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Methylocella silvestris sp. nov., a novel methanotroph isolated from an acidic forest cambisol

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Two strains of Gram-negative, aerobic, non-pigmented, non-motile, rod-shaped, methane-oxidizing bacteria were isolated from an acidic forest cambisol near Marburg, Germany, and were designated as strains BL2^T and A1. These bacteria were morphologically and phenotypically similar to *Methylocella palustris* K^T. The cells possess a highly specific bipolar appearance. They lack the intracytoplasmic membranes common to all methane-oxidizing bacteria except *Methylocella*, but contain a vesicular membrane system connected to the cytoplasmic membrane. A soluble methane monooxygenase was present, but no particulate methane monooxygenase could be detected. These bacteria utilize the serine pathway for carbon assimilation. Strains BL2^T and A1 are moderately acidophilic, mesophilic organisms capable of growth at pH values between 4·5 and 7 (with an optimum at pH 5·5) and at temperatures between 4 and 30 °C. Compared with *Methylocella palustris* K^T, these strains have greater tolerance of cold temperatures, dissolved salts and methanol. On the basis of 16S rRNA gene sequence identity, of species with validly published names, strain BL2^T is most closely related to *Methylocella palustris* K^T (97·3 % identity), *Beijerinckia indica* subsp. *indica* ATCC 9039^T (97·1 %) and *Methylocapsa acidiphila* B2^T (96·2 %). The DNA G+C content is 60 mol% and the major phospholipid fatty acid is 18 : 1ω7. Strain BL2^T showed only 21–22 % DNA–DNA hybridization with *Methylocella palustris* K^T. The data therefore suggest that strains BL2^T and A1 represent a novel species of *Methylocella*; the name *Methylocella silvestris* sp. nov. is proposed, with strain BL2^T (=DSM 15510^T=NCIMB 13906^T) as the type strain.

INTRODUCTION

Aerobic methanotrophic bacteria are able to use methane (CH_4) as a sole carbon and energy source. The 11 currently recognized genera of methanotrophs belong to the γ- and α-subclasses of the *Proteobacteria*; these are respectively designated as type I or type II methanotrophs. The two types differ physiologically in several ways, most notably in their carbon-assimilation pathways [ribulose monophosphate (RuMP) versus serine pathway] and the arrangement of intracytoplasmic membranes (ICMs) (Hanson & Hanson, 1996).

The existence of methanotrophs adapted to high or low extremes of temperature, pH and salinity has been demonstrated by cultivation-independent molecular ecology techniques and by the isolation and characterization of pure cultures (Murrell *et al.*, 1998; Trotsenko & Khmelenina, 2002). Much of the Earth's surface, including most wetlands and forest soils of the temperate and boreal zones, is acidic. Recently, novel acidophilic methanotrophs were isolated from *Sphagnum* peat bogs and described as the new genera and species *Methylocella palustris* (Dedysh *et al.*, 1998, 2000) and *Methylocapsa acidiphila* (Dedysh *et al.*, 2002). These isolates grow optimally at about pH 5·5. They belong to the α-subclass of the *Proteobacteria* but do not form a monophyletic group with the previously known type II methanotrophs of the genera *Methylosinus* and *Methylocystis*.

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Abbreviations: DNMS, dilute nitrate mineral salts; ICM, intracytoplasmic membrane; pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the partial gene sequences of *mmoX*, *mxaF* and *nifH* of *Methylocella silvestris* strain BL2^T are respectively AJ491847–AJ491850.

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Instead, they are closely related to the acidophilic, non-methanotrophic bacterium *Beijerinckia indica*. *Methylocapsa acidiphila* B2^T, like most other known methanotrophs, possesses the particulate methane monooxygenase enzyme (pMMO) and contains a well-developed ICM system in which pMMO is bound. *Methylocella palustris* does not contain an ICM system, which makes it unique among methanotrophs. The genes encoding the pMMO enzyme, which are present in all other methanotrophs, could not be detected in *Methylocella palustris* with either a PCR assay considered universal for the *pmoA* gene or hybridization with a *pmoA* gene fragment of *Methylococcus capsulatus* Bath. *Methylocella palustris*, therefore, appears to possess a soluble form of methane monooxygenase (sMMO) only.

Here, we describe a third organism belonging to this acidophilic group of methanotrophs. This organism was isolated from acidic forest soil rather than from *Sphagnum* peat.

METHODS

Isolation and maintenance of strains BL2^T and A1. Soil cores in plastic tubes (3 cm diameter, 13 cm depth) were sampled in December 1999 from an acidic (pH 3·8–4·3) cambisol under a beech-dominated forest stand near Marburg, Germany. Details of the soil, its methane-oxidation rate and the methanotrophic community detectable with molecular methods were published previously (Henckel *et al.*, 2000). Intact cores were sealed at the bottom and placed in a gas-tight chamber containing 20 % (v/v) CH_4 in air. The headspace in the chamber was replaced periodically.

After 6 months, soil crumbs from the mineral A horizon (8–13 cm from the soil surface) of the cores were sprinkled on plates of dilute nitrate mineral salts (DNMS) medium. This was nitrate mineral salts medium, as described previously for the isolation of methanotrophic bacteria (Hanson *et al.*, 1991), diluted 1 : 5 with distilled water and containing 1 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 5·8). DNMS was solidified with Bacto agar (15 g l^{-1}; Difco). After incubation for 2 months in an atmosphere containing 20 % CH_4, slimy, semi-transparent masses developed around most soil crumbs on the plates. These were carefully picked with a loop and restreaked onto fresh DNMS plates. Single colonies were successively selected from plates for restreaking until pure cultures were obtained. Two such cultures were designated BL2^T and A1.

