Methylobacterium populi sp. nov., a novel aerobic, pink-pigmented, facultatively methylotrophic, methane-utilizing bacterium isolated from poplar trees (*Populus deltoides* × *nigra* DN34)

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A pink-pigmented, aerobic, facultatively methylotrophic bacterium, strain BJ001^T, was isolated from internal poplar tissues (*Populus deltoides* × *nigra* DN34) and identified as a member of the genus *Methylobacterium*. Phylogenetic analyses showed that strain BJ001^T is related to *Methylobacterium thiocyanatum*, *Methylobacterium extorquens*, *Methylobacterium zatmanii* and *Methylobacterium rhodesianum*. However, strain BJ001^T differed from these species in its carbon-source utilization pattern, particularly its use of methane as the sole source of carbon and energy, an ability that is shared with only one other member of the genus, *Methylobacterium organophilum*. In addition, strain BJ001^T is the only member of the genus *Methylobacterium* to be described as an endophyte of poplar trees. On the basis of its physiological, genotypic and ecological properties, the isolate is proposed as a member of a novel species of the genus *Methylobacterium*, *Methylobacterium populi* sp. nov. (type strain, BJ001^T = ATCC BAA-705^T = NCIMB 13946^T).

Species of the genus *Methylobacterium* are strictly aerobic, facultatively methylotrophic, Gram-negative, rod-shaped bacteria that are able to grow on one-carbon compounds (e.g. methanol or methylamine), as well as on a variety of C₂, C₃ and C₄ substrates (Green, 1992). Only the type species, *Methylobacterium organophilum*, has been shown to use methane as the sole source of carbon and energy (Patt *et al.*, 1976). The genus *Methylobacterium* belongs to

the $\alpha 2$ subclass of the *Proteobacteria* and currently consists of 14 species with validly published names (Heumann, 1962; Ito & Iizuka, 1971; Kouno & Ozaki, 1975; Patt *et al.*, 1976; Rock *et al.*, 1976; Austin & Goodfellow, 1979; Green & Bousfield, 1983; Urakami & Komagata, 1984; Bousfield & Green, 1985; Green *et al.*, 1988; Urakami *et al.*, 1993; Wood *et al.*, 1998; Doronina *et al.*, 2000, 2002; McDonald *et al.*, 2001; Sy *et al.*, 2001). Members of the genus *Methylobacterium* are distributed in a wide variety of natural and man-made environments, including soil, air, dust, fresh- and marine water and sediments, water supplies, bathrooms, air-conditioning systems and masonry (Hiraishi *et al.*, 1995; Trotsenko *et al.*, 2001). Some species have been described as opportunistic human pathogens (Truant *et al.*, 1998; Hornei *et al.*, 1999). In addition, methylotrophic bacteria are frequently associated with terrestrial and aquatic plants, where they colonize roots and leaf surfaces (Austin *et al.*, 1978; Yoshimura, 1982; Corpe & Rheem, 1989; Trotsenko *et al.*, 2001; Lidstrom & Chistoserdova, 2002). The association of *Methylobacterium* species with plants seems to rely on a symbiotic relationship between the bacterium and the plant host. Plants produce methanol

Published online ahead of print on 20 February 2004 as DOI 10.1099/ijs.0.02796-0.

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Abbreviations: IGS, intergenic spacer; SEM, scanning electron microscope.

The GenBank/EMBL/DDBJ accession number for the 16S and 16S–23S IGS rDNA sequence of strain BJ001^T is AY251818.

Tissue culture images, photomicrographs, 16S and 16S–23S IGS rDNA sequences of BJ001^T, sequence similarity matrices, carbon- and nitrogen-source utilization data and enzymic reactions of BJ001^T are available as supplementary material in IJSEM Online.

Dedicated to Olivier Van Aken (1964–1980).

