Methylibium fulvum sp. nov., a member of the *Betaproteobacteria* isolated from ginseng field soil, and emended description of the genus *Methylibium*

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Two bacterial strains, designated Gsoil 322^T and Gsoil 328, were isolated from soil of a ginseng field in Pocheon province (Republic of Korea). The strains were Gram-negative, motile, aerobic rods that showed nearly identical physiological profiles and similar chemotaxonomic profiles. The two strains were oxidase-positive but catalase-negative, reduced nitrate to nitrite and had fatty acid profiles in which C_{16:0}, C_{17:0} cyclo and C_{16:1ω7c}/iso-C_{15:0} 2-OH were predominant. The DNA G+C contents of Gsoil 322^T and Gsoil 328 were 66.6 and 66.7 mol%, respectively. Q-8 was observed as the major quinone. Comparative 16S rRNA gene sequence analysis showed that strain Gsoil 322^T belongs to the class *Betaproteobacteria* and was most closely related to *Methylibium petroleiphilum* ATCC BAA-1232^T (97.5 % sequence similarity). On the basis of its phenotypic properties and phylogenetic distinctiveness, strain Gsoil 322^T (=KCTC 12591^T =LMG 23394^T) was classified in the genus *Methylibium* as the type strain of a novel species, for which the name *Methylibium fulvum* sp. nov. is proposed.

The genus *Methylibium* was recently proposed by Nakatsu *et al.* (2006) and, at the time of writing, it comprises only one recognized species, *Methylibium petroleiphilum*. During the course of a study on the culturable aerobic and facultatively anaerobic bacterial community in the soil of the ginseng fields in Pocheon province (Republic of Korea), a large number of novel bacterial strains were isolated. In the present study, we have characterized two of these isolates, strains Gsoil 322^T and Gsoil 328. Phenotypic, chemotaxonomic and phylogenetic analyses establish the affiliation of the isolates to the genus *Methylibium*. The data obtained also suggest that strain Gsoil 322^T represents a novel species of the genus.

Strains Gsoil 322^T and Gsoil 328 were originally isolated from a soil sample of a ginseng field in Pocheon province. The soil sample was suspended in 50 mM phosphate buffer (pH 7.0) and serial decimal dilutions of the suspension were spread on modified R2A agar plates (per litre: 0.25 g tryptone, 0.25 g peptone, 0.25 g yeast extract, 0.125 g malt extract, 0.125 g beef extract, 0.25 g Casamino acids, 0.25 g soytone, 0.5 g glucose, 0.3 g soluble starch, 0.2 g xylan,

0.3 g sodium pyruvate, 0.3 g K₂HPO₄, 0.05 g MgSO₄, 0.05 g CaCl₂, 15 g agar). The plates were incubated at 30 °C for 1 month. Single colonies on the plates were purified by subculturing on modified R2A medium. Purified colonies were tentatively identified by partial sequencing of the 16S rRNA gene (Im *et al.*, 2005). Strains Gsoil 322^T and Gsoil 328 grew on modified R2A agar plates under aerobic conditions. The isolates were cultured routinely on R2A agar at 25 °C and maintained as a glycerol suspension (20 %, w/v) at –70 °C.

For phylogenetic analysis of strains Gsoil 322^T and Gsoil 328, DNA was extracted by using a genomic DNA extraction kit (Core Biosystem), the 16S rRNA gene was amplified by PCR and sequencing of the purified PCR product was carried out according to the method of Kim *et al.* (2005). The 16S rRNA gene sequences were compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Multiple alignments were performed via the CLUSTAL_X program (Thompson *et al.*, 1997). Gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated according to the Kimura two-parameter model (Kimura, 1983) and phylogenetic trees were constructed by using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971)

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains Gsoil 322^T and Gsoil 328 are AB245356 and AB245357, respectively.

methods in the MEGA3 program (Kumar *et al.*, 2004) with bootstrap values based on 1000 replications (Felsenstein, 1985).

