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Methylibium petroleiphilum gen. nov., sp. nov., a novel methyl *tert*-butyl ether-degrading methylotroph of the *Betaproteobacteria*

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A Gram-negative, rod-shaped, motile, non-pigmented, facultative aerobe that grew optimally at pH 6·5 and 30 °C (strain PM1^T) was isolated for its ability to completely degrade the gasoline additive methyl *tert*-butyl ether. Analysis of the 16S rRNA gene sequence indicated that this bacterium was a member of the class *Betaproteobacteria* in the *Sphaerotilus–Leptothrix* group. The 16S rRNA gene sequence identity to other genera in this group, *Leptothrix*, *Aquabacterium*, *Roseateles*, *Sphaerotilus*, *Ideonella* and *Rubrivivax*, ranged from 93 to 96 %. The chemotaxonomic data including Q-8 as the major quinone, C16 : 1ω7*c* and C16 : 0 as the major fatty acids and a DNA G+C content of 69 mol%, support the inclusion of strain PM1^T in the class *Betaproteobacteria*. It differed from other members of the *Sphaerotilus–Leptothrix* group by being a facultative methylotroph that used methanol as a sole carbon source, and by also being able to grow heterotrophically in defined media containing ethanol, toluene, benzene, ethylbenzene and dihydroxybenzoates as sole carbon sources. On the basis of the morphological, physiological, biochemical and genetic information, a new genus and species, *Methylibium petroleiphilum* gen. nov., sp. nov., is proposed, with PM1^T (=ATCC BAA-1232^T=LMG 22953^T) as the type strain.

Organisms that can use one-carbon compounds as energy sources are called methylotrophs (Lidstrom, 2001). A subset of this group, the methanotrophs, can use methane as their sole carbon source. Methylotrophs have been extensively studied because of their potential use in biotechnology and bioremediation (Lidstrom & Stirling, 1990; Hanson & Hanson, 1996). The aerobic methylotrophs have representatives in the *Proteobacteria*, high-G+C and low-G+C Gram-positive bacteria that have been isolated from diverse environments. Within the *Proteobacteria*, the majority of the methylotrophs that have been isolated belong to either the *Alphaproteobacteria* or *Gammaproteobacteria*. Three genera, *Methylobacillus* (Urakami & Komagata, 1986), *Methylophilus* (Jenkins *et al.*, 1987) and *Methylovorus* (Govorukhina & Trotsenko, 1991), in the class *Betaproteobacteria* are considered to be restricted facultative methylotrophs because they can use methanol but not methane as a sole carbon source, and can use only a limited number of other carbon sources such as glucose and fructose. Phylogenetic analysis based on their 16S rRNA gene sequence resulted in all of them being grouped in the order *Methylophilales* (Bratina *et al.*, 1992; Garrity & Holt, 2001). Currently, none of the described methanotrophs belong to the class *Betaproteobacteria*. However, comparison of the 16S rRNA gene sequence indicated that isolate PM1^T was most closely related to the class *Betaproteobacteria* in the *Sphaerotilus–Leptothrix* group (Bruns *et al.*, 2001). In this study, morphological, physiological, biochemical and genetic information is used to propose a new genus and species, *Methylibium petroleiphilum* gen. nov., sp. nov.

Strain PM1^T was isolated from a mixed bacterial culture enriched with methyl *tert*-butyl ether (MTBE) using a bench-scale biofilter inoculated with material from a

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Abbreviations: MTBE, methyl *tert*-butyl ether; PHB, poly-β-hydroxybutyrate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain PM1^T is AF176594.