(representing nearly 50 % of the total volatile atmospheric organic carbon), which is toxic and is used by *Methylobacterium* species as the sole source of carbon and energy (i.e. the methanol cycle; Trotsenko *et al.*, 2001). In response, *Methylobacterium* species produce phytohormones (cytokinins and auxins), which are known to stimulate plant growth (Ivanova *et al.*, 2001; Koenig *et al.*, 2002), fix atmospheric nitrogen (Sy *et al.*, 2001) or help plants to fight pathogens (Holland & Polacco, 1994). Bacteria are often pink to red, due to the presence of carotenoids, and are referred to as pink-pigmented facultative methylotrophs. Members of the genus *Methylobacterium* are highly resistant to dehydration, freezing, chlorine, UV and ionizing radiation and elevated temperatures (Trotsenko *et al.*, 2001). *Methylobacterium* species are known to metabolize a range of toxic organic chemicals, such as methyl chloride (McDonald *et al.*, 2001), methyl bromide (Goodwin *et al.*, 2001), methyl iodide (Schaefer & Oremland, 1999), dichloromethane (Doronina *et al.*, 2000), ethylated sulfur-containing compounds (de Zwart *et al.*, 1996), methylated amines (Trotsenko *et al.*, 2001), methyl *tert*-butyl ether (Mo *et al.*, 1997) and cyanate and thiocyanate (Wood *et al.*, 1998). In this paper, the formal taxonomic description of a novel *Methylobacterium* strain, BJ001^T, isolated from poplar tissues and able to use methane as the sole source of carbon and energy, is reported.

Methylobacterium sp. strain BJ001^T was isolated from poplar plantlets and from tissue cultures (*Populus deltoides* × *nigra* DN34) that were developed initially from surface-sterilized explants and maintained under axenic conditions (Van Aken & Schnoor, 2002). Images of tissue cultures containing strain BJ001^T are available in Supplementary Fig. A in IJSEM Online. Poplar plantlets were surface-sterilized before bacterial isolation. The isolated bacterium was maintained routinely on Luria–Bertani (LB) medium, supplemented with fructose (5 g l⁻¹), or on selective methanol mineral medium (Green, 1992). Morphological properties were studied according to general protocols (Gerhardt *et al.*, 1994). Scanning electron microscope (SEM) observations were performed on fixed material that was prepared for routine examination by glutaraldehyde fixation, osmium tetroxide post-fixation and graded ethanol dehydration (Bozzola & Russell, 1998). Samples were critical point-dried, mounted on stubs, sputter-coated with gold/palladium and visualized by using a Hitachi S-4000 SEM equipped with a field-emission electron source. Carbon-source utilization tests were performed by using a standard protocol described by Green & Bousfield (1982). Cellular fatty acids were extracted according to Bligh & Dyer (1959), purified on Sephadex beads (Amersham Biosciences) and analysed by GC-mass spectroscopy (Gerhardt *et al.*, 1994). DNA G+C content was determined by HPLC analysis of individual nucleosides, resulting from DNA hydrolysis and dephosphorylation (Mesbah *et al.*, 1989). DNA manipulations were carried out according to standard protocols (Ausubel *et al.*, 1999; Sambrook & Russell, 2000). 16S and 16S–23S intergenic

spacer (IGS) rDNA analyses were performed by PCR amplification using the following primers: 27f (positions 11–27 of bacterial 16S rDNA, *Escherichia coli* numbering), 1522r (positions 1492–1522), 926f (positions 901–926) and 115r/23S (positions 97–115 of bacterial 23S rDNA, *E. coli* numbering) (Hurek *et al.*, 1997; Tan *et al.*, 2001). PCR conditions were as described by Tan *et al.* (2001). PCR products were cloned in a pGEM vector (Promega) and submitted to the University of Iowa DNA Core (Iowa City, IA, USA) for sequencing. Determined rDNA and reference sequences from GenBank were aligned by using CLUSTAL_W multiple alignment and BIOEDIT (version 5.0.9) software. The tree topology was inferred by the parsimony method (heuristic search) using PAUP (version 4.0) software (Sinauer Associates). DNA–DNA hybridization was carried out according to Doronina *et al.* (2002), using a method based on that of Denhardt (1966). Unlabelled, denatured DNA of *Methylobacterium* species was immobilized on nitrocellulose membranes (Bio-Rad) (Ausubel *et al.*, 1999; Sambrook & Russell, 2000). Reference DNA from strain BJ001^T was labelled with deoxy[1',2',5'-³H]cytidine 5'-triphosphate (57 Ci mmol⁻¹ = 2.1 × 10⁶ MBq mmol⁻¹) by using a Nick Translation kit (N5500; Amersham Biosciences). After labelling, reference DNA exhibited a specific activity of 1.47 × 10⁵ Bq µg⁻¹ and a mean size of 400–600 bp (as determined on 2.0 % agarose gel). Labelled DNA was denatured at 100 °C for 10 min. Hybridization was performed according to Denhardt (1966), with a ratio of labelled to immobilized DNA of 1:100 (Doronina *et al.*, 2002). Low-stringency (pre)-hybridization solution consisted of 30 % (v/v) formamide, 1 × SSC, 5 × Denhardt's solution, 1.0 % SDS and 100 µg denatured salmon sperm DNA ml⁻¹. Radioactivity retained on the membrane was determined by liquid scintillation counting.

