***Methylocella palustris* gen. nov., sp. nov., a new methane-oxidizing acidophilic bacterium from peat bogs, representing a novel subtype of serine-pathway methanotrophs**

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A new genus, *Methylocella*, and a new species, *Methylocella palustris*, are proposed for three strains of methane-oxidizing bacteria isolated from acidic *Sphagnum* peat bogs. These bacteria are aerobic, Gram-negative, colourless, non-motile, straight and curved rods that utilize the serine pathway for carbon assimilation, multiply by normal cell division and contain intracellular poly- β -hydroxybutyrate granules (one at each pole). These strains use methane and methanol as sole sources of carbon and energy and are moderately acidophilic organisms with growth between pH 4.5 and pH 7.0, the optimum being at pH 5.0–5.5. The temperature range for growth is 10–28 °C with the optimum at 15–20 °C. The intracytoplasmic membrane system is different from those of type I and II methanotrophs. Cells contain an extensive periplasmic space and a vesicular membrane system connected to the cytoplasmic membrane. The strains grew only on media with a low salt content (0.2–0.5 g l⁻¹). All three strains were found to possess soluble methane monooxygenase and are able to fix atmospheric nitrogen via an oxygen-sensitive nitrogenase. No products were observed in a PCR with particulate methane monooxygenase-targeted primers; hybridization with a *pmoA* probe was also negative. The major phospholipid fatty acids are 18:1 acids. The G+C content of the DNA is 61.2 mol%. The three strains share identical 16S rRNA gene sequences and represent a novel lineage of methane-oxidizing bacteria within the α -subclass of the class *Proteobacteria* and are only moderately related to type II methanotrophs of the *Methylocystis*–*Methylosinus* group. The three strains are most closely related to the acidophilic heterotrophic bacterium *Beijerinckia indica* subsp. *indica* (96.5% 16S rDNA sequence similarity). Collectively, these strains comprise a new species and genus *Methylocella palustris* gen. nov., sp. nov.; strain K^T (= ATCC 700799^T) is the type strain.

Keywords: *Methylocella palustris* gen. nov., sp. nov., acidophilic methane-oxidizing bacteria, serine-pathway methanotrophs

INTRODUCTION

Methane-oxidizing bacteria (MOB) are a ubiquitous group of micro-organisms possessing the unique ability to utilize methane as the sole source of carbon

Abbreviations: FAME, fatty acid methyl ester; ICM, intracytoplasmic membrane; MOB, methane-oxidizing bacteria; pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase.

The EMBL/GenBank/DBJ accession number for the 16S rRNA gene of *Methylocella palustris* strain K^T is Y17144.

and energy. The current classification separates all the known methanotrophs into three groups (types I, II and X) by multiple criteria, including cell morphology, the arrangement of intracytoplasmic membranes, the pathway for formaldehyde assimilation, the DNA G + C content and the major phospholipid fatty acids. Both 5S rRNA and 16S rRNA sequence analyses indicated that all known MOB form phylogenetically coherent clusters within the α - and γ -subclasses of the *Proteobacteria* (Hanson & Hanson, 1996).

Although MOB have been isolated from diverse

environments including soils, sediments, wetlands, freshwater and marine habitats (Whittenbury *et al.*, 1970a; Hanson & Hanson, 1996; King, 1992), the known ecophysiological types of methanotrophs are quite limited. Most MOB available in pure cultures are both mesophilic and neutrophilic; however, thermophilic (Bodrossy *et al.*, 1997), psychrophilic (Omelchenko *et al.*, 1993; Bowman *et al.*, 1997) and alkaliphilic (Khmelenina *et al.*, 1997) methanotrophs have been described.

Until recently, MOB that colonize acidic environments remained unknown and pure cultures of acidophilic methanotrophs were absent from culture collections. Last year, however, we reported on the enrichment of methanotrophic communities from acidic peat bogs of four boreal sites in West Siberia and European North Russia (Dedys *et al.*, 1998a) and on the isolation of three pure cultures of MOB from these enrichments (Dedys *et al.*, 1998b). These isolates are the first representatives of a new group of previously unculturable MOB and initial studies have revealed them to be considerably different from other methanotrophs. The 16S rRNA gene sequence data indicate that the acidophilic strains represent a novel lineage of MOB only moderately related to the known cluster of α -proteobacterial methanotrophs, i.e. *Methylosinus*–*Methylocystis* spp. (Dedys *et al.*, 1998b). Furthermore, the acidophilic strains are most closely affiliated with the heterotrophic bacterium *Beijerinckia indica* subsp. *indica*, while no other known group of methanotrophs has any close relatives that are not methanotrophs (Hanson & Hanson, 1996). The nucleotide sequence of the PCR-amplified fragment of the *mmoX* gene [which encodes the α -subunit of the soluble methane monooxygenase (sMMO) hydroxylase] in these acidophilic methanotrophs diverges significantly from these gene fragments in known methanotrophs and forms a branch that is distinct from the two known *mmoX* sequence groups [the *Methylocystis*–*Methylosinus*-like *mmoX* sequences and the *Methylococcus capsulatus* (Bath) *mmoX* sequence].

In this paper we describe the cell ultrastructure and physiology of these three methanotrophic isolates from peat bogs, as well as their biochemical, chemotaxonomic and genotypic characteristics. We propose a new genus, *Methylocella* gen. nov., and the species *Methylocella palustris* sp. nov. for these strains.

METHODS

Bacterial strains and culture conditions. Strains K^T, S6 and M131 were isolated from methanotrophic enrichments of three different *Sphagnum* peat bogs (Dedys *et al.*, 1998a). The isolation procedure and the culture conditions used were described elsewhere (Dedys *et al.*, 1998b). The fivefold dilution of minimal mineral medium M1 (Dedys *et al.*, 1998a), designated as medium M2 was used for strain cultivation. Solid M2 medium was prepared by adding 1.5% (w/v) purified agar (Difco). After sterilization, the medium was adjusted to pH 5.0–5.5 with 0.1 M H₃PO₄. The isolates were maintained both on M2-agar medium slants and in

liquid cultures. In the latter case, M2 medium was supplemented with 0.5% (v/v) of a vitamin stock solution containing the following (mg per 100 ml): biotin (0.4); nicotinic acid (10); riboflavin (10); and inositol (10). To study the ultrastructure of cells grown in Cu-rich medium, copper was added aseptically as CuCl₂ · 2H₂O to M2 medium after autoclaving to achieve a copper concentration of 5 μ M. For growth, screw-cap 500 ml serum bottles were used with a headspace:liquid-space ratio of 4:1. After inoculation, the bottles were sealed with silicone rubber septa and methane was added aseptically by a syringe equipped with a disposable filter (0.22 μ m) to achieve a 15–20% concentration in the headspace. Bottles were incubated on a rotary shaker (200 r.p.m.) at 20 °C. Methanotrophic bacteria were subcultured at 4–5-week intervals.