Reference bacterial strains. *Methylocella palustris* K^T (=ATCC 700799^T), *Methylocapsa acidiphila* B2^T (=DSM 13967^T=NCIMB 13765^T) and *B. indica* subsp. *indica* ATCC 9039^T were used as reference strains. *Methylocapsa acidiphila* B2^T was grown on half-strength nitrogen-free M1 medium (Dedysh *et al.*, 2002). *Methylocella palustris* K^T was cultivated on M1 medium supplemented with 250 mg KNO_3 l^{-1} and solidified with 1·5 % (w/v) agar. Both methanotrophs were grown under a gas phase of 10 % (v/v) CH_4 in air. *B. indica* subsp. *indica* ATCC 9039^T was grown on nitrogen-free mineral medium containing glucose (Becking, 1984).

Verification of strain purity. Culture purity was ensured by examination under phase-contrast and electron microscopy and by plating on media containing the following different organic substrates: nutrient agar (Difco), nutrient agar diluted 1 : 10 or DNMS supplemented with either 0·1 % (w/v) sucrose or glucose. Plates were incubated in an atmosphere not supplemented with CH_4. To ensure detection of slowly growing heterotrophic satellites, growth was assessed after incubation for 4 weeks at 25 °C.

Finally, DNA was extracted from two separate batches of strain BL2^T and PCR-amplified partial fragments of the 16S rRNA gene were analysed by denaturing gradient gel electrophoresis to determine whether only a single gene product was detectable in the cultures. All steps were performed as described previously (Henckel *et al.*, 1999) except that the PCR mixtures all contained premix F (Epicenter Technologies) instead of 1·5 mM $MgCl_2$, and the denaturing gradient gel electrophoresis gel was run at 150 V for 6 h.

Morphological observations. Cell morphology was examined in batch cultures grown in liquid DNMS for 2 weeks (in vials sealed with butyl-rubber stoppers and injected with CH_4 at 10 %, v/v). The presence of cysts or exospores was examined using stationary-phase liquid and plate cultures (up to 4–5 months old) (Dedysh *et al.*, 2000). The Gram-stain reaction and lysis in 3 % (w/v) KOH (Gerhardt, 1981) were performed on both 1- and 3-week-old cultures. Lysis by 0·2 and 2 % (w/v) SDS was determined by direct microscopic observation.

Electron microscopy. Cells were collected by centrifugation and fixed for 1 h at 4 °C in 0·15 M cacodylate buffer (pH 7·3) containing 0·1 % (w/v) ruthenium red and 1·2 % (w/v) glutaraldehyde. Cells were then washed three times with 0·15 M cacodylate buffer (pH 7·3) by centrifugation (3 min at 3000 ***g***). Cells were additionally fixed for 3 h at 20 °C in a solution containing 1·5 % (w/v) OsO_4 and 0·1 % (w/v) ruthenium red in 0·05 M cacodylate buffer (pH 7·3) and then washed with 0·15 M cacodylate buffer (pH 7·3). Thin-sectioning, staining and transmission electron microscopy were performed as described previously (Dedysh *et al.*, 2000).

Physiological tests. To test the pH range, DNMS containing 1·25 mM phosphate buffer was adjusted to pH 4–7·5 (in steps of 0·5 pH units) with H_3PO_4. Duplicate 120 ml serum vials (40 ml medium) at each pH were capped with butyl-rubber stoppers and injected with 10 % (v/v) CH_4. The OD_{600} was measured in an Eppendorf BioPhotometer at 2-day intervals for 2 weeks. Growth at temperatures from 4 to 45 °C and tolerance of NaCl concentrations from 0·01 to 1 % (w/v) were also determined in liquid DNMS cultures monitored for 2 weeks (Dedysh *et al.*, 2000).

Nitrogen fixation was assayed using the acetylene-reduction procedure of Takeda (1988). Batch cultures in nitrogen-free DNMS medium containing 0·5 % (v/v) methanol were assayed in the mid-exponential growth phase. Incubations with acetylene were performed under both aerobic and microaerobic conditions, using the protocol described for *Methylocella palustris* (Dedysh *et al.*, 2000).

Range of utilizable carbon and nitrogen sources. The range of potential growth substrates was examined using 0·05 % (w/v) concentrations of the following carbon sources: formate, formamide, methylamine, dimethylamine, trimethylamine, glucose, fructose, sucrose, lactose, galactose, xylose, sorbose, maltose, raffinose, arabinose, ribose, lactate, oxalate, citrate, mannitol and sorbitol. The capacity to utilize methanol at concentrations from 0·01 to 6 % (v/v) was determined in DNMS liquid medium supplemented with methanol (vials were capped with butyl-rubber stoppers to prevent loss of methanol by vaporization). Nitrogen sources were tested by replacing KNO_3 in DNMS with 0·05 % (w/v) $NaNO_2$, NH_2OH, $(NH_4)_2SO_4$, NH_4Cl, glycine, L-alanine, L-serine, L-isoleucine, L-proline, L-cystine, L-methionine, L-glutamine, L-histidine, L-arginine, L-lysine or yeast extract. Growth was examined, after incubation for 30 days, in comparison with a negative control (i.e. growth on nitrogen-free DNMS medium).

Preparation of cell-free extract. Cells grown under CH_4 (1–2 weeks) were harvested by centrifugation (15 000 ***g***, 20 min) and washed in 0·05 M Tris/HCl buffer (pH 7·2) containing 5 mM $MgCl_2$. Cells (1 g fresh weight) were resuspended in 5 ml of the same buffer; 1 ml of a solution containing 1 mg lysozyme (Sigma) was added and the resulting mixture was incubated for 3 h at 20 °C. The suspension was centrifuged (15 000 ***g***, 20 min), washed in 0·05 M

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Tris/HCl buffer (pH 7·2) containing 5 mM $MgCl_2$ and 0·1 mM PMSF, resuspended in the same buffer, sonicated (3× 30 s) and centrifuged (15 000 ***g***, 40 min). The soluble fraction was used to measure enzyme activities.

