Methylibium aquaticum sp. nov., a betaproteobacterium isolated from a eutrophic freshwater pond

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A freshwater bacterium, designated IMCC1728^T, was isolated from a eutrophic pond. The strain was Gram-negative, chemoheterotrophic and facultatively aerobic, forming non-motile rods that contained poly-β-hydroxybutyrate granules. Based on 16S rRNA gene sequence comparisons, the most closely related species to strain IMCC1728^T was *Methylibium petroleiphilum* (97.0 % similarity). Phylogenetic trees generated using 16S rRNA gene sequences showed that this isolate formed an independent phyletic line of the genus *Methylibium* clade of the class *Betaproteobacteria*. The DNA G+C content of the strain was 66.2 ± 0.4 mol%. The predominant cellular fatty acid constituents were $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH (43.1 %), $C_{16:0}$ (20.3 %), $C_{12:0}$ (13.4 %) and $C_{10:0}$ 3-OH (7.3 %). The strain contained Q-8 as the predominant ubiquinone. Several phenotypic characteristics, including flagellation, temperature range for growth and carbon source utilization patterns, differentiated strain IMCC1728^T from species of the genus *Methylibium.* Therefore, it is proposed that strain IMCC1728^T represents a novel species, *Methylibium aquaticum* sp. nov. The type strain is IMCC1728^T (=KCCM 42364^T=NBRC 102349^T).

The genus *Methylibium* of the *Sphaerotilus–Leptothrix* group (Spring, 2002) in the class *Betaproteobacteria* was recently established by Nakatsu *et al.* (2006) and the genus currently contains one species, *Methylibium petroleiphilum*. *M. petroleiphilum* was isolated from a compost biofilter. This species is a Gram-negative, rod-shaped, facultative methylotrophic bacterium that is motile with one polar flagellum and can degrade methyl *tert*-butyl ether. Characterization of strain IMCC1728^T, a novel species of the genus *Methylibium* isolated from a eutrophic freshwater pond, is described in this report.

Strain IMCC1728^T was isolated from an artificial freshwater pond located inside Inha University (Incheon, Korea) by using a standard dilution plating method on R2A agar (Reasoner & Geldreich, 1985) diluted in distilled water (1 : 10, v/v) after aerobic incubation at 20 °C for 5 days. After determination of optimum medium and temperature for growth, bacterial cultures were routinely maintained on R2A agar at 30 °C.

Methods for DNA extraction, PCR and 16S rRNA gene sequencing have been described in a previous study (Cho & Giovannoni, 2003). The resultant almost-complete 16S rRNA gene sequence (1484 bp) of strain IMCC1728^T was aligned with sequences of nearest neighbours using the ARB software package (Ludwig *et al.*, 2004) and 16S rRNA gene sequence similarities were calculated based on this alignment. Unambiguously aligned 1294 nt positions, determined from 13 betaproteobacterial 16S rRNA gene sequences, were used for phylogenetic analyses in PAUP* 4.0 beta 10 (Swofford, 2002). Phylogenetic trees were generated by neighbour-joining (Saitou & Nei, 1987) with the Jukes–Cantor distance (Jukes & Cantor, 1969), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods. Robustness of the neighbour-joining and maximum-parsimony trees was confirmed by bootstrap analyses based on 100 resamplings of the sequences. Preliminary sequence comparison with 16S rRNA gene sequences deposited in GenBank and RDP-II (Cole *et al.*, 2005) indicated that strain IMCC1728^T was closely related to members of the genus *Methylibium* in the class *Betaproteobacteria*. Strain IMCC1728^T showed highest 16S rRNA gene sequence similarity to *M. petroleiphilum* ATCC BAA-1232^T (97.0 %), followed by *Ideonella dechloratans* ATCC 51718^T (96.1 %), *Aquabacterium commune* ATCC BAA-209^T (95.7 %) and *Aquabacterium parvum* ATCC BAA-208^T (95.6 %). In all phylogenetic trees generated in this study (Fig. 1), strain IMCC1728 ^{T}, uncultured groundwater bacterium 300A-D08 (GenBank accession no. AY662010; Fields *et al.*, 2005) and uncultured iron-oxidizing lagoon bacterium 015 (GenBank accession

Abbreviations: PHB, poly-β-hydroxybutyrate; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IMCC1728^T is DQ664244.