Reference bacterial strains. *Methylococcus capsulatus* (Bath) ATCC 33009^T, *Methylosinus sporium* NCIMB 11126^T, *Methylosinus trichosporium* OB3b NCIMB 11131^T, *Methylocystis parvus* OBBP NCIMB 11129^T, *Methylomicrobium album* BG8 ATCC 33003, *Beijerinckia indica* subsp. *indica* ATCC 9039^T and *Rhodopseudomonas acidophila* ATCC 25092^T were used as reference strains. The methanotrophs were cultivated on NMS medium with methane as the growth substrate (Whittenbury *et al.*, 1970a), while *Beijerinckia indica* was grown on nitrogen-free mineral medium with glucose (Becking, 1984) and *Rhodopseudomonas acidophila* was grown in the light under anaerobic conditions on succinate/mineral medium (Pfennig, 1969).

Verification of strain purity. Both phase-contrast and electron microscopy were used to check the acidophilic isolates for contamination. The absence of heterotrophic satellites was checked by plating methanotrophic isolates on several media containing different organic substrates. These media were as follows: standard undiluted and 10-fold-diluted Luria–Bertani agar (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl) and M2-agar media (Noble agar) amended with 0.1% (w/v) glucose, fructose or sucrose. As a control, the same agar mineral medium was used without any organic substrates. To prove the homogeneity of isolated methanotrophic strains, the cells were grown on methane and methanol with subsequent RFLP analysis of the cloned 16S rRNA genes to examine possible shifts in population structure. For RFLP analysis, a single colony of every strain was used to inoculate a liquid culture. This culture was grown on methane to the mid-exponential phase. Then cells were collected by centrifugation and divided into two parts, one of which was again grown in liquid culture with methane, while the other sample was transferred to M2 medium containing 0.3% (w/v) methanol. After 5 d incubation, the cells were harvested, genomic DNA was extracted from both cultures by using an SDS-based method (see Methods). PCR-mediated amplification of 16S rRNA genes was performed with an oligonucleotide primer set useful for most members of the domain *Bacteria* (Weisburg *et al.*, 1991). The PCR-amplified 16S rRNA gene products were quantified and ligated into the pCR II vector provided with the TA cloning kit (Invitrogen). Screening of recombinant clones was performed by direct amplification of the cloned inserts using vector-specific primers (Zhou *et al.*, 1997). The constructed 16S rDNA clone library was then reamplified and restriction digested by two sets of tetrameric endonucleases, i.e. *MspI* + *RsaI* and *HhaI* + *HaeIII* (Gibco–BRL Life Technologies). The resulting restriction products were separated by gel electrophoresis in 3.5% (w/v) Metaphor agarose (FMS Bioproducts) stained with 0.5 μ g ethidium bromide ml⁻¹ in 1 × Tris borate/EDTA at 4 °C for 5 h.

Morphological observations. Cell size measurement and shape classification were performed by using the image-analysis system developed at the Center for Microbial Ecology at Michigan State University (Liu *et al.*, 1998). The Gram-stain reaction and lysis in 3% (w/v) KOH (Gerhardt, 1981) were carried out with both 2- and 4-week-old cultures. Capsules were stained with 2.5% (w/v) nigrosine and observed using oil-immersion phase-contrast microscopy. Poly- β -hydroxybutyrate inclusions were determined by staining with 0.03% (w/v) Sudan black and 0.5% safranin (Gerhardt, 1981). The observations of rosette formation, as well as checks for the presence of exospores or cysts, were carried out during all phases of growth on both liquid and agar media. Exospore formation was also assayed by determining cell viability after heating a 2-week-old cell suspension at 80 °C for 20 min and then observing micro-colony formation on M2 agar medium after 5 and 10 d incubation with methane. The same procedure was used to determine the proportion of viable cells after measuring cell resistance to desiccation (Whittenbury *et al.*, 1970b). Lysis by 0.2 and 2% (w/v) SDS was determined spectrophotometrically (OD_{410}) and by direct microscopic observation. Colony morphology and pigmentation were examined with 2, 4 and 6-week-old M2-agar-plate cultures.

Electron microscopy. Thin sections were examined using batch cultures grown to the early exponential, late-exponential and stationary growth phases. Cells were collected by centrifugation and pre-fixed with 1.5% (w/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 6.5) for 1 h at 4 °C and then fixed in 1% (w/v) OsO_4 in the same buffer for 4 h at 20 °C. After dehydration in an ethanol series, the samples were embedded in a Spurr epoxy resin. Thin sections were cut on an LKB-4800 microtome, stained with 3% (w/v) uranyl acetate in 70% (v/v) ethanol and then post-stained with lead citrate (Reynolds, 1963). The specimen samples were examined with a JEOL JEM 100B transmission electron microscope at an accelerating voltage of 80 kV.

Cell morphology was examined using batch cultures in the late-exponential growth phase. The procedure used for sample fixation and preparation was as described previously (Dedysh *et al.*, 1998a). Cells were examined with a JEOL JSM-6400V scanning electron microscope.

Physiological tests. Growth at various temperatures ranging from 4 to 45 °C as well as tolerance to NaCl at concentrations ranging from 0.01 to 1% (w/v) was determined with M2 agar cultures. Tolerance to dyes [0.001% (w/v) malachite green, 0.01% methylene blue and 0.001% crystal violet] was tested by incorporating dyes into M2 agar plates. Sensitivity to antibiotics was examined by spreading cells on to an M2 agar medium and applying filter discs containing the following antibiotics: erythromycin (15 μ g ml⁻¹), linkomycin (2 μ g ml⁻¹), gentamicin (10 μ g ml⁻¹), novobiocin (5 μ g ml⁻¹), nalidixic acid (30 μ g ml⁻¹) and streptomycin (10 μ g ml⁻¹). Growth under methane was assessed after 2 weeks. The ability to grow under microaerobic conditions was determined in growth experiments with 5% O_2 concentration in the headspace of incubation flasks. Nitrogen fixation was assayed on nitrogen-free M2 medium with batch cultures in the mid-exponential growth phase by using the acetylene-reduction procedure of Takeda (1988). Prior to acetylene injection, the incubation flasks were divided into two groups: one group of flasks was flushed with sterile air to remove any remaining methane, whereas the other flasks were purged with sterile dinitrogen to remove methane and oxygen, with oxygen subsequently being added by syringe to a 5% (v/v) concentration in the headspace.

Methanol was added to the flasks at a concentration of 0.01% (v/v). The amount of ethylene produced was measured after 15 h incubation on a rotary shaker (200 r.p.m.) at 20 °C.