Enzyme assays. Enzymic studies were performed as described for *Methylocella palustris* K^T (Dedysh *et al.*, 2000). To test for sMMO activity, naphthol formation from naphthalene was monitored with tetrazotized *o*-dianisidine according to the procedure of Phelps *et al.* (1992). sMMO activity was measured in whole cells grown in either copper-deficient medium (with no added copper) or at copper excess (2·5 μM Cu^{2+}). MMO activity was also estimated as the velocity of radioactivity incorporation from $^{14}CH_4$ into both cells and CO_2. Cells were grown to an OD_{600} of 0·3–0·5, harvested by centrifugation (10 000 ***g***, 20 min), washed in 10 mM potassium phosphate buffer (pH 6·5) and then resuspended in the same buffer containing 10 mM sodium formate. Aliquots (1 ml) of the cell suspension were placed into 15 ml vials, which were capped and sealed; 100 μl $^{14}CH_4$ (2 μCi μmol^{-1}) was injected into the gas phase and the vials were incubated at 20 °C. After 10, 20 or 30 min, 1 ml 2 M NaOH was added with a syringe to stop all metabolic processes and to absorb all $^{14}CO_2$ evolved. After 1 day, 0·2 ml culture samples were taken, placed onto Whatman GF/F glass-fibre papers (1·5 × 1·5 cm) and air-dried. Papers were rinsed with 0·2 ml distilled water and dried again. Radioactivity was measured on an Intertechnique SL-30 liquid scintillation spectrometer.

Comparative sequence analysis. DNA to be used in PCRs was extracted from strains $BL2^T$ and A1 using a mechanical disruption procedure (Henckel *et al.*, 1999). PCR-mediated amplification of the 16S rRNA gene from position 28 to 1491 (numbering according to the International Union of Biochemistry nomenclature for *Escherichia coli* 16S rRNA), as well as of a partial fragment of the *mxaF* gene, was carried out as described previously (Dedysh *et al.*, 2000). Partial fragments of the *mmoX* gene (encoding a subunit of sMMO) and of the *nifH* gene (encoding dinitrogenase reductase H) were amplified using primers and PCR programs described, respectively, by Auman *et al.* (2000) and Auman *et al.* (2001). In each case, PCR mixtures contained 0·5 μM each primer, 1× Premix F (Epicenter Technologies) and 1 U *Taq* polymerase (QBioGene). Sequencing was performed as described previously (Dedysh *et al.*, 2000) and comparative phylogenetic analysis was carried out using the ARB program package (Strunk & Ludwig, 1996).

DNA base composition and DNA–DNA hybridization. Cultures of strain $BL2^T$ and *Methylocella palustris* K^T were grown on DNMS plates for 8 weeks. Cells were separated from their capsular matrix by a combination of heating to 50 °C and vigorous shaking. DNA extraction and DNA–DNA hybridization of the two strains were performed as described by Martin *et al.* (1997). The G+C content of the DNA was analysed as described by Groth *et al.* (1996).

Analysis of fatty acid methyl esters. Fatty acid methyl esters were extracted from cells and analysed by GC as described by Kämpfer & Kroppenstedt (1996).

RESULTS

Colony morphology of strains $BL2^T$ and A1 and growth in liquid medium

Raised, white, circular colonies with an entire edge and a smooth surface developed on DNMS agar after incubation for 1–2 weeks. Six-week-old colonies were 2–4 mm in diameter. With continued growth, the colonies developed a semi-transparent layer over the original white colony form and eventually merged together to form an amorphous slimy cover on the agar surface. Liquid cultures displayed white turbidity; a surface pellicle was not formed. In high concentrations of methanol (0·5–5 %, v/v), liquid cultures first developed white turbidity and later became semi-transparent and highly viscous.

Cell morphology and ultrastructure

Cells of strains $BL2^T$ and A1 were Gram-negative, non-motile, short, slightly curved rods, 0·6–0·8 μm in width and 1·2–1·5 μm in length. They reproduced by normal cell division. Similarly to *Methylocella palustris*, the cells had a distinctive bipolar appearance which was more or less pronounced depending on the culture conditions. Cells occurred singly or as aggregates in which each cell was separated from the others by the capsular material. This material stained with ruthenium red (Luft, 1964) and was therefore polysaccharidic in nature (Fig. 1a–c). The formation of rosettes was not observed. Although both plate and liquid cultures up to 5 months old were thoroughly examined, no spores or other resting stages were observed.

Thin sections were prepared from both methane- and methanol-grown cells. Methane-grown cells had an ICM composed of flattened, ovoid or tube-shaped vesicles oriented irregularly on the periphery of the cytoplasm (Fig. 1d, e). Ovoid vesicles were occasionally combined into chains (Fig. 1e). It is possible that these ovoid vesicles originated through division of the long tube-shaped vesicles. All three types of vesicle (flattened, tube-shaped and ovoid) were bounded by three-layered membranes, and each contained a homogeneous matrix of lower electron density with respect to the cytoplasm. In methanol-grown cells, small ovoid vesicles combined into chains on the cell periphery were predominant (Fig. 1a, b). These ICM structures were similar to those of *Methylocella palustris* (Dedysh *et al.*, 2000), except that the vesicles in *Methylocella palustris* were smaller and more spherical. Methanol-grown cells contained one large inclusion of poly-β-hydroxybutyrate per cell (Fig. 1f).

Culture purity

Both isolates displayed a uniform cell morphology in cultures of different age. No growth was observed either on any of the complex organic plating media tested or on various multicarbon substrates in liquid culture. Finally, only a single 16S rRNA gene product (as determined by denaturing gradient gel electrophoresis analysis) could be amplified by PCR from DNA of cultures of strain $BL2^T$.