compost biofilter from the Los Angeles County Joint Water Pollution Control Plant (Carson, CA, USA) (Hanson *et al.*, 1999). Isolates were obtained on minimal medium (Mu & Scow, 1994) with MTBE (25 mg HPLC grade, 99·9 % pure; Fisher Scientific) as the sole carbon source. MTBE utilization was confirmed by monitoring the disappearance of the substrate using gas chromatography (Shimadzu GC-14A, equipped with a photonionization detector). MTBE mineralization was determined by measuring $^{14}CO_2$ production using uniformly labelled [^{14}C]MTBE (NEN Life Science Products). Strain PM1^T and its relatives have been found to completely mineralize this compound and can do so at rates that have made it an appealing choice for use in the bioremediation of contaminated sites (Hristova *et al.*, 2001). MTBE is a gasoline additive that is not readily degraded in all environments and therefore has become a widespread contaminant of groundwater in the USA (Squillace *et al.*, 1996). The compound consists of four methyl groups surrounding a carbon monoxide and is produced from chemically reacting methanol and isobutylene. Two pathways for the degradation of this compound have been described to date. The initial step for both pathways is the conversion of MTBE to hydroxymethyl *tert*-butyl ether; then, in the pathway described for propane-oxidizing bacteria, *tert*-butyl alcohol and formaldehyde are formed (Steffan *et al.*, 1997). In the degradation pathway used by *Mycobacterium* species, MTBE is converted to *tert*-butyl ether and then hydrolysed to *tert*-butyl alcohol and formate (François *et al.*, 2002; Smith *et al.*, 2003). Formaldehyde and formate both enter the C_1 metabolic cycle, involved in the cycling of one-carbon compounds, where CO_2 and NADH are generated (Ellis *et al.*, 2001). Strain PM1^T grows on *tert*-butyl alcohol, formaldehyde and formate (K. Hristova and K. M. Scow, unpublished results), suggesting that at least part of its MTBE biodegradation pathway is similar to that reported for cometabolizers.

Cell morphology and motility were examined under a phase-contrast microscope (Olympus AX80T) and a Zeiss EM 109 transmission electron microscope (TEM) operated at 80 kV. Cells were grown in minimal medium and a mixture of sodium glutamate, sodium succinate, sodium acetate, yeast extract and Casamino acids (each 0·5 g l^{-1}), or minimal medium with MTBE as the sole carbon source. For TEM examination the cells were suspended in Millipore-filtered distilled water, and single drops of the cell suspension were placed on carbon- and Formvar-coated copper grids. Single drops of 1 % (w/v) aqueous uranyl acetate were added to the grid for 10–20 s. The cell ultrastructure was also examined using thin sections observed using a model H-7000 electron microscope operating at 75 kV (Hitachi). The thin sections were prepared by rapidly freezing concentrated cells in liquid nitrogen, followed by fixation with an acetone/osmium tetroxide (2 %, w/v) solution by freeze-substitution. The fixed cells were then embedded in Spurr resin (Quetol 653). The thin sections were double-stained with uranyl acetate and lead citrate.

Media for the detection of starch, casein, DNA and gelatinase hydrolysis, and for nitrate reduction, were prepared according to the *Difco Manual*. Modified aesculin agar contained 0·01 % (w/v) aesculin, 0·05 % (w/v) iron(III) citrate, 5 g beef extract l^{-1}, 5 g peptone l^{-1} and 15 % (w/v) agar. Urea hydrolysis was determined by using commercially available urea agar (Remel). Cells for catalase and oxidase tests were grown on nutrient agar. API 20NE biochemical tests were conducted according to the manufacturer's instructions (bioMérieux). Quinones were analysed as described previously (Tamaoka *et al.*, 1983), by using reversed-phase HPLC (Beckman System Gold with a Hewlett Packard Zorbox ODS column). Cellular fatty acid methyl esters were analysed by using a Hitachi M7200A GC/3DQMS system, equipped with a DB-5ms capillary column (30 m × 0·25 mm) coated with 5 % phenyl-methylpolysiloxane (J & W Scientific) (Hanada *et al.*, 1995). The G + C content was measured by HPLC, as described previously (Kamagata & Mikami, 1991).