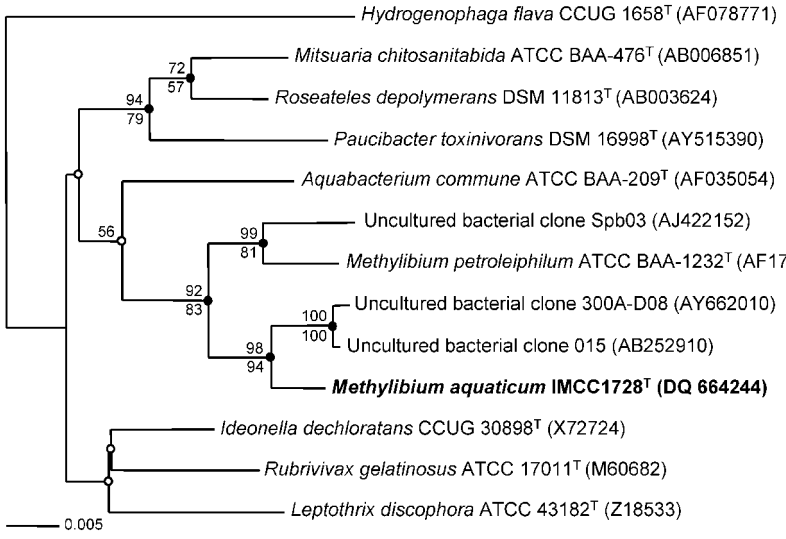

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationship between strain IMCC1728^T and its relatives. Bootstrap percentages (above 50 %) from both neighbour-joining (above nodes) and maximum-parsimony (below nodes) are shown. Nodes recovered reproducibly by all treeing methods (filled circles) or by two treeing methods (open circles) are indicated. Bar, 0.005 substitutions per nucleotide position.

no. AB252910) formed a monophyletic clade. This clade was anchored to the clade containing *M. petroleiphilum* with high bootstrap values (92 % in neighbour-joining and 83 % in maximum-parsimony trees), indicating that strain IMCC1728^T represents a novel species of the genus *Methylibium*.

Growth at various temperatures (4–42 °C) and optimum growth temperature were determined on R2A agar. In further phenotypic and chemotaxonomic tests, strain IMCC1728^T was routinely maintained at 30 °C on R2A agar, unless otherwise noted. The pH range and optimum for growth were examined at pH 4.0–12.0. The requirement of NaCl and the optimum for growth were determined in R2A agar supplemented with 0–15 % (w/v) NaCl. Cell morphology was observed under a phase-contrast microscope (Nikon 80i) and by transmission electron microscopy (TEM) (CM200; Philips). TEM was also used to determine the presence of flagella and intracellular granules. The presence of poly-β-hydroxybutyrate (PHB) granules was confirmed using the Nile Blue A staining method described by Ostle & Holt (1982). Colony morphology, size and colour were examined from cultures grown for 3 days. Motility was tested by using wet mounts of exponential-phase cells. Anaerobic growth was tested employing Anaerocult C mini (EM science) and AnaeroPack Anaero (Mitsubishi Gas Chemical). Catalase activity was determined by the reaction of 3 % hydrogen peroxide on fresh colonies and the oxidase test was performed using Kovacs' solution (Kovacs, 1956). Other biochemical tests were carried out by using the API 20NE and API ZYM (both bioMérieux) systems according to the manufacturer's instructions. Sole carbon source utilization tests were performed using custom-made 48-well microplates. Approximately 1.4×10^4 cells ml^{-1} in PBS (pH 7.4) were inoculated into each well, which contained one of 47 different carbon compounds (list of compounds is given in the species description) at a final concentration of 0.2 % (w/v or v/v). After incubation of the microplates for 1 week at 30 °C, cellular growth and purity were checked as described previously (Cho & Giovannoni, 2003). Susceptibility to 10 different antimicrobial agents (list given in the species description) was determined using the diffusion plate method (Jorgensen *et al.*, 1999). The DNA G+C content was analysed by using HPLC according to Mesbah *et al.* (1989) with a Discovery C18 column (5 μm, 15 cm × 4.6 mm; Supelco). Cellular fatty acid methyl esters were collected from fresh cultures grown on R2A agar at 30 °C for 4 days and analysed according to the instructions of the Microbial Identification System (MIDI) by the Korean Culture Center of Micro-organisms (KCCM). Respiratory quinone content was determined by using reverse-phase HPLC analysis (Komagata & Suzuki, 1987) by the KCCM.

