First publ. in: International Journal of Systematic and Evolutionary Microbiology 57 (2007), 1073-1080

Methylosoma difficile gen. nov., sp. nov., a novel methanotroph enriched by gradient cultivation from littoral sediment of Lake Constance

Monali Rahalkar, Ingeborg Bussmann and Bernhard Schink

LS Mikrobielle Ökologie, Fachbereich Biologie, Universität Konstanz, Fach M 654, 78457 Konstanz, Germany

Correspondence
Bernhard Schink
bernhard.schink@uni-konstanz.de

A novel methanotroph, strain LC 2^T, was isolated from the littoral sediment of Lake Constance by enrichment in opposing gradients of methane and oxygen, followed by traditional isolation methods. Strain LC 2^T grows on methane or methanol as its sole carbon and energy source. It is a Gram-negative, non-motile, pale-pink-coloured methanotroph showing typical intracytoplasmic membranes arranged in stacks. Cells are coccoid, elliptical or rod-shaped and occur often in pairs. Strain LC 2^T grows at low oxygen concentrations and in counter-gradients of methane and oxygen. It can grow on medium free of bound nitrogen, possesses the *nifH* gene and fixes atmospheric nitrogen at low oxygen pressure. It grows at neutral pH and at temperatures between 10 and 30 °C. Phylogenetically, it is most closely related to the genus *Methylobacter*, with the type strains of *Methylobacter tundripaludum* and *Methylobacter psychrophilus* showing 94 and 93.4 % 16S rRNA gene sequence similarity, respectively. Furthermore, the *pmoA* gene sequence of strain LC 2^T is most closely related to *pmoA* gene sequences of *Methylobacter* strains (92 % similar to *Methylobacter* sp. LW 12 by deduced amino acid sequence identity). The DNA G+C content is 49.9 mol% and the major cellular fatty acid is 16 : 1ω7*c* (60 %). Strain LC 2^T (=JCM 14076^T=DSM 18750^T) is described as the type strain of a novel species within a new genus, *Methylosoma difficile* gen. nov., sp. nov.

INTRODUCTION

Aerobic methane-oxidizing bacteria (MOB) or methanotrophs are a unique and important group of bacteria which act as natural filters, controlling the release of methane, an important greenhouse gas, from anoxic sediments and soils. Aerobic MOB in freshwater environments such as lake sediments are active at the zone where methane and oxygen meet. In a previous study from our lab, a specific cultivation set-up was developed in which methanotrophic bacteria were grown in opposing gradients of methane and oxygen (Bussmann *et al.*, 2006). A novel bacterial strain, LC 2^T, was isolated which was found to be moderately related to the *Methylobacter–Methylosarcina* group (92–94 % similarity) and less related to the other genera of MOB (similarity 90–91 %), e.g. *Methylomonas* and *Methylomicrobium*, as determined by 16S rRNA gene sequence comparison. The closest relatives of this new strain were *Methylobacter* species such as *Methylobacter psychrophilus* (Tourova *et al.*, 1999) and the recently described *Methylobacter tundripaludum* SV96^T (Wartiainen *et al.*, 2006), which are either psychrophilic or have been isolated from cold habitats. In culture-independent studies, we found that littoral sediment of Lake Constance is dominated by type I MOB (Bussmann *et al.*, 2006; Pester *et al.*, 2004). Among these, *Methylobacter*-like MOB (clone group B1) dominated clone libraries of the partial *pmoA* gene (Bussmann *et al.*, 2006) amplified with primers A189f–A682r (Holmes *et al.*, 1995) as well as with primers A189f–mb661r (Costello & Lidstrom, 1999) and contributed to the major peaks observed in T-RFLP studies from the littoral sediment of Lake Constance (Pester *et al.*, 2004). Recently, many *pmoA* and 16S rRNA gene sequences related to the genus *Methylobacter* have been reported as dominant groups in other freshwater habitats such as freshwater wetland marshes (Bodelier *et al.*, 2005) and lakes such as Mono Lake (Lin *et al.*, 2005) and Lake Washington (Nercessian *et al.*, 2005), estuarine habitats (McDonald *et al.*, 2005), as well as in chironomid larvae in lake sediments (Eller *et al.*, 2005). To date, a total of 13 genera of methanotrophs have been described, which belong to either the class *Alphaproteobacteria* (type II MOB) or *Gammaproteobacteria* (type I MOB). Recently, it was discovered that *Crenothrix polyspora* Cohn, a well-known, uncultured filamentous bacterium, is a methane oxidizer, and its 16S rRNA genes (total of four clusters) formed a new group within the family

Abbreviations: MOB, methane-oxidizing bacteria; sMMO, soluble methane monooxygenase.

The GenBank/EMBL/DDBJ accession numbers for the complete 16S rRNA gene sequence and partial *pmoA* and *nifH* gene sequences of strain LC 2^T are respectively DQ119050, DQ119047 and DQ665842.

Methylococcaceae which also branched near *Methylobacter psychrophilus* and related methanotrophs (Stoecker *et al.*, 2006).

The purpose of the present study is to characterize strain LC 2^T formally and to determine its correct taxonomical position. Although this strain was isolated from a gradient culture, it could also grow on solid and in liquid media.

