Methylobacterium platani sp. nov., isolated from a leaf of the tree *Platanus orientalis*

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A novel bacterial strain, designated PMB02^T, was isolated from a leaf of the tree *Platanus orientalis*. Colonies grown on TYG agar plates were circular, pink-pigmented and slow-growing, being 0.2–1.5 mm in diameter after 3 days growth. The cells of strain PMB02^T were Gram-negative, aerobic, motile rods that possessed oxidase and catalase activities and grew at 20–30 °C, pH 6–8 and in media containing less than 1 % NaCl. The major respiratory quinone was identified as Q-10. A phylogenetic analysis based on 16S rRNA gene sequence comparisons indicated that strain PMB02^T was related to members of the genus *Methylobacterium*. A comparative 16S rRNA gene sequence-based phylogenetic analysis placed the strain in a clade with the species *Methylobacterium aquaticum* and *Methylobacterium variable*, with which it showed sequence similarities of 97.7 and 97.4 %, respectively. The values for DNA–DNA hybridization between strain PMB02^T and *M. aquaticum* CCM 7218^T and *M. variable* GR3^T were less than 32 %. On the basis of the phenotypic characterization, the phylogenetic analysis and the DNA–DNA relatedness data, strain PMB02^T is considered to represent a novel species of the genus *Methylobacterium*, for which the name *Methylobacterium platani* sp. nov. is proposed. The type strain is PMB02^T (=KCTC 12901^T=JCM 14648^T).

The genus *Methylobacterium* mainly consists of pink-pigmented, facultatively methylotrophic bacteria. The genus belongs to the class *Alphaproteobacteria* and the cells are strictly aerobic, Gram-negative rods that are able to grow on a range of carbon substrates and are also able to utilize single-carbon (C₁) compounds (such as formate, formaldehyde and methanol) as sole sources of carbon and energy (Lidstrom & Chistoserdova, 2002). Members of the genus *Methylobacterium* are ubiquitous in nature, being found in soils, dust and lake sediments. They have also been found in association with plants, specifically leaf surfaces, and it has been hypothesized that they potentially dominate phyllospheric bacterial populations (Corpe & Basile, 1982; Hirano & Upper, 1991). The genus *Methylobacterium* was first described by Patt *et al.* (1976) and at the time of writing consists of the following 24 recognized species (<http://www.bacterio.cict.fr/m/methylobacterium.html>):

Methylobacterium adhaesivum (Gallego *et al.*, 2006), *M. aminovorans* (Urakami *et al.*, 1993), *M. aquaticum* (Gallego *et al.*, 2005a), *M. chloromethanicum* (McDonald *et al.*, 2001), *M. dichloromethanicum* (Doronina *et al.*, 2000), *M. extorquens* (Bousfield & Green, 1985), *M. fujisawaense* (Green *et al.*, 1988), *M. hispanicum* (Gallego *et al.*, 2005a), *M. isbiliense* (Gallego *et al.*, 2005c), *M. jeotgali* (Aslam *et al.*, 2007), *M. lusitanum* (Doronina *et al.*, 2002), *M. mesophilicum* (Green & Bousfield, 1983), *M. nodulans* (Jourand *et al.*, 2004), *M. organophilum* (Patt *et al.*, 1976), *M. oryzae* (Madhaiyan *et al.*, 2007), *M. podarium* (Anesti *et al.*, 2004), *M. populi* (Van Aken *et al.*, 2004), *M. radiotolerans* (Green & Bousfield, 1983), *M. rhodesianum* (Green *et al.*, 1988), *M. rhodinum* (Green & Bousfield, 1983), *M. suomiense* (Doronina *et al.*, 2002), *M. thiocyanatum* (Wood *et al.*, 1998), *M. variable* (Gallego *et al.*, 2005b) and *M. zatmanii* (Green *et al.*, 1988). Here, we provide a detailed taxonomic characterization of a *Methylobacterium*-like bacterial strain, PMB02^T, which was isolated from a tree leaf.

