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

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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). A novel bacterial strain, LC 2T, 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 Methylomonas, 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 tundriplus.

The GenBank/EMBL/DDBJ accession numbers for the complete 16S rRNA gene sequence and partial pmoA and nifH gene sequences of strain LC 2T are respectively DQ119050, DQ119047 and DQ665842. Strain LC 2T grows on methane or methanol as its sole carbon and energy source. 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 tundriplus and Methylobacter psychrophilus showing 94 and 93.4 % 16S rRNA gene sequence similarity, respectively. Furthermore, the pmoA gene sequence of strain LC 2T 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 16:07C (60 %). Strain LC 2T (=DSM 14076T = JCM 18750T) is described as the type strain of a novel species within a new genus, Methylosoma difficile gen. nov., sp. nov.

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

A novel methanotroph, strain LC 2T, 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 2T 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 2T 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 strain of Methylobacter tundriplus and the recently described Methylosoma difficile gen. nov., sp. nov., a novel methanotroph enriched by gradient cultivation from littoral sediment of Lake Constance. 

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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% 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$O, 0.05 g KCl, 0.005 g CaCl$_2$, 0.016 g Na$_2$SO$_4$ and trace element solution SL 10 (1 ml 1$^{-1}$) (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 1$^{-1}$). Strain LC $2^T$ 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 $2^T$ was grown in 100 ml flasks or 15 ml glass tubes with 25–30 or 5 ml medium, respectively, in closed desiccators. 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 a year after isolation, strain LC $2^T$ was grown in closed desiccators 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 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 presence of heterotrophic contaminants was checked every time by platting 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 preservation 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 confirmed by phase-contrast microscopy.

**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, arabinohe, 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 $2^T$. 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 $2^T$ 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 optimal 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 2T 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), then 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 1 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 2T**

Strain LC 2T was isolated after enrichment in a gradient culture system obtained after a final dilution of $8 \times 10^{-4}$ 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 2T 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 2T 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 2T resembled Methylobacter tundripaludum SV96T in colony colour and cell size (Table 1). Comparison of strain LC 2T with members of other methanotrophic genera is shown in Table 1.

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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Methyloccus</th>
<th>Methylobacterium</th>
<th>Methylosarcina*</th>
<th>Methylosphaera</th>
<th>Methylobacter</th>
<th>Strain LC 2T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell morphology</td>
<td>Rods</td>
<td>Rods</td>
<td>Sarcina-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</td>
<td>49.9</td>
</tr>
</tbody>
</table>

*Except Methylosarcina lacus.*
†Methylobacter tundripaludum.

http://ijs.sgmjournals.org
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). 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 $2^T$ 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.
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, Methylophilium 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 not been found so far in other type I methanotrophs.

Absence of sMMO and presence of nitrogenase

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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 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 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 other described species of Methylobacter. The amino acid similarities were 92 % to Methylobacter sp. LW12 and Methylomonas buryatense 5B T 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

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Methylomonas</th>
<th>Methylomonas</th>
<th>Methylomonas</th>
<th>Methylomonas</th>
<th>Methylomonas</th>
<th>Methylomonas</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>2.74</td>
</tr>
<tr>
<td>14:0</td>
<td>22 ± 3</td>
<td>1 ± 1</td>
<td>15 ± 0.5</td>
<td>&lt;1</td>
<td>9 ± 2</td>
<td>8.55</td>
<td></td>
</tr>
<tr>
<td>16:1ω8c</td>
<td>30 ± 11</td>
<td>16 ± 3</td>
<td>15 ± 1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></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>
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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>
<td>ND</td>
<td></td>
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<tr>
<td>16:1ω5r</td>
<td>12 ± 4</td>
<td>20 ± 10</td>
<td>25 ± 5</td>
<td>&lt;1</td>
<td>11 ± 1</td>
<td>ND</td>
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<tr>
<td>16:0</td>
<td>7 ± 2</td>
<td>15 ± 3</td>
<td>19 ± 1</td>
<td>4 ± 8</td>
<td>16 ± 1</td>
<td>8.5</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>
<td></td>
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<td>16:1ω11c</td>
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</tbody>
</table>
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* [Me.thy.lo.so.ma. N.Gr. prefix *methylene* referring to methyl groups; Gr. neut. *n. soma* body; N.L. neut. *n. Methylosoma* a methyl group (-utilizing) body].

Obligately aerobic, *C*,-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*. 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.

**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, 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 growing best at low (2 %) oxygen tensions. Uses methane 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 16:1 o7ωc, followed by 16:1 o6ωc, 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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