METHODS

Isolation and growth conditions. Strain LC 2^T was isolated from littoral sediment of Lake Constance after an initial enrichment in a gradient culture followed by transfer into a liquid dilution series and finally on solid agarose medium, as described in detail by Bussmann *et al.* (2006). Cells were grown in dilute mineral medium (Bussmann *et al.*, 2006) either in liquid medium, on solid medium (with 1.2 % agarose NEEO, Ultraquality; Roth) with 0.01 % cyclo heximide or in a gradient system (0.2 % agarose) with opposing gradients of methane and oxygen as described before (Bussmann *et al.*, 2006). Growth on 1.5 % agar (BD Biosciences) was also checked. The diluted medium as described in Bussmann *et al.* (2004) contained the following salts (per litre): 0.1 g NaCl, 0.04 g $MgCl_2.6H_2O$, 0.05 g KCl, 0.015 g $CaCl_2.2H_2O$, 0.016 g Na_2SO_4 and trace element solution SL 10 (1 ml l^{-1}) (Widdel, 1988). Potassium/sodium phosphate buffer (pH 7.2) and KNO_3 were added to final concentrations of 150 μM and 50 μM, respectively, and the medium was buffered to pH 7.2 with 0.01 M HEPES. In addition, a seven vitamin solution (Widdel & Pfennig, 1981) was added (1 ml l^{-1}). Strain LC 2^T was checked also for growth in: (i) classical nitrate mineral salt medium (Whittenbury *et al.*, 1970) with the trace ele ment solution replaced by SL 10 solution, (ii) low nitrate mineral salt medium (1/10 concentration of potassium nitrate, pH 7.0 with HEPES buffer) and (iii) medium with 10 fold concentrated version of the above mentioned mineral medium (Bussmann *et al.*, 2004). The effect of copper on growth was checked by inoculating the cul ture with additional 1 5 μM $CuSO_4$ in liquid medium. The basal concentration of copper in the mineral medium was very low (0.011 μM). Strain LC 2^T was grown in 100 ml flasks or 15 ml glass tubes with 25 30 or 5 ml medium, respectively, in closed desicca tors. The strain was maintained by streaking single colonies on solid agarose plates or by inoculating a single colony in liquid medium and then streaking the grown liquid culture on agarose plates. Until about a year after isolation, strain LC 2^T was grown in closed desic cators under a gas atmosphere of 24 % methane, 2 % CO_2, 17 % O_2 and the balance N_2 (Bussmann *et al.*, 2006). As the strain grew well in gradients and appeared to grow at low oxygen tension (Bussmann *et al.*, 2006), the gas atmosphere for growing this culture was modi fied slightly to 20 % methane, 2 % CO_2, 30 % air (around 6 % O_2) and the balance N_2. All growth experiments performed in this study were performed with this modified gas atmosphere in the dark at 16 or 21 °C, unless otherwise mentioned. The strain was also tested for growth in closed 150 ml bottles with different volumes of liquid medium and a gas phase of methane and air (20 : 80). Possible pre sence of heterotrophic contaminants was checked every time by plat ing on 1 : 10 diluted nutrient agar plates with an additional 0.05 % yeast extract. For long term storage, glycerol stocks were prepared and preserved at 70 °C. Survival of the strain after such preserva tion was checked by inoculation into fresh medium. Cultures were also preserved at 4 °C for long term storage. Ten per cent inoculum was used in all growth experiments and the incubation period was 8 weeks. Since the bacteria tended to grow as a faint pink biofilm at the bottom, the tube or flask was shaken well to suspend the cells uniformly before the OD_{570} was recorded. Growth was always con firmed by phase contrast microscopy.

Morphological characterization and electron microscopy. Cells were observed under a phase contrast microscope (Axiophot; Zeiss) and photographed with a cooled charge couple device camera (Magnafire; INTAS). For fixation for electron microscopy, 50 ml of an exponentially growing culture was spun down at 5000 ***g***. The cell pellet was washed once with PBS (50 mM potassium phosphate buffer, pH 7.5, plus 0.9 % NaCl) and then suspended in 1 ml PBS. Glutaraldehyde (140 μl of a 25 % aqueous solution) was added to the cell suspension, which was then incubated overnight at 4 °C, before the cells were centrifuged again at 5000 ***g***. The pellet was washed again in PBS and finally suspended in around 150 μl PBS. After chemical fixation, cells were embedded in 1.5 % (w/v) molten agar (final concentration). The agar block was cut into small pieces of 1 mm^3 and the pieces were dehydrated in a graded methanol series (v/v: 15 and 30 % for 15 min; 50, 75 and 95 % for 30 min; 100 % for 1 h) under concomitant temperature reduction to 40 °C. The samples were infiltrated with Lowicryl K4M resin [in methanol (v/v): 50 % for 1 h; 66 % for 2 h; 100 % for 10 h] and then polymer ized for 24 h at 40 °C and for 3 days at room temperature (Roth *et al.*, 1981; Hoppert & Holzenburg, 1998). Resin sections of 80 100 nm thickness were cut with glass knives. Electron microscopy was performed in a Philips EM 301 transmission electron micro scope at 80 kV with calibrated magnifications. To check for the pre sence of cellular appendages like flagella or pili, negative stainings were prepared with fresh cell material.

