Methylocapsa acidiphila gen. nov., sp. nov., a novel methane-oxidizing and dinitrogen-fixing acidophilic bacterium from *Sphagnum* bog

Svetlana N. Dedysh,[1,4] Valentina N. Khmelenina,[2] Natalia E. Suzina,[2] Yuri A. Trotsenko,[2] Jeremy D. Semrau,[3] Werner Liesack[4] and James M. Tiedje[5]

[1] Institute of Microbiology, Russian Academy of Sciences, Moscow 117811, Russia

[2] Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow region, 142292, Russia

[3] Department of Civil and Environmental Engineering, The University of Michigan, Ann Arbor, MI 48109-2125, USA

[4] Max-Planck-Institut für Terrestrische Mikrobiologie, D-35043 Marburg, Germany

[5] Center for Microbial Ecology, Michigan State University, East Lansing, MI 48824-1325, USA

Author for correspondence: Svetlana N. Dedysh. Tel: +7 95 135 0591. Fax: +7 95 135 6530. e-mail: vdhome@bolshe.msk.ru

A novel genus and species, *Methylocapsa acidiphila* gen. nov., sp. nov., are proposed for a methane-oxidizing bacterium isolated from an acidic *Sphagnum* peat bog. This bacterium, designated strain B2^T, represents aerobic, Gram-negative, colourless, non-motile, curved coccoids that form conglomerates covered by an extracellular polysaccharide matrix. The cells use methane and methanol as sole sources of carbon and energy and utilize the serine pathway for carbon assimilation. Strain B2^T is a moderately acidophilic organism with growth between pH 4·2 and 7·2 and at temperatures from 10 to 30 °C. The cells possess a well-developed system of intracytoplasmic membranes (ICM) packed in parallel on only one side of the cell membrane. This type of ICM structure represents a novel arrangement, which was termed type III. The resting cells are *Azotobacter*-type cysts. Strain B2^T is capable of atmospheric nitrogen fixation; it possesses particulate methane monooxygenase and does not express soluble methane monooxygenase. The major phospholipid fatty acid is 18:1ω7*c* and the major phospholipids are phosphatidylglycerols. The G+C content of the DNA is 63·1 mol%. This bacterium belongs to the α-subclass of the *Proteobacteria* and is most closely related to the acidophilic methanotroph *Methylocella palustris* K^T (97·3% 16S rDNA sequence similarity). However, the DNA–DNA hybridization value between strain B2^T and *Methylocella palustris* K^T is only 7%. Thus, strain B2^T is proposed to comprise a novel genus and species, *Methylocapsa acidiphila* gen. nov., sp. nov. Strain B2^T (= DSM 13967^T = NCIMB 13765^T) is the type strain.

Keywords: *Methylocapsa acidiphila* gen. nov., sp. nov., acidophilic methane-oxidizing bacterium, serine-pathway methanotrophs

INTRODUCTION

Recently, the use of acidic media with a low salt content enabled the enrichment of acidophilic methanotrophic communities from *Sphagnum* peat bogs of four boreal sites in West Siberia and European North Russia (Dedysh *et al.*, 1998a). Three of the four enriched communities were obtained on nitrogen-sufficient media and three pure cultures of methane-oxidizing bacteria (MOB) were isolated from these enrichments (Dedysh *et al.*, 1998b). These isolates, strains K^T, S6 and M131, were subsequently described as the first representatives of a novel genus of serine-pathway methanotrophs within the α-subclass of the *Proteobacteria*, namely *Methylocella palustris* (Dedysh *et al.*, 2000). These moderately acidophilic MOB grow only on media with a low salt content (below 1 g l^{-1}) and the pH optimum for growth is 5·0–5·5. Cells of *Methylocella* lack the intracytoplasmic membrane (ICM) structures that are highly characteristic for other methanotrophs, but contain a vesicular membrane system connected to the cytoplasmic membrane. In contrast to all other MOB that form phylogeneti-

Abbreviations: ICM, intracytoplasmic membrane; MOB, methane-oxidizing bacterium; PHB, poly-β-hydroxybutyrate; pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Methylocapsa acidiphila* strain B2^T is AJ278726.

cally coherent clusters within the α- and γ-subclasses of the *Proteobacteria*, *Methylocella* is closely affiliated to the heterotrophic bacterium *Beijerinckia indica* subsp. *indica*. *Methylocella palustris* K^T was the first and so far the only methanotrophic species for which the presence of genes encoding particulate methane monooxygenase (pMMO) could not be demonstrated based on a PCR assay considered universal for *pmoA* genes encoding the active-site polypeptide of pMMO. These bacteria possess soluble MMO (sMMO) and the nucleotide sequence of the PCR-amplified fragment of *mmoX*, which encodes the α-subunit of the sMMO hydroxylase, was identical to the sequence of *mmoX* clones obtained from all three communities enriched on media supplemented with nitrate (Dedysh *et al.*, 1998a).

The fourth acidophilic methanotrophic enrichment was obtained on nitrogen-free medium from Bakchar bog in West Siberia. In contrast to enrichments from samples of the other sites, no PCR products were obtained with *mmoX*-targeted primers and template DNA from this methanotrophic community. We also failed to isolate any MOB from this enrichment using the procedure developed for the isolation of *Methylocella palustris* K^T. In this paper, we report the isolation of a pure culture of an acidophilic methanotrophic bacterium from this enrichment using another isolation strategy. This novel methanotrophic isolate, designated strain $B2^T$, represents a genotype and phenotype clearly different from *Methylocella palustris* K^T. Here, we describe the cell ultrastructure, physiological properties and metabolic pathways of this methanotrophic bacterium, as well as its biochemical, chemotaxonomic and genotypic features. We propose a novel genus and species, *Methylocapsa acidiphila* gen. nov., sp. nov., for this strain.

METHODS

Methanotrophic community and culture conditions. The methanotrophic community was enriched from an acidic peat bog (pH 3·6–4·5) underlying a *Sphagnum–Carex* plant community (Bakchar bog, Plotnikovo field station in West Siberia, 56° N, 82° E) as described previously using nitrogen-free M2 medium at pH 4·0–4·5 (Dedysh *et al.*, 1998a). This community was grown in 120-ml serum bottles containing 25 ml M2 nitrogen-free medium at pH 4·1. After inoculation, methane was added aseptically through silicone rubber septa to achieve a gas-mixing ratio in the headspace of approximately 25%. The bottles were then incubated for 1 week at 24 °C and shaken at 200 r.p.m.