The bacterium isolated from poplar tissues, strain BJ001^T, was identified by phylogenetic analysis as a member of the genus *Methylobacterium* (Fig. 1). Members of this genus are known to colonize the rhizosphere and phyllosphere of a variety of plant species (Austin *et al.*, 1978; Yoshimura, 1982; Corpe & Rheem, 1989; Holland & Polacco, 1994; Trotsenko *et al.*, 2001). However, this is the first report of an endophytic association with a poplar tree (*Populus* sp.). The isolate had the following characteristics of the genus *Methylobacterium* (Green, 1992). Cells were rod-shaped (0.8–1.0 × 1.0–10.0 µm), frequently branched and occurred singly or in rosettes (Fig. 2). They exhibited polar growth and were motile by a single polar or lateral flagellum. Photomicrographs of strain BJ001^T are available in Supplementary Figs B and C in IJSEM Online. Cells stained Gram-negative and colonies were pink to red. Cells were strictly aerobic and catalase- and oxidase-positive (Gerhardt *et al.*, 1994). Due to the chemotaxonomic homogeneity of the genus *Methylobacterium*, phylogenetic analyses constitute a critical tool for species identification (Green & Bousfield, 1982; Doronina *et al.*, 2002). According to 16S rDNA sequences, the closest relatives of strain BJ001^T were *Methylobacterium thiocyanatum*, *Methylobacterium*

Fig. 1. Phylogenetic tree based on 16S rDNA sequences of members of the genus *Methylobacterium* and other representatives of the *Proteobacteria*, showing the location of strain BJ001^T. GenBank accession numbers are provided in parentheses. Distances reflect the number of pairwise character differences.

extorquens, *Methylobacterium zatmanii* and *Methylobacterium rhodesianum*, with 99.3, 99.1, 98.6 and 98.5% 16S rDNA sequence similarity, respectively, corresponding to the interspecies separation level of the genus *Methylobacterium* (94.2–99.4%; Doronina *et al.*, 2002). On the basis of 16S–23S IGS rDNA sequences, strain BJ001^T shared 78.7–82.1% similarity with *M. extorquens* (GenBank accession nos AF293375 and AF338180) and 66.5% similarity with the type species, *M. organophilum* (GenBank accession no. AF338181). The 16S and 16S–23S IGS rDNA sequence of strain BJ001^T and sequence similarity matrices are available in Supplementary Tables A

and B in IJSEM Online. Levels of DNA relatedness between strain BJ001^T and its closest relatives, as determined by DNA–DNA hybridization, were 15–59%, which indicates that strain BJ001^T can be separated from other members of the genus *Methylobacterium* (Table 1). Phenotypic differences between *Methylobacterium* species are limited and often rely on utilization of carbon and energy sources (Green, 1992). Like other members of the genus, strain BJ001^T grew on C₁ substrates, such as methanol, methylamine, formate and formaldehyde. In addition, strain BJ001^T utilized methane, an ability that is shared with only one other species of the genus, *M. organophilum* (Patt *et al.*, 1976). Strain BJ001^T may play an important ecological role by consuming methane, the greenhouse effect of which is 20 times higher than that of carbon dioxide (Trotsenko *et al.*, 2001). Strain BJ001^T differed from its closest relatives (i.e. *M. thiocyanatum*, *M. extorquens*, *M. zatmanii* and *M. rhodesianum*) in several other carbon-source utilization features (summarized in Table 2). *M. thiocyanatum* grows on glucose, arabinose, glutamate, citrate, cyanate and thiocyanate, *M. zatmanii* grows on trimethylamine and *M. rhodesianum* grows on dimethylamine, none of which support growth of strain BJ001^T. On the other hand, *M. extorquens* does not use fructose, *M. zatmanii* does not use betaine and *M. rhodesianum* does not use tartrate, all of which are substrates for strain BJ001^T (Rock *et al.*, 1976; Urakami & Komagata, 1984; Green, 1992; Wood *et al.*, 1998). Fructose, the first hexose synthesized by plant photosynthesis, was by far the best carbon substrate for strain BJ001^T, which may be related to its association with poplar trees. Tables of carbon- and nitrogen-source utilization and enzymic reactions of BJ001^T are available as Supplementary Tables D, E and F in IJSEM Online.