Utilizable carbon and nitrogen sources. Utilization of various carbon sources was studied in liquid mineral medium supplemented with one of the following filter sterilized substrates (0.1 %, w/v): formate, formamide, arabinose, raffinose, lactose, maltose, xylose, glucose, fructose and sucrose. The ability of the strain to grow on methanol and formaldehyde was tested at lower concentrations (10 50 mM methanol and 1 50 mM formaldehyde). Other sub strates such as acetate (2 and 10 mM) were tested for growth in liquid medium to check for heterotrophic growth (Dedysh *et al.*, 2005) of strain LC 2^T. Nitrogen sources were tested with liquid medium in which KNO_3 was replaced by one of the following com pounds at 0.05 % (w/v): NH_4Cl, urea, glycine, serine, valine, aspara gine, aspartate, L glutamic acid, glutamate, peptone and yeast extract. To test the ability of the culture to fix atmospheric N_2, media free of bound nitrogen compounds were used. The acetylene reduction test was done with cultures grown with 10 mM methanol without any bound nitrogen source, as modified by Auman *et al.* (2001). Briefly, 25 ml of such a grown culture was transferred to a bottle with a rubber stopper and was gassed with nitrogen; air and acetylene were added (89 % N_2: 9 % air: 1 % acetylene) and incubated over night. The gas phase was checked for ethylene production by gas chromatography.

Optimum pH, temperature and salt content. The optimum pH and temperature ranges were determined in liquid medium. Growth at pH 2.6 9.0 was checked after buffering the medium with citrate/phosphate buffer (pH 2.6 6.6), HEPES buffer (pH 7 and 7.5) and glycine buffer (pH 8 and 9). Growth was also checked without using any buffer, but using only HCl or NaOH to adjust the pH. Strain LC 2^T was grown at a temperature range of 4 37 °C in liquid medium. To determine the optimum salt concentration, additional NaCl (0.5, 1, 1.5 and 2.0 %, w/v) was added to the mineral medium. The basal NaCl concentration in the mineral medium was 0.01 % (w/v).

Resistance to desiccation and heat. Heat resistance was tested by heating cell suspensions at 50, 60, 70 or 80 °C for 10 min each followed by plating onto solid medium and incubating under opti mal conditions for 2 3 weeks. Desiccation resistance was assessed according to Whittenbury *et al.* (1970) by air drying suspensions of strain LC 2^T on glass slides and then inoculating into medium after an interval of 1 4 weeks. Formation of exospores was checked for

by heating a 3–4 week old culture at 80 °C for 20 min and then looking for colony formation after incubation under standard conditions (Bowman *et al.*, 1993). Cysts were stained according to Vela & Wyss (1964).

Cellular fatty acid analysis. Phospholipid fatty acid analyses were performed at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). Strain LC 2^T was grown in 200 ml flasks, in closed desiccators. Cells were pelleted, freeze dried and sent for phospholipid fatty acid analysis. There, the cells were saponified and methylated and the methyl esters were extracted and subjected to GC. The GC elution profile of the fatty acid methyl esters was compared with the fatty acid patterns stored in the fatty acid database of the Microbial Identification System (MIDI Inc.) and qualitative and quantitative compositions of the pattern were given.

Presence of soluble methane monooxygenase (sMMO) and nitrogenase. The presence of sMMO was checked by PCR amplification of the *mmoX* gene with primers *mmoX*A *mmoX*B (Auman *et al.*, 2000) as well as by colorimetric assay (Graham *et al.*, 1992). To check for the presence of nitrogenase, the *nifH* gene was amplified as described by Poly *et al.* (2001), partially sequenced and subjected to BLAST search at the NCBI (http://www.ncbi.nlm.nih.gov/) (Altschul *et al.*, 1990).

DNA extraction, phylogenetic analysis and G+C content. DNA was extracted and the complete 16S rRNA gene sequence was determined as described previously (Bussmann *et al.*, 2006). 16S rRNA gene sequences of type I methanotrophs of the family *Methylococcaceae* along with newly described sequences of *Crenothrix polyspora* and sequences of some clones were obtained after BLAST search. Phylogenetic analysis was done using the ARB software package (version 2.5b) (http://www.arb-home.de; Ludwig *et al.*, 2004). The new sequences were added to the ARB database and aligned using the FAST Aligner tool as implemented in ARB. Alignments were checked and corrected manually where necessary. Sequences of 1419 nucleotides were used for alignment. Only those positions that were identical in at least 50 % of all sequences were used to create a filter. Phylogenetic analysis was done using the maximum likelihood, neighbour joining and maximum parsimony algorithms as implemented in ARB (Ludwig *et al.*, 2004). Phylogenetic distances were also determined by using the similarity matrix in ARB without using any filter and also with a sequence of *Escherichia coli* as the filter. Phylogenetic analysis of the *pmoA* gene was done as described earlier (Bussmann *et al.*, 2006), and phylogenetic trees were constructed based on 164 amino acids. G+C content was measured at the DSMZ by HPLC by a method adapted from Tamaoka & Komagata (1984) and calculated according to the method of Mesbah *et al.* (1989).