Isolation and maintenance of methane-oxidizing strain $B2^T$. Cell suspensions of the exponentially growing Bakchar microbial community were serially diluted in tenfold steps in nitrogen-free M2 medium of pH 4·5–5·0 using screw-cap 35-ml serum vials with a headspace/liquid space ratio of 6:1. After inoculation, methane was added aseptically to attain a final mixing ratio of approximately 25%. The inoculated vials were then incubated at 24 °C and 200 r.p.m. The cultures were examined by phase-contrast microscopy and, if morphologically uniform, the cells were transferred to fresh medium and grown again under the same growth conditions. After repeating this process of serial dilution 20 times over 2 years, a methane-oxidizing isolate was purified, designated strain $B2^T$. Once isolated, this bacterium was maintained in nitrogen-free M2 medium and was subcultured at 2–3 week intervals.

Verification of purity of strain $B2^T$. Both phase-contrast and electron microscopy were used to examine cultures of strain $B2^T$ for contamination throughout the various stages of growth as well as under different cultivation conditions (pH range of 4 to 6, nitrogen-free and nitrogen-sufficient M2 media). The absence of heterotrophic satellite colonies was confirmed by plating $B2^T$ culture on several media containing different organic substrates. These were standard undiluted and tenfold-diluted Luria–Bertani agar [1·0% (w/v) tryptone, 0·5% (w/v) yeast extract, 1·0% (w/v) NaCl] and M2 agar media [1·5% (w/v) Noble agar] amended with 0·1% (w/v) glucose, fructose or sucrose. Examination of the $B2^T$ culture for the presence of heterotrophic satellites was also performed using liquid M2 media supplemented with the following carbon sources at 0·05% (w/v): glucose, fructose, sucrose, galactose, arabinose, ribose, lactate, pyruvate, citrate, acetate, succinate, ethanol and mannitol. To ensure detection of slowly growing heterotrophic satellites, growth was assessed after 4 weeks of incubation at 24 °C and 200 r.p.m.

Morphological observations. Cell morphology and the presence of cysts or exospores were examined by using batch cultures grown to the early-exponential, late-exponential and stationary growth phases. The Gram-stain reaction and lysis in 3% (w/v) KOH (Gerhardt, 1981) were tested with both 1- and 3-week-old cultures. Lysis by 0·2 and 2% (w/v) SDS was determined by direct microscopic observation.

Electron microscopy. Preparation of thin sections and electron microscopy were done as described previously (Khmelenina *et al.*, 1997, 1999). For freeze-fractured preparations, exponentially growing cells were collected and frozen in liquid propane cooled to −196 °C with liquid nitrogen. Cells were fractured according to Suzina *et al.* (1995) in a JEE-4X vacuum evaporator at 3×10^{-4} Pa and a sample temperature of −100 °C. The fracture faces were shadowed with a platinum/carbon mixture and coated with carbon. Replicas and ultrathin sections were imaged in a JEM-100B transmission electron microscope operating at 60 kV.

Effect of pH on growth of strain $B2^T$. Cells of strain $B2^T$, growing exponentially at pH 5·0, were collected by centrifugation, washed twice with fresh nitrogen-free M2 medium and inoculated into 120-ml serum bottles containing 25 ml nitrogen-free M2 medium at pH values ranging from 3·9 to 8·0. To maintain constant ionic strength with varying pH, 0·1 M solutions of H_3PO_4, KH_2PO_4 and K_2HPO_4 were used. After inoculation and addition of methane, the samples were incubated for 10 d at 24 °C and 200 r.p.m. Growth, monitored by nephelometry at 410 nm, and pH of the culture were measured over time in triplicate bottles.

Other physiological tests. Growth of strain $B2^T$ was monitored for 2 weeks with methane as the sole growth substrate under a variety of conditions, including temperatures ranging from 4 to 37 °C and NaCl at concentrations of 0·01–1% (w/v) using liquid cultures. The ability to grow under microaerobic conditions was also determined in growth experiments with 5% O_2 (v/v) in the headspace of incubation flasks.

Range of utilizable carbon and nitrogen sources. The

following carbon sources at 0·05% (w/v) were examined to determine the range of potential growth substrates of strain B2^T: formate, formamide, urea, methylamine, dimethylamine, trimethylamine, tetramethylammonium chloride, ethanol, glucose, fructose, sucrose, galactose, arabinose, ribose, lactate, pyruvate, citrate, acetate, succinate and mannitol. The capacity to utilize methanol at concentrations from 0·01 to 1% (v/v) was determined on M2 medium. Nitrogen sources were also tested using M2 medium with addition of the following compounds at 0·02% (w/v): $NaNO_2$, $(NH_4)_2SO_4$, DL-alanine, glycine, 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 and yeast extract. Growth was examined after 2 weeks of incubation and confirmed by comparison with growth on nitrogen-free M2 medium.

Naphthalene assay of sMMO activity. In order to monitor sMMO activity, the naphthalene assay developed by Brusseau *et al.* (1990) was used as described previously (Lontoh & Semrau, 1998) for all methanotrophic reference strains as well as the novel methanotrophic isolate, strain B2^T.

Acetylene-reduction assay of nitrogen-fixation activity. 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 injection of acetylene, 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) mixing ratio in the headspace. Methanol was added to the flasks at a concentration of 0·05% (v/v). The amount of ethylene produced was measured after 3 h incubation on a rotary shaker (200 r.p.m.) at 24 °C.

Reference bacterial strains. *Methylosinus trichosporium* OB3b^T (= ATCC 35070^T), *Methylococcus capsulatus* Bath (= ATCC 33009), *Methylocella palustris* K^T (= ATCC 700799^T), '*Methylobacter bovis*' 89 and *Methylosinus trichosporium* 19 were used as reference strains. The last two strains were obtained from the collection of methanotrophic bacteria of the Institute of Biochemistry and Physiology of Microorganisms (Pushchino, Russia). All the methanotrophic strains except *Methylocella palustris* K^T were cultivated on NMS medium with methane as the growth substrate (Whittenbury *et al.*, 1970a). *Methylocella palustris* K^T was grown on M2 medium supplemented with vitamins (Dedysh *et al.*, 2000).

Analytical techniques. CH_4 and C_2H_4 were measured with a GC equipped with a flame-ionization detector. CO_2 and O_2 were analysed by GC with a thermoconductivity detector. All measurements were performed using triplicate samples.