Range of utilizable carbon and nitrogen sources. The following carbon sources (each at a concentration of 0.1%, w/v) were examined to determine the range of substrates that can be utilized for growth and energy by the acidophilic methanotrophic isolates: formate, formamide, urea, methylamine, dimethylamine, trimethylamine, tetramethylammonium chloride, glucose, fructose, sucrose, arabinose, lactose, xylose, maltose, raffinose, ribose, galactose, acetate, citrate, oxalate, mannitol and sorbitol. The capacity to utilize methanol at concentrations ranging from 0.01 to 0.5% (v/v) was determined by measuring the growth dynamics (OD_{410} and CO_2 production) of all three strains on M2 liquid medium with methanol. Nitrogen sources were also tested using M2 agar medium in which KNO_3 was replaced with one of the following compounds at a concentration of 0.05% (w/v): $NaNO_2$, $(NH_4)_2SO_4$, glycine, DL-alanine, L-serine, L-threonine, L-valine, L-leucine, L-isoleucine, L-proline, L-cysteine, L-methionine, L-glutamate, L-aspartate, L-glutamine, L-asparagine, L-histidine, L-phenylalanine, L-tryptophan, L-arginine, L-lysine, L-ornithine, L-citrulline or yeast extract. Growth was examined after 30 d incubation and confirmed by comparison with the negative control (grown on nitrogen-free M2 agar medium).

Analytical techniques. CH_4 and C_2H_4 were measured with a gas chromatograph equipped with a flame-ionization detector. CO_2 and O_2 were analysed by GC with a thermconductivity detector. All measurements were performed using triplicate samples. Cell biomass was measured by converting optical density at 410 nm to cell carbon, using the appropriate calibration curve.

Genomic bacterial DNA isolation. Chromosomal DNA from methanotrophic isolates and reference bacterial strains was extracted using the SDS-based assay as described previously (Dedysh *et al.*, 1998a).

Comparative sequence analysis of the 16S rRNA gene. The 16S rRNA gene sequences from *Methylosinus sporium* (NCIMB 11126^T), *Methylosinus trichosporium* OB3b (NCIMB 11131^T) and *Methylocystis parvus* ÖBBP (NCIMB 11129^T) were determined for reference purposes. PCR-mediated amplification of the 16S rRNA genes from positions 28–1491 (using numbering according to the International Union of Biochemistry nomenclature for *Escherichia coli* 16S rRNA) and sequencing were carried out as already described for the acidophilic strains K^T, M131 and S6 (Dedysh *et al.*, 1998b). The phylogenetic position of the three acidophilic methanotrophs was determined by comparing their 16S rRNA gene sequences with α -proteobacterial reference sequences for which at least 1400 determined nucleotide sequence positions were available. Distance matrix [ARB (Strunk *et al.*, 1996) and PHYLIP (Felsenstein, 1993)], maximum-likelihood [ARB and fastDNAMl (Maidak *et al.*, 1997)] and maximum-parsimony methods [ARB and PHYLIP (Felsenstein, 1993)] were used for tree reconstruction. Several trees were generated for each of these treeing methods: these trees differed in terms of (i) the α -proteobacterial reference sequences used and (ii) the set of alignment positions used for tree reconstruction. These sets were determined for a sequence stretch at *E. coli* positions 28–1477 and were based on a 50% invariance criterion (Friedrich *et al.*, 1996) by using the complete data set consisting of 424 reference sequences or by using subsets of

these sequences and the appropriate tool of the ARB program package. The statistical significance of interior branch points was tested in bootstrap analyses by using neighbour-joining methods (ARB; 1000 data resamplings).

PCR amplification of the *pmoA* gene and hybridization experiments. Two sets of primers were used to amplify specific fragments of the *pmoA* gene encoding the 27 kDa polypeptide of particulate methane monooxygenase (pMMO) from the chromosomal DNA of *Methylococcus capsulatus* (Bath), *Methylosinus trichosporium* OB3b, *Methylomicrobium album* BG8 and all three acidophilic isolates, strains K^T, S6 and M131. Primers A189 (5'-GGNGACTGGGACTTCTGG-3') and A682 (5'-GAASGCNGAGAAGAASGC-3') were used to amplify a 525 bp portion of *pmoA* (Holmes *et al.*, 1995) and primers A189 and mb661 (5'-CCGGMGCAACGTCYTTACC-3') were used to amplify a 510 bp fragment of *pmoA* (Costello & Lidstrom, 1999). Amplification was carried out on an Eppendorf Mastercycler using the reaction conditions described by MacDonald *et al.* (1995).

Genomic DNA was collected from *Methylococcus capsulatus* (Bath), *Methylosinus trichosporium* OB3b, *Methylomicrobium album* BG8 and strain K^T, using the SDS procedure described earlier (Dedysh *et al.*, 1998a). The chromosomal DNA was digested with restriction enzymes *Sal*I, *Pst*I, *Bam*HI, *Eco*RI and *Hind*III as recommended by the supplier (Boehringer Mannheim). The digested DNA was separated by using standard procedures on 1% (w/v) agarose Tris borate gels and then transferred to nylon membranes (Roche Molecular Biolabs) as outlined by Sambrook *et al.* (1989). Hybridizations were performed using the 510 bp PCR product of the *pmoA* gene of *Methylococcus capsulatus* (Bath), with primers A189 and mb661. This PCR product was labelled using digoxigenin-dUTP according to the instructions of the manufacturer (Boehringer Mannheim) and hybridization was performed overnight at 42 °C. The membranes were then washed twice in 2 × SSC (Sambrook *et al.*, 1989)/0.1% SDS at 60 and 62 °C for 15 min and then exposed to X-ray film.

Whole-cell fatty acid analysis. Methanotrophic isolates were grown on M2 mineral medium with methane (Dedysh *et al.*, 1998a) and *Beijerinckia indica* was grown on mineral medium with glucose (Becking, 1984). Cells were collected in the late-exponential growth phase. Saponification, methylation and extraction of fatty acids were performed by using the procedure described by Sasser (1990). Analysis of the fatty acid methyl esters (FAMES) was carried out using an HP 5890 series II gas chromatograph (Hewlett Packard) equipped with a flame-ionization detector and a 7673 automatic sampler. Samples were separated on an HP ultra2 (cross-linked 5% phenyl methyl silicone) column 25 m × 0.2 mm diameter with a film thickness of 0.33 µm. The carrier gas was ultrahigh purity hydrogen at 50 ml min⁻¹ with a split ratio of 50:1. An initial temperature of 170 °C was ramped to 270 °C at a rate of 5 °C min⁻¹. The column was baked after each run for 2 min at 300 °C. Injector and detector temperatures were held at 250 and 300 °C, respectively. Fatty acid profiles were quantified and identified using the Sherlock Microbial Identification system (MIDI).

Enzyme assays. For enzymic studies, the cultures were harvested in the late exponential or early stationary growth phase. Cells were sedimented by centrifugation (4000 g, 30 min), washed with 0.05 M K/Na-phosphate (pH 6.8) or Tris/HCl (pH 7.2) buffer, resuspended in the same buffer and disrupted in cooled 10 ml cuvettes using an MSE

ultrasonic disintegrator (150 W, 20 kHz, 4 × 30 s). Cell debris was removed by centrifugation at 6000 g for 30 min. The resulting supernatant was centrifuged again at 30000 g for 1 h for separation of the membrane fraction.