The 16S rRNA gene sequences of strains Gsoil 322^T and Gsoil 328 were continuous stretches of 1477 and 1489 bp, respectively. The level of 16S rRNA gene sequence similarity between the two strains was 99.9% (Fig. 1). Preliminary comparison against the 16S rRNA gene sequences in GenBank indicated that strains Gsoil 322^T and Gsoil 328 belonged to the *Sphaerotilus–Leptothrix* group in the *Betaproteobacteria*. On the basis of 16S rRNA gene sequence similarity data, it was found that the closest recognized relative of strain Gsoil 322^T was *M. petroleiphilum* ATCC BAA-1232^T (97.5% sequence similarity). Levels of sequence similarity of strain Gsoil 322^T with other recognized members of the *Betaproteobacteria* were less than 95.6%. The relationship between the new isolates and other members of the *Betaproteobacteria* was also evident in the phylogenetic tree (Fig. 1). Strains Gsoil 322^T and Gsoil 328 and *M. petroleiphilum* ATCC BAA-1232^T formed a monophyletic clade with a high bootstrap value (100%), which was supported by the neighbour-joining and maximum-parsimony methods employed. Because the 16S rRNA gene sequence similarity of the two novel strains with *M. petroleiphilum* ATCC BAA-1232^T was above 97%, DNA–DNA hybridizations were carried out with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989), by using an FLX 800 microplate fluorescence reader (Bio-Tek) for fluorescence measurements. The hybridization temperature was 50 °C, and reciprocal experiments were performed for *M. petroleiphilum* ATCC BAA-1232^T, Gsoil 322^T and Gsoil 328.

The Gram stain reaction was identified by the non-staining method as described by Buck (1982). Cellular morphology was examined by light microscopy (Nikon)

and transmission electron microscopy with cells grown for 3 days at 30 °C on R2A agar. For the latter, cells were negatively stained with 1% (w/v) phosphotungstic acid and, following air-drying, the grids were examined by using a model CM-20 transmission electron microscope (Philips). Catalase and oxidase tests were performed as outlined by Cappuccino & Sherman (2002). Single carbon-source assimilation studies and nitrate reduction tests under aerobic and anaerobic conditions were carried out as described by Liu *et al.* (2006). Additional carbon sources, such as methanol, ethanol and butanol, at 0.1% (v/v) and a number of aromatic hydrocarbons, including MTBE (methyl *tert*-butyl ether), phenol, toluene, benzene, ethyl benzene, benzoate, 2,4-dihydroxybenzoate, 2,5-dihydroxybenzoate, 2,6-dihydroxybenzoate and 3,4-dihydroxybenzoate, were tested in base salt medium as described by Liu *et al.* (2006). Some physiological characteristics were determined with API 20E galleries according to the manufacturer's instructions (bioMérieux). Tests for degradation of DNA [DNase agar (Scharlau), with DNase activity determined by flooding plates with 1 M HCl], casein, chitin, starch (Atlas, 1993), lipid (Kouker & Jaeger, 1987), xylan and cellulose (Ten *et al.*, 2004) were performed and evaluated after 10 days. Growth at different temperatures and pH was assessed after 5 days incubation. Salt tolerance was tested on R2A medium supplemented with 1–10% (w/v) NaCl after 5 days incubation. Growth on nutrient agar, trypticase soy agar (TSA) and MacConkey agar was evaluated at 25 °C.

Strains Gsoil 322^T and Gsoil 328 had virtually identical characteristics with regard to their cellular, morphological, physiological and biochemical properties. Cells of the two strains were Gram-negative, motile by means of a single polar flagellum and rod-shaped, 0.4–0.5 µm in width and 0.8–1.3 µm in length (Fig. 2). Colonies that formed after 2 days incubation on R2A agar (Difco) at 25 °C were

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strains Gsoil 322^T and Gsoil 328 among neighbouring species selected from the class *Betaproteobacteria* on the basis of 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) of $\geq 70\%$ are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. Bar, 0.01 substitutions per nucleotide position.

Fig. 2. Transmission electron micrographs of negatively stained single cells of strain Gsoil 322^T (a) and strain Gsoil 328 (b), showing single polar flagella. Bars, 1 µm.

circular, smooth, convex and yellowish. Neither of the strains grew under anaerobic conditions and neither required NaCl for growth. The isolates grew in the pH range 5.5–8.5, with optimal growth between pH 6.5 and 7.0; the optimum growth temperature was 25 °C. In contrast to Gsoil 328, strain Gsoil 322^T was negative for succinate utilization. Other physiological characteristics of strains Gsoil 322^T and Gsoil 328 are summarized in the species description below. Characteristics useful for differentiating between strains Gsoil 322^T, Gsoil 328 and *M. petroleiphilum* are summarized in Table 1.