DNA isolation and characterization. Genomic DNA from strain B2^T was extracted using the SDS-based assay as described previously (Dedysh *et al.*, 1998a). The DNA base composition of strain B2^T was determined by thermal denaturation using a Unicam SP1800 spectrophotometer at a heating rate of 0·5 °C min^{-1}. The G+C content was calculated with the equation of Owen *et al.* (1969): G+C content (mol%) = $2·08T_m - 106·4$. DNA of *Escherichia coli* K-12 was used as the standard. DNA–DNA hybridization of strain B2^T and *Methylocella palustris* K^T was done on nitrocellulose membrane filters (Hybond-N, Amersham) according to Lysenko *et al.* (1988). Genomic DNA fractions were labelled using deoxy[1′,2′,5′-^{3}H]cytidine 5′-triphosphate and the nick translation kit N 5500 following the manufacturer's instructions (Amersham; Technical Bulletin, 1980). Hybridization was performed with five replications in an incubation mixture containing 2×SSC (0·3 M NaCl, 0·03 M trisodium citrate, pH 7·0) and 50% (v/v) formamide at 49 °C for 24 h.

Comparative sequence analysis of the 16S rRNA gene. PCR-mediated amplification of the 16S rRNA gene from positions 28 to 1491 (numbering according to the International Union of Biochemistry nomenclature for *Escherichia coli* 16S rRNA), sequencing and phylogenetic inference were carried out as described for *Methylocella palustris* K^T (Dedysh *et al.*, 1998b, 2000).

Whole-cell fatty acid and phospholipid analysis. Fatty acid analyses for both *Methylocella palustris* K^T and strain B2^T were performed by Microbial Insights, Inc. (Rockford, TN, USA). Extractions were performed using one-phase chloroform/methanol/buffer extractant. Lipids were recovered, dissolved in chloroform and fractionated on disposable silicic acid columns into neutral-, glyco- and polar-lipid fractions. The polar lipid fraction was transesterified with mild alkali to recover the phospholipid ester-linked fatty acids (PLFA) as methyl esters in hexane. PLFA were analysed by GC with peak confirmation performed by electron impact mass spectrometry.

Intact phospholipid profiles were prepared as described previously (Fang *et al.*, 2000). Phospholipids were extracted overnight at 4 °C from liquid cultures using a 2:1:0·8 mixture of methanol, dichloromethane (DCM) and phosphate buffer. Lipids were then partitioned into organic solvent by adding DCM and water such that the final DCM/methanol/water ratio was 1:1:0·9. The organic phase was then filtered and the total lipid extract was dried and redissolved in methanol. Intact phospholipid profiles were determined using liquid chromatography/electrospray ionization–mass spectrometry analysis with an HP 1090 liquid chromatography/HP 5989B single quadrupole mass spectrometer with an electrospray interface. The concentrations of phospholipids were calculated based on the chromatographic area response of individual phospholipids relative to that of an internal standard (18:1-lyso-phosphatidylglycerol) and reported as μg ml^{-1} liquid culture. The reproducibility of the analysis was better than 87% ($n = 5$).

Enzyme assays. Enzymic studies were performed as described for *Methylocella palustris* K^T (Dedysh *et al.*, 2000).

RESULTS

Isolation and cell morphology of the methane-oxidizing strain B2^T

Despite the large spectrum of pH and incubation conditions, all attempts to isolate MOB from the Bakchar community by surface plating were unsuccessful. However, the isolation of the methanotrophic strain B2^T in pure culture was achieved by using multiple repeated serial dilutions in liquid medium. Cells of strain B2^T were Gram-negative, non-motile, curved coccoids, 0·7–1·0 μm in width and 0·8–1·2 μm in

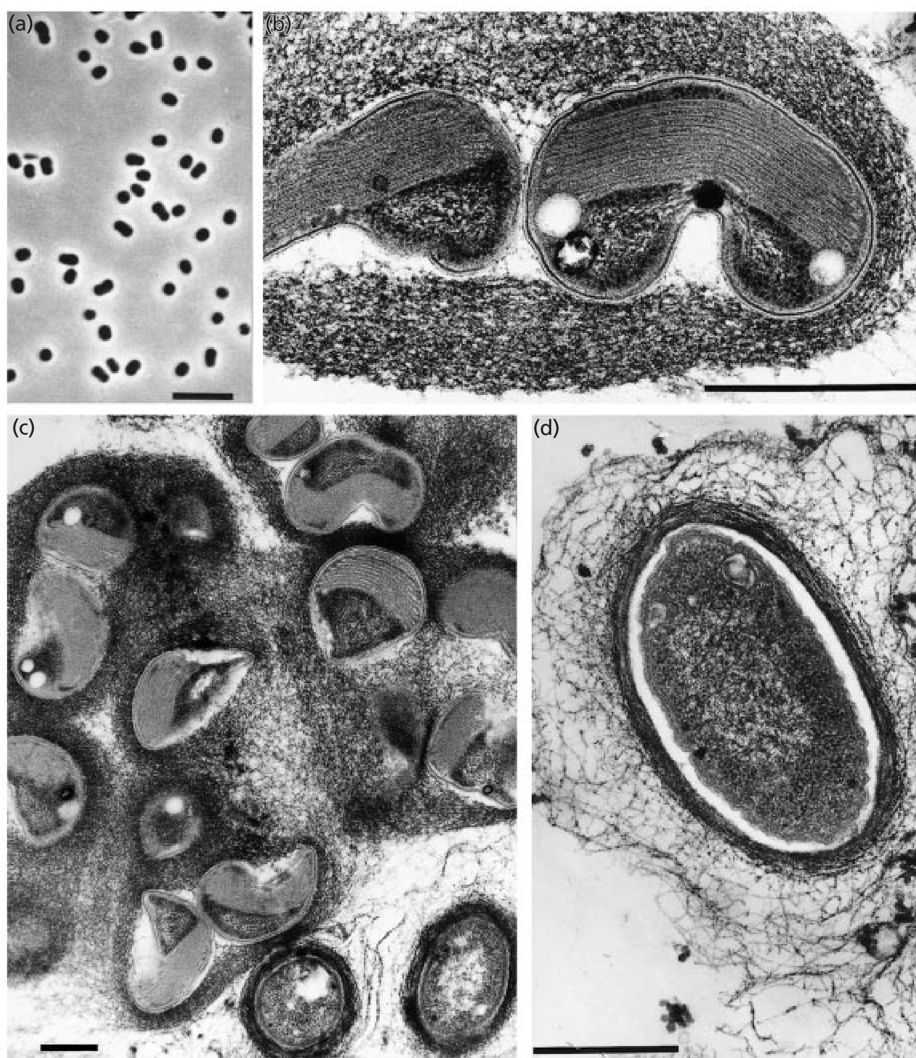

Fig. 1. (a) Phase-contrast micrograph of strain B2^T grown on methane for 4 d at pH 4·5. Bar, 5 μm. (b) Negatively stained thin section of vegetative cells of strain B2^T prefixed by ruthenium red. Cells are included in a fibrous polysaccharide matrix. Bar, 0·5 μm. (c) Negatively stained thin section of cellular aggregates of strain B2^T prefixed with ruthenium red. Vegetative cells with well-developed ICM and PHB granules are included in the polysaccharide matrix. Separate lipid cysts without ICM structures are located on the periphery of the vegetative cell conglomerates. Bar, 0·5 μm. (d) Thin section of a cyst of strain B2^T. Bar, 0·5 μm.