Growth of strain PM1^T in mineral salts medium (MSM) (Mu & Scow, 1994) with MTBE as the sole carbon source or 0·5 × strength liquid tryptic soy broth (Difco) was tested at various temperatures (25, 30 and 37 °C) and pH values (5·0–10·0 in 0·5 increments), and with various vitamins and trace metals (mixture and individual metals). Additional carbon sources such as pyruvate, acetate, citrate, L-asparagine, DL-alanine and glycine at 0·01 % (w/v), and butanol, ethanol and methanol at 0·1 % (v/v), utilized by PM1^T were also tested in MSM. Growth in the presence of a number of aromatic hydrocarbons, including benzoate, benzene, 2,3-dihydroxybenzoate, 2,4-dihydroxybenzoate, 2,5-dihydroxybenzoate, 2,6-dihydroxybenzoate, 3,4-dihydroxybenzoate, 3,5-dihydroxybenzoate, ethylbenzene, naphthalene, toluene and xylene was also investigated. Anaerobic growth was tested at 30 °C using DSMZ334 liquid culture medium (DSMZ, 2001), with acetate as the carbon source and sparged with an H_2/CO_2 mixture (80 : 20 ratio). All growth tests were performed in triplicate at 28 °C for 24–77 h, and turbidity was monitored by spectrophotometry (Spectronic 20 D +; Milton Roy Analytical Div.) at 525 nm.

DNA was extracted from cultures, after growth in MSM plus MTBE as the carbon source, using a Fast DNA extraction kit (Q-BIOgene), according to the manufacturer's instructions. The presence of genes encoding aromatic oxygenase that could potentially be involved in the degradation of some of the aromatic hydrocarbon growth substrates tested was determined using previously described PCR primers and conditions (Baldwin *et al.*, 2003). Primers and conditions previously described by Holmes *et al.* (1995) and Miguez *et al.* (1997) were also used to determine the presence of genes encoding particulate methane monooxygenase (pMMO) and soluble methane monooxygenase (sMMO), respectively.

The 16S rRNA gene sequence of strain PM1^T was determined previously (Bruns *et al.* 2001) (GenBank accession

no. AF176594). Phylogenetic trees were constructed by calculating distances (Kimura and maximum-likelihood) and clustering [neighbour-joining method (Saitou & Nei, 1987) with 1000 bootstrap, using DNAMAN version 4.1 software (Lynnon Biosoft)]. Reference sequences (GenBank; Benson *et al.*, 2003) chosen were those of previously described methylotrophic members of the class *Betaproteobacteria* and the closest phylogenetic relatives of strain PMI^T that represent strains from species with validly published names. Only nearly complete 16S rRNA gene sequences (approximately 1500 bp) with a minimal number of ambiguous bases were chosen, and were optimally aligned prior to tree construction.

Under all growth conditions tested, the cells grew singly as $0{\cdot}5 \times 1\text{–}2$ μm rods, without a sheath (Fig. 1), were motile by means of a single polar flagellum (Fig. 1b) and reproduced by normal cell division. Strain PM1^T was Gram-negative, oxidase-positive and catalase-negative, and was capable of hydrolysing urea but not starch, gelatin, aesculin, casein or DNA. Nitrate was reduced to nitrite, but nitrite was not reduced. API 20NE test results were negative for *o*-nitrophenyl β-D-galactopyranoside, lysine decarboxylase, ornithine decarboxylase, citrate, indole, Voges–Proskauer test, glucose, rhamnose, sucrose, melibiose, arabinose and xylose. Centrally located intracellular granules were observed, which were considered to be poly-β-hydroxybutyrate (PHB) granules (Fig. 1c). This reserve material is commonly found in members of the *Sphaerotilus–Leptothrix* group (Spring, 2002). The characteristic cell morphology of members of the *Sphaerotilus–Leptothrix* group of sheathed cells growing filamentously with oxidized manganese or iron deposits was not observed (Table 1). Instead, the cell morphology was more similar to that of members of the genus *Aquabacterium*, which was also a member of this clade (Kalmbach *et al.*, 1999), except that a surficial fibrillar matrix was not observed. Additionally, intracytoplasmic membrane structures of any type indicative of most methanotrophs were not observed (Bowman, 2000).

Within 2–3 days on nutrient agar, strain PM1^T formed cream-coloured, flat colonies with smooth margins, of 2–3 mm in diameter. Colonies were white in colour when the strain was grown on minimal media with MTBE as the sole carbon source. No pink or orange colony pigmentation was observed, which is often indicative of some methanotrophs (Bowman, 2000). Vitamins were not required for growth; subculture of strain PM1^T in medium without vitamins had no effect on growth. Trace metals required for the use of MTBE as a sole carbon source were Co, Cu, Mn, Zn, Mo, Ni and Fe. Strain PM1^T could grow both aerobically and anaerobically. Other genera in the *Leptothrix* group whose members are also facultative aerobes are *Rubrivivax*, *Ideonella* and *Aquabacterium* (Spring, 2002). Facultatively anaerobic methylotroph representatives also occur (Lidstrom, 2001); therefore the occurrence of this phenotype is not unusual.