In summary, cells of strain IMCC1728^T were Gram-negative, chemoheterotrophic, facultatively aerobic, non-motile, short rods that contained PHB granules (Fig. 2). Detailed results of phenotypic and biochemical tests are given in the species description and Table 1. As shown in Table 1, strain IMCC1728^T shared similar characteristics with *M. petroleiphilum* ATCC BAA-1232^T, including DNA G+C content, major fatty acid constituents, Q-8 as the major quinone type and the presence of PHB granules. However, differences in several phenotypic properties (Table 1) together with a phylogenetically distinct relationship (Fig. 1) between the two strains indicate that strain IMCC1728^T represents a novel species of the genus *Methylibium*, for which the name *Methylibium aquaticum* sp. nov. is proposed.

Description of *Methylibium aquaticum* sp. nov.

Methylibium aquaticum (a.qua′ti.cum. L. neut. adj. *aquaticum* living in water, aquatic).

Gram-negative and non-motile. Cells are short rods (0.8–1.9 × 0.4–0.7 μm). After 4 days incubation, colonies on R2A agar are circular, umbonate, beige-milkish, smooth

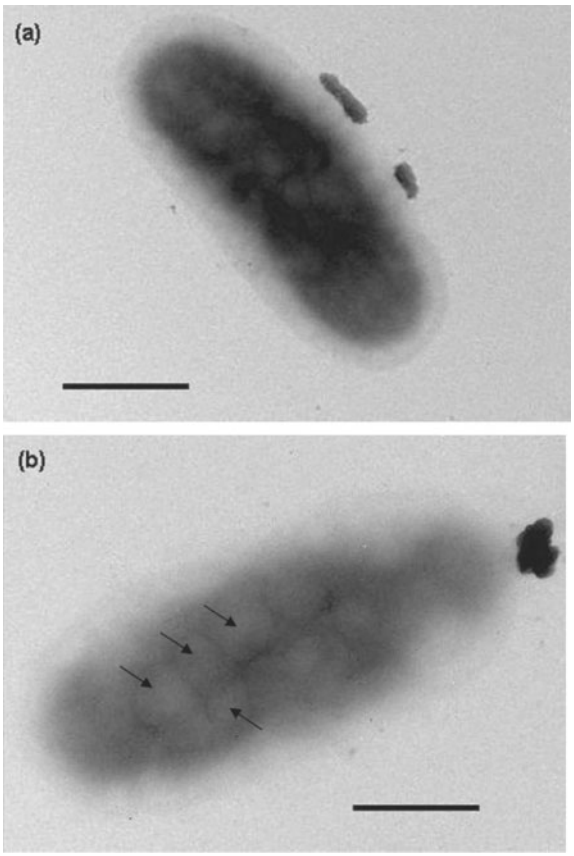

Fig. 2. (a, b) Negatively stained TEMs of cells of strain IMCC1728^T. Arrows in panel (b) indicate PHB granules. Bars, 0.5 μm