The activities of methanol dehydrogenase (EC 1.1.88.8), phenazine methosulfate-, NAD- and glutathione-dependent formaldehyde dehydrogenases (EC 1.2.1.1), formate dehydrogenase (EC 1.2.1.2), NADH-dehydrogenase, hexulosephosphate synthase, pyrophosphate-dependent 6-phosphofructokinase (EC 2.7.1.90), ATP-dependent 6-phosphofructokinase (EC 2.7.1.11), fructose-1,6-bisphosphate aldolase (EC 4.1.2.13), 6-phosphogluconate dehydratase/2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14), hydroxypyruvate reductase (EC 1.1.1.29), serine-glyoxylate aminotransferase (EC 2.6.1.45), ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39), phosphoribulokinase (EC 2.7.1.19) isocitrate lyase (EC 1.2.4.1), malate synthase, (EC 4.1.3.2), malate dehydrogenase (EC 1.1.1.37), isocitrate dehydrogenase (EC 1.1.1.42), pyruvate dehydrogenase (EC 1.2.4.1), α-ketoglutarate dehydrogenase (EC 1.2.4.2), glutamate dehydrogenase (EC 1.4.1.2), alanine dehydrogenase (EC 1.4.1.1), glutamate synthase (EC 2.6.1.53) and glutamine synthetase (EC 6.3.1.2) were assayed as described previously (Shishkina & Trotsenko, 1979, 1982). The sMMO activity was measured by using a colorimetric assay with tetrazotized *o*-dianisidine (Phelps *et al.*, 1992).

DNA base composition analysis. The DNA base composition was determined by using the thermal denaturation method with an SP1800 spectrophotometer (Uicam) at a heating rate of 0.5 °C min⁻¹. The G + C content of the DNA (mol %) was calculated using the equation of Owen *et al.* (1969): mol % G + C = $(T_m \times 2.08) - 106.4$. The DNA of *E. coli* K-12 was used as the standard.

Nucleotide sequence accession numbers. The nucleotide sequence of the 16S rRNA gene of *Methylocella palustris* strain K^T has already been deposited in the EMBL, GenBank and DDBJ nucleotide sequence databases under accession number Y17144 (Dedysh *et al.*, 1998b). The nucleotide sequences of the 16S rRNA genes of *Methylocystis parvus* NCIMB 11129^T, *Methylosinus sporium* NCIMB 11126^T and *Methylosinus trichosporium* NCIMB 11131^T, determined for reference purposes, have EMBL/GenBank/DDBJ nucleotide sequence databases accession numbers Y18945, Y18946 and Y18947, respectively. The accession numbers of the 16S rRNA gene sequences used as references for the reconstruction of the tree are as follows: *Ancylobacter aquaticus* ATCC 25396^T, M62790; *Azorhizobium caulinodans* ATCC 43989^T, X67221; *Azospirillum lipoferum* ATCC 29707^T, M59061; *Beijerinckia indica* ATCC 9039^T, M59060; *Blastochloris viridis* ATCC 19567^T, D25314; *Brucella suis* ATCC 23444^T, L26169; *Methylobacterium organophilum* ATCC 27886^T, D32226; *Rhodoplanes elegans* ATCC 51906^T, D25311; *Rhodopseudomonas acidophila* ATCC 25092^T, M34128; *Rhodopseudomonas palustris* ATCC 17001^T, D25312.

RESULTS

Colony morphology

Visible, but very small (about 0.1–0.3 mm), colonies were observed on M2 agar after at least 2 weeks incubation. After growth on plates for 6 weeks, the colonies were highly raised, had a tough slime con-

Fig. 1. (a) Six-week-old colonies of strain K^T on M2 agar medium; bar, 0.5 cm. (b) Phase-contrast micrograph of strain K^T grown on methane for 10 d, showing the typical bipolar appearance of the cells; bar, 10 μm. (c) Scanning electron micrograph of cells of strain S6.

sistency, were circular with an entire margin and a smooth surface and were 1–2 mm in diameter (Fig. 1a). Colonies 2–3 months old occasionally had a folded or slightly plicated surface. Initially, the colonies were semi-transparent or uniformly turbid, but became opaque white over time.

Cell morphology and ultrastructure

The cells of strains K^T, S6 and M131 were Gram-negative, non-motile, polymorphic, straight or curved rods with rounded ends occurring singly or in shapeless aggregates, but did not form rosettes during any growth stage (Fig. 1c). The cells were 0.6–1.0 μm wide by 1.0–2.5 μm long and reproduced by normal cell division. The major distinctive feature of the cells was their highly specific bipolar appearance, which was easily observable even under phase-contrast microscopy (Fig. 1b). This was due to large, highly refractile, intracellular granules of poly-β-hydroxybutyrate formed at each cell pole (Fig. 2a). When grown on agar media, cells produced capsules approximately 0.2–1 μm thick. Exospores resembling those of *Methylosinus* were observed in very low numbers in cultures more than 4 months old (Fig. 2d). The examination of a younger cultures did not reveal exospores and these cultures were not heat resistant. Although no cysts or cyst-like cells were observed regardless of cell age and culture conditions, the cells were found to be resistant to desiccation. After 7 and 30 d desiccation, 13 and 9% of the cells, respectively, were able to form microcolonies. The cells were not lysed by 2% SDS.

The ultrastructure of all three strains was examined throughout the various stages of growth. Interestingly, regardless of the growth phase, the intracytoplasmic membrane (ICM) structures of acidophilic isolates were different from those seen in type I and type II MOB (Fig. 2). When grown under normal oxygen tension, the cells possessed an extensive periplasmic space containing a heterogeneous matrix of lower electron density relative to that of cytoplasm (Fig. 2a, b). The periplasmic space on cross-sections was 20–80 nm in width. Also, small spherical vesicles (60–100 nm in diameter) bounded by three layered membranes were located on the periphery of the cytoplasm (Fig. 2c). The micrographs showed that these vesicles were formed by cytoplasmic membrane invaginations. The vesicular matrix had the same density as periplasmic matrix. In many cells of these isolates, however, the ICM system was either poorly developed or unobservable. The effect of different culture conditions on the cell ultrastructure was also examined in this study. Regardless of whether growth was oxygen-limited (5% O₂ in the headspace), the medium was supplemented with 5 μM copper, or methanol was provided as a carbon and energy source (0.03%, v/v), neither type I nor type II ICM structures were formed. When the strains were grown under oxygen-limiting conditions, approximately one-third of the cells were observed to have large inclusions with periodic macromolecular structure (Fig. 2e, f).

Purity criteria

Examinations using both phase-contrast- and electron microscopy failed to reveal any contamination of the methanotrophic isolates by other bacteria, as judged from cell morphology. No growth was observed on

Fig. 2. For legend see facing page.

any of the complex organic media tested, whether diluted or undiluted. All three strains, when tested on agar mineral media with individual sugars, exhibited

weak growth of the same low magnitude as that observed on control plates lacking any organic substrates. To further verify the purity, RFLPs between

Table 1. Cellular fatty acid compositions of the three acidophilic methanotrophic isolates, *Beijerinckia indica* subsp. *indica* and type II methylotrophic bacteria

The numbers of carbon atoms and unsaturated carbon bonds, respectively, are given here in the form 12:0 (for example). ND, Not detected; N, no data.