Strain PM1^T could utilize a number of organic acids and carbohydrates as sole carbon sources. Of the various substrates tested, PM1^T grew on the following compounds as sole carbon sources (in decreasing order of turbidity): ethanol, pyruvate, L-asparagine, acetate, butanol, DL-alanine, methanol and MTBE. Growth was not observed on citrate or DL-glycine. The number of aromatic hydrocarbons that could be utilized was striking, many of which would co-occur with MTBE in gasoline-contaminated sites. Of those tested, growth was observed (in decreasing order of turbidity) in toluene, benzene, phenol, ethylbenzene, 3,4-dihydroxybenzoate, 2,5-dihydroxybenzoate, 3,5-dihydroxybenzoate, 2,6-dihydroxybenzoate and 2,3-dihydroxybenzoate. The optical densities of cultures were maintained in 2,4-dihydroxybenzoate, naphthalene and xylene; there was no substantial increase in turbidity. Typically, the range of substrates that can be used by methylotrophs is limited (Lidstrom, 2001). Other physiological traits of the strain when grown in MTBE have been reported previously (Hanson *et al.*, 1999). Concentrations of MTBE as the sole carbon and energy source of as high as 500 μg ml^{-1} could be degraded, and support growth. At

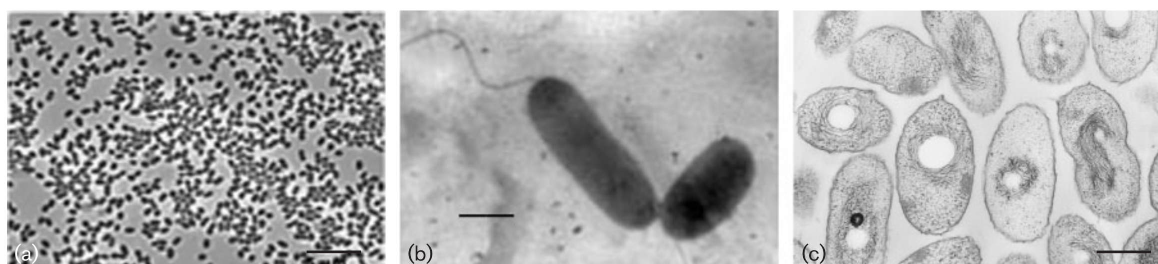

Fig. 1. Photomicrographs of *Methylibium petroleiphilum* strain PM1^T. (a) Phase-contrast micrograph of unstained cells of strain PM1^T after 2 days of growth on minimal medium plus a mixed carbon source. (b) Electron micrograph of negatively stained cells of strain PM1^T after growth on minimal medium plus MTBE showing a single polar flagellum. (c). Electron micrograph of thin sections of negatively stained cells of strain PM1^T after 2 days of growth on minimal medium plus MTBE. Bars, 5 μm (a) and 0·5 μm (b and c).

Table 1. Comparison of characteristics of the genera phylogenetically close to strain PM1^T

Taxa: 1, [*Alcaligenes*] *latus*; 2, [*Pseudomonas*] *saccharophila*; 3, *Roseateles*; 4, *Rubrivivax*; 5, *Ideonella*; 6, *Leptothrix*; 7, *Sphaerotilus*; 8, *Aquabacterium*; 9, strain PM1^T. Adapted from Spring (2002). +, Present in all species; −, absent from all species; NR, not reported.

