**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

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 *Methyllobacter 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 : 1 ω7c (80 %). 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.

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**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; Pester et al., 2004). Among these, *Methylhabacter*-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 I MOB) or *Gammaproteobacteria* (type II 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 *Methylloclustus*. 

**Abbreviations:** MOB, methane-oxidizing bacteria; sMMO, soluble methane monoxygenase.
*Methylococcaceae* which also branched near *Methylobacter psychrophilus* and related methanotrophs (Stoeker et al., 2006).

The purpose of the present study is to characterize strain LC 2\(^1\) 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\(^1\) was isolated from litoral 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 % cycloheximide 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\(_2\)O, 0.05 g KCl, 0.015 g CaCl\(_2\)-2H\(_2\)O, 0.016 g Na\(_2\)SO\(_4\) 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\(^1\) was checked also for growth in (i) classical nitrate mineral salt medium (Whittenbury et al., 1970) with the trace element 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 culture 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\(^1\) was grown in 100 ml flasks or 15 ml glass tubes with 25–30 or 5 ml medium, respectively, in closed descicators. 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\(^1\) was grown in closed descicators 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 modified 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 \(\pm\) 1 °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 microscope at 80 kV with calibrated magnifications. To check for the presence 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 substrates, 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\(^1\). Nitrogen sources were tested with liquid medium in which KNO\(_3\) was replaced by one of the following compounds at 0.05 % (w/v): NH\(_4\)Cl, urea, glycine, serine, valine, asparagine, 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 overnight. 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\(^1\) was grown at a temperature range of 4–37 °C in the mineral 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 optimal conditions for 2–3 weeks. Desiccation resistance was assessed according to Whittenbury et al. (1970) by air-drying suspensions of strain LC 2\(^1\) on glass slides and then inoculating into medium after an interval of 1–4 weeks. Formation of exosporia was checked for

Morphological characterization and electron microscopy. Cells were observed under a phase-contrast microscope (Axioskop; Zeiss) and photographed with a cooled charge-couple device camera (Magnarite; 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 polymerized 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 microscope at 80 kV with calibrated magnifications. To check for the presence of cellular appendages like flagella or pili, negative stainings were prepared with fresh cell material.
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<sup>T</sup> 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 monoxygenase (sMMO) and nitrogenase.** The presence of sMMO was checked by PCR amplification of the mmoX gene with primers mmoXa–mmoXB (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 wherever 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<sup>T</sup>**

Strain LC 2<sup>T</sup> was isolated after enrichment in a gradient culture system obtained after a final dilution of 8 × 10<sup>-4</sup> 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<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup> resembled *Methylobacter tundripaludum* SV96<sup>T</sup> in colony colour and cell size (Table 1). Comparison of strain LC 2<sup>T</sup> with members of other methanotrophic genera is shown in Table 1.

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**Table 1.** Comparison of strain LC 2<sup>T</sup> with other methanotrophic genera

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>Methylosomas</em></th>
<th><em>Methylocrobiium</em></th>
<th><em>Methylosarcina</em>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>Methylophaera</em></th>
<th><em>Methylobacter</em></th>
<th>Strain LC 2&lt;sup&gt;T&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell morphology</td>
<td>Rods</td>
<td>Rods</td>
<td>Sarccina-shaped or cocci</td>
<td>Cocci</td>
<td>Rods or cocci</td>
<td>Cocci, elliptical or rods</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>+</td>
<td>Variable</td>
<td>-</td>
<td>Variable</td>
<td>-</td>
</tr>
<tr>
<td>Cyst formation</td>
<td>+</td>
<td>-</td>
<td>Variable</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrogenase genes</td>
<td>Variable</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pigmentation</td>
<td>Pink, yellow–ochre</td>
<td>White</td>
<td>Light brown–buff</td>
<td>Yellow, brown or pale pink†</td>
<td>Pale pink</td>
<td></td>
</tr>
<tr>
<td>G+C content (mol%)</td>
<td>51–59</td>
<td>50–60</td>
<td>53–54</td>
<td>43–46</td>
<td>49–54–49</td>
<td>49,9</td>
</tr>
</tbody>
</table>

<sup>a</sup>Except *Methylosarcina lacus.*

<sup>†</sup>*Methylobacter tundripaludum.*

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http://ijs.sgmjournals.org
Strain LC 2T 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 2T 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). Coccolid 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 2T 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 2T 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 2T 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 2T 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 OD₅₇₀ 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.
Resistance to desiccation and heat and formation of exospores

Strain LC 2T 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 2T showed a pattern of fatty acids unique compared with representatives of the related type I methanotrophic genera Methylobacter, Methylosarcina, Methylophilus 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 16:1ω7c. Strain LC 2T also contained 12:0 fatty acids, which have been observed only in the genus Methylomicrobium. The proportions of 16:1ω7c, 14:0 and 16:0 were comparable to those of members of the neighbouring genus Methylobacter. Strain LC 2T contained 15% 16:1ω6c, which was similar to the values found in the genera Methylophilus and Methylomonas. There was no 16:1ω5c or ω5 fatty acid, as seen in almost all other genera except Methylomicrobium, which has a very low percentage of 16:1ω5c. In addition, strain LC 2T also showed minor amounts of 15:0, 16:1ω11c 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 2T 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 SV96T was 86% similar to that of strain LC 2T.

