6890; Hewlett Packard) using the Microbial Identification software package (Sasser, 1990).

Determination of DNA G+C content

For measurement of the G+C content of chromosomal DNA, the genomic DNA of strain TR6-03^T was extracted and purified as described by Moore and Dowhan (1995) and enzymatically degraded into nucleosides. The G+C content was then determined as described by Mesbah *et al.* (1989), using a reverse-phase HPLC system (Younglin).

Results and Discussion

Morphological and phenotypic characteristics

Cells of strain TR6-03^T were Gram-negative, strictly aerobic, non-spore-forming, non-motile, rod shaped, oxidase-positive and catalase-negative. The colonies grown on R2A agar plates for 2 days were smooth, circular, light-yellowish in color, convex, and 2-3 mm in diameter. On R2A agar, TR6-03^T was able to grow at 4-42°C, but not at 45°C. The isolate grew well on nutrient agar and TSA, but not on MacConkey agar. The phenotypic and chemotaxonomic characteristics that differentiate the strain TR6-03^T from other closely related *Mucilaginibacter* species are listed in Table 1.

Biotransformation of ginsenosides

Results from a time course study of the biotransformation of the ginsenosides Re and Rg_1 are shown in Fig. 1. Ginsenoside Re was transformed into Rg_2(S) (line 4). Ginsenoside Rg_1 was transformed into compound Rh_1(S) (line 5). Ginsenosides Re and Rg_1 were almost completely hydrolyzed into Rg_2(S) and Rh_1 (S) after 3 days, respectively.

Phylogenetic analysis

The 16S rRNA gene sequences of the strain TR6-03^T determined in this study were continuous stretches of 1480 bp

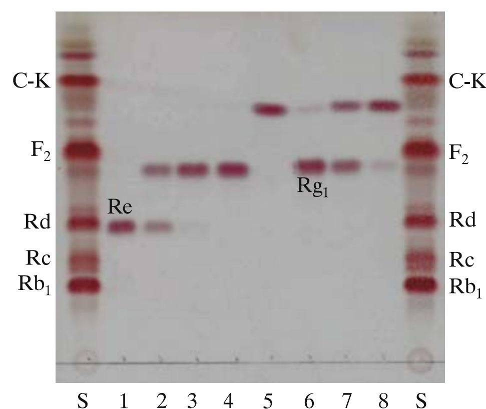

Fig. 1. TLC analyses of time-course transformation of ginsenoside Re and Rg_1 by strain TR6-03^T. Developing solvent: $CHCl_3$/MeOH/H_2O (65:35:10, by vol.). Lanes: S, saponin standards; 1, reaction mixture of Re after 1 day; 2, 2 days; 3, 3 days; 4, Rg_2(S); 5, Rh_1(S); 6, reaction mixture of Rg_1 after 1 day; 7, 2 days; 8, 3 days. Abbreviations: C-K, compound K.

Table 1. Differentiating characteristics of *M. composti* TR6-03^T and the type strains of related *Mucilaginibacter* species
Strains: 1, *Mucilaginibacter composti* TR6-03^T; 2, *Mucilaginibacter lappiensis* ANJLI2^T; 3, *Mucilaginibacter rigui* WPCB133^T; 4, *Mucilaginibacter dorajii* FR-f4^T
Data were from this study, Männistö *et al*. (2010), Baik *et al*. (2010), and Kim *et al*. (2010). All strains were Gram-negative, non-motile, rod shaped, positive for oxidase activities. In API 20 NE and API ID 32 GN kits, all strains were positive for assimilation of D-glucose and L-arabinose. All strains were negative for nitrate reduction, indole production, acid production from glucose, hydrolysis of xylan, and assimilation of the following substrates: D-mannitol, *N*-acetylglucosamine, caprate, citrate, phenyl-acetate, 4-hydroxybenzoate, L-rhamnose, inositol, itaconate, suberate, acetate, L-alanine, 5-ketogluconate, 3-hydroxybenzoate and L-serine. In API ZYM kits, all the strains were positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *α*-galactosidase, *β*-galactosidase, *α*-glucosidase and *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase activities. All strains were negative for lipase (C14) and α-chymotrypsin activities. +, Positive; -, negative.