Characteristic	1	2	3	4	5	6	7	8	9
Flagellation	Peritrichous	One polar	Several polar	One polar	Several, polar or subpolar	One polar; subpolar tuft	Subpolar tuft	One polar	One polar
Formation of sheaths	−	−	−	−	−	+	+	−	−
Carotenoid pigments	−	−	+	+	−	−	−	−	−
Photoautotrophic growth	−	−	−	+	−	−	−	−	−
Oxidation of Mn^{2+}	−	NR	NR	NR	NR	+	−	−	NR
Methanol as sole carbon source	NR	NR	NR	NR	NR	NR	NR	−	+
G+C content (mol%)	NR	69	66	70–72	68	68–71	69	65–66	69
Isolation source	Soil	Mud	River water	Mud	Activated sludge	Freshwater, sediment	Freshwater, activated sludge	Drinking water	Biofilter

the time of the study by Hanson *et al.* (1999), protein analysis indicated that a substantial increase in biomass did not occur when MTBE was used as a carbon source, approximately 0·18 mg cells (mg MTBE)$^{-1}$. Higher yields have been obtained since this initial value was published, with optimization of the culture conditions for growth of strain PM1^T (K. Hristova and K. M. Scow, unpublished results).

To gain insight into the novelty of the genes potentially involved in the utilization of some of the growth substrates tested, PCR was performed using a variety of primers that had been used previously to detect genes encoding oxygenase (Baldwin *et al.*, 2003). PCR products were obtained with primers specific for genes encoding ring-hydroxylating toluene monooxygenase and phenol hydroxylase. The presence of these genes suggested that degradation of at least some of the aromatic hydrocarbons involved catabolic pathways that have been previously described in other bacteria. No products were observed with the other oxygenase gene primers used. Also, no PCR amplicons were produced using primers for the sMMO or pMMO genes that are typically found in methanotrophs. The 16S rRNA gene sequence of isolate L013.11 was found to closely match that of strain PM1^T (Fig. 2). This isolate has not been cultivated, but was found in peat soil after methane enrichment with $^{13}CH_4$ (Morris *et al.*, 2002). The authors of that study speculated that this strain was a novel representative of methanotrophs within the class *Betaproteobacteria*. The close phylogenetic relationship of isolate L013.11 to strain PM1^T suggests that it is at least methylotrophic, but further research is needed to clarify the role played by this strain in methane-enriched communities.

Phylogenetic analysis using the 16S rRNA gene indicated that strain PM1^T fell into the *Sphaerotilus–Leptothrix* sub-cluster within the class *Betaproteobacteria* (Spring, 2002). The sequence formed a separate branch from those of the described genera in this group, *Leptothrix*, *Aquabacterium*, *Roseateles*, *Sphaerotilus*, *Ideonella* and *Rubrivivax* (Fig. 2). Using 16S rRNA gene sequence identity, the most closely related bacterium with a validly published name was *Aquabacterium commune* DSM 11901^T (96 %), followed closely by others in the same clade, *Aquabacterium citratiphilum* DSM 11900^T (95 %), *Aquabacterium parvum* DSM 11968^T (95 %), *Ideonella dechloratans* ATCC 51718^T (95 %), *Leptothrix discophora* ATCC 51168 (95 %), *Leptothrix cholodnii* strain CCM 1827 (95 %), *Leptothrix mobilis* DSM 10617^T (94 %), *Rubrivivax gelatinosus* ATCC 17011^T (95 %), *Roseateles depolymerans* DSM 11813^T (94 %) and *Sphaerotilus* sp. IF5 (93 %). Isolates from this group have been obtained from a wide variety of environments and display diverse physiologies. However, there are no reports of any of these bacteria being able to use methanol as a sole carbon source. The sequence identity to four methylotrophic species that belong to the class *Betaproteobacteria* was much lower: *Methylobacillus pratensis* NCIMB 13994^T had 85 % identity; '*Methylophilus freyburgensis*' strain I 42T, 85 %; *Methylophilus leisingeri* DSM 6813^T, 83 %; and *Methylophilus methylotrophus* ATCC 53528^T, 80 %. The 16S rRNA gene sequence analysis indicates that strain PM1^T represents a new genus and species.