RESULTS AND DISCUSSION

Isolation and growth characters of strain LC 2^T

Strain LC 2^T was isolated after enrichment in a gradient culture system obtained after a final dilution of 8×10^{-4} of the littoral sediment, after further transfers on liquid and solid media (Bussmann *et al.*, 2006). The strain was isolated from a mixed culture in which it was associated with thin rods. When isolated, the colonies were much bigger and more mucoid and watery than after extended cultivation. This could be due to a tendency of the cells to form aggregates or to the presence of an initially contaminating bacterium. The strain grew in liquid culture at low oxygen tensions, up to 10 % air, i.e. 2 % oxygen. In gradients, it formed two bands, the deepest one being 32 mm from the air-exposed end (Bussmann *et al.*, 2006). Strain LC 2^T also grew well on 1.5 % agar, although agarose was used routinely. The strain could not be maintained on solid medium by repeated transfers. Therefore, a single colony was always grown in liquid culture and then streaked on solid medium. No active motility could be detected 2 years after isolation. Cells occurred singly or in pairs or sometimes in chains of four or five cells (especially on solid medium) or in aggregates. In liquid medium, strain LC 2^T formed a mucoid pink biofilm at the bottom of the flask. If the flask was shaken, a whorl of mucoid biofilm appeared which was held together at a small point in the centre of the flask. After shaking, the cells could be suspended uniformly. Cells also became oval or elliptical in shape and changed from coccoid to rod-shaped forms in older cultures. Liquid cultures grew better in magnetically stirred flasks inside a desiccator. Strain LC 2^T resembled *Methylobacter tundripaludum* SV96^T in colony colour and cell size (Table 1). Comparison of strain LC 2^T with members of other methanotrophic genera is shown in Table 1.

Table 1. Comparison of strain LC 2^T with other methanotrophic genera

Characteristic	*Methylomonas*	*Methylomicrobium*	*Methylosarcina**	*Methylosphaera*	*Methylobacter*	Strain LC 2^T
Cell morphology	Rods	Rods	Sarcina shaped or cocci	Cocci	Rods or cocci	Cocci, elliptical or rods
Motility	+	+	Variable		Variable	
Cyst formation	+		Variable		+	+
Nitrogenase genes	Variable			+	+	+
Pigmentation	Pink, yellow ochre	White	Light brown buff		Yellow, brown or pale pink†	Pale pink
G+C content (mol%)	51–59	50–60	53–54	43–46	49–54	49.9

*Except *Methylosarcina lacus*.
†*Methylobacter tundripaludum*.

Strain LC 2^T did not survive in glycerol stock cultures. After initial growth at optimum temperature, the strain survived well at 4 °C for 2–3 months. Poor growth was observed at increased copper concentrations. The strain grew well in dilute nitrate mineral salt medium and undiluted mineral medium but grew poorly in the classical nitrate mineral salt medium. It also grew well in non-shaken closed bottles (150 ml), with 15–20 ml medium and 20 % methane in the gas phase.

Morphological characterization and electron microscopy

Strain LC 2^T formed pale-pink colonies, around 1–2 mm diameter on agarose plates and 2–3 mm diameter on agar, after incubation for 2–3 weeks (Table 1). Coccoid cells were observed (often in pairs) by phase-contrast microscopy which were 1.5–2 μm in length and approximately 1 μm in diameter (Fig. 1a). Intracytoplasmic membranes were arranged in stacks, mainly at the cell periphery (Fig. 1b), which is a feature typical of type I methanotrophs (Whittenbury *et al.*, 1970). Cells contained large polyhydroxyalkanoate granules and, very probably, also glycogen granules, which stained dark.

Ultrathin sections of the cell periphery exhibited an appearance typical of a Gram-negative cell envelope, with two dark, thin layers of the outer membrane, a peptidoglycan layer located in the periplasm and a cytoplasmic membrane (Fig. 1c). Flagella were absent, but pili approximately 5 nm in width and up to 2 μm in length could be detected infrequently.

Utilizable carbon and nitrogen sources

Strain LC 2^T grew only on methane or methanol (10–50 mM). No other carbon substrates were utilized. Of the different nitrogen sources checked, it utilized nitrate, L-glutamine, L-glutamic acid, L-asparagine and L-aspartic acid. Growth on organic nitrogen sources such as peptone and yeast extract was better and faster than growth on nitrate. Strain LC 2^T grew without any bound nitrogen source under standard gas conditions. The acetylene reduction test was positive, although the ethylene peak was very small and the reaction required overnight incubation.

Effect of pH, temperature and NaCl concentration on growth

Strain LC 2^T grew at pH 5–9 when no additional buffers were used, with the best growth at pH 6–8. When the medium was buffered, growth was observed only around neutral pH, which might be due to the sensitivity of the strain to high concentrations of organic compounds. Strain LC 2^T grew in a temperature range of 16–30 °C, the optimum growth temperature being around 25 °C. The specific growth rates at 16, 25 and 30 °C were 0.0024, 0.0041 and 0.0065 h^{-1}. Although growth was fast at 30 °C, growth declined after reaching an OD_{570} of around 0.15, which might be due to an imbalance of its metabolism. Little growth was observed at 10 °C, and no growth was observed at 37 or 4 °C. NaCl added to the medium to concentrations of 0.5–2 % inhibited growth.