length (Fig. 1a), that reproduced by binary fission with cross constriction formation. Cells occurred singly or formed aggregates that were surrounded by an extracellular matrix of polysaccharide (Fig. 1b, c) as revealed by contrasting the acid mucopolysaccharides with ruthenium red (Luft, 1964).

Characterization of growth

Strain B2^T grew in liquid mineral media only and did not form colonies on solid media made with any purity grade agar or with agarose. Liquid cultures displayed a white turbidity that mostly sedimented and a surface pellicle was not formed. Cellular conglomerates were predominantly formed when cells were grown in acidic conditions on nitrogen-free media.

Cell ultrastructure

A well-developed system of ICM was revealed in thin sections of the cells (Fig. 1b, c). Extended layers of membrane vesicles were packed in parallel to each other on only one side of the cell membrane. The number of membrane vesicles was approximately seven to ten per cell. Freeze-fractured preparations showed an intermediate type of ICM arrangement in cells of strain B2^T in comparison with the characteristic type I ICM arrangement (stacks of vesicles) in

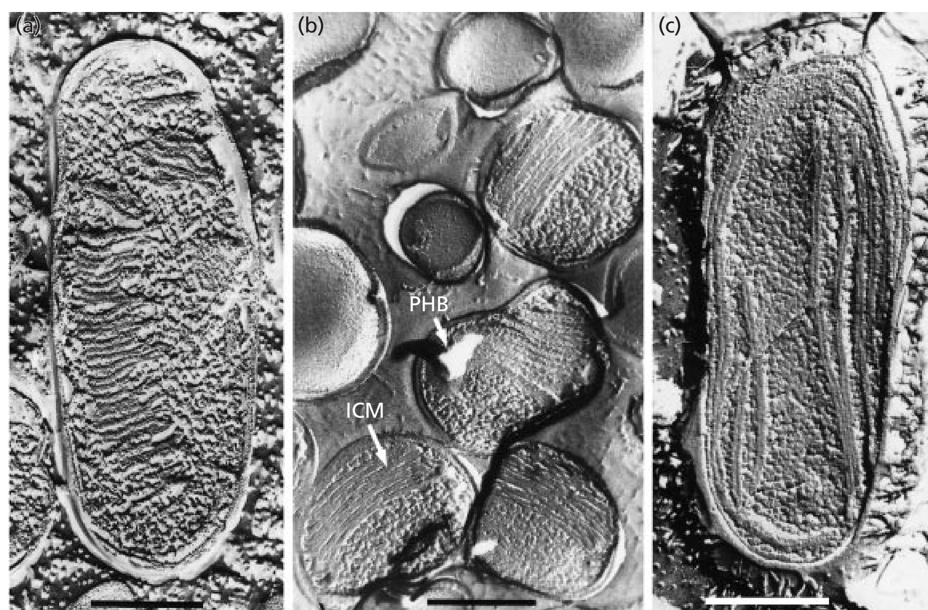

Fig. 2. Freeze-fractured cells of '*Methylobacter bovis*' 89 (a), strain B2^T (b) and *Methylosinus trichosporium* 19 (c) with characteristic type I, intermediate type and type II ICM arrangements. Bars, 0·5 μm.

'*Methylobacter bovis*' 89 and type II ICM arrangement (peripherally and loosely arranged membranes aligned parallel to cytoplasmic membrane) in *Methylosinus trichosporium* 19 (Fig. 2). Intracellular inclusions were observed as low electron-transparent spherical granules of poly-*β*-hydroxybutyrate (PHB), which was confirmed by freeze-fracture methods (Dunlop & Robards, 1973). PHB in the granules was stretched during cutting, as seen on the freeze-etched preparations (Fig. 2b).

As seen in thin sections, the cells formed a large amount of extracellular polysaccharide matrix, revealed by contrasting the acid mucopolysaccharides with ruthenium red. Several methanotrophic cells were found embedded in this matrix, implying that cell division occurred inside the matrix. On the periphery of the cellular aggregates, but included in the matrix, were morphologically different cells (Fig. 1c, d). These cells did not possess complex membrane systems and were surrounded by an additional two-layered envelope or coat. The inner part of the envelope near the outer membrane of these cells contained closely packed fibrillae with high electron density. The more extensive outer coat also consisted of fibrillae but had lower electron density. These cells are apparently the resting stage of the bacterium and are more similar to the cysts of the *Azotobacter*-like type than to the 'lipid cysts' formed by representatives of the genus *Methylocystis*.

Purity criteria

Microscopic checks confirmed the uniform cell morphology of strain B2^T at different stages of culture development and under different growth conditions (pH 4·5, 5·5 and 6·5 as well as growth on nitrogen-free and nitrogen-sufficient media). Electron microscopy examinations also failed to reveal any contamination of isolate B2^T by other bacteria. Finally, no growth was obtained on complex organic media or basal medium M2 supplemented with individual sugars.