Characteristics	1	2	3	4
Isolation source	Compost	Lichen	Wet land freshwater	Rhizosphere
Colony colour on R2A	Light yellow	Light pink to reddish	Pale pink	Light yellow
Cell Size	0.4-0.6×1.8-3.0	0.3-0.5×1-3	0.3-0.4×1.0-1.7	0.6-0.8×1.1-1.8
Growth temperature (°C)	4-42	0-31	4-37	4-30
pH range	6.0-8.0	4.5-8.0	5.0-10.0	5.0-8.0
Salt tolerance at 1% (w/v)	-	+	-	+
Catalase	-	+	+	+
Arginine dihydrolase	+	+	+	-
Urease	+	+	+	-
Hydrolysis of				
Carboxymethyl-cellulose	-	+	-	+
Starch	-	-	-	+
Gelatin	-	+	-	-
Tween 80	-	-	-	+
Carbon utilization of[a]				
Gluconate	-	+	-	-
Adipate	-	+	-	-
Malate	-	+	+	-
Salicin	+	-	-	+
D-Melibiose	-	+	+	+
L-Fucose	-	+	-	-
D-Sorbitol	-	+	-	-
Propionate	-	+	-	-
Caprate	-	-	-	-
Valerate	-	+	-	-
L-Histidine	-	+	-	-
2-Ketogluconate	-	+	-	-
3-Hydroxy-butyrate	-	+	-	-
L-Proline	-	+	+	-
D-Ribose	-	+	-	-
D-Sucrose	-	-	+	+
D-Mannose	-	+	+	+
D-Maltose	-	+	+	+
Malonate	-	+	-	-
Lactate	-	+	-	-
Glycogen	-	-	+	w
API ZYM test results[a]				
Trypsin	-	+	+	-
β-Glucuronidase	-	+	-	-
α-Mannosidase	-	+	+	+
α-Fucosidase	-	+	+	+
Quinone	MK-7	MK7, MK6	MK-7	MK-7
G+C content (mol%)	45.6	43.5	47	42.6

[a] All these data from this study.

(base position 9-1505 with respect to the *Escherichia coli* numbering system), which were deposited in a GenBank database (accession numbers AB267719). Sequence similarity values between TR6-03^T and other *Mucilaginibacter* species using the

EzTaxon server [http://www.eztaxon.org/; Chun *et al*. (2007)] ranged from 96.3% to 93.3%. Strain TR6-03^T showed highest similarity to *Mucilaginibacter lappiensis* ANJLI2^T (96.3%), followed by *M. paluda* TPT56^T (96.2%) and *M. dorajii* FR-f4^T (96.1%). Since strains differing by >3.0% at the 16S rRNA gene level are likely to exhibit <70% relatedness at the whole-genome level (Stackebrandt and Goebel, 1994), strain TR6-03^T appears to be a different species.

The relationship between strain TR6-03^T and other members of the genus *Mucilaginibacter* was also evident in the neighbor-joining tree, which used over 1350 nt (Fig. 2). In this phylogenetic tree, the isolate formed a distinct lineage. Strain TR6-03^T, *M. lappiensis* ANJLI2^T, *M. dorajii* FR-f4^T, and *M. rigui* WPCB133^T formed a monophyletic group although it didn't have a high bootstrap value, which was supported by the maximum-parsimony tree-making method.

Chemotaxonomy and G+C content

The predominant respiratory quinone of strain TR6-03^T was menaquinone 7 (MK-7), in line with all other members of the genus *Mucilaginibacter*. The cellular fatty acids of strain TR6-03^T and related type strains are listed in Table 2. The major fatty acids of all 4 compared strains were summed feature 3 (comprising $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2OH), iso-$C_{15:0}$ and iso-$C_{17:0}$ 3OH. As shown in Table 2, qualitative and quantitative differences in fatty acid content could be observed between strain TR6-03^T and its phylogenetically closest relatives. The DNA G+C content of strain TR6-03^T was 45.6 mol%, similar to those of *M. lappiensis* ANJLI2^T, *M. dorajii* FR-f4^T, and *M. rigui* WPCB133^T, which were in the range of 42.6-47.0 mol%.