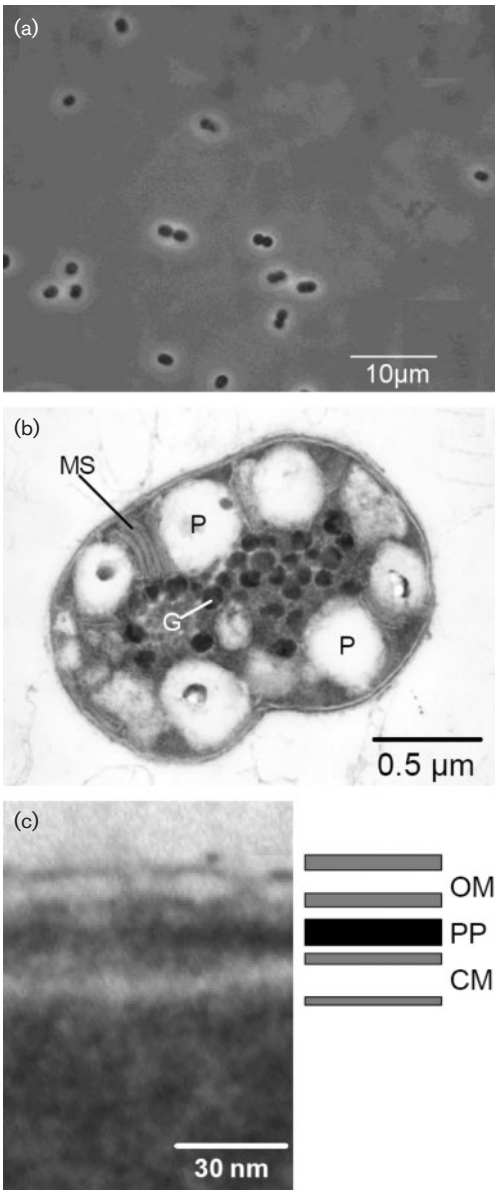

Fig. 1. Morphology of strain LC 2^T. (a) Phase-contrast micrograph of cells grown in liquid medium with methane. (b) Ultrathin sections showing membrane stacks (MS), polyhydroxyalkanoate (P) granules and glycogen (G) granules. (c) Ultrathin section of the cell periphery with the typical appearance of a Gram-negative cell envelope: two dark, thin layers of the outer membrane (OM), one dark, thick layer of peptidoglycan located in the periplasm (PP) and two dark layers of the cytoplasmic membrane (CM).

Resistance to desiccation and heat and formation of exospores

Strain LC 2^T grew after a heat shock at 50 and 60 °C for 10 min, but did not grow after a heat shock at 70 °C. It formed neither microcolonies after exposure to 80 °C nor exospores. Even though cysts were frequently observed in older cultures, they were not resistant to desiccation for 1 week.

Cellular fatty acid analysis

Strain LC 2^T showed a pattern of fatty acids unique compared with representatives of the related type I methanotrophic genera *Methylobacter*, *Methylosarcina*, *Methylomicrobium* and *Methylomonas*, although the patterns of the genus *Methylobacter* were the closest (Table 2). Fatty acid patterns of *Crenothrix polyspora* were not available and thus could not be compared. The major fatty acid was 16 : 1ω7*c*. Strain LC 2^T also contained 12 : 0 fatty acids, which have been observed only in the genus *Methylosarcina*. The proportions of 16 : 1ω7*c*, 14 : 0 and 16 : 0 were comparable to those of members of the neighbouring genus *Methylobacter*. Strain LC 2^T contained 15 % 16 : 1ω6*c*, which was similar to the values found in the genera *Methylomicrobium* and *Methylomonas*. There was no 16 : 1ω5*c* or 5*t* fatty acid, as seen in almost all other genera except *Methylosarcina*, which has a very low percentage of 16 : 1ω5*c*. In addition, strain LC 2^T also showed minor amounts of 15 : 0, 16 : 1ω11*c* and 16 : 0 3-OH fatty acids, which have not been found so far in other type I methanotrophs.

Absence of sMMO and presence of nitrogenase

Strain LC 2^T did not show the presence of sMMO as determined by PCR of *mmoX* genes or colorimetric assay. The presence of nitrogenase was confirmed by PCR amplification of a partial *nifH* gene. After NCBI BLAST, the partial sequence of around 300 nt was found to be 90 % similar to the *nifH* gene of an uncultured bacterium NR1620 (GenBank accession no. AF035504) and 86–88 % similar to partial *nifH* gene sequences of *Methylobacter* species, the closest being *Methylobacter luteus* (88 %). The partial *nifH* gene sequence of *Methylobacter tundripaludum* SV96^T was 86 % similar to that of strain LC 2^T.