Strain PMB02^T was isolated from a leaf from a *Platanus orientalis* tree at Korea University, Seoul, Korea, and cultivated on a potato dextrose agar (Difco) plate at room temperature. After initial cultivation, cells were maintained

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain PMB02^T is EF426729.

Photomicrographs of cells of strain PMB02^T, DNA–DNA hybridization data and fatty acid profiles for strain PMB02^T and related strains are available as supplementary material with the online version of this paper.

on TYG medium (0.5 % tryptone, 0.25 % yeast extract and 0.1 % glucose) at 30 °C. For phenotypic characterization, growth on TYG medium at various temperatures (4, 10, 20, 25, 30, 33 and 37 °C) and different pHs (4, 5, 6, 7, 8 and 9) was measured for 3 days. The optimum temperature range was 25–30 °C, but growth did occur at 20–30 °C. The optimum pH for growth was between 6.0 and 7.0, but growth could occur between pH 6.0 and 8.0. Cellular morphology and motility were determined microscopically (Filter set 20 Rhod; Carl Zeiss). Cells of strain PMB02^T were Gram-negative, aerobic, motile rods (1.4–1.5 × 2.0–6.5 µm) that occurred singly, in pairs or in rosettes (on TYG medium for 48 h at 30 °C). When strain PMB02^T was incubated on TYG medium (pH 7.0) at 30 °C at 220 r.p.m., the growth rate was 0.24 ± 0.04 cells h⁻¹. Colonies of strain PMB02^T were circular in shape, pink in colour and 0.2–1.5 mm in diameter when grown on TYG agar for at least 2–3 days. Interestingly, colonies of some *Methylobacterium* species, such as *M. jeotgali* (Aslam *et al.*, 2007) and *M. nodulans* (Jourand *et al.*, 2004), are non-pigmented. MSB medium (Stanier *et al.*, 1966) was used for the utilization of sole C₁ sources, such as methanol and formaldehyde. Each carbon source was tested at concentrations of 0.01, 0.05, 0.1, 0.5 and 1.0 % (v/v). Strain PMB02^T was able to grow well with 0.1 % methanol and 0.01 % formaldehyde as sole carbon sources. To evaluate NaCl tolerance, cells were grown for 3 days in TYG

medium containing 0, 0.5, 1, 2 and 3 % NaCl. Strain PMB02^T is a slow-growing organism that does not grow in the presence of ≥ 1 % NaCl. *M. aquaticum* CCM 7218^T (Gallego *et al.*, 2005a) and *M. variable* GR3^T (Gallego *et al.*, 2005c) also do not grow in the presence of ≥ 1 % NaCl, but *M. lusitanum* (Doronina *et al.*, 2002) and *M. suomiense* (Doronina *et al.*, 2002) can grow in the presence of 3 % NaCl (Table 1). To investigate whether strain PMB02^T produced polyhydroxyalkanoates, cells were grown in TYG medium for 72 h, then washed twice with PBS and transferred to modified MSB medium containing succinate (10 mM). After 16 h, cells were harvested, stained with Nile blue A (Ostle & Holt, 1982) and then washed with water (to remove excess stain) and then with 100 % ethanol for 1 min; the cells were then observed using a fluorescence microscope (Filter set 20 Rhod). Strain PMB02^T accumulated polyhydroxyalkanoates when succinate was used as a carbon source (see Supplementary Fig. S1, available in IJSEM Online). Oxidase activity was tested using 1 % (w/v) *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride: 10 µl reagent was dropped onto culture on a glass slide. A positive reaction is shown by a colour change to violet or purple within 20 s. Catalase activity was investigated by checking for the production of bubbles in a 3 % (v/v) hydrogen peroxide solution. Oxidase and catalase activities were detected in strain PMB02^T. Nutritional characteristics were determined

Table 1. Differential characteristics of strain PMB02^T and the type strains of related species of *Methylobacterium*

Strains: 1, PMB02^T; 2, *M. variable* GR3^T (Gallego *et al.*, 2005c); 3, *M. aquaticum* CCM 7218^T (Gallego *et al.*, 2005a); 4, *M. lusitanum* NCIMB 13799^T (Doronina *et al.*, 2002); 5, *M. suomiense* NCIMB 13778^T (Doronina *et al.*, 2002); 6, *M. populi* NCIMB 13946^T (Van Aken *et al.*, 2004). All strains are motile. ND, Not determined; +, positive; –, negative.