Table 1. Differential phenotypic characteristics of strains Gsoil 322^T and Gsoil 328 and their nearest phylogenetic neighbour *M. petroleiphilum* ATCC BAA-1232^T

Strains: 1, Gsoil 322^T; 2, Gsoil 328 (data from the present study); 3, *M. petroleiphilum* ATCC BAA-1232^T (data from Nakatsu *et al.*, 2006). All strains are positive for the following: motility, nitrate reduction, utilization of acetate, L-asparagine, methanol, ethanol, butanol and aromatic hydrocarbons, including MTBE, phenol, toluene, benzene, ethyl benzene, benzoate, 2,4-dihydroxybenzoate, 2,5-dihydroxybenzoate, 2,6-dihydroxybenzoate and 3,4-dihydroxybenzoate, as sole carbon sources. All strains are negative for the following: growth at 37 °C, catalase, β-galactosidase, lysine decarboxylase, ornithine decarboxylase, indole production, utilization of citrate and glycine, acid production from D-glucose, L-rhamnose, sucrose, D-melibiose and L-arabinose and hydrolysis of starch, casein, gelatin and DNA. +, Positive; (+), weakly positive; –, negative; ND, no data available.

Characteristic	1	2	3
Colony colour	Yellow	Yellow	Cream
Anaerobic growth	–	–	+
Nitrite reduction	–	+	–
Growth at 4 °C	–	+	ND
Urease	–	–	+
Voges-Proskauer test	(+)	(+)	–
Aesculin hydrolysis	+	+	–
Assimilation of pyruvate	–	–	+
DNA G + C content (mol%)	66.6	66.7	69.0

Cellular fatty acid profiles were determined for strains grown on R2A agar (Difco) for 2 days. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acids were then analysed by GC (Hewlett Packard 6890) with the Microbial Identification software package (Sasser, 1990). Mean values for each component were obtained from duplicate experiments. 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 by using Sep-Pak Vac cartridges silica (Waters) and subsequently analysed by HPLC as described by Hiraishi *et al.* (1996). For measurement of the G + C content of the chromosomal DNA, genomic DNA was extracted and purified as described by Moore & Dowhan (1995) and enzymically degraded into nucleosides and the G + C content of the DNA was then determined as described by Mesbah *et al.* (1989) by using reversed-phase HPLC.

The cellular fatty acids of strains Gsoil 322^T and Gsoil 328 ranged from C₁₅ to C₁₈ in length and included saturated, cyclo and monoenic components. In the two strains, C_{16:0}, C_{17:0} cyclo and summed feature 4 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) were the major fatty acids. A comparison of the cellular fatty acid compositions of strains Gsoil 322^T and Gsoil 328 and *M. petroleiphilum* ATCC BAA-1232^T is given in Table 2. All three contained C_{16:0} and C_{16:1}ω7c as predominant components, but the novel strains could be differentiated from *M. petroleiphilum* based on high levels of C_{17:0} cyclo and absence of any hydroxy fatty acids. Q-8 was observed as the major quinone. The DNA G + C contents of strains Gsoil 322^T and Gsoil 328 were 66.6 and 66.7 mol%, respectively, and these values are similar to that reported for *M. petroleiphilum* (Nakatsu *et al.*, 2006).

DNA–DNA relatedness values between strains Gsoil 322^T and Gsoil 328 were 95–98 %. Strains Gsoil 322^T and Gsoil 328 showed low levels of DNA–DNA relatedness, 10 and 12 %, respectively, with *M. petroleiphilum* ATCC BAA-1232^T. High levels of DNA–DNA relatedness between strains Gsoil 322^T and Gsoil 328 and low levels with *M. petroleiphilum* ATCC BAA-1232^T clearly indicated that the

Table 2. Fatty acid compositions (%) of strains Gsoil 322^T and Gsoil 328 and their nearest phylogenetic neighbour *M. petroleiphilum* ATCC BAA-1232^T

Strains: 1, Gsoil 322^T; 2, Gsoil 328 (data from the present study); 3, *M. petroleiphilum* ATCC BAA-1232^T (data from Nakatsu *et al.*, 2006). –, Not detected. Data for *M. petroleiphilum* ATCC BAA-1232^T scored as: ++, major fatty acid; +, minor fatty acid.