Physiological characteristics

Strains $BL2^T$ and A1 grew on methane or methanol as the sole carbon and energy source. Slow growth was also observed on ammonium formate and on methylamine. In contrast to *Methylocella palustris*, which did not grow at methanol concentrations above 0·3 %, the isolates from forest soil utilized methanol at a wide range of concentrations (0·01–5 %). The optimum was between 0·5 and 2 %. Cells

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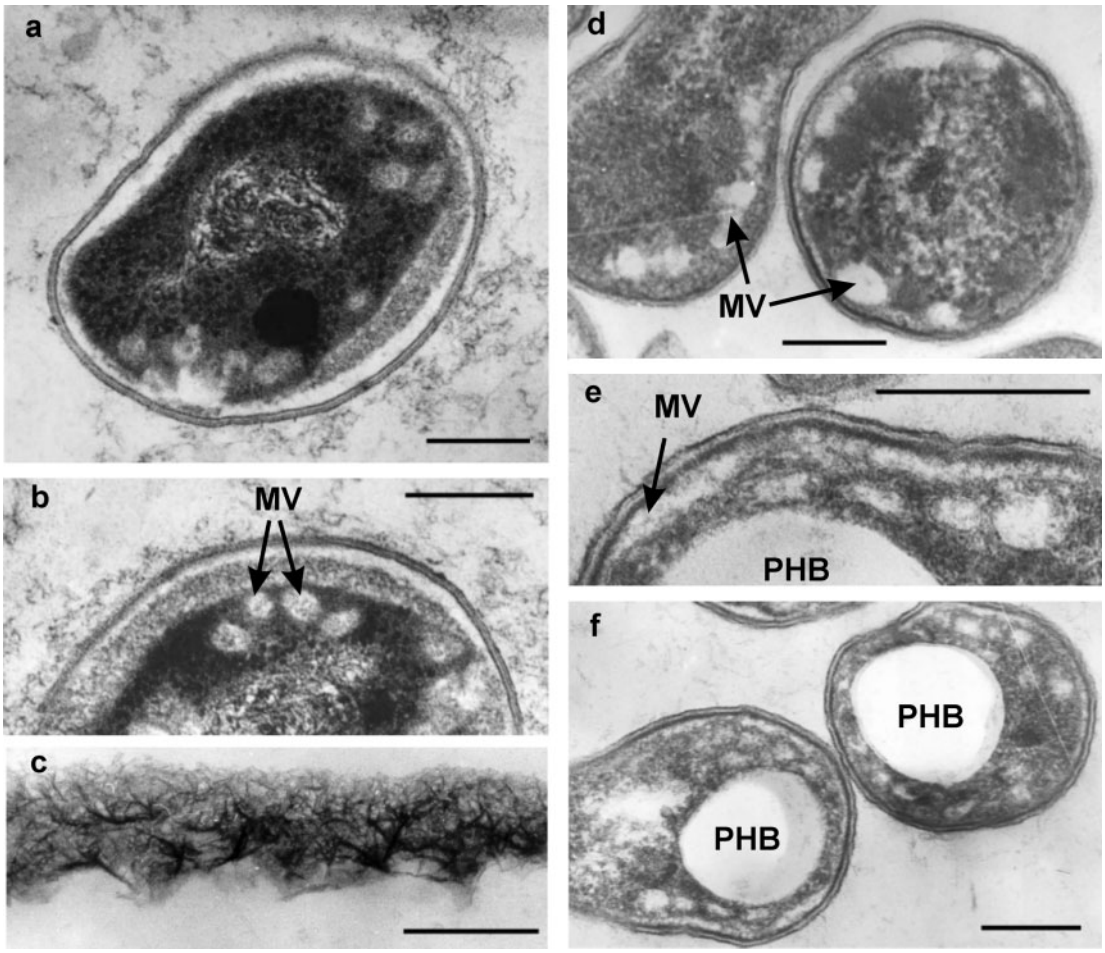

Fig. 1. Electron micrographs of ultrathin sections of cells of strain BL2^T grown on methanol (a, b, f) and on methane (c–e). Chains of flattened and ovoid membrane vesicles located on the cell periphery are shown (a, b, d–f) (MV, membrane vesicles). Large granules of poly-β-hydroxybutyrate (PHB) are visible in methanol-grown cells (f). The fibrous structure of the polysaccharide capsule of strain BL2^T can be seen (c). The polysaccharide capsule is not visible around all cells (a, b, d–f), as it became detached during cell preparation. Bars, 0·2 μm.

grown at higher methanol concentrations (3–5 %) had large capsules up to 5–8 μm in diameter. Growth did not occur on the other C_1 or multicarbon compounds tested. Growth factors were not required. Both strains utilized ammonium salts, nitrates and yeast extract as nitrogen sources. Weak growth also occurred when L-alanine, L-serine, L-cystine, L-glutamine and L-proline were provided as nitrogen sources. Although the isolates were also capable of growth on nitrogen-free agar medium, they grew poorly under aerobic conditions in liquid nitrogen-free medium. Acetylene-reduction activity by strains BL2^T and A1 was detectable only in microaerobic conditions and ranged from 4·0 to 5·5 nmol C_2H_4 (mg dry biomass)$^{-1}$ h^{-1}. The amplification of a *nifH* gene from strain BL2^T provides additional evidence that the genetic potential for nitrogen fixation is present.

The pH of the medium was altered by the growth of the culture, so the strict pH dependence was difficult to determine. However, no growth occurred at pH 4 or 7·5. The fastest growth occurred at pH 5·5 and, after growth for 2 weeks in media with initial pH values between 5 and 6·5, the final pH tended to be about 5·8. The temperature range for growth was 4–30 °C, with the optimum at 15–25 °C. No growth occurred at 37 °C. The specific growth rate of culture under CH_4 (10 %, v/v), calculated from increases in OD_{600} in the exponential phase (10 days) of growth (a mean of 10 vials), was 0·33 day^{-1} (equal to a doubling time of 2·1 days). Like *Methylocella palustris*, the forest isolates grew better on diluted media, but the optimum salt concentration was about twice as high (0·4–1·0 g salts l^{-1}) (Dedysh *et al.*, 2000). Growth was inhibited completely by NaCl concentrations above 0·8 %.