Physiological characteristics

Strain B2^T was capable of growth on methane and methanol as the sole carbon and energy sources. Methanol supported growth only when used at concentrations below 0·05% (v/v). We did not observe growth either on other C_1 substrates or on the range of multicarbon compounds tested. Growth factors were not required. Strain B2^T utilized ammonium salts, nitrates and yeast extract as nitrogen sources, although it was able to fix atmospheric nitrogen and grew well in nitrogen-free medium. The acetylene reduction activity of strain B2^T in microaerobic conditions ranged from 5·7 to 7·0 nmol C_2H_4 (mg dry biomass)$^{-1}$ h^{-1}, while under aerobic conditions it fell to 0·2–0·3 nmol C_2H_4 (mg dry biomass)$^{-1}$ h^{-1}.

sMMO activity, as determined by the production of naphthol from naphthalene, was never observed in strain B2^T. The isolate grew in the pH range 4·2–7·2 with the optimum at pH 5·0–5·5. The temperature range for growth was 10–30 °C with the optimum at approximately 20 °C; no growth occurred at 37 °C. The generation time was in the range 15–40 h. Similar to *Methylocella palustris*, strain B2^T grew in media with low salt content (up to 1–1·5 g l^{-1}), while the optimum salt concentrations were in the range 0·2–

Table 1. Cellular fatty acid compositions of the novel acidophilic methanotroph B2^T, *Methylocella palustris* K^T and other type II methanotrophs

Taxa are indicated as: 1, *Methylocapsa acidiphila* B2^T; 2, *Methylocella palustris* K^T; 3, *Methylosinus* and *Methylocystis* spp. Values are percentages of total fatty acids. br, Branched; cy, cyclo.

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

* Data taken from Bowman *et al.* (1993).

Table 2. Activities of the enzymes of primary and intermediate metabolism in cell extracts of strain B2^T

Activities are given in nmol min^{-1} (mg protein)$^{-1}$.

Enzyme	Cofactor*	Activity
sMMO	–	0
pMMO	–	100
Methanol dehydrogenase	PMS	219
Formaldehyde dehydrogenase	PMS	20
	GSH, NAD	0
Formate dehydrogenase	PMS	42
	NAD	28
Ribulose-1,5-bisphosphate carboxylase	–	0
Hexulose-phosphate synthase	–	0
6-Phosphofructokinase	ATP	0
	PPi	75
Fructose-1,6-bisphosphate aldolase	–	43
Glucose-6-phosphate dehydrogenase	NAD	0
	NADP	32
6-Phosphogluconate dehydrogenase	NAD	16
	NADP	33
KDPG aldolase	–	0
Hydroxypyruvate reductase	NADH	38
	NADPH	0
Serine-glyoxylate aminotransferase	–	33
Pyruvate kinase	–	0
Pyruvate dehydrogenase	NAD	0
Isocitrate dehydrogenase	NAD	46
	NADP	40
α-Ketoglutarate dehydrogenase	NAD	13
Malate dehydrogenase	NAD	19
Glutamate dehydrogenase	NAD	0
	NADP	0
Glutamate synthase	NADH	33
	NADPH	0
Glutamine synthetase	ATP, Mn^{2+}	92

* GSH, Reduced glutathione; PMS, phenazine methosulfate; PPi, pyrophosphate.

0·5 g l^{-1}. Growth was inhibited completely in the presence of NaCl at concentrations above 0·5 % (w/v).

Cellular fatty acid and phospholipid profiles

The cellular fatty acid composition was determined for both strain B2^T and *Methylocella palustris* K^T (Table 1). These two methanotrophs possessed highly similar PLFA profiles. The major component of their profiles was 18:1ω7*c* fatty acid, which comprised 78 % of the total PLFAs. Interestingly, both organisms lacked 18:1ω8*c*, a common and specific fatty acid for the genera *Methylosinus* and *Methylocystis* (Guckert *et al.*, 1991; Bowman *et al.*, 1993).

Although similar, the PLFA profiles of strain B2^T and *Methylocella palustris* K^T were not identical. The total content of 16-carbon fatty acids in *Methylocella palustris* K^T exceeded the corresponding value in strain B2^T. Specifically, strain B2^T possessed 16:1ω7*c* acid only, while *Methylocella palustris* K^T contained both 16:1ω7*c* and 16:1ω7*t* acids in almost equal amounts. The content of 18:0 fatty acid was also significantly higher in B2^T than in *Methylocella palustris* K^T. Another distinctive feature of the strain B2^T PLFA profile was the presence of 17:1ω7*c* and i17:0 fatty acids, which are not common for type II methanotrophs.

The intact phospholipid profile analyses showed that strain B2^T and *Methylocella palustris* K^T have significantly different phospholipids. The major phospholipids (63 %) in strain B2^T were phosphatidylglycerols, which were not detected in *Methylocella palustris* K^T. The minor phospholipids in strain B2^T were phosphatidylethanolamines (17 %) and phosphatidylmethylethanolamines (3·5 %). In contrast to strain B2^T, 94 % of the total phospholipids in *Methylocella palustris* K^T were represented by phosphatidylmethylethanolamine (Fang *et al.*, 2000).

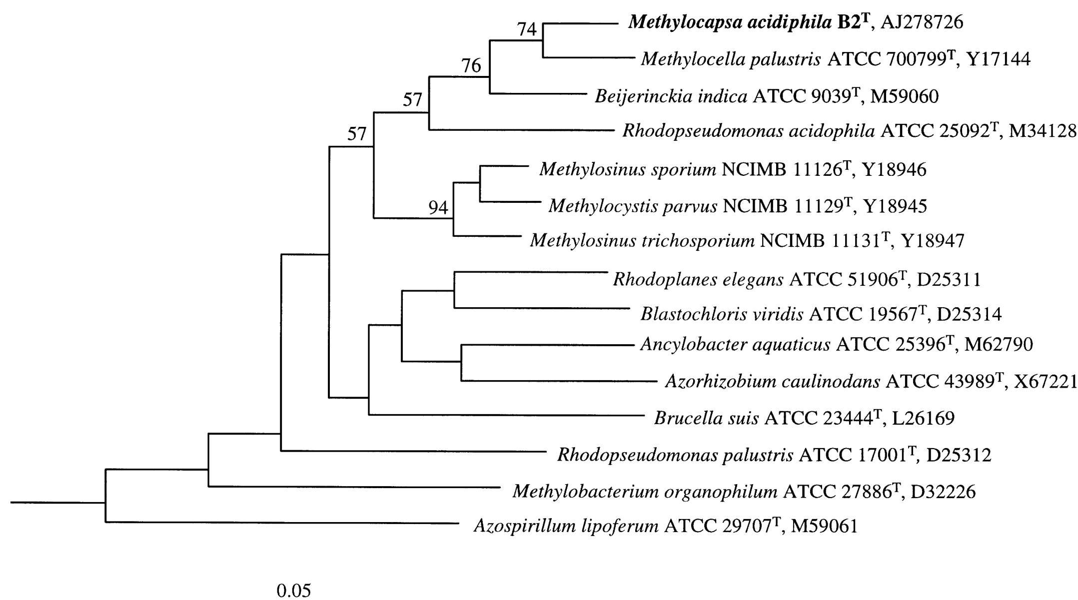

Fig. 3. 16S rDNA-based dendrogram showing the phylogenetic position of strain B2^T in relation to *Methylocella palustris* K^T and other representative members of the α-subclass of the *Proteobacteria*. The dendrogram was reconstructed by using distance matrix methods [calculation of the distance matrix with the equation of Jukes & Cantor (1969) and construction of the distance tree by the neighbour-joining method (Saitou & Nei, 1987)]. Numbers indicate the percentage significance values for interior nodes, as derived from a bootstrap test in which 1000 data resamplings were used. The root was determined by using the 16S rDNA sequence of the γ-proteobacterium *Methylococcus capsulatus* as the outgroup reference. Bar, 0·05 substitutions per nucleotide sequence position.