Strain	Percentage of total fatty acids						
	12:0	13:0	16:1*	16:0	17:0	18:1†	18:0
<i>Methylocella palustris</i> K ^T	ND	ND	18.20	8.80	ND	71.20	1.80
<i>Methylocella palustris</i> S6	ND	ND	18.59	8.62	ND	71.47	1.33
<i>Methylocella palustris</i> M131	ND	ND	13.66	7.47	ND	75.94	2.93
<i>Beijerinckia indica</i> subsp. <i>indica</i>	1.53	0.65	1.23	4.69	1.41	89.02	1.47
<i>Methylosinus</i> and <i>Methylocystis</i> spp.‡	N	N	0.3–1.5	0–1.5	ND	75–87	0.4–5
<i>Methylobacterium</i> spp.‡	N	N	0–6.4	3–6.4	0.2–0.4	81–85	6.9–8.6

* Mixture of $\omega 7c/\omega 7t$.

† Mixture of $\omega 7c/\omega 7t/\omega 8c/\omega 8t/\omega 9c$.

‡ Data are from Bowman *et al.* (1991) and Guckert *et al.* (1991).

individual 16S rRNA gene clones obtained from two batches of strain K^T grown on different substrates were examined. In total, 86 16S rDNA clones (46 clones were retrieved from the culture incubated with methane and 40 clones were from the culture grown on methanol) were obtained and the restriction patterns compared. No differences were observed between any of the clones investigated using two different sets of tetrameric endonucleases. From these results, it can be concluded that strain K^T is a homogeneous 16S rDNA-defined population and, thus, represents a pure culture.

Physiological characteristics

The strains K^T, S6 and M131 were capable of growth on methane and methanol as the sole carbon and energy sources. Methanol supported growth only when used in low concentrations (0.01–0.1%, v/v), while it inhibited growth at concentrations above 0.3% (v/v). Growth did not occur either on other C₁ substrates or on the range of multicarbon compounds tested. The growth factors were not strictly required, but growth was stimulated by the addition of vitamins (biotin, nicotinic acid, riboflavin and inositol). All three strains utilized ammonium salts, nitrates and yeast extract as nitrogen sources; they were also able to fix atmospheric nitrogen when grown in nitrogen-free media. The acetylene-reduction activity of strains in microaerobic conditions was found to range from 5.0 to 8.2 nmol

C₂H₄/(mg dry biomass × h), while under aerobic conditions it fell to 3.2–4.3 nmol C₂H₄/(mg dry biomass × h).

The strains grew in the pH range 4.5–7.0, the optimum being pH 5.0–5.5 (Dedysh *et al.*, 1998b). The temperature range for growth was 10–28 °C, with the optimum at approximately 20 °C; no growth occurred at 30 °C. All three strains grew well in microaerobic conditions; moreover, their specific growth rate was 10–20% higher under a reduced partial pressure of oxygen than during growth under high dissolved oxygen concentrations. The generation time was in the range 17–46 h.

Cell growth occurred only in media with a low salt content (up to 1–1.5 g salt l⁻¹), the optimum salt concentration being in the range 0.2–0.5 g l⁻¹. Growth was completely inhibited in the presence of NaCl at concentrations above 0.5% (w/v). All strains were resistant to erythromycin, linkomycin, gentamicin, novobiocin and nalidixic acid, but sensitive to streptomycin at 10 µg ml⁻¹. Strains tolerated 0.001% crystal violet and 0.01% methylene blue, while 0.001% malachite green inhibited growth.

Cellular fatty acid profiles

The major fatty acid components of these strains of acidophilic methanotrophs were 18:1 fatty acids

Fig. 2. Electron micrographs of ultrathin sections of vegetative cells of the acidophilic methanotrophs grown under different conditions. Cells grown under normal oxygen tension showed an extensive periplasmic space on the cell periphery (a, b), inclusions of poly- β -hydroxybutyrate and polyphosphate granules formed at each cell pole (a). Spherical vesicles were formed by cytoplasmic membrane invaginations on the cytoplasm periphery (c). (d) Exospore found in 1-year-old culture. Cells grown under oxygen-limiting conditions showed inclusions of supra-molecular organization: (e) longitudinal section; (f) cross-section. Bars, 0.5 µm.

Table 2. Activities of enzymes of primary and intermediate metabolism in cell extracts of acidophilic methanotrophs [nmol min⁻¹ (mg protein)⁻¹]

PMS, Phenyl methyl sulfonate.

Enzyme	Strain		
	S6	K ^T	M131
Methane monooxygenase (soluble)	73	83	53
Methanol dehydrogenase (PMS)	21	15	35
Formaldehyde dehydrogenase			
PMS	3	2	2
SH, NAD	0	0	0
Formate dehydrogenase			
PMS	133	311	235
NAD	112	232	207
NADH-dehydrogenase	207	230	137
Ribulose-1,5-bisphosphate carboxylase	0	0	0
Hexulosephosphate synthase	0	0	0
6-Phosphofructokinase			
ATP	0	0	0
PPi	37	88	110
Fructose-1,6-bisphosphate aldolase	80	186	243
Glucose-6-phosphate dehydrogenase			
NAD	25	30	7
NADP	8	18	6
6-Phosphogluconate dehydrogenase			
NAD	20	10	9
NADP	0	0	0
KDPG aldolase	0	0	0
Hydroxypyruvate reductase			
NADH	160	92	148
NADPH	187	122	207
Serine-glyoxylate aminotransferase	161	126	136
Pyruvate kinase	20	76	10
Pyruvate dehydrogenase (NAD)	0	0	0
Isocitrate dehydrogenase			
NAD	0	0	0
NADP	10	10	104
α-Ketoglutarate dehydrogenase (NAD)	15	33	12
Malate dehydrogenase (NADPH)	130	974	311
Glutamate dehydrogenase			
NADH	27	13	12
NADPH	0	0	0
Glutamate synthase (NADH)	10	19	8
Glutamine synthetase Mn ²⁺ , ATP	23	24	7

(Table 1). This is a characteristic feature of all known type II methylotrophic bacteria (Bowman *et al.*, 1991; Guckert *et al.*, 1991). Nevertheless, the fatty acid patterns of our isolates did not match the fatty acid patterns either of the serine-pathway methanotrophs or of the serine-pathway methylotrophic bacteria unable to use methane as a growth substrate. The most distinctive feature of the FAME patterns of strains K^T, S6 and M131 was the relatively large amount of 16:1 and 16:0 fatty acids (Table 1). Overall, the content of 16-carbon fatty acids in these peat-bog isolates was

21–27 mol%, whereas this value never exceeded 15 mol% for any of the known type II methylotrophs.