Cellular fatty acid profiles

Overall, the cellular fatty acid composition of strain BL2^T was quite similar to those of *Methylocella palustris* K^T and *Methylocapsa acidiphila* B2^T (Table 1). The distinguishing

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features of the phospholipid fatty acid profile of strain BL2^T compared with *Methylocella palustris* K^T were the presence of the i17 : 0, i18 : 0 and 19 : 0 fatty acids and a lower total content of C_{16} fatty acids.

Metabolic pattern

Cell suspensions of strain BL2^T grown under CH_4 oxidized ^{14}C-methane at 20 nmol min^{-1} (mg protein)$^{-1}$, regardless of the copper content of the medium. Cells grown in medium without added copper or with a low Cu^{2+} concentration (0·3 μM) oxidized naphthalene at the same rate, implying that sMMO was responsible for methane oxidation. A twofold lower naphthalene-oxidation rate [10 nmol min^{-1} (mg protein)$^{-1}$] was observed in cells grown in copper excess (2·5 μM). However, this occurred without a decrease in total MMO activity (measured with $^{14}CH_4$). These data may imply the presence of pMMO in this organism.

Cell-free extracts of strain BL2^T displayed activities of serine-pathway-specific enzymes (hydroxypyruvate reductase and serine-glyoxylate aminotransferase) but not of the key RuMP-cycle enzyme hexulosephosphate synthase or of key enzymes of the ribulose 1,5-bisphosphate cycle (ribulose bisphosphate carboxylase/oxygenase and phosphoribulokinase) (Table 2). Activity of 2-oxoglutarate dehydrogenase was also detected, indicating that the complete tricarboxylic acid cycle operates in this methanotroph. However, glyoxylate-cycle enzymes were absent. Pyrophosphate-dependent 6-phosphofructokinase and fructose-1,6-bisphosphate aldolase were present. Also, high activities of the pentose-phosphate-cycle enzymes (glucose-6-phosphate and 6-phosphogluconate dehydrogenases) were measured in cell extracts. In general, the metabolic pattern of BL2^T is similar to those of *Methylocella palustris* and *Methylocapsa acidiphila* (Dedysh *et al.*, 2000, 2002).

Table 1. Cellular fatty acid compositions of isolate BL2^T and other type II methanotrophs

Taxa/strains: 1, *Methylocella silvestris* sp. nov. BL2^T; 2, *Methylocella palustris* K^T (data from Dedysh *et al.*, 2002); 3, *Methylocapsa acidiphila* B2^T (Dedysh *et al.*, 2002); 4, *Methylosinus* and *Methylocystis* spp. (Bowman *et al.*, 1993). a, Anteiso; br, branched; cy, cyclo; i, iso.

Fatty acid	1	2	3	4
i15 : 0	1·2	0·2	0·1	0–0·9
a15 : 0	0	0	0	0–3·5
15 : 0	0	0·1	0	0–0·7
i16 : 0	0	0	0	0–1·4
16 : 1ω7*c*	8·8	6·8	4·7	0·3–14·2
16 : 1ω7*t*	0	5·8	0	0
16 : 1ω6*c*	0	0	0	0–0·3
16 : 1ω5*c*	0	0·1	0·1	0–0·2
16 : 0	3·0	5·9	7·3	0·7–5·1
i17 : 1ω7*c*	0	0·1	0	0
i17 : 0	2·5	0	0·6	0–0·3
a17 : 0	0	0·3	0	0–0·6
17 : 1ω8*c*	0	0·3	0	0–0·2
17 : 1ω7*c*	0	0	1·0	0–0·7
17 : 1ω6*c*	0	0·1	0	0–0·1
17 : 0	0	0·1	0·1	0–0·4
i18 : 0	0·5	0	0	0
cy18 : 0	0	0	0	0–6·5
18 : 1ω8*c*	0	0	0	52·9–73·6
18 : 1ω7*c*	82·2	78·6	78·3	14·8–37·7
18 : 1ω7*t*	0	0	0	0–4·6
18 : 1ω5*c*	0	0·1	0	0–0·2
18 : 0	1·2	0·9	7·6	0–5·0
br19 : 1	0	0	0	0–0·2
cy19 : 0	0	0	0	0–0·4
19 : 0	0·6	0	0	0

Phylogenetic analysis and DNA–DNA hybridization

A phylogenetic tree constructed on the basis of nearly complete (at least 1350 bases) 16S rRNA gene sequences (Fig. 2) indicated that strains BL2^T and A1 belong to the α-subclass of the *Proteobacteria*. The closest neighbours are *Methylocella palustris* K^T (2·7 % sequence difference), *B. indica* subsp. *indica* ATCC 9039^T (2·9 %) and *Methylocapsa acidiphila* B2^T (3·8 %). The DNA–DNA hybridization value of strain BL2^T with its closest phylogenetic neighbour (*Methylocella palustris* K^T) was therefore determined and was found to be only 21–22 %. DNA–DNA hybridization was not carried out with *B. indica* because this bacterium is a non-methanotrophic heterotroph. This critical phenotypic difference alone is sufficient to rule out the inclusion of the isolates in the genus *Beijerinckia*.

A phylogenetic analysis based on partial *mmoX* sequences also indicated that, among sMMO-containing methanotrophs, the closest neighbour of strains BL2^T and A1 was *Methylocella palustris* K^T (10·3–10·5 % nucleotide sequence difference, 3·3–3·4 % derived amino acid sequence difference), followed by other type II methanotrophs of the genera *Methylosinus* and *Methylocystis* (Fig. 3). The partial *mxaF* nucleotide sequences of strains BL2^T and A1 differed from that of *Methylocella palustris* K^T by 18·8–20·5 % (14·9–15·9 % derived amino acid sequence difference) and it was not evident from *mxaF* sequence-based phylogeny that they were closer phylogenetically to *Methylocella palustris* than to other methylotrophic bacteria (data not shown).

A *pmoA* product (the active-site polypeptide of pMMO) could not be amplified by PCR with any of the following three primer sets for this gene: A189/A682 (Holmes *et al.*, 1995), A189/mb661r (Costello & Lidstrom, 1999) and A189/A648b (Dunfield *et al.*, 2002).