Characteristic	1	2	3	4	5	6
Cells form pairs	+	+	+	Rarely	Rarely	+
Cells form rosettes	+	+	+	–	–	+
Cell length (µm)	2.0–6.5	2–6	4.5–8	1.7–2	1.2–2.5	1–10
Cell width (µm)	1.4–1.5	1–1.5	1.5–1.7	1–1.2	0.8–1	0.8–1
Pigmentation	Pink	Pink	Pink, red	Pink	Pink	Pink, red
Colony diameter (mm)	0.2–1.5	2–7	1–2	1	0.5–1	0.1–0.2
Oxidase	+	–	–	+	+	+
Tolerance of NaCl (%)	1	1	1	3	3	2
Ubiquinone	Q-10	ND	ND	Q-10	Q-10	ND
Optimal temperature (°C)	25–30	28	28	30–37	25–30	20–30
pH range	6.0–8.0	5.0–8.0	5.0–7.0	6.9–7.5	6.9–7.5	5.0–8.0
Major fatty acid	<i>cis</i> -Vaccenic acid	<i>cis</i> -Vaccenic acid	<i>cis</i> -Vaccenic acid	<i>cis</i> -Vaccenic acid	<i>cis</i> -Vaccenic acid	Oleic acid
DNA G + C content (mol%)	68.5	69.2	67.5	66.5	65.8	70.4
Hydrolysis of:						
Tween 40	–	–	+	ND	ND	ND
Tween 80	–	–	+	ND	ND	ND
Assimilation of:						
L-Arabinose	+	–	–	–	–	–
L-Asparagine	–	+	+	ND	ND	ND
L-Fucose	–	+	+	ND	ND	–
D-Galactose	–	+	+	ND	ND	ND
D-Glucose	–	+	+	–	+	–

using the Biolog GN2 system (Gallego *et al.*, 2005b): Biolog GN microplates were inoculated with 125 µl cell suspension per well and then incubated at 28 °C for 7 days. A summary of the phenotypic characteristics of PMB02^T in comparison with those of closely related *Methylobacterium* species is presented in Table 1.

16S rRNA genes were PCR-amplified from DNA extracts using universal primers 27f (5'-AGAGTTTGTATCCTGGC-TCAG-3') and 1492r (5'-GGCTACCTTGTTACGACTT-3'), and were purified as described previously (Kim *et al.*, 2005). The resulting 16S rRNA gene sequence (1432 nt) of strain PMB02^T was compared with available 16S rRNA gene sequences in GenBank by using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>) to determine an approximate phylogenetic affiliation. Gene sequences were then aligned with those of closely related species using CLUSTAL W (Thompson *et al.*, 1994). Phylogenetic trees were constructed using the neighbour-joining, maximum-likelihood and maximum-parsimony algorithms, which are available in PHYLIP, version 3.6 (Felsenstein, 2002). 16S rRNA gene sequence comparisons for similarity calculations were made between the novel strain and related members of the genus *Methylobacterium*, using the FASTA3 program (European Bioinformatics Institute; <http://www.ebi.ac.uk/fasta33/nucleotide.html>). Comparative 16S rRNA gene sequence analyses revealed that strain PMB02^T was phylogenetically affiliated to the genus *Methylobacterium*. Furthermore, the phylogenetic analysis based on 16S rRNA gene sequences indicated that strain PMB02^T formed a phyletic lineage, within the genus *Methylobacterium*, with *M. aquaticum* CCM 7218^T at a bootstrap value

of 63.0 % (Fig. 1). The topologies of the phylogenetic trees built using the maximum-likelihood and maximum-parsimony algorithms also indicated that the isolate belongs to the genus *Methylobacterium* (data not shown). Comparative 16S rRNA gene sequence analyses showed that the isolate was most closely related to *M. aquaticum* CCM 7218^T and *M. variabile* GR3^T, with 16S rRNA gene sequence similarities of 97.7 and 97.4 %, respectively. The sequence similarities with respect to other *Methylobacterium* species were less than 96.0 %. It has been suggested that bacterial strains with less than 97 % 16S rRNA gene sequence identity represent novel species (Stackebrandt & Goebel, 1994).