The fatty acid profile of *Beijerinckia indica* subsp. *indica* was more similar to those of members of the genus *Methylobacterium* than to the FAME profile of these acidophilic methanotrophs. The major fatty acids were also 18:1, but the total amount of acids with 16-carbon atoms was only 6 mol%. Finally, a number of minor fatty acids (12:0, 13:0 and 17:0) were present in *Beijerinckia indica* subsp. *indica*,

Fig. 3. Hybridization of the *pmoA* fragment to the chromosomal DNAs of reference methane-oxidizing bacteria strains and an acidophilic methanotroph. DNAs were digested with *Sall*, *PstI*, *BamHI*, *EcoRI* and *HindIII* and hybridized to the 510 bp PCR product of the *pmoA* gene of *Methylococcus capsulatus* (Bath) labelled with digoxigenin (DIG)-dUTP. The membrane was washed at 60 °C (a). Lanes: 1, DIG-labelled molecular mass standards; 2, *Methylobacterium album* BG8 *Sall* digest; 3, *Methylobacterium album* BG8 *PstI* digest; 4, *Methylococcus capsulatus* (Bath) *Sall* digest; 5, *Methylococcus capsulatus* (Bath) *PstI* digest; 6, *Methylosinus trichosporium* OB3b *Sall* digest; 7, *Methylosinus trichosporium* OB3b *PstI* digest; 8, strain K^T *Sall* digest; 9, strain K^T *PstI* digest; 10, *Pseudomonas putida* *PstI* digest. The membrane was washed at 62 °C (b). Lanes: 1, DIG-labelled molecular mass standards; 2, *Methylococcus capsulatus* (Bath) *BamHI* digest; 3, *Methylococcus capsulatus* (Bath) *EcoRI* digest; 4, *Methylococcus capsulatus* (Bath) *HindIII* digest; 5, *Methylococcus capsulatus* (Bath) *PstI* digest; 6, strain K^T *BamHI* digest; 7, strain K^T *EcoRI* digest; 8, strain K^T *HindIII* digest; 9, strain K^T *PstI* digest; 10, *Methylobacterium album* BG8 *HindIII* digest; 11, *Methylobacterium album* BG8 *PstI* digest; 12, *P. putida* *PstI* digest (10 µg DNA per lane).

whereas the acidophilic methanotrophs lacked all such fatty acids.

Enzymological analysis

The enzymic profiles given in Table 2 show that all three acidophilic isolates oxidize CH₄ to CO₂ by sequential action of sMMO and the appropriate dehydrogenases of methanol, formaldehyde and for-

mate. The isolates possessed the activities of the serine-pathway-specific enzymes (hydroxypyruvate reductase and serine-glyoxylate aminotransferase), whereas the activities of the key enzymes of the ribulose monophosphate pathway (hexulose phosphate synthase) or the Calvin-Benson-cycle enzymes (ribulose biphosphate carboxylase/oxygenase and phosphoribulokinase) were not detectable. The presence of the α -ketoglutarate dehydrogenase activity indicates that a complete tricarboxylic acid cycle exists in these methanotrophs. The acidophilic methanotrophs also have a relatively high activity of pyrophosphate-dependent 6-phosphofructokinase (PPi-PFK), which is common to all methanotrophic strains tested. The presence of glutamine synthetase and glutamate synthase indicated that the isolates utilize the glutamate cycle for ammonia assimilation.

PCR amplification of *pmo* genes and hybridization

Using the two primer sets described in the Methods, the expected PCR products were observed from *Methylococcus capsulatus* (Bath), *Methylosinus trichosporium* OB3b and *Methylobacterium album* BG8 (data are not shown). These strains were chosen as they represent the major phylogenetic categories of methanotrophs. No PCR products were obtained with template DNA extracted from strains K^T, M131 and S6, regardless of the primer set used. Such a finding suggests either that the *pmoA* gene does not exist in acidophilic strains or that it is sufficiently divergent to render the primers unsuitable for amplification of this gene in acidophilic methanotrophs.

To determine if the gene encoding the 27 kDa polypeptide of the pMMO was either present or absent from this acidophilic methanotroph, the 510 bp PCR product encoding part of the *pmoA* gene was hybridized to the chromosomal DNA of *Methylococcus capsulatus* (Bath), *Methylosinus trichosporium* OB3b, *Methylobacterium album* BG8 and strain K^T. Clear hybridization of the *pmoA* fragment was seen for the well-characterized methanotrophs, but only weak hybridization was found with the digests of strain K^T chromosomal DNA on the membrane washed at low stringency (60 °C) (Fig. 3). A weak hybridization of the same strength was observed with the digests of *Pseudomonas putida* DNA used as a negative control. Upon more stringent washing, the hybridization of the *pmoA* probe to strain K^T DNA was not seen. Such weak binding may indicate either non-specific hybridization or that this gene, if present, is very divergent from that of *Methylococcus capsulatus* (Bath).

Comparative 16S rDNA sequence analysis

Irrespective of the α -proteobacterial reference sequences, the set of alignment positions and the treeing method used for tree construction, strains K^T, M131 and S6 always grouped with the heterotrophic species *Beijerinckia indica* subsp. *indica* as the most

Fig. 4. 16S rDNA sequence-based dendrogram showing the phylogenetic position of strain K^T in relation to representative members of the α -subclass of the *Proteobacteria*. Evolutionary distances were calculated by the method of Jukes & Cantor (1969). The dendrogram was reconstructed using the treeing algorithm of De Soete (1983). The root was determined by using the 16S rDNA sequence of *Methylococcus capsulatus* ATCC 19069^T (accession no. X72770) as the outgroup reference. The numbers indicate the significance values for interior nodes as derived from a bootstrap test (1000 data resamplings). Bar, 5% estimated difference in nucleotide sequences.

closely related organism and were clearly distinct from the type II methanotrophs of the *Methylocystis*–*Methylosinus* group (Fig. 4). The level of 16S rDNA similarity between the acidophilic methanotrophs and *Beijerinckia indica* was 96.5%. Because our earlier detailed analysis of the 16S rDNA sequence data currently available for type II methanotrophs in public databases indicated a large amount of ambiguous nucleotide sequence positions, we sequenced the almost full-length 16S rRNA gene for the type strains of the type species of the genera *Methylocystis* and *Methylosinus*, i.e. *Methylocystis parvus* OBBP and *Methylosinus trichosporium* OB3b, as well as for the type strain of *Methylosinus sporium* (NCIMB 11126^T). The three strains formed a tight cluster, having 16S rDNA sequence similarity values between 97.5 and 98.0%. The level of 16S rDNA similarities between the three representatives of the *Methylocystis*–*Methylosinus* group and the novel acidophilic methanotrophs ranged from 93.3 to 93.8%.