DISCUSSION

Until now, the methanotrophic genus *Methylocella* has been represented by a single species isolated from peat bogs in

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Table 2. Activities of enzymes of primary and intermediate metabolism in cell extracts of isolate BL2^T

PMS, Phenazine methosulfate; PPi, pyrophosphate.

Enzyme	Co-factor	Activity [nmol min^{-1} (mg protein)$^{-1}$]
Methane monooxygenase (soluble)	–	20
Methanol dehydrogenase	PMS	137
Formaldehyde dehydrogenase	PMS	4
Formate dehydrogenase	NAD	10
	PMS	22
Ribulose-1,5-bisphosphate carboxylase	–	0
Hexulosephosphate synthase	–	0
Phosphoribulokinase	ATP	0
Hydroxypyruvate reductase	NADH	64
	NADPH	0
Serine-glyoxylate aminotransferase	–	28
6-Phosphofructokinase	PPi	27
	ATP	0
Fructose-1,6-bisphosphate aldolase	–	22
Glucose-6-phosphate dehydrogenase	NAD	133
	NADP	16
6-Phosphogluconate dehydrogenase	NAD	113
	NADP	15
2-Keto-3-deoxy-6-phosphogluconate aldolase	–	0
Isocitrate dehydrogenase	NAD	0
	NADP	8
2-Oxoglutarate dehydrogenase	NAD	12
Glutamate dehydrogenase	NADH	8
Malate synthase	–	0
Isocitrate lyase	–	0

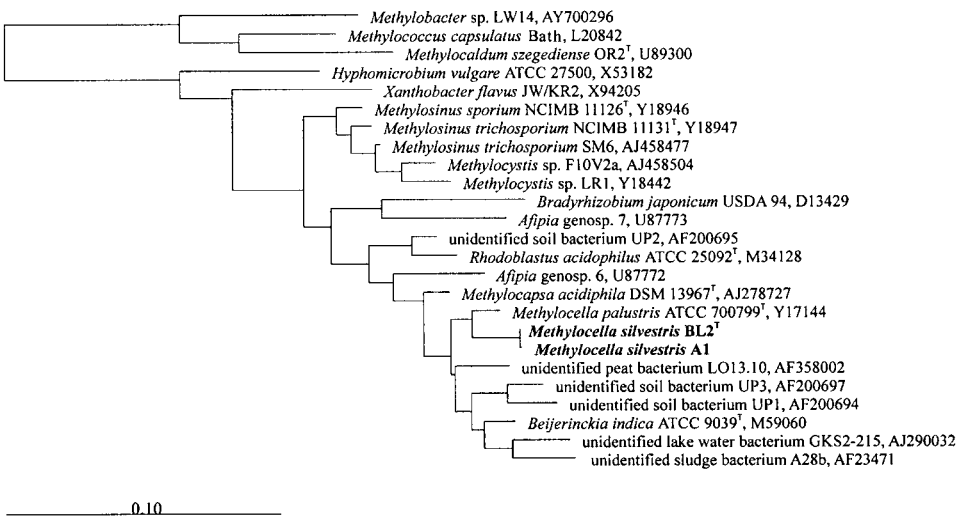

Fig. 2. Maximum-likelihood phylogenetic tree based on nearly complete 16S rRNA gene sequences, showing the positions of strains BL2^T and A1 relative to selected *α-Proteobacteria* and relative to gene sequences retrieved in cultivation-independent studies. The type I methanotrophs *Methylococcus capsulatus* Bath, *Methylobacter* sp. LW14 and *Methylocaldum szegediense* OR2^T were used as an outgroup. Bar, 0·1 changes per nucleotide position.

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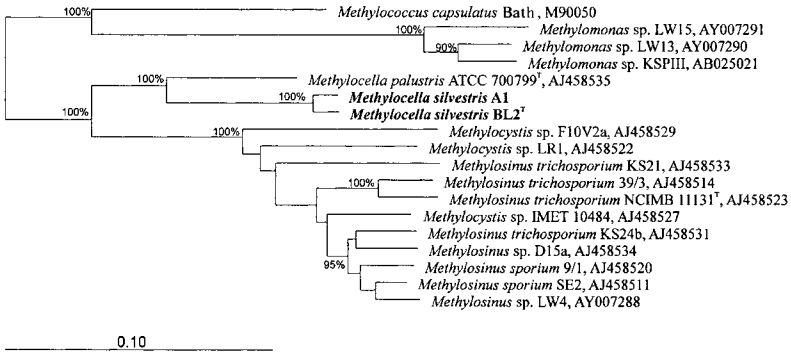

Fig. 3. Neighbour-joining tree (Jukes–Cantor correction) constructed using partial *mmoX* nucleotide sequences, showing the positions of strains A1 and BL2^T relative to other type I and II methanotrophs. Bootstrap values (100 data resamplings) >90 % are shown. Bar, 0·1 changes per nucleotide position.

western Siberia and northern Russia (Dedysh *et al.*, 1998, 2000). The isolation of *Methylocella* strains BL2^T and A1 from a forest soil extends the known phylogenetic and ecological diversity of this genus. The forest strains were phenotypically and phylogenetically most similar to *Methylocella palustris* among known bacteria. However, minor phenotypic differences were evident between *Methylocella palustris* K^T and strain BL2^T, sequence-based phylogenetic identities were low compared with identities among other closely related methanotrophic genera and the DNA–DNA hybridization was only 21–22 %, indicating that the forest strains should be considered as representing a novel species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

Bacteria of the genus *Methylocella* lack an extensive ICM system and appear to consume methane primarily via sMMO instead of the pMMO that is associated with the ICM systems of other methanotrophs. The sMMO activity in strain BL2^T was only partially decreased by a level of copper (2·5 μM) that completely inhibited sMMO activity in *Methylosinus trichosporium* OB3b^T (Morton *et al.*, 2000). It is reasonable that inhibition or repression of sMMO should not occur in *Methylocella* strains if they do not contain pMMO as an alternative to sMMO. However, we cannot rule out the presence of pMMO in strain BL2^T. In $^{14}CH_4$ experiments, MMO activity remained constant under conditions that lowered sMMO activity by 50 %.