DNA–DNA hybridization was conducted to investigate the relatedness of strain PMB02^T with respect to its closest relatives, *M. aquaticum* CCM 7218^T and *M. variabile* GR3^T, using ³²P-labelled DNA probes and the methods described by Ezaki *et al.* (1989). The genomic DNA of strain PMB02^T or *M. aquaticum* CCM 7218^T was extracted using a genomic DNA-purification kit (Promega) and used as a probe (see Supplementary Table S1, available in IJSEM Online). Purified DNA (1 mg) from strain PMB02^T or *M. aquaticum* CCM 7218^T was labelled with ³²P-labelled dCTP (PerkinElmer) and a random primer labelling kit (Invitrogen). The ³²P-labelled DNA probe was then purified with a Nick column (Amersham Biosciences) through TE buffer (Tris-EDTA, pH 7.5). The genomic DNA was denatured by adding 0.4 M NaOH and 10 mM EDTA and then heating the sample at 100 °C for 10 min (to ensure complete denaturation). The DNA was then neutralized by adding an equal volume of cold 2 M

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain PMB02^T and related taxa. Bootstrap percentages (based on 1000 replicates) are shown if greater than 50 %. *Methylorhabdus multivorans* DM13^T was used as an outgroup. Bar, 0.01 changes per nucleotide position.

ammonium acetate (pH 7.0). The DNA solutions (200 ng) were transferred onto a nylon membrane (Nytran N; Schleicher & Schuell) using dot-blot apparatus (Bio-Rad). After the DNA had been filtered, the same volume of 0.4 M NaOH was added to each well. The blotted membrane was rinsed in $2 \times$ SSC, air-dried and then irradiated at 254 nm to cross-link the DNA. Hybridization with the ^{32}P -labelled DNA probes was conducted at 65 °C. After hybridization, the nylon membrane was washed twice, first with $2 \times$ SSC and then with 0.1 % SDS for 10 min at room temperature, then with $0.1 \times$ SSC and 0.5 % SDS for 30 min at 65 °C, to remove the unbound probe. The level of DNA–DNA relatedness (%) was calculated as $100(I_{\text{R}}/I_{\text{T}})$, where I_{R} is the intensity of hybridization of reference strains and I_{T} is the intensity of hybridization of the tested strain. The DNA–DNA hybridization data showed that strain PMB02^T had DNA–DNA relatedness values of 31.3 and 29.0 % with *M. aquaticum* CCM 7218^T and *M. variable* GR3^T, respectively, when ^{32}P -labelled PMB02^T DNA probes were used (see Supplementary Table S1). *M. aquaticum* CCM 7218^T had DNA–DNA hybridization levels of 28.9 and 33.3 % with strain PMB02^T and *M. variable* GR3^T, respectively, when ^{32}P -labelled *M. aquaticum* CCM 7218^T DNA probes were used. A hybridization value below 70 % has been suggested as the threshold value for delineating a bacterial species (Wayne *et al.*, 1987), so these data indicate that strain PMB02^T represents a novel species of the genus *Methylobacterium*.