DISCUSSION

The combination of phenotypic traits, metabolic pathways and cellular fatty acid composition, together with the results of comparative sequence analysis of 16S rRNA genes, enables the acidophilic isolates described here to be characterized as similar to (yet distinct from) the type II methanotrophs. The type II methanotrophs characterized to date comprise validated species in the genera *Methylosinus* and *Methylocystis*. Members of these two genera are very similar in their physiological, metabolic and chemo-

taxonomic features, although some differences have been reported in cell morphology, the types of resting cells formed (cysts or exospores) and motility (Bowman *et al.*, 1993; Hanson & Hanson, 1996). On the basis of their genomic properties, all known type II methanotrophs represent a very tight group. The results of analysis of 5S rRNA (Bulygina *et al.*, 1990) and 16S rRNA (Bratina *et al.*, 1992) sequences indicated that these methanotrophs form a distinct and tight cluster, within the α -subclass of the *Proteobacteria*, which is separate from the rest of the serine-pathway methylotrophs. Subsequent investigations of the functional genes encoding key enzymes in the methane-oxidation pathway of type II methanotrophs provided further proof of this cluster homogeneity. Examination of the sMMO gene cluster of *Methylocystis* sp. strain M (McDonald *et al.*, 1997) has revealed a high degree of sequence identity with the corresponding genes from *Methylosinus trichosporium* OB3b (Cardy *et al.*, 1991). Comparative sequence analysis of *pmoA*, which encodes the 27 kDa polypeptide of the pMMO, also placed all studied *Methylosinus* and *Methylocystis* representatives into one firm cluster (Semrau *et al.*, 1995; Murrell & Holmes, 1996; McDonald *et al.*, 1997). The same conclusions were drawn from sequence analysis of the methanol dehydrogenase gene *mxoF*, encoding the large subunit of the enzyme, of a number of representative methano- and methylotrophs (McDonald & Murrell, 1997).

Acidophilic isolates, although similar to type II methanotrophs, had significant differences that dis-

tinguished them from representatives of both the genus *Methylosinus* and the genus *Methylocystis*. The most distinctive morphological feature of these isolates was a highly specific bipolar cell appearance not previously observed in type II methanotrophs. Furthermore, in most cases resting cells were not formed by these isolates, while some exospores were found only in several-month-old cultures. The cell ultrastructure of acidophilic methanotrophs was also radically different from those of known methanotrophs. All known MOB, regardless of type classification, develop characteristic ICM structures; the type of membrane arrangement has been used as a key taxonomic criterion since the very first classification of MOB was proposed (Whittenbury *et al.*, 1970a; Davies & Whittenbury, 1970). According to this criterion methanotrophs were separated into those having stacks of vesicular discs in the cytoplasmic space (type I) and those with paired membranes aligned to the periphery of the cell (type II). The ICM content is dependent on the growth conditions and is more extensive under oxygen-limiting cultivation and in media with high copper concentrations (Scott *et al.*, 1981; Suzina *et al.*, 1985; Phelps *et al.*, 1992). The extent of growth is also important, with *Methylosinus trichosporium* OB3b shown to have a more developed ICM system during the mid-exponential, late-exponential and stationary growth phases (Best & Higgins, 1981), while ICM degeneration has been described for *Methylococcus capsulatus* (Texas) as these cells age (Hyder *et al.*, 1979). Furthermore, the ICM in type II methanotrophs has been shown to be less well ordered than that of type I methanotrophs and these cells can possess a poorly developed ICM array represented by membrane-bound vesicular structures on the cell periphery (Best & Higgins, 1981; Scott *et al.*, 1981; Suzina *et al.*, 1985; Phelps *et al.*, 1992). With these observations in mind, the acidophilic methanotrophs were examined at different stages of growth and under different culture conditions, including growth under oxygen limitation and in copper-sufficient media. To exclude subjectivity during specimen examination, the strains were investigated by specialists from three different institutions (the Center for Electron Optics at Michigan State University, the Institute of Biochemistry and Physiology of Microorganisms and the Institute of Microbiology at the Russian Academy of Sciences). Nonetheless, the peripheral membranes of the type II arrangement were not observed in any of the strains under any of the conditions tested. The characteristic feature of the cell ultrastructure was an extensive periplasmic space lacking any distinctive structures and a more-or-less developed vesicular membrane system connected to the cytoplasmic membrane and located on the cytoplasm periphery. A similar vesicular membrane system connected to the cytoplasmic membrane was described for some strains of hyphomicrobia (Conti & Hirsch, 1965) and for the phototrophic bacteria of the genera *Rhodospirillum* and *Rhodopseudomonas* (Oelze & Drews, 1971). The questions regarding the specifics of ICM in acidophilic

methanotrophs remain to be investigated, along with the nature and function of the inclusions revealed in cells grown under oxygen limitation. The latter resemble the toxin inclusions found in some *Bacillus thuringiensis* cells (Cooksey, 1971) and peroxisomal structures in methylotrophic yeasts (Sulter *et al.*, 1993). In view of all the above data, the cell ultrastructure of acidophilic methanotrophs appears to differ from those of other MOB and the type of ICM arrangement should be considered to be new and distinct.

The physiology of bog methanotrophs is quite consistent with the conditions of their natural habitat. The *Sphagnum* peat bogs are representative of an acidic and extra-dilute environment with a salt content of approximately 5–50 mg (l peat water)⁻¹. Our isolates exhibited a high level of sensitivity to salt stress and grew better in media with a low salt content (0.2–0.5 g l⁻¹). The conventional mineral media (with a salt content of approx. 1.5–3 g l⁻¹) would not support the growth of these isolates. This criterion clearly distinguishes bog isolates from the known MOB and places them at the opposite end of the spectrum from the recently discovered alkaliphilic methanotrophs (which prefer high salt concentrations; Khmelenina *et al.*, 1997). The acidophilic nature of bog isolates is another characteristic that makes them different from known MOB. While these bacteria can be grown over a wide range of pH values (including semi-neutral media), optimal growth occurs at pH values between 5.0 and 5.5, which is typical of moderately acidophilic organisms.

Analysis of the metabolic pathways in these cells has shown that the acidophilic isolates use the serine pathway for carbon assimilation and the glutamate cycle for NH₄⁺ assimilation in much the same way as all known type II methanotrophs. The bog isolates contain P_i-PFK, a highly specific MOB characteristic, but uncommon in methylotrophic bacteria. Some peculiarities of central metabolism, however, were found in the acidophilic methanotrophs. Both NADH- and NADPH-specific hydroxypyruvate reductase activities were detected whereas the NADH-specific enzyme is more common in type II methanotrophs. Also, the acidophilic isolates were found to possess enzymes for the pentose phosphate pathway (i.e. glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) at levels almost equal to those found in type I methanotrophs. These enzymes have not been previously detected in MOB with the serine pathway for carbon assimilation. The presence of pyruvate kinase activity is another distinctive metabolic feature as this enzyme has not been found in any known obligate MOB. Our recent study shows that the pattern of carbohydrate metabolism in these acidophilic methanotrophs shows some similarity to that of *Beijerinckia indica* subsp. *indica* (V. N. Khmelenina & Y. A. Trotsenko, unpublished results), further supporting the conclusion that these bacteria are closely related.