The almost full-length 16S rRNA gene sequences with the highest identity to that of strain PM1^T were from clones made from PCR amplicons of DNA obtained directly from polluted locations that represent uncultivated bacteria. As mentioned above, one of the best-matching sequences was that of strain L013.11 (97 % identity) (Fig. 2), which was amplified from peat soil after methane enrichment with $^{13}CH_4$ (Morris *et al.*, 2002). This suggests that the methylotrophic trait of strain PM1^T is not unique. Examples of other closely matching sequences were those of strain HTCC304 isolated from trichloroethylene- and *cis*-dichloroethylene-contaminated groundwater (GenBank accession no. AY429720; 99 % identity), clone Spb03 isolated from biofilms in the extremely polluted Spittelwasser River (AJ422152; 98 % identity) (Brummer *et al.*, 2003) and clone S1B10 isolated from activated sludge enriched with nonylphenol (AY382153; 96 % identity).

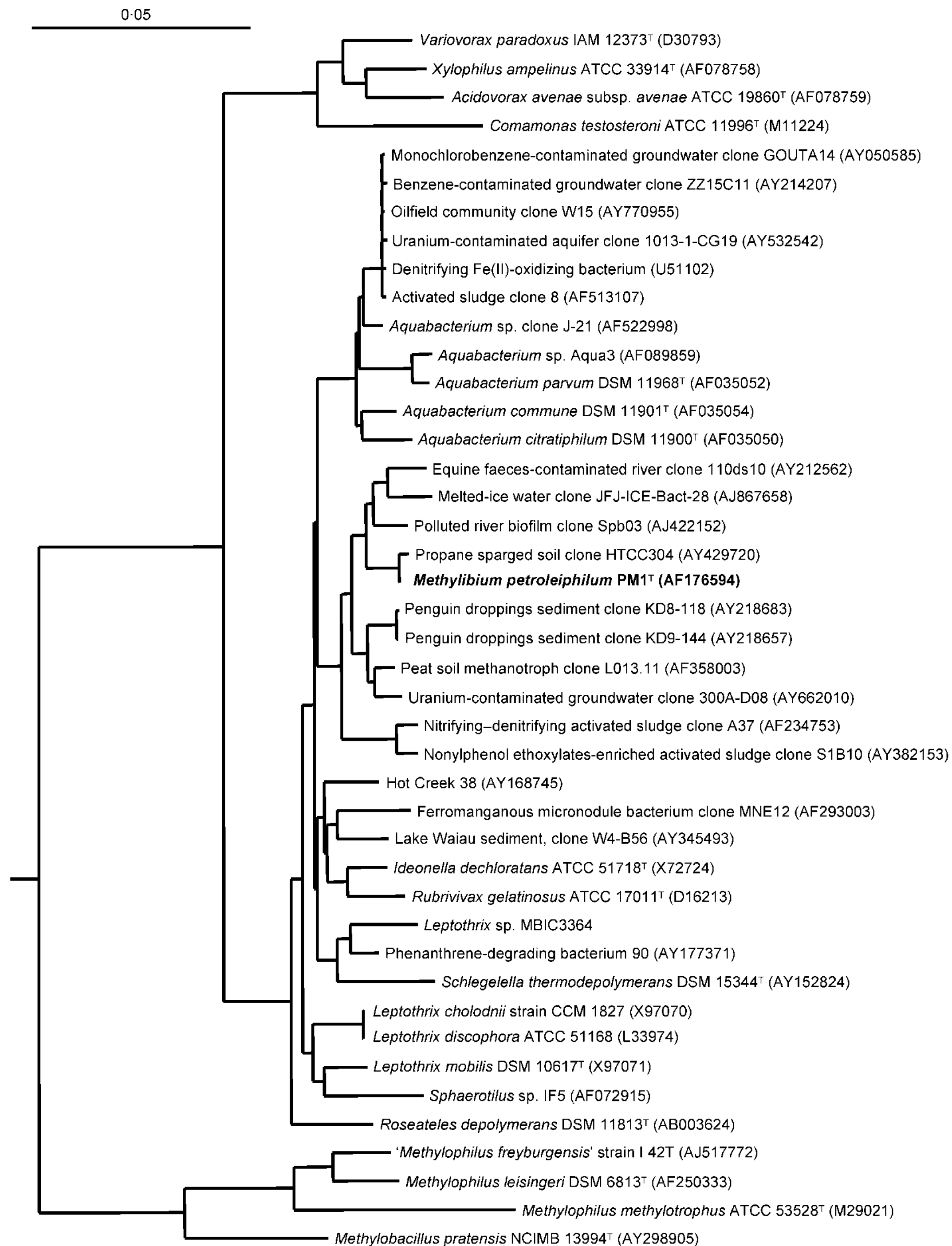

Fig. 2. Phylogenetic position of strain $PM1^T$ among neighbouring species selected from the class *Betaproteobacteria*. Bar, 0·05 substitution per nucleotide position in 16S rRNA gene sequences. GenBank accession numbers and culture collection numbers (where available) used in the tree construction are included on the figure.