Taxonomic conclusions

The characteristics of strain TR6-03^T were consistent with the description of the genus *Mucilaginibacter* with regard to morphological, biochemical and chemotaxonomic properties. However, the phylogenetic distance between strain TR6-03^T and recognized *Mucilaginibacter* species, the relatively low 16S rRNA gene sequence similarity (<96.3%) and the combination of unique phenotypic characteristics (Table 1) warrant assignment of strain TR6-03^T to the genus *Mucilaginibacter* as the type strain of a novel species, for which the name *Mucilaginibacter composti* sp. nov. is proposed.

Description of *Mucilaginibacter composti* sp. nov.

Mucilaginibacter composti (N.L. n. compostum -i, compost; N.L. gen. n. composti, of compost).

Cells are Gram-negative, strictly aerobic, non-motile and non-spore-forming rods (0.4-0.6 μm in diameter and 1.8-3.0 μm in length) after culture on R2A agar for 2 days. Colonies are smooth, transparent, convex, circular with regular margins, light-yellowish in color, and 2-3 mm in diameter after 2 days on R2A agar. Growth also occurs on nutrient agar and TSA,

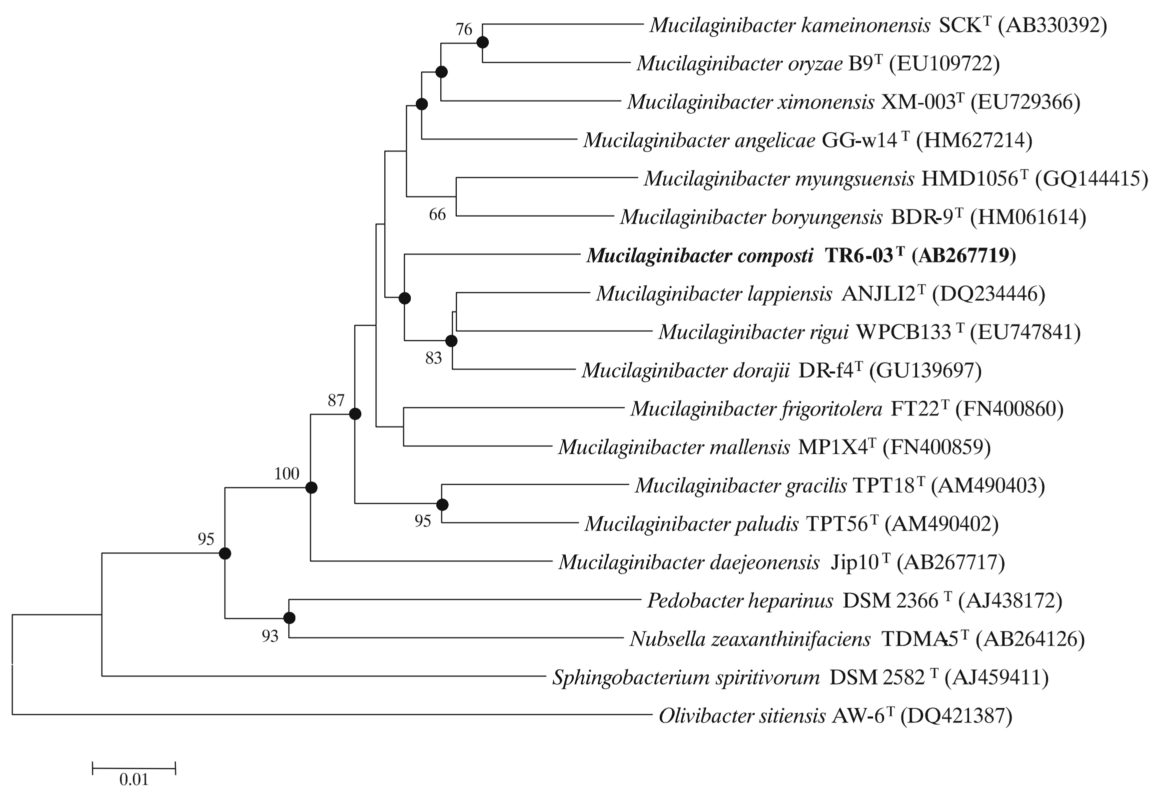

Fig. 2. Phylogenetic tree constructed from a comparative analysis of 16S rRNA gene sequences showing the relationships of *M. composti* TR6-03^T with other related species. This tree was made using the neighbor-joining method (Saitou and Nei, 1987) with a Kimura (1983) two-parameter distance matrix and pairwise deletion. Dots indicate generic branches that were also recovered by using maximum-parsimony algorithms. Bootstrap values (expressed as percentages of 1,000 replications) greater than 65% are shown at the branch points. Bar, 0.01 substitutions per 1 nucleotide position.