Table 3. Major characteristics that distinguish *Methylocella* gen. nov. and other genera of type II methanotrophs

Characteristic	<i>Methylosinus</i>	<i>Methylocystis</i>	<i>Methylocella</i>
Cell morphology	Vibrioid or pyriform	Coccobacillary or curved rods	Bipolar straight or curved rods
Motility	+ / -	-	-
Rosette formation	+	-	-
Type of resting cells	Exospores	Cysts	Exospores
Arrangement of intracytoplasmic membrane structure	Paired membranes aligned to periphery of cell (type II)	Paired membranes aligned to periphery of cell (type II)	Vesicular membrane system connected to the cytoplasmic membrane
Growth at 30 °C	+	+	-
Growth at pH 5.0	-	-	+
Growth at 0.05 % NaCl	+	+	-
Possess sMMO	+ / -	+ / -	+
Possess pMMO	+	+	-
Possess pyruvate kinase	-	-	+
G + C composition (mol %)	62-67	62-67	61
Closest phylogenetic relative	<i>Methylocystis</i>	<i>Methylosinus</i>	<i>Beijerinckia</i>

The fatty acid pattern of the acidophilic methanotrophs had a predominance of 18:1 acids, similar to that found in all type II methylotrophic bacteria (Bowman *et al.*, 1991; Guckert *et al.*, 1991). Despite such a basic similarity, the complete fatty acid profile did not match the fatty acid patterns either of the serine-pathway methanotrophs or of the serine-pathway methylotrophic bacteria unable to use methane. Interestingly, perhaps the most distinctive feature of the FAME patterns of the bog isolates was the relatively large amount of both 16:1 and 16:0 fatty acids (see the corresponding section in Results).

The updated 16S rDNA sequence data of the type strains of *Methylosinus sporium*, *Methylosinus trichosporium* and *Methylocystis parvus* clearly indicate that the *Methylosinus-Methylocystis* cluster is an even less phylogenetically diverse group than was previously thought. This finding provides further evidence of the evolutionary distinction between the acidophilic methanotrophs and the *Methylosinus-Methylocystis* cluster. The *mmoX* gene sequence data strongly support the above conclusion. As reported earlier, the *mmoX* gene sequence in acidophilic methanotrophs is significantly divergent from that in other methanotrophs (Dedysh *et al.*, 1998b). The DNA sequences of the gene cluster encoding the sMMO proteins of *Methylococcus capsulatus* (Bath), *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M (Stainthorpe *et al.*, 1990; Cardy *et al.*, 1991; McDonald *et al.*, 1997) are highly conserved. For the *mmoX* gene fragment investigated in our

study, the amino acid sequences of *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M had 93.7% identity, whereas the levels of identity with the same fragment of *Methylococcus capsulatus* (Bath) are 73.0 and 77.4%, respectively. The *mmoX* gene fragment of acidophilic isolates showed an equal level of divergence from the *Methylosinus-Methylocystis* group and from *Methylococcus capsulatus* (Bath). The levels of sequence divergence (76.5-78.6 and 78.0%, respectively) indicated that the *mmoX* gene of the acidophilic isolates forms a separate branch clearly distinct from the *mmoX* sequences of the *Methylocystis-Methylosinus* and *Methylococcus* groups.

PCR amplification of *pmoA* gene fragments from DNA of all three acidophilic strains was unsuccessful using two sets of primers, although both sets were clearly useful for amplifying the corresponding fragment from type I, II and X methanotrophs. These results were confirmed by specialists of three different institutions (the Center for Microbial Ecology at Michigan State University, the Max-Planck-Institut für Terrestrische Mikrobiologie in Marburg and the Department of Civil and Environmental Engineering at the University of Michigan). Furthermore, the hybridization of the 510 bp *pmoA* probe was only slightly visible with low-stringency washing, suggesting that the DNA fragment to which this probe is bound in strain K^T has only slight similarity to that of the probe generated from *Methylococcus capsulatus* (Bath). These results are very intriguing and suggest that the

gene encoding the 27 kDa polypeptide, if present in strain K^T, is very divergent from that of other methanotrophs. As these acidophilic isolates are relatively dissimilar to other known methanotrophs phenotypically, metabolically and on the basis of 16S rDNA and *mmoX* sequence data, the possibility that the cells do not have the capacity to express the pMMO should be considered and investigated further with other probes based on other genes in the *pmo* operon.

On the basis of these results, it is our conclusion that these isolates be placed in a separate taxon, for which we propose the generic name *Methylocella*, with the type species *Methylocella palustris*. The major characteristics differentiating the genus *Methylocella* from the other genera of the serine pathway methanotrophs are summarized in Table 3.

Description of *Methylocella* gen. nov.

Methylocella (Me.thyl.o.cel'la. M.L. n. *methyl* the methyl group; L. n. *cella* a cell; M.L. n. *Methylocella* methyl-using cell).

Gram-negative, polymorphic, straight or curved short rods that are 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 shapeless aggregates, but do not form rosettes. Non-motile. Encapsulated. Resting cells are exospores. Cells are not lysed by 2% SDS. The ICM system is different from those of both type I and type II methanotrophic bacteria. Cells contain an extensive periplasmic space and a vesicular membrane system connected to the cytoplasmic membrane. Possess sMMO. Optimal growth occurs at temperatures between 15 and 20 °C; no growth occurs at temperatures above 30 °C. Growth occurs between pH 4.5 and 7.0. Highly sensitive to salt stress; prefers diluted media with a low salt content. Obligate utilizer of C₁ compounds via the serine pathway. Does not contain the RuMP and RuBP enzymes. The tricarboxylic acid cycle is complete. Fixes atmospheric nitrogen via an oxygen-sensitive nitrogenase. The major phospholipid ester-linked fatty acids are 18:1 acids. The G+C content of the DNA is 61.2 mol%. Phylogenetically related to, but clearly distinct from, the coherent group of type II methanotrophs in the α-subclass of the *Proteobacteria*; more closely affiliated with the acidophilic heterotrophic bacterium *Beijerinckia indica* subsp. *indica*. Contains one species, *Methylocella palustris*, which includes the type strain, K^T. Habitats are the acidic wetlands, particularly *Sphagnum* peat bogs.

Description of *Methylocella palustris* sp. nov.

Methylocella palustris (pa.lus'tris. L. n. *palus* a bog; M.L. adj. *palustris* bog-inhabiting).

The description is as for the genus but with the following additional traits. Optimal growth at 20 °C

and at pH 5.5. Carbon sources used include methane and methanol. NaCl inhibits growth at a concentration of 0.5% (w/v). The type strain is strain K^T, which was isolated from *Sphagnum* peat from the Kyrgyznoye ombrotrophic bog in West Siberia (56° N, 85° E) and is deposited in the American Type Culture Collection (Manassas, VA) as strain 700799^T. The species also includes strains S6 and M131.

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