Phylogenetic analysis and G+C content

The complete 16S rRNA gene sequence and partial *pmoA* gene sequence of strain LC 2^T were determined in previous studies; the 16S rRNA gene sequence was confirmed again after 1.5 years of cultivation, by cloning, screening of 20 clones by RFLP as described before (Bussmann *et al.*, 2006) and sequencing of two clones. The sequences were 99.7 % identical to the deposited sequence, with only two or three bases different, confirming the sequence and the stability of the culture under the present growth conditions. In the phylogenetic tree, the position of strain LC 2^T was between the group of *Methylobacter psychrophilus*-like bacteria and *C. polyspora* (Stoecker *et al.*, 2006) (Fig. 2). According to similarity values calculated using the ARB program (with or without *E. coli* as a filter), the closest cultured relatives are *Methylobacter tundripaludum* SV96^T (94 % similarity) and *Methylobacter psychrophilus* Z-0021^T (93.4 %). Different gene clusters of *C. polyspora* and other *Methylobacter* species showed similarity of 92–93 %. BLAST results revealed that the *pmoA* nucleotide sequence of strain LC 2^T differed by 14 % from the *pmoA* sequences of *Methylobacter* sp. strain LW12 and *Methylobacter* sp. strain BB5.1 and by more than 15 % from the other described species of *Methylobacter*. The amino acid similarities were 92 % to *Methylobacter* sp. LW 12 and *Methylomicrobium buryatense* 5B^T and 93 % to clone B67 and clone A55 from Lake Constance littoral sediment

Table 2. Cellular fatty acids of strain LC 2^T in comparison with other type I methanotrophs

Values are percentages of total fatty acids. Data for reference genera were taken from Bowman *et al.* (1995) (*Methylomonas*, *Methylomicrobium*, *Methylococcus* and *Methylobacter*) and Wise *et al.* (2001) (*Methylosarcina*). NR, Not reported; ND, not detectable.

Fatty acid	*Methylomonas*	*Methylomicrobium*	*Methylosarcina*	*Methylococcus*	*Methylobacter*	Strain LC 2^T
12 : 0	NR	NR	3 ± 0.4	NR	NR	2.74
14 : 0	22 ± 3	1 ± 1	1.5 ± 0.5	< 1	9 ± 2	8.55
16 : 1ω8*c*	30 ± 11	16 ± 3	15 ± 1	ND	ND	ND
16 : 1ω7*c*	11 ± 4	17 ± 3	28 ± 3	28 ± 10	57 ± 1	60
16 : 1ω6*c*	9 ± 4	10 ± 4	8 ± 1	3 ± 2	5 ± 1	15
16 : 1ω5*c*	4 ± 2	6 ± 1	< 1	3 ± 2	7 ± 1	ND
16 : 1ω5*t*	12 ± 4	20 ± 10	25 ± 5	< 1	11 ± 1	ND
16 : 0	7 ± 2	15 ± 3	19 ± 1	44 ± 8	8 ± 1	8.5
15 : 0	NR	NR	NR	NR	NR	0.79
16 : 1ω11*c*	NR	NR	NR	NR	NR	2.44
16 : 1 3 OH	NR	NR	NR	NR	NR	1.31

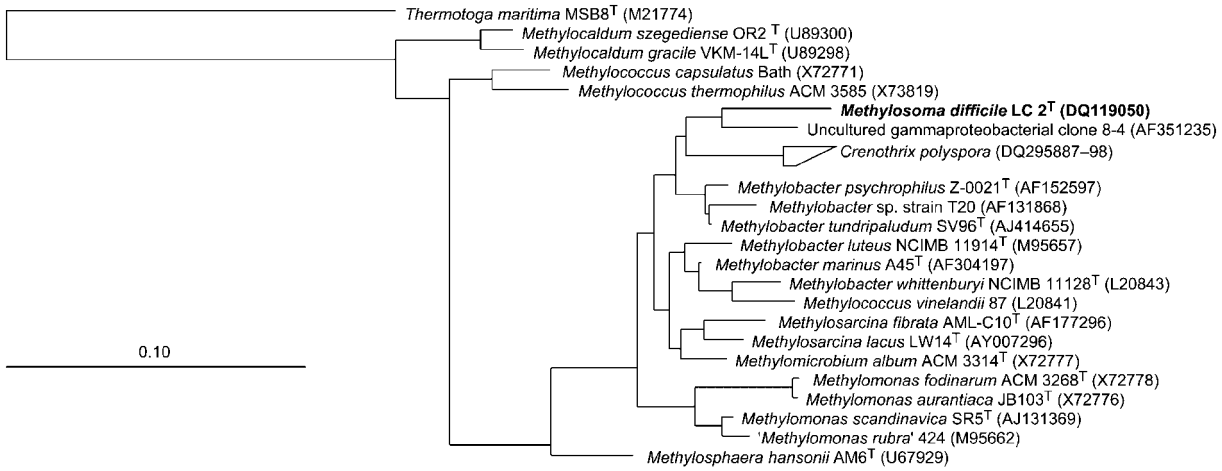

Fig. 2. Maximum-likelihood tree of the 16S rRNA gene sequence of strain LC 2^T in comparison with other methanotrophic cultured members and clones. Strain numbers are indicated for species and GenBank accession numbers are given in parentheses. Bar, 10 % sequence divergence.