Table 2. Cellular fatty acid profiles of strain TR6-03[T] and recognized *Mucilaginibacter* species
Strains: 1, *Mucilaginibacter composti* TR6-03[T]; 2, *Mucilaginibacter lappiensis* ANJLI2[T]; 3, *Mucilaginibacter rigui* WPCB133[T]; 4, *Mucilaginibacter dorajii* FR-f4[T]
All data were from this study except those of *M. dorajii* FR-f4[T] which were taken from Kim *et al*. (2010). All cells were cultured on R2A agar for 2 days with MIDI version 6.0. -, not detected. tr, trace amount (<0.5%); ECL, equivalent chain length. Major fatty acids are shown in bold type. Some fatty acids that account for less than 0.5% of the total fatty acids in all strains are excluded. Therefore, the percentages do not add up to 100%.

Fatty acid	1	2	3	4
Saturated				
$C_{14:0}$	0.9	0.6	0.6	0.6
$C_{15:0}$	-	-	-	2.8
$C_{16:0}$	8.2	7.3	7.7	7.8
Unsaturated				
$C_{14:1}$ ω5c	3.2	1.4	2.4	-
$C_{15:1}$ ω6c	-	-	0.6	tr
$C_{16:1}$ ω5c	9.5	3.4	4.6	6.8
$C_{17:1}$ ω8c	0.5	-	0.9	0.6
Branched-chain fatty acid				
iso-$C_{13:0}$	-	-	0.6	-
iso-$C_{15:0}$	19.1	23.2	25.6	15.0
iso-$C_{15:0}$ 3OH	3.1	2.1	2.1	1.8
iso-$C_{16:0}$		0.5	1.2	tr
iso-$C_{17:0}$	1.4	0.8	1.0	tr
iso-$C_{17:0}$ 3OH	13.0	14.9	9.9	7.0
anteiso-$C_{15:0}$	2.2	0.8	1.7	-
anteiso-$C_{17:0}$	1.7	0.8	1.4	-
iso-$C_{17:1}$ ω9c	4.1	4.2	4.6	1.8
Hydroxy fatty acids				
$C_{15:0}$ 2OH	-	-	0.7	0.5
$C_{15:0}$ 3OH	-	-	0.5	tr
$C_{16:0}$ 3OH	-	2.1	0.8	1.6
Summed feature				
3; $C_{16:1}$ ω7c/iso-$C_{15:0}$ 2OH	32.8	37.0	31.8	45.6
Unknown				
ECL 13.565	-	-	-	1.2
ECL 16.582	-	-	-	0.5
ECL 18.81	-	-	-	0.6

* Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed features consist of: 3, $C_{16:1}$ ω7c/iso-$C_{15:0}$ 2OH

but not on MacConkey. Grows on R2A at 4-42°C but not at 45°C, and at pH 6.0-8.0. Optimum growth occurs at 30-37°C and at pH 7.0. Growth occurs well without NaCl supplement. Catalase is negative, oxidase is positive. Does not hydrolyze DNA, casein, starch, pullulan, laminarin, xylan, and carboxymethyl-cellulose. Carbon assimilation tests using single carbon sources (API ID 32 GN, API 20 NE) and enzyme activities (API ZYM) are listed in Table 1. MK-7 is the predominant respiratory quinone, and summed feature 3 (comprising $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2OH), iso-$C_{15:0}$ and iso-$C_{17:0}$ 3OH are the major cellular fatty acids (>10%). The G+C content of the genomic DNA is 45.6 mol%.

The type strain, TR6-03[T] (=KACC 14956[T] =KCTC 12642[T] =LMG 23497[T]) was isolated from compost in Chungwon province, Korea.

Abbreviations used

Re, 6-*O*-[α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl]-20-*O*-*β*-D-glucopyranosyl-20(S)-protopanaxatriol; Rg_1, 6-*O*-*β*-D-glucopyranosyl-20-*O*-*β*-D-glucopyranosyl-20(S)-protopanaxatriol; Rg_2(S), 6-*O*-[α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl]-20(S)-protopanaxatriol; Rh_1(S), 6-*O*-*β*-D-glucopyranosyl-20(S)-protopanaxatriol

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