Other environments from which related clones have been obtained are polychlorinated biphenyl-polluted soil (Nogales *et al.*, 1999), groundwater contaminated with high levels of nitric acid-bearing uranium waste (clone 300A-D08; AY662010), a stream contaminated with equine faecal material (Simpson *et al.*, 2004), benzene-contaminated groundwater (clone ZZ15C11; AY214207), penguin droppings (clone KD9-144; AY218657) and melted-ice water (clone JFJ-ICE-Bact-28; AJ867658). A better understanding of the physiology of this genus

will probably result in the cultivation of more representatives of this group from a wide range of habitats.

Direct analysis of 16S rRNA genes from MTBE-contaminated sites where MTBE-biodegradation potential has been demonstrated indicated that bacteria with $PM1^T$-like sequences are common in these locations (Kane *et al.*, 2001; Hristova *et al.*, 2003; Smith *et al.*, 2005). Sequences deposited in GenBank (AF409035, AF409034) from another MTBE study also indicate the possible presence of strains related to $PM1^T$. Using quantitative real-time PCR, changes in the density of $PM1^T$-like sequences in the native community of contaminated groundwater and sediment have been shown to correspond to MTBE removal (Hristova *et al.*, 2003). These studies suggest that this bacterium is widespread and is a major component of microbial communities in MTBE-contaminated sediment and groundwater.

Description of *Methylibium* gen. nov.

Methylibium (Me.thy.li.bi′um. N.L. n. *methyl* the methyl radical, the methyl group; Gr. n. *bios* life; N.L. neut. n. *Methylibium* referring to methylotroph).

Cells are motile, Gram-negative straight rods. Oxidase-positive. Negative for gelatinase and catalase. Hydrolyse urea and reduce nitrate to nitrite. Cells possess PHB granules as a storage material and reproduce by binary fission. Growth occurs heterotrophically under aerobic conditions. Facultative methylotrophs able to use methanol as a sole carbon source in addition to a variety of other more complex carbon sources. The major quinone is Q-8. The major fatty acids are C16 : 1ω7*c* and C16 : 0, and in lesser amounts C10 : 0 3-OH, C12 : 0, C12 : 0 2-OH, C12 : 0 3-OH, C14 : 0, C17 : 0ω cyclo7–8*c*, C18 : 1ω7*c* and C18 : 0. On the basis of the results of 16S rRNA gene sequence comparison, the bacteria belong to the class *Betaproteobacteria*. The DNA G + C content of the type species is 69 mol%. The type species is *Methylibium petroleiphilum*.

Description of *Methylibium petroleiphilum* sp. nov.

Methylibium petroleiphilum (pe.tro.lei.phi′lum. Gr. n. *petra* stone, rock; L. n. *oleum* oil; Gr. adj. *philos* loving; N.L. neut. adj. *petroleiphilum* petrol loving).

Exhibits the following properties in addition to those given in the genus description. Colonies are cream in colour under conditions suitable for MTBE degradation. Grows well heterotrophically in media containing ethanol, methanol, toluene, benzene, ethylbenzene and dihydroxybenzoates as the sole carbon source. Vitamins are not required for growth. Optimum pH and temperature for growth are 6·5 and 30 °C, respectively. Does not grow at 37 °C. The genome size is 4·6 Mb (http://genome.jgi-psf.org/finished_microbes/metpe/metpe.home.html). Inhabits subsurface environments highly contaminated with MTBE.

The type strain is $PM1^T$ (=ATCC BAA-1232^T=LMG 22953^T), which was isolated from a mixed bacterial culture enriched using a bench-scale biofilter inoculated with some solid support material from a compost biofilter located at the Los Angeles County Joint Water Pollution Control Plant (Carson, CA, USA).

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