In contrast to alkaliphilic species, which are represented mostly by type I methanotrophs and grow well at high external salinities (Trotsenko & Khmelenina, 2002), acidophilic methanotrophs grow only in media with a low dissolved salt content. This interesting feature deserves further study, which may lead to a better understanding of the main principles of adaptation and survival of acidophilic and alkaliphilic methanotrophs.

The cambisol from which strains BL2^T and A1 were obtained has a pH of 3·8–4·3. This is typical of most forested soils of the temperate zone, so it is possible that members of the genus *Methylocella* are widespread geographically, in terrestrial soil as well as in wetlands. The methanotroph community of this cambisol has been characterized using cultivation-independent molecular methods (Henckel *et al.*, 2000). This study failed to detect methanotrophs related to *Methylocella* species. However, the primer systems used targeted only (i) the *pmoA* gene, which is not detectable in strain BL2^T, and (ii) the 16S rRNA gene, from which only the most abundant members of the entire bacterial community are expected to be retrieved. Cultivation of *Methylocella* strains adds to the known diversity of the methanotroph community in this forest soil; it also demonstrates the method-inherent limitations of cultivation-independent techniques in the exhaustive characterization of a microbial community. Cultivation-independent analysis did identify a *pmoA* sequence cluster somewhat related to the *pmoA* of *Methylocapsa acidiphila* B2^T (Henckel *et al.*, 2000), leading to the hypothesis that more, as yet uncultivated, acidophilic methanotrophs exist.

Forest soils periodically produce methane in anaerobic microsites, especially when water-saturated (e.g. Yavitt *et al.*, 1990; Wang & Bettany, 1997; Andersen *et al.*, 1998), and are therefore a potential habitat for methanotrophic bacteria. These soils also consume methane from the atmosphere, but it is unlikely that methanotrophs possessing only sMMO are responsible for this activity. The pMMO form of the enzyme has a higher affinity for methane than does sMMO (Hanson & Hanson, 1996) and is a more probable candidate for the consumption of the trace (1·7 p.p.m.v.) level of CH_4 in the atmosphere. When batch cultures of strain BL2^T were cultivated in closed flasks, the threshold CH_4 concentration below which no more consumption occurred was always >100 p.p.m.v. (data not shown). On the basis of the growth conditions used, it is therefore unlikely that strains BL2^T and A1 are capable of surviving on, or even of transiently consuming, atmospheric methane. We propose that these strains live on methanol or on methane produced in anaerobic soil microsites.

Emended description of *Methylocella* Dedysh *et al.* 2000

Methylocella (Me.thy.lo.cel′la. N.L. n. *methylum* the methyl group; L. n. *cella* a chamber, a cell; N.L. n. *Methylocella* methyl-using cell).

Cells are Gram-negative, polymorphic, straight or curved short rods, 0·6–1·0 μm wide by 1·0–2·5 μm long, with rounded ends. Produce large, highly refractile, intracellular poly-β-hydroxybutyrate granules, one at each pole. Reproduce by normal cell division. Cells occur singly or in

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irregularly shaped aggregates, but do not form rosettes. Non-motile. Encapsulated. Resting cells (if they occur) are exospores. Cells are not lysed by 2 % SDS. The ICM system is different from those of both types I and II methanotrophic bacteria. Cells contain an extensive periplasmic space and a vesicular membrane system connected to the cytoplasmic membrane. Possess sMMO. The temperature range for growth is 4–30 °C with an optimum at 15–25 °C; no growth occurs at 37 °C. Growth occurs at pH 4·5–7·0. Highly sensitive to salt stress: prefer diluted media with a low salt content. Utilize C_1 compounds via the serine pathway. Do not contain the enzymes of the RuMP and ribulose 1,5-bisphosphate pathways. The tricarboxylic acid cycle is complete. Fix atmospheric nitrogen via an oxygen-sensitive nitrogenase. The major phospholipid fatty acids are 18 : 1 acids. The G+C content of the DNA is 60–61 mol%. Phylogenetically related to, but clearly distinct from, the type II methanotroph genera *Methylocystis* and *Methylosinus* in the α-subclass of the *Proteobacteria*; more closely affiliated with the acidophilic methanotrophic bacterium *Methylocapsa acidiphila* and the acidophilic heterotrophic bacterium *B. indica*. Distinguished from *B. indica* by the ability to consume methane. Habitats are acidic wetlands, particularly *Sphagnum* peat bogs, and acidic soils. The type species is *Methylocella palustris*.

Description of *Methylocella silvestris* sp. nov.

Methylocella silvestris (sil.ves′tris. L. adj. *silvestris* of the forest).

The description is as for the genus, with the following additional traits. Optimal growth occurs at 15–25 °C and at pH 5·5. Capable of slow growth at 4 °C. Carbon sources used include methane, methanol and methylamines. Utilizes methanol in a wide concentration range, from 0·01 to 5 % (v/v). NaCl inhibits growth at concentrations above 0·8 % (w/v). The type strain is strain BL2^T (=DSM 15510^T= NCIMB 13906^T), which was isolated from an acidic cambisol under a beech-dominated forest near Marburg, Germany. The species also includes strain A1.

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REFERENCES

Andersen, B. L., Bidoglio, G., Leip, A. & Rembges, D. (1998). A new method to study simultaneous methane oxidation and methane production in soils. *Global Biogeochem Cycles* **12**, 587–594.

Auman, A. J., Stolyar, S., Costello, A. M. & Lidstrom, M. E. (2000). Molecular characterization of methanotrophic isolates from freshwater lake sediment. *Appl Environ Microbiol* **66**, 5259–5266.