Enzymological analysis

The methane-oxidizing activity was mainly located in the ICM, since sMMO was not expressed by strain B2^T. The other enzyme activities involved in the sequential oxidation of CH_4 to CO_2 (dehydrogenases of methanol, formaldehyde and formate) were also observed, as shown in Table 2. Activities of both hydroxypyruvate reductase and serine-glyoxylate aminotransferase were revealed, indicating that strain B2^T assimilates formaldehyde via the serine pathway. Activities of the Calvin cycle enzymes (phosphoribulokinase and ribulose-bisphosphate carboxylase) as well as the key ribulose monophosphate cycle enzyme (hexulose-phosphate synthase) were not found. The presence of α-ketoglutarate dehydrogenase indicated that the complete tricarboxylic acid cycle operates in this bacterium. The glyoxylate shunt enzymes, isocitrate lyase and malate synthase, were not detectable. Relatively high activities were found of the pentose phosphate cycle enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The finding of pyrophosphate-dependent 6-phosphofructokinase and fructose-1,6-bisphosphate aldolase activities suggested carbohydrate metabolism through the reversible glycolytic sequence. However, no activity of pyruvate kinase was detected. Finally, strain B2^T used the glutamate cycle enzymes, glutamine synthetase and glutamate synthase, for ammonium assimilation. In general, the metabolic pattern of this acidophilic strain was the same as in other neutrophilic type II methanotrophs that possess the isocitrate lyase-negative variant of the serine pathway.

Phylogenetic analysis and DNA–DNA reassociation study

Comparative sequence analysis of the 16S rRNA gene showed that strain B2^T is most closely related to the acidophilic methane-oxidizing bacterium *Methylocella palustris* K^T, a member of the α-subclass of the *Proteobacteria* (Fig. 3). The overall 16S rDNA sequence similarity between strain B2^T and *Methylocella palustris* K^T is 97·3 %. The next closest related micro-organism is the non-methanotrophic bacterium *Beijerinckia indica* subsp. *indica*, which exhibits overall 16S rDNA sequence similarity of 96·2 and 96·5 % to strain B2^T and *Methylocella palustris* K^T, respectively.

The DNA G + C content of strain B2^T was 63·1 mol %. The DNA–DNA hybridization value of strain B2^T and *Methylocella palustris* K^T was 7 %, while the homologous DNA fractions showed 100 % hybridization.

DISCUSSION

Given all the characteristic features of B2^T, we believe that this novel acidophilic isolate should be classified as a type II methanotrophic bacterium. It is the second culturable representative of the novel subtype of serine-pathway methanotrophs to which *Methylocella palustris* K^T also belongs. Strain B2^T displayed pheno-

typic traits very similar to those of *Methylocella palustris* K^T, e.g. pH and temperature ranges for growth, as well as sensitivity to salt stress. These traits are defined by the physico-chemical characteristics of the acidic, oligotrophic habitat from which both methanotrophs were isolated. However, a closer look at many of the other key features showed that these two methanotrophs should be considered two different taxa.

Both the cell morphology and ultrastructure of strain $B2^T$ were profoundly different from those of *Methylocella palustris* K^T. The different cell shape, lack of bipolar cell appearance and formation of cellular conglomerates covered by a polysaccharide matrix made strain $B2^T$ clearly distinguishable from *Methylocella palustris* K^T. Also, the types of resting cells formed by strain $B2^T$ and *Methylocella palustris* K^T were clearly different. *Methylocella palustris* K^T was shown to form exospores, while strain $B2^T$ formed an unusual type of cyst. The ultrastructure of these cysts somewhat resembled the 'lipid cysts' of *Methylocystis parvus* (Wittenbury *et al*., 1970b). The key difference, however, was the presence of an additional extensive outer coat in the cysts of strain $B2^T$. The structure of these resting cells was more similar to the *Azotobacter*-like cysts that are characteristic of type I methanotrophs. However, the structure differed from the *Azotobacter*-like cysts in the presence of outer cell membranes and the presence of intine (inner wall as a thick homogeneous layer) (Socolofsky & Wyss, 1961).

The most striking ultrastructural distinction between strain $B2^T$ and *Methylocella palustris* K^T was that strain $B2^T$, like most of the other known MOB, contained a very well-developed ICM system, while *Methylocella palustris* K^T did not. This ICM system, which was observed in cells of strain $B2^T$ under all growth conditions, appeared as stacks of membrane vesicles packed in parallel on only one side of the cell membrane. This pattern of ICM arrangement is different from the ICM of both type I and type II methanotrophs. To confirm the observations obtained with ultrathin sections, cell structure studies were also performed by the freeze-fracture method, which prevents any mechanical damage to cell structures and provides the most native pictures of ICM arrangements. The freeze-fracture images clearly showed the difference between the ICM arrangement in cells of strain $B2^T$ and those in known types of methanotrophs. The membranes of strain $B2^T$ were also aligned parallel to the cell wall (similar to type II ICM), but they were always located on only one side of the cell membrane and never formed uninterrupted vesicles, while they were also closely packed in stacks like ICM of type I methanotrophs. We suggest this membrane pattern as a type III of ICM arrangement in methanotrophic bacteria.

The two acidophilic methanotrophs, *Methylocella palustris* and strain $B2^T$, were obtained using different enrichment and isolation procedures. The *Methylocella*-specific isolation approach included enrichment on nitrogen-sufficient liquid medium followed by agar surface plating, while strain $B2^T$ was enriched on nitrogen-free medium and isolated by serial dilution, as it did not form colonies on agar media. These two isolation strategies resulted in the selection of two micro-organisms with different growth characteristics. Although *Methylocella palustris* K^T was capable of dinitrogen fixation, growth on nitrogen-free medium was poor and occurred only under microaerobic conditions. In contrast, strain $B2^T$ grew very well on nitrogen-free medium under both aerobic and microaerobic conditions. This could be due to the protective effect of the polysaccharide matrix covering cell conglomerates of strain $B2^T$.