Phylogenetic analysis and G+C content

The complete 16S rRNA gene sequence and partial pmoA gene sequence of strain LC 2T 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 97.7% 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 2T 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 SV96T (94% similarity) and Methylobacter psychrophilus Z-0021T (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 2T 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 described species of Methylobacter. The amino acid similarities were 92% to Methylobacter sp. LW12 and Methylomicrobium buryatense 5B5 and 93% to clone B67 and clone A55 from Lake Constance littoral sediment.

Table 2. Cellular fatty acids of strain LC 2T 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, Methylophilus, Methylomonas and Methylobacter) and Wise et al. (2001) (Methylomicrobium). NR, Not reported; ND, not detectable.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Methylophilus</th>
<th>Methylomonas</th>
<th>Methylophilus</th>
<th>Methylomicrobium</th>
<th>Methylobacter</th>
<th>Methylobacter</th>
<th>Methylobacter</th>
<th>Methylobacter</th>
<th>Strain LC 2T</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:0</td>
<td>NR</td>
<td>NR</td>
<td>3 ± 0.4</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>2.74</td>
</tr>
<tr>
<td>14:0</td>
<td>22 ± 3</td>
<td>1 ± 1</td>
<td>1.5 ± 0.5</td>
<td>&lt;1</td>
<td>9 ± 2</td>
<td>8.55</td>
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<td>16:1ω8c</td>
<td>30 ± 4</td>
<td>16 ± 3</td>
<td>15 ± 1</td>
<td>ND</td>
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<tr>
<td>16:1ω7c</td>
<td>11 ± 4</td>
<td>17 ± 3</td>
<td>28 ± 3</td>
<td>28 ± 10</td>
<td>57 ± 1</td>
<td>60</td>
<td></td>
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<tr>
<td>16:1ω6c</td>
<td>9 ± 4</td>
<td>10 ± 4</td>
<td>8 ± 1</td>
<td>3 ± 2</td>
<td>5 ± 1</td>
<td>15</td>
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<tr>
<td>16:1ω5c</td>
<td>4 ± 2</td>
<td>6 ± 1</td>
<td>&lt;1</td>
<td>3 ± 2</td>
<td>7 ± 1</td>
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<td></td>
<td></td>
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<tr>
<td>16:1ω5t</td>
<td>12 ± 4</td>
<td>20 ± 10</td>
<td>25 ± 5</td>
<td>&lt;1</td>
<td>11 ± 1</td>
<td>ND</td>
<td></td>
<td></td>
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<tr>
<td>16:0</td>
<td>7 ± 2</td>
<td>15 ± 3</td>
<td>19 ± 1</td>
<td>44 ± 8</td>
<td>8 ± 1</td>
<td>8.5</td>
<td></td>
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<tr>
<td>15:0</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>0.79</td>
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<td>16:1ω11c</td>
<td>NR</td>
<td>NR</td>
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<td></td>
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<td>2.44</td>
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<tr>
<td>16:1 3-OH</td>
<td>NR</td>
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(Bussmann et al., 2006). The unique position of the strain LC 2T sequence in the pmoA tree is shown in Fig. 3.

The G + C content of strain LC 2T as determined by HPLC was 49.9 mol%. Comparison of strain LC 2T with other genera of methanotrophs is shown in Table 1. The sequence differences in the 16S rRNA genes of strain LC 2T and species of Methylobacter with validly published names are between 6 and 8 % and are therefore too large to allow strain LC 2T to be accommodated in the genus Methylobacter. Differences in 16S rRNA gene sequences between C. polyspora and strain LC 2T 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 2T 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* [M.ethyl.oso.ma. N.Gr. prefix *methyo* referring to methyl groups; Gr. neut. n. *soma* body; N.L. neut. n. *Methylosoma* a methyl group (-utilizing) body].

Obligately aerobic, C1-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*.  

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**Fig. 2.** Maximum-likelihood tree of the 16S rRNA gene sequence of strain LC 2T 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.

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**Fig. 3.** Neighbour-joining tree based on the derived amino acid sequences of the pmoA gene of strain LC 2T 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* (diffi.ci’le. L. neut. adj. difficile difficult, referring to difficulties in cultivating the type strain).

Large, cocccoid 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. *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 16:1ω6c, followed by 16:1ω6c, 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 the type strain is 49.9 mol% (HPLC determination).

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

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