with entire margin and 0.5–2.0 mm in diameter. Growth occurs at 15–42 °C and pH 6–10 and in 0–1 % NaCl; optimal growth is observed at 30 °C, pH 7.0 and in the absence of NaCl. Contains PHB granules. Chemoheterotrophic and facultatively aerobic. Anaerobic growth is much slower than aerobic growth. Catalase-negative and oxidase-positive. Gelatinase activity and aesculin hydrolysis are weakly positive. Nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, urease and β-galactosidase are negative (API 20NE). Using the API ZYM system, alkaline phosphatase and leucine arylamidase activities are positive; esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are negative. Of the 47 different carbon sources in the custom-made 48-well plate assay, the following carbon substrates are utilized: methanol, glycerol, D-ribose, D-galactose, *N*-acetyl-D-glucosamine, rhamnose, adonitol, D-xylitol, gluconic acid, malonic acid, propionic acid, L-alanine, L-arginine, L-histidine, L-proline and L-serine.

Table 1. Characteristics that differentiate strain IMCC1728^T from *Methylibium petroleiphilum* ATCC BAA-1232^T

Data from this study and Nakatsu *et al.* (2006). +, Positive; −, negative; W, weakly positive. Both strains were positive for oxidase activity, anaerobic growth, presence of PHB, and utilization of methanol and alanine. Both strains were negative for catalase activity, indole production, and utilization of arabinose, xylose, melibiose, sucrose, glycine, glucose and citric acid. The major quinone of both strains was Q-8.

Characteristic	*M. aquaticum* IMCC1728^T	*M. petroleiphilum* ATCC BAA-1232^T
Isolation source	Eutrophic freshwater	Compost biofilter
Flagellation	−	+
Growth at 37 °C	+	−
Nitrate reduction	−	+
Hydrolysis of:		
Urea	−	+
Aesculin	W	−
Gelatin	W	−
Carbon utilization:		
Ethanol	−	+
Rhamnose	+	−
Pyruvic acid	−	+
Major fatty acid (%):*		
$C_{16:1}\omega7c$	43.1†	43.4
$C_{16:0}$	20.3	39.0
$C_{12:0}$	13.4	4.7
$C_{10:0}$ 3-OH	7.3	2.9
cyclo $C_{17:0}$	4.2	−
$C_{18:1}\omega7c$	1.5	7.6
DNA G+C content (mol%)	66.2	69

*The major fatty acid proportions of *M. petroleiphilum* were graciously provided by Cindy H. Nakatsu.
†$C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH.

However, methylamine, ethanol, DL-glyceraldehyde, D-arabinose, D-xylose, D-fructose, D-glucosamine, D-glucose, D-mannose, D-cellobiose, D-lactose, maltose, melibiose, sucrose, trehalose, D-melezitose, D-raffinose, arabitol, D-mannitol, *myo*-inositol, D-sorbitol, citric acid, glucuronic acid, itaconic acid, pyruvic acid, succinic acid, L-glutamic acid, L-glycine, L-leucine, L-lysine and L-ornithine are not utilized. Susceptible to (μg) chloramphenicol (25), erythromycin (15), streptomycin (10), gentamicin (10), kanamycin (30), penicillin G (10) and vancomycin (30). Resistant to (μg) ampicillin (10), rifampicin (50) and tetracycline (30). The cellular fatty acids are composed of $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH (43.1 %), $C_{16:0}$ (20.3 %), $C_{12:0}$ (13.4 %), $C_{10:0}$ 3-OH (7.3 %), cyclo $C_{17:0}$ (4.2 %), $C_{18:1}\omega7c$ (1.5 %), $C_{15:0}$ (1.4 %), $C_{17:0}$ (1.4 %), $C_{14:0}$ (1.0 %), $C_{15:1}\omega6c$ (0.9 %), $C_{18:0}$ (0.8 %) and 11-methyl $C_{18:1}\omega7c$ (0.6 %). The major quinone type is Q-8. The DNA G+C content of the type strain is 66.2 ± 0.4 mol%.

The type strain, IMCC1728^T (=KCCM 42364^T=NBRC 102349^T), was isolated from an artificial freshwater pond located inside Inha University, Incheon, Korea.

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