The metabolic pattern of strain $B2^T$ was more similar to that of *Methylocella* than to those of *Methylocystis* and *Methylosinus*. However, only NADH-specific hydroxypyruvate reductase activity was detected in cell extracts of strain $B2^T$, while all three strains of *Methylocella palustris* showed both NADH- and NADPH-specific activities. Enzyme activities related to the pentose phosphate cycle were found in strain $B2^T$, while such activities were not detected in members of the *Methylocystis*/*Methylosinus* group.

Like all members of the *Methylosinus*/*Methylocystis* group, the two acidophilic methanotrophs contained 18:1 fatty acids as the predominant PLFAs. However, the predominant PLFA in strain $B2^T$ and *Methylocella palustris* K^T was 18:1ω7*c*, while 18:1ω8*c*, characteristic of the *Methylocystis*/*Methylosinus* group, was not detected. Previously, 18:1ω8*c* PLFA was considered highly indicative of type II methanotrophs and thus widely used as a signature fatty acid in environmental studies on MOB. Our data show that the applicability of this marker is restricted to members of the genera *Methylocystis* and *Methylosinus* and, consequently, should not be considered universal for all type II MOB.

In contrast to all previously known methanotrophs, we also found 17-carbon PLFAs in detectable amounts (up to 1 %) in the acidophilic strains, i.e. i17:0 and 17:1ω7*c* in strain $B2^T$ and 17:0 and 17:1ω8*c* in *Methylocella palustris* K^T. Interestingly, such 17-carbon PLFAs, as well as large amounts of 18:1ω7*c*, which may both be indicative of this novel type II subgroup of acidophilic methanotrophs, were found to be ^{14}C-labelled in soil samples that showed atmospheric methane uptake after incubation with $^{14}CH_4$ (Holmes *et al*., 1999; Roslev & Iversen, 1999). These $^{14}CH_4$-PLFA fingerprinting studies provide evidence for the presence of active methanotrophs in those soils that act as sinks for atmospheric methane and, by inference, provide some evidence for the wide ecological distribution of acidophilic methanotrophs and their probable contribution to the oxidation of atmospheric methane.

In contrast to the PLFA profiles, which were shown to be very similar for the two acidophilic methanotrophs,

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

Characteristic	*Methylosinus*	*Methylocystis*	*Methylocella*	*Methylocapsa*
Cell morphology	Vibrioid or pyriform	Coccobacillary or curved rods	Bipolar straight or curved rods	Curved coccoids
Motility	+/−	−	−	−
Rosette formation	+	−	−	−
Type of resting cell	Exospores	Cysts	Exospores	*Azotobacter*-type cysts
ICM arrangement	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	Membrane vesicles packed in parallel on one side of the cell membrane (type III)
Growth at:				
30 °C	+	+	−	+
pH 5	−	−	+	+
0·5 % NaCl	+	+	−	−
Activity of:				
sMMO	+/−	+/−	+	−
pMMO	+	+	−	+
Pyruvate kinase	−	−	+	−
Major phospholipids	Phosphatidylglycerol	Phosphatidylglycerol	Phosphatidylmethylethanolamine	Phosphatidylglycerol
Major PLFAs	18:1*ω*8*c*	18:1*ω*8*c*	18:1*ω*7*c*	18:1*ω*7*c*
G+C content (mol%)	62–67	62–67	61	63

the phospholipid analysis confirmed a profound difference between strain B2^T and *Methylocella palustris* K^T. Similar to *Methylosinus*, the major phospholipids in strain B2^T were represented by phosphatidylglycerols, while this type of phospholipid could not be detected in *Methylocella palustris* K^T. In contrast, 94 % of total phospholipids in *Methylocella palustris* K^T were phosphatidylmethylethanolamines, which were not found in *Methylosinus* and were present only at a very low level (about 3 %) in cells of strain B2^T.

The 16S rRNA gene sequence similarity between strain B2^T and *Methylocella palustris* K^T was 97·3 %. Although this value often indicates members of the same genus, it is in the range of 16S rDNA sequence similarities that separate members of the genera *Methylosinus* and *Methylocystis*. For instance, sequence similarities between 16S rDNA of *Methylocystis parvus* OBBP^T, *Methylosinus trichosporium* OB3b^T and *Methylosinus sporium* are in the range 97·5–98 % (Dedysh *et al*., 2000). The profound genotypic distinctness between strain B2^T and *Methylocella palustris* K^T was revealed by only 7 % DNA–DNA hybridization, a value far below 70 %, which has been defined as the threshold for considering two strains as belonging to the same genospecies (Wayne *et al*., 1987). According to this criterion, strain B2^T would have to be considered at least a novel species. Moreover, the DNA–DNA hybridization value determined for strain B2^T and *Methylocella palustris* K^T falls into the range of such values observed between members of different genera of type II MOB. Indeed, the values between the type species of the genera *Methylosinus* and *Methylocystis* range from 7 to 21 % (Bowman *et al*., 1993).

Taking into consideration the obvious morphological, ultrastructural, biochemical and genotypic differences between strain B2^T and *Methylocella palustris* K^T, we believe that strain B2^T, although phylogenetically related to *Methylocella palustris* K^T, should be considered a novel genus of serine pathway methanotroph. We propose the generic name *Methylocapsa* gen. nov., with the type species *Methylocapsa acidiphila* sp. nov. In this context, an additional, important feature for their differentiation into different genera is that strain B2^T and *Methylocella palustris* K^T, although inhabiting the same habitat (i.e. *Sphagnum* peat bogs), probably occupy different ecological niches. These acidophilic methanotrophs seem to express different types of MMO, i.e. sMMO by *Methylocella palustris* K^T and pMMO by strain B2^T. The characteristics that differentiate the genus *Methylocapsa* from the genus *Methylocella* as well as from other genera of the serine-pathway methanotrophs are summarized in Table 3.

Description of *Methylocapsa* gen. nov.

Methylocapsa [Me.thy.lo.cap′sa. N.L. n. *methyl* the methyl group; L. n. *capsa* a cover or capsule (receptacle, container); N.L. fem. n. *Methylocapsa* methyl-using cell covered by a capsule].