For the analysis of quinones and fatty acid methyl esters, cells were grown on TYG liquid medium at 30 °C for 48 h. Fatty acids were extracted and prepared according to standard protocols provided with the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The following fatty acids were detected in strain PMB02^T (see Supplementary Table S2, available in IJSEM Online): $\text{C}_{18:1\omega7\text{c}}$ (81.0 %), $\text{C}_{16:0}$ (7.6 %), $\text{C}_{18:0}$ 3-OH (3.46 %), summed feature 3 (2.24 %) comprising $\text{C}_{16:1\omega7\text{c}}$, iso- $\text{C}_{15:0}$ 2-OH or both, summed feature 2 ($\text{C}_{14:0}$ 3-OH/iso- $\text{C}_{16:1}$ I, 1.98 %) and $\text{C}_{18:0}$ (1.71 %). Traces (≤ 1.0 %) of $\text{C}_{14:0}$, $\text{C}_{18:1\omega9\text{c}}$, $\text{C}_{12:0}$, $\text{C}_{19:0}$ 10-methyl, $\text{C}_{14:1\omega5\text{c}}$, $\text{C}_{16:0}$ N alcohol and $\text{C}_{18:3\omega6\text{c}}$ were detected. The major quinone of strain PMB02^T was Q-10. The G+C content of the genomic DNA was determined using HPLC analysis methods described previously (Mesbah *et al.*, 1989). The DNA G+C content of strain PMB02^T was 68.5 mol%, which falls within the range described for the genus *Methylobacterium* (Green, 1992).

Description of *Methylobacterium platani* sp. nov.

Methylobacterium platani (pla.ta'ni. L. gen. n. *platani* of *Platanus orientalis*, referring to the tree species from which the micro-organism was isolated).

Cells are Gram-negative rods measuring $1.4\text{--}1.5 \times 2.0\text{--}6.5$ µm, occurring singly, in pairs or in rosettes. Cells are motile and non-spore-forming. Cells are slow-growing on nutrient agar and TYG agar and produce small, smooth,

pink-pigmented colonies. Methanol, formaldehyde and formate are utilized by strain PMB02^T as sole carbon sources. Does not grow in the presence of ≥ 1 % NaCl. Positive for catalase and cytochrome oxidase. Optimum temperature range for growth is 25–30 °C, but growth does occur at 20–30 °C. Optimum pH for growth is between 6.0 and 7.0, but growth does occur between pH 6.0 and 8.0. Oxidizes the following compounds as sole carbon and energy sources: L-arabinose, D-fructose, methyl pyruvate, monomethyl succinate, acetic acid, *cis*-aconitic acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, α -hydroxybutyric acid, β -hydroxybutyric acid, γ -hydroxybutyric acid, α -ketobutyric acid, α -ketoglutaric acid, DL-lactic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, L-aspartic acid, L-glutamic acid, L-proline and L-threonine. Does not utilize the following compounds as a carbon and energy source: α -cyclodextrin, dextrin, glycogen, Tweens 40 and 80, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, D-arabitol, cellobiose, L-erythritol, L-fucose, D-galactose, gentiobiose, α -D-glucose, myo-inositol, α -D-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melibiose, methyl β -D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, xylitol, citric acid, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, *p*-hydroxyphenylacetic acid, itaconic acid, α -ketovaleric acid, malonic acid, glucuronamide, alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxyl L-proline, L-leucine, L-ornithine, L-phenylalanine, L-pyrogutamic acid, D-serine, L-serine, DL-carnitine, γ -aminobutyric acid, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, DL- α -glycerol phosphate, glucose 1-phosphate or glucose 6-phosphate. Predominant cellular fatty acids are *cis*-vaccenic acid ($\text{C}_{18:1\omega7\text{c}}$, 81.0 %), $\text{C}_{16:0}$ (7.6 %), $\text{C}_{18:0}$ 3-OH (3.46 %), summed feature 3 (2.24 %) comprising $\text{C}_{16:1\omega7\text{c}}$, iso- $\text{C}_{15:0}$ 2-OH or both, summed feature 2 ($\text{C}_{14:0}$ 3-OH/iso- $\text{C}_{16:1}$ I, 1.98 %) and $\text{C}_{18:0}$ (1.71 %). The major ubiquinone is Q-10. The DNA G+C content (by HPLC) is 68.5 mol% (T_{m}).

The type strain, PMB02^T (=KCTC 12901^T=JCM 14648^T), was isolated from a leaf from a tree (*Platanus orientalis*).

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