Strain BJ001^T has been shown to mineralize the toxic explosives hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tertanitro-1,3,5,7-tetrazocine (HMX),

Fig. 2. Scanning electron micrograph of strain BJ001^T isolated from *Populus deltoides* × *nigra* DN34. Bar, 5.0 μm.

Table 1. DNA–DNA hybridization values between *Methylobacterium populi* BJ001^T and its closest relatives

The hybridization procedure was carried out according to the method of Denhardt (1966).

Species	DNA–DNA hybridization (%)
<i>Methylobacterium populi</i> BJ001 ^T	100
<i>Methylobacterium thiocyanatum</i> DSM 11490 ^T	47
<i>Methylobacterium extorquens</i> ATCC 14718	59
<i>Methylobacterium zatmanii</i> JCM 2819	34
<i>Methylobacterium rhodesianum</i> JCM 2810	25
<i>Methylobacterium suomiense</i> VKM B-2238 ^T	33
<i>Methylobacterium lusitanum</i> VKM B-2239 ^T	15
<i>Methylobacterium organophilum</i> JCM 2833 ^T	28
<i>Agrobacterium tumefaciens</i> NCPPB 2437 ^T	7

Table 2. Differential carbon-substrate utilization among *Methylobacterium* species

Utilization of various compounds as sole sources of carbon and energy is shown. Adapted from Green (1992). Species: 1, BJ001^T; 2, *M. aminovorans* (Urakami *et al.*, 1993); 3, *M. chloromethanicum* (McDonald *et al.*, 2001); 4, *M. dichloromethanicum* (Doronina *et al.*, 2000); 5, *M. extorquens* (Urakami & Komagata, 1984); 6, *M. fujisawaense* (Kouno & Ozaki, 1975); 7, *M. lusitanum* (Doronina *et al.*, 2002); 8, *M. mesophilicum* (Austin & Goodfellow, 1979); 9, *M. organophilum* (Patt *et al.*, 1976); 10, *M. radiotolerans* (Ito & Iizuka, 1971); 11, *M. rhodesianum* (Rock *et al.*, 1976); 12, *M. rhodinum* (Heumann, 1962); 13, *M. suomiense* (Doronina *et al.*, 2002); 14, *M. thiocyanatum* (Wood *et al.*, 1998); 15, *M. zatmanii* (Rock *et al.*, 1976). +, Growth; –, no growth; v, variable; w, weak growth; ND, not determined.

Carbon source	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
D-Glucose	–	–	–	–	–	+	–	+	+	+	–	w	+	+	–
D-Fucose	–	ND	ND	ND	–	+	ND	+	–	+	–	–	ND	ND	–
D-Xylose	–	–	–	–	–	+	–	–	–	+	–	–	–	ND	–
L-Arabinose	–	ND	ND	ND	–	+	–	+	–	+	–	–	–	w	–
D-Fructose	+	+	–	+	–	v	+	–	+	–	+	+	+	+	+
L-Aspartate/L-glutamate	–	+	+	–	v	+	–	+	–	+	v	+	–	+	–
Citrate	–	–	+	+	–	+	–	+	–	+	–	+	–	+	–
Sebacate	–	ND	ND	ND	–	+	ND	v	–	+	–	–	ND	ND	–
Acetate	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+
Betaine	+	+	+	+	+	–	–	–	–	+	+	+	+	ND	–
Tartrate	+	ND	ND	ND	v	v	–	–	–	–	–	–	–	ND	v
Ethanol	+	ND	ND	ND	+	+	+	+	+	v	+	+	+	ND	+
Methane	+	ND	–	–	–	–	–	–	v	–	–	–	–	ND	–
Methylamine	+	+	+	+	+	–	+	–	+	–	+	+	+	+	+
Dimethylamine	–	+	ND	ND	–	–	–	–	–	–	+	–	–	–	–
Trimethylamine	–	+	–	–	–	–	–	–	+	–	–	–	–	–	+
Cyanate	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	ND
Thiocyanate	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	ND
Nutrient agar	+	ND	ND	ND	v	+	ND	–	+	+	+	+	ND	+	+