Gram-negative, curved coccoids, 0·5–0·8 µm wide by 0·8–1·2 µm long. Cells occur singly or form round-shaped conglomerates surrounded by extracellular polysaccharide matrix; do not form rosettes. Repro-

duce by binary fission. Non-motile. Produce intracellular PHB granules. Resting cells are *Azotobacter*-type cysts. Cells are not lysed by 2% SDS. Cells possess a well-developed ICM system of type III which appears as stacks of membrane vesicles packed in parallel on only one side of the cell membrane. Does not express sMMO. The temperature range for growth is 10–30 °C with the optimum at 20–24 °C; no growth at 37 °C. Growth occurs between pH 4·2 and 7·2. Prefers dilute media of low salt content. Obligate utilizer of C_1 compounds via the serine pathway. Does not contain the ribulose monophosphate and ribulose bisphosphate enzymes. Tricarboxylic acid cycle is complete. Capable of atmospheric nitrogen fixation. The major PLFA is $18:1\omega7c$ and the major phospholipids are phosphatidylglycerols. The G+C content is 63 mol%. Belongs to the α-subclass of the *Proteobacteria*; phylogenetically related to the acidophilic methanotrophic bacterium *Methylocella palustris* K^T and the acidophilic heterotrophic bacterium *Beijerinckia indica* subsp. *indica*. The type and only species is *Methylocapsa acidiphila*. Habitat is acidic wetlands, particularly *Sphagnum* peat bogs.

Description of *Methylocapsa acidiphila* gen. nov., sp. nov.

Methylocapsa acidiphila (a.ci.di′phi.la. N.L. n. *acidum* acid from L. adj. *acidus* sour; Gr. adj. *philus* loving; N.L. fem. adj. *acidiphila* acid-loving).

Description as for the genus plus the following traits. Optimal growth at 20–24 °C and at pH 5·0–5·5. Carbon sources used include methane and methanol. Methanol supports growth only when used at concentrations below 0·05% (v/v). NaCl inhibits growth at a concentration of 0·5% (w/v).

The type strain is strain $B2^T$ (=DSM 13967^T = NCIMB 13765^T), which was isolated from *Sphagnum–Carex* peat of Bakchar bog, West Siberia (56° N, 82° E).

ACKNOWLEDGEMENTS

This research was supported in part by the Russian Fund of Basic Research (grants no. 99-04-48725 and 99-04-04035), the EC CONGAS Project and by the Deutsche Forschungsgemeinschaft (436 RUS 113/543/0). We also thank Jaisong Fang (University of Michigan) for assistance with intact phospholipid analysis.

REFERENCES

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.

Brusseau, G. A., Tsien, H. C., Hanson, R. S. & Wackett, L. P. (1990). Optimization of trichloroethylene oxidation by methanotrophs and the use of a colorimetric assay to detect soluble methane monooxygenase activity. *Biodegradation* **1**, 19–29.

Dedysh, S. N., Panikov, N. S. & Tiedje, J. M. (1998a). Acidophilic methanotrophic communities from *Sphagnum* peat bogs. *Appl Environ Microbiol* **64**, 922–929.

Dedysh, S. N., Panikov, N. S., Liesack, W., Großkopf, R., Zhou, J. & Tiedje, J. M. (1998b). 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.

Dunlop, W. F. & Robards, A. W. (1973). Ultrastructural study of poly-β-hydroxybutyrate granules from *Bacillus cereus*. *J Bacteriol* **114**, 1271–1280.

Fang, J., Barcelona, M. J. & Semrau, J. D. (2000). Characterization of methanotrophic bacteria on the basis of intact phospholipid profiles. *FEMS Microbiol Lett* **189**, 67–72.

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

Guckert, J. B., Ringelberg, D. B., White, D. C., Hanson, R. S. & Bratina, B. J. (1991). Membrane fatty acids as phenotypic markers in the polyphasic taxonomy of methylotrophs within the Proteobacteria. *J Gen Microbiol* **137**, 2631–2641.

Holmes, A. J., Roslev, P., McDonald, I. R., Iversen, N., Henriksen, K. & Murrell, J. C. (1999). Characterization of methanotrophic bacterial populations in soils showing atmospheric methane uptake. *Appl Environ Microbiol* **65**, 3312–3318.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Khmelenina, V. N., Kalyuzhnaya, M. G., Starostina, N. G., Suzina, N. E. & Trotsenko, Y. A. (1997). Isolation and characterization of halotolerant alkaliphilic methanotrophic bacteria from Tuva soda lakes. *Curr Microbiol* **35**, 257–261.

Khmelenina, V. N., Kalyuzhnaya, M. G., Sakharovsky, V. G., Suzina, N. E., Trotsenko, Y. A. & Gottschalk, G. (1999). Osmoadaptation in halophilic and alkaliphilic methanotrophs. *Arch Microbiol* **172**, 321–329.

Lontoh, S. & Semrau, J. D. (1998). Methane and trichloroethylene degradation by *Methylosinus trichosporium* OB3b expressing particulate methane monooxygenase. *Appl Environ Microbiol* **64**, 1106–1114.

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

Lysenko, A. M., Gal'chenko, V. F. & Chernykh, N. A. (1988). Taxonomic study of obligate methanotrophic bacteria using the DNA–DNA hybridization technique. *Microbiology* (English translation of *Mikrobiologiya*) **57**, 653–658.

Owen, R. J., Lapage, S. P. & Hill, L. R. (1969). Determination of base composition from melting profiles in dilute buffers. *Biopolymers* **7**, 503–516.

Roslev, P. & Iversen, N. (1999). Radioactive fingerprinting of microorganisms that oxidize atmospheric methane in different soils. *Appl Environ Microbiol* **65**, 4064–4070.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Socolofsky, M. D. & Wyss, O. (1961). Cysts of *Azotobacter*. *J Bacteriol* **81**, 946–954.

Suzina, N. E., Duda, V. I., Anisimova, L. A., Dmitriev, V. V. & Boronin, A. M. (1995). Cytological aspects of resistance to potassium tellurite conferred on *Pseudomonas* cells by plasmids. *Arch Microbiol* **163**, 282–285.

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

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

Whittenbury, R., Phillips, K. C. & Wilkinson, J. F. (1970a). Enrichment, isolation and some properties of methane-utilizing bacteria. *J Gen Microbiol* **61**, 205–218.

Whittenbury, R., Davies, S. L. & Davey, J. F. (1970b). Exospores and cysts formed by methane-utilizing bacteria. *J Gen Microbiol* **61**, 219–226.