(Bussmann *et al.*, 2006). The unique position of the strain LC 2^T sequence in the *pmoA* tree is shown in Fig. 3.

The G+C content of strain LC 2^T as determined by HPLC was 49.9 mol%. Comparison of strain LC 2^T with other genera of methanotrophs is shown in Table 1. The sequence differences in the 16S rRNA genes of strain LC 2^T and species of *Methylobacter* with validly published names are between 6 and 8 % and are therefore too large to allow strain LC 2^T to be accommodated in the genus *Methylobacter*. Differences in 16S rRNA gene sequences between *C. polyspora* and strain LC 2^T are around 7 %. Beyond this, the morphological difference of filamentous versus non-filamentous growth prevents us from including our strain in the genus *Crenothrix*. Thus, strain LC 2^T has to be described as a member of a new genus, *Methylosoma*, within the type I MOB, as *Methylosoma difficile* gen. nov., sp. nov.

Description of *Methylosoma* gen. nov.

Methylosoma [Me.thy.lo.so′ma. N.Gr. prefix *methylo* referring to methyl groups; Gr. neut. n. *soma* body; N.L. neut. n. *Methylosoma* a methyl group (-utilizing) body].

Obligately aerobic, C_1-compound-utilizing bacteria. Cells possess a typical membrane system with stacks of intracytoplasmic membranes, typical of type I methanotrophs. Gram-negative. Phylogenetic analysis and morphological characters place the genus close to the genus *Methylobacter*. The type and only known species is *Methylosoma difficile*.

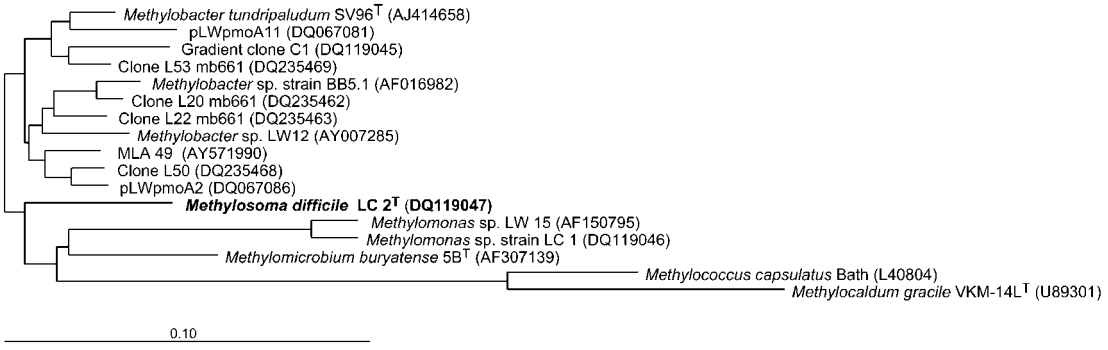

Fig. 3. Neighbour-joining tree based on the derived amino acid sequences of the *pmoA* gene of strain LC 2^T and from other cultured and uncultured methanotrophs. Strain numbers are indicated for species and GenBank accession numbers are given in parentheses. Bar, 10 % sequence divergence.

Description of *Methylosoma difficile* sp. nov.

Methylosoma difficile (dif.fi.ci'le. L. neut. adj. *difficile* difficult, referring to difficulties in cultivating the type strain).

Large, coccoid cells, 1.5–2 μm in length and approximately 1 μm in diameter, often occurring in pairs, chains or aggregates; sometimes also slightly elongated rod-shaped forms. Non-motile; division by binary fission, forming cysts which are not resistant to heat or desiccation. Cells are pale-pink-pigmented and do not possess sMMO. Microaerobic, growing best at low (2 %) oxygen tensions. Uses methane or methanol (10–50 mM) as the sole carbon source and nitrate, L-glutamine, L-glutamic acid, L-asparagine or L-aspartic acid as the nitrogen source. Fixes atmospheric nitrogen; *nifH* gene present. Grows best at 16–30 °C and at neutral pH. Does not grow with enhanced concentrations of NaCl or copper. The major fatty acid is 16 : 1ω7*c*, followed by 16 : 1ω6*c*, 14 : 0 and 16 : 0. G+C content of the DNA of the type strain is 49.9 mol% (HPLC determination). Habitat: sediments of freshwater lakes, at the interface of oxic and methane-supplied sediment layers.

The type strain, LC 2^T (=DSM 18750^T=JCM 14076^T), was isolated from littoral sediment of Lake Constance in May 2004.

ACKNOWLEDGEMENTS

This study was supported by Sonderforschungsbereich (SFB) 454 on the Deutsche Forschungsgemeinschaft, Bonn, and research funds of the Universität Konstanz. We wish to thank especially Dr Michael Hoppert, University of Göttingen, for performing the electron microscopic characterization.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403 410.

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

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

Bodelier, P. L. E., Meima-Franke, M., Zwart, G. & Laanbroek, H. J. (2005). New DGGE strategies for the analyses of methanotrophic microbial communities using different combinations of existing 16S rRNA based primers. *FEMS Microbiol Ecol* **52**, 163 174.

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.