which contaminate numerous military sites in the USA, to CO₂ (data not shown). Whereas this extends the list of organic pollutants that are potentially biodegraded by *Methylobacterium* species, this particular ability has not been tested among other members of the genus and cannot be regarded as a diagnostic test. According to their widespread distribution in a diversity of environments, *Methylobacterium* species may be suited for natural attenuation or *in situ* bioremediation (including phytoremediation) of polluted sites.

On the basis of its 16S and 16S–23S IGS rDNA sequence similarity data, DNA–DNA hybridization values, carbon-source utilization pattern (including the use of methane) and endophytic association with poplar trees, strain BJ001^T is proposed as the type strain of a novel *Methylobacterium* species, with the name *Methylobacterium populi* sp. nov.

Description of *Methylobacterium populi* sp. nov.

Methylobacterium populi (po'pu.li. L. gen. n. *populi* of poplar).

Cells are aerobic, Gram-negative, asporogenous rods (0.8–1.0 × 1.0–10.0 µm) that occur singly, in pairs or in rosettes. Cells are motile by one single polar or lateral flagellum. Colonies are pink to red, slow-growing and 0.1–0.2 mm in

diameter after 4 days at 28 °C on LB or nutrient agar (NA) plates. The pink pigment is water-insoluble and has absorption maxima at 390, 473, 505 and 534 nm in chloroform/methanol (1:1). Positive for the following enzymic reactions: catalase, oxidase, alkaline phosphatase, esterases (C₄ and C₈), valine arylamidase, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Carbon sources utilized are D-fructose, glycerol, methanol, ethanol, formate, acetate, succinate, lactate, tartrate, pyruvate, fumarate, salicylate, formaldehyde, methylamine, methane and betaine. Grows on LB and NA plates at 28 °C. Does not use D- or L-arabinose, D-fucose, D-galactose, D-glucose, D-lactose, D-mannose, D-xylose, sucrose, propan-2-ol, n-butanol, inositol, mannitol, sorbitol, L-aspartate, L-glutamate, glycine, citrate, sebacate, dimethylamine, trimethylamine, chloromethane, dichloromethane, cyanate or thiocyanate. Nitrogen sources utilized are ammonium, nitrate, L-alanine, L-aspartate, L-glutamate, L-glutamine, glycine, L-tryptophan and methylamine. Cellular fatty acids are: hexadecanoate (palmitic acid, C_{16:0}), 6.4 ± 0.4 % (n = 3); *cis*-9-octadecenoate (oleic acid, C_{18:1}), 81.6 ± 2.1 % (n = 3); and octadecanoate (stearic acid, C_{18:0}), 11.9 ± 0.3 % (n = 3). Optimal pH for growth is 7.0; does not grow at pH 4.0 or 9.0. Optimal temperature for growth is 20–30 °C; does not grow at 15 or 40 °C. Does not grow in the presence of 2.0 % NaCl. DNA G + C content is 70.4 ± 0.3 mol% (n = 3).

The type strain, BJ001^T (=ATCC BAA-705^T=NCIMB 13946^T), was isolated from internal poplar tissues (*Populus deltoides* × *nigra* DN34) obtained from Hramoor Nursery (Manistee, MI, USA).

Acknowledgements

This is a contribution of the W. M. Keck Phytotechnology Laboratory at the University of Iowa, supported by a gift from the W. M. Keck Foundation. We thank SERDP (Strategic Environmental Research and Development Program award number 02 CU13-17) for financial support. We acknowledge C. S. Harwood (University of Iowa, IA, USA) and M. E. Lidstrom (University of Washington, WA, USA) for relevant discussion.

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