Fatty acid	1	2	3
C _{12:0}	–	–	+
C _{14:0}	–	–	+
C _{15:0}	0.9	1.2	–
C _{16:0}	44.3	39.1	++
C _{17:0}	1.0	1.1	–
C _{18:0}	–	–	+
C _{10:0} 3-OH	–	–	+
C _{12:0} 2-OH	–	–	+
C _{12:0} 3-OH	–	–	+
C _{17:0} cyclo 7–8c	–	–	+
C _{17:0} cyclo	22.0	25.2	–
C _{16:1} ω7c/iso-C _{15:0} 2-OH	25.5	25.3	+ +/–
C _{18:1} ω7c/C _{18:1} ω9t/C _{18:1} ω12t	6.4	8.0	+ / – / –

two new strains should be classified as representing a novel species (Stackebrandt & Goebel, 1994).

On the basis of morphological, physiological and chemotaxonomic characteristics, together with data from 16S rRNA gene sequence comparisons, strains Gsoil 322^T and Gsoil 328 are considered to represent a single novel species, for which the name *Methylibium fulvum* sp. nov. is proposed.

Emended description of the genus *Methylibium*

The genus *Methylibium* was described by Nakatsu *et al.* (2006). According to the original description, members of the genus are able to hydrolyse urea. However, *Methylibium fulvum* is unable to hydrolyse urea; therefore, the hydrolysis of urea is not a distinguishing characteristic of the genus *Methylibium*.

Description of *Methylibium fulvum* sp. nov.

Methylibium fulvum (ful'vum. L. neut. adj. *fulvum* deep yellow, gold-coloured).

Cells are Gram-negative and motile by means of a single polar flagellum. Oxidase-positive but catalase-negative. Reduces nitrate to nitrite. Growth occurs on TSA, but not on MacConkey agar. Hydrolyses aesculin, but not starch, chitin, xylan, CM-cellulose, casein or DNA. The following substrates are utilized for growth: D-fructose, D-xylose, L-xylose, sucrose (weakly), acetate, 3-hydroxybutyrate, 3-hydroxybenzoate, citrate, lactate, D-mannitol, D-sorbitol, L-asparagine, L-aspartate, L-glutamine, L-glutamate, L-phenylalanine and L-proline. The following substrates are not utilized for growth: D-glucose,

D-galactose, D-mannose, D-xylose, L-arabinose, trehalose, maltose, D-cellobiose, D-melibiose, D-raffinose, D-fucose, L-rhamnose, L-sorbose, D-arabinose, D-ribose, D-lactose, D-adonitol, dulcitol, xylitol, inositol, glycerol, amygdalin, glycogen, inulin, N-acetylglucosamine, dextran, pyruvate, valerate, fumarate, salicin, malate, formate, propionate, tartrate, gluconate, caprate, maleic acid, phenylacetate, 4-hydroxybenzoate, malonate, glutarate, itaconate, adipate, suberate, oxalate, succinate, L-alanine, L-arginine, L-histidine, L-cysteine, glycine, L-isoleucine, L-leucine, L-threonine, L-lysine, L-methionine, L-serine, L-tryptophan, L-tyrosine and L-valine. In API 20E tests, the Voges-Proskauer reaction is weakly positive; gelatin hydrolysis, β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, urease, hydrogen sulfide and indole production are all negative. Acid is not produced from L-arabinose, D-mannitol, inositol, D-sorbitol, L-rhamnose, sucrose, D-melibiose, D-glucose or amygdalin. Major fatty acids are C_{16:0}, C_{17:0} cyclo and summed feature 4 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH). DNA G + C content is 66.6–66.7 mol%.

The type strain, Gsoil 322^T (=KCTC 12591^T =LMG 23394^T), was isolated from soil of a ginseng field in Pocheon province, Republic of Korea.

Acknowledgements

This study was supported by a grant from the Korea Science and Engineering Foundation, Ministry of Science and Technology, Republic of Korea.

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