Bowman, J. P., Sly, L. I. & Stackebrandt, E. (1995). The phylogenetic position of the family *Methylococcaceae*. *Int J Syst Bacteriol* **45**, 182 185.

Bussmann, I., Pester, M., Brune, A. & Schink, B. (2004). Preferential cultivation of type II methanotrophic bacteria from littoral sediments (Lake Constance). *FEMS Microbiol Ecol* **47**, 179 189.

Bussmann, I., Rahalkar, M. & Schink, B. (2006). Cultivation of methanotrophic bacteria in opposing gradients of methane and oxygen. *FEMS Microbiol Ecol* **56**, 331 344.

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

Dedysh, S. N., Knief, C. & Dunfield, P. F. (2005). *Methylocella* species are facultatively methanotrophic. *J Bacteriol* **187**, 4665 4670.

Eller, G., Deines, P., Grey, J., Richnow, H.-H. & Krüger, M. (2005). Methane cycling in lake sediments and its influence on chironomid larval δ^{13}C. *FEMS Microbiol Ecol* **54**, 339 350.

Graham, D. W., Korich, D. G., Leblanc, R. P., Sinclair, N. A. & Arnold, R. G. (1992). Applications of a colorimetric plate assay for soluble methane monooxygenase activity. *Appl Environ Microbiol* **58**, 2231 2236.

Holmes, A. J., Owens, N. J. P. & Murrell, J. C. (1995). Detection of novel marine methanotrophs using phylogenetic and functional gene probes after methane enrichment. *Microbiology* **141**, 1947 1955.

Hoppert, M. & Holzenburg, A. (1998). *Electron Microscopy in Microbiology*. Oxford: BIOS Scientific.

Lin, J.-L., Joye, S. B., Scholten, J. C. M., Schäfer, H., McDonald, I. R. & Murrell, J. C. (2005). Analysis of methane monooxygenase genes in Mono Lake suggests that increased methane oxidation activity may correlate with a change in methanotroph community structure. *Appl Environ Microbiol* **71**, 6458 6462.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S. & other authors (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363 1371.

McDonald, I. R., Smith, K. & Lidstrom, M. E. (2005). Methanotrophic populations in estuarine sediment from Newport Bay, California. *FEMS Microbiol Lett* **250**, 287 293.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159 167.

Nercessian, O. G., Noyes, E., Kaluyzhnaya, M. G., Lidstrom, M. E. & Chistoserdova, L. (2005). Bacterial populations active in metabolism of C1 compounds in the sediment of Lake Washington, a freshwater lake. *Appl Environ Microbiol* **71**, 6885 6899.

Pester, M., Friedrich, M. W., Schink, B. & Brune, A. (2004). *pmoA* based analysis of methanotrophs in a littoral lake sediment reveals a diverse and stable community in a dynamic environment. *Appl Environ Microbiol* **70**, 3138 3142.

Poly, F., Monrozier, L. J. & Bally, R. (2001). Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. *Res Microbiol* **152**, 95 103.

Roth, J., Bendayan, M., Carlemalm, E., Villiger, W. & Garavito, M. (1981). Enhancement of structural preservation and immunocytochemical staining in low temperature embedded pancreatic tissue. *J Histochem Cytochem* **29**, 663 671.

Stoecker, K., Bendinger, B., Schoning, B., Nielsen, P. H., Nielsen, J. L., Baranyi, C., Toenshoff, E. R., Daims, H. & Wagner, M. (2006). Cohn's *Crenothrix* is a filamentous methane oxidizer with an unusual methane monooxygenase. *Proc Natl Acad Sci U S A* **103**, 2363 2367.

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed phase high performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125 128.

Tourova, T. P., Omel'chenko, M. V., Fegeding, K. V. & Vasiléva, L. V. (1999). The phylogenetic position of *Methylobacter psychrophilus*

sp. nov. *Microbiology* (English translation of *Mikrobiologiia*) **68**, 568 570.

Vela, G. R. & Wyss, O. (1964). Improved stain for visualization of *Azotobacter* encystment. *J Bacteriol* **87**, 476 477.

Wartiainen, I., Hestnes, A. G., McDonald, I. R. & Svenning, M. M. (2006). *Methylobacter tundripaludum* sp. nov., a methane oxidizing bacterium from Arctic wetland soil on the Svalbard islands, Norway (78° N). *Int J Syst Evol Microbiol* **56**, 109 113.

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

Widdel, F. (1988). Microbiology and ecology of sulfate and sulfur reducing bacteria. In *Biology of Anaerobic Microorganisms*, pp. 469 585. Edited by A. J. Zehnder. Chichester: Wiley.

Widdel, F. & Pfennig, N. (1981). Studies on dissimilatory sulfate reducing bacteria that decompose fatty acids. I. Isolation of a new sulfate reducer enriched with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov. sp. nov. *Arch Microbiol* **129**, 395 400.

Wise, M. G., McArthur, J. & Shimkets, L. J. (2001). *Methylosarcina fibrata* gen. nov., sp. nov. and *Methylosarcina quisquiliarum* sp. nov., novel type I methanotrophs. *Int J Syst Evol Microbiol* **51**, 611 621.