Auman, A. J., Speake, C. C. & Lidstrom, M. E. (2001). *nifH* sequences and nitrogen fixation in type I and type II methanotrophs. *Appl Environ Microbiol* **67**, 4009–4016.

Becking, J.-H. (1984). Genus *Beijerinckia* Derx 1950, 145^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 311–321. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Bowman, J. P., Sly, L. I., Nichols, P. D. & Hayward, A. C. (1993). Revised taxonomy of the methanotrophs: description of *Methylobacter* gen. nov., emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group I methanotrophs. *Int J Syst Bacteriol* **43**, 735–753.

Costello, A. M. & Lidstrom, M. E. (1999). Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. *Appl Environ Microbiol* **65**, 5066–5074.

Dedysh, S. N., Panikov, N. S., Liesack, W., Großkopf, R., Zhou, J. & Tiedje, J. M. (1998). Isolation of acidophilic methane-oxidizing bacteria from northern peat wetlands. *Science* **282**, 281–284.

Dedysh, S. N., Liesack, W., Khmelenina, V. N., Suzina, N. E., Trotsenko, Y. A., Semrau, J. D., Bares, A. M., Panikov, N. S. & Tiedje, J. M. (2000). *Methylocella palustris* gen. nov., sp. nov., a new methane-oxidizing acidophilic bacterium from peat bogs, representing a novel subtype of serine-pathway methanotrophs. *Int J Syst Evol Microbiol* **50**, 955–969.

Dedysh, S. N., Khmelenina, V. N., Suzina, N. E., Trotsenko, Y. A., Semrau, J. D., Liesack, W. & Tiedje, J. M. (2002). *Methylocapsa acidiphila* gen. nov., sp. nov., a novel methane-oxidizing and dinitrogen-fixing acidophilic bacterium from *Sphagnum* bog. *Int J Syst Evol Microbiol* **52**, 251–261.

Dunfield, P. F., Tchawa Yimga, M., Dedysh, S. N., Berger, U., Liesack, W. & Heyer, J. (2002). Isolation of a *Methylocystis* strain containing a novel *pmoA*-like gene. *FEMS Microbiol Ecol* **41**, 17–26.

Gerhardt, P. (1981). *Manual of Methods for General Bacteriology*. Washington, DC: American Society for Microbiology.

Groth, I., Schumann, P., Weiss, N., Martin, K. & Rainey, F. A. (1996). *Agrococcus jenensis* gen. nov., sp. nov., a new genus of actinomycetes with diaminobutyric acid in the cell wall. *Int J Syst Bacteriol* **46**, 234–239.

Hanson, R. S. & Hanson, T. E. (1996). Methanotrophic bacteria. *Microbiol Rev* **60**, 439–471.

Hanson, R. S., Netrusov, A. I. & Tsuji, K. (1991). The obligate methanotrophic bacteria *Methylococcus*, *Methylomonas*, and *Methylosinus*. In *The Prokaryotes*, pp. 2350–2365. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.

Henckel, T., Friedrich, M. & Conrad, R. (1999). Molecular analyses of the methane-oxidizing microbial community in rice field soil by targeting the genes of the 16S rRNA, particulate methane monooxygenase, and methanol dehydrogenase. *Appl Environ Microbiol* **65**, 1980–1990.

Henckel, T., Jäckel, U., Schnell, S. & Conrad, R. (2000). Molecular analyses of novel methanotrophic communities in forest soil that oxidize atmospheric methane. *Appl Environ Microbiol* **66**, 1801–1808.

Holmes, A. J., Costello, A., Lidstrom, M. E. & Murrell, J. C. (1995). Evidence that particulate methane monooxygenase and ammonium monooxygenase may be evolutionarily related. *FEMS Microbiol Lett* **132**, 203–208.

Kämpfer, P. & Kroppenstedt, R. M. (1996). Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* **42**, 989–1005.

Luft, J. H. (1964). Electron microscopy of cell extraneous coats as revealed by ruthenium red staining. *J Cell Biol* **23**, 54A–55A.

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Martin, K., Schumann, P., Rainey, F. A., Schuetze, B. & Groth, I. (1997). *Janibacter limosus* gen. nov., sp. nov., a new actinomycete with *meso*-diaminopimelic acid in the cell wall. *Int J Syst Bacteriol* **47**, 529–534.

Morton, J. D., Hayes, K. F. & Semrau, J. D. (2000). Effect of copper speciation on whole-cell soluble methane monooxygenase activity in *Methylosinus trichosporium* OB3b. *Appl Environ Microbiol* **66**, 1730–1733.

Murrell, J. C., McDonald, I. R. & Bourne, D. G. (1998). Molecular methods for the study of methanotroph ecology. *FEMS Microbiol Ecol* **27**, 103–114.

Phelps, P. A., Agarwal, S. K., Speitel, G. E., Jr & Georgeou, E. (1992). *Methylosinus trichosporium* OB3b mutants having constitutive expression of soluble methane monooxygenase in the presence of high levels of copper. *Appl Environ Microbiol* **58**, 3701–3708.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Strunk, O. & Ludwig, W. (1996). ARB: a software environment for sequence data. Munich: Technische Universität München. http://www.arb-home.de/

Takeda, K. (1988). Characteristics of a nitrogen-fixing methanotroph, *Methylocystis* T-1. *Antonie van Leeuwenhoek* **54**, 521–534.

Trotsenko, Y. A. & Khmelenina, V. N. (2002). Biology of extremophilic and extremotolerant methanotrophs. *Arch Microbiol* **177**, 123–131.

Wang, F. L. & Bettany, J. R. (1997). Methane emission from Canadian prairie and forest soils under short-term flooding conditions. *Nutr Cycl Agroecosyst* **49**, 197–202.

Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Yavitt, J. B., Downey, D. M., Lang, G. E. & Sexstone, A. J. (1990). Methane consumption in two temperate forest soils. *Biogeochemistry* **9**, 39–52.

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