Methylosarcina lacus sp. nov., a methanotroph from Lake Washington, Seattle, USA, and emended description of the genus Methylosarcina

Marina G. Kalyuzhnaya,1 Sergey M. Stolyar,2 Ann J. Auman,3 Jimmie C. Lara,4 Mary E. Lidstrom1,4 and Ludmila Chistoserdova1

An obligately methanotrophic bacterial strain, LW14T, isolated from the sediment of Lake Washington, Seattle, USA, is described taxonomically. The isolate is an aerobic, Gram-negative, non-motile bacterium capable of growth on methane, and possesses type I intracytoplasmic membranes (i.e. it is a type I methanotroph). The strain possesses particulate methane monoxygenase (MMO) and has no soluble MMO. Formaldehyde is assimilated via the ribulose monophosphate cycle. The isolate grows within a pH range of 4–8, with the optimum between pH 5.5 and 6.5. The cellular fatty acid profile is dominated by C16:0, C16:1ω7c and C18:1ω5t fatty acids. The DNA G+C content is 53.3 ± 0.4 mol%. On the basis of sequence analysis of the 16S rRNA gene, isolate LW14T is related most closely to representatives of the genus Methylosarcina. However, DNA–DNA hybridization analysis reveals only a distant relationship between isolate LW14T and the previously described Methylosarcina species. On the basis of its phenotypic and genotypic characteristics, LW14T represents a novel species of the genus Methylosarcina, for which the name Methylosarcina lacus sp. nov. is proposed, with LW14T (≡ ATCC BAA-1047T = JCM 13284T) as the type strain.

Obligate methanotrophs are a specialized group of bacteria capable of utilizing methane as the sole source of carbon and energy. These bacteria play a major role in the global cycling of methane, oxygen and nitrogen (King, 1992; Hanson & Hanson, 1996) and have a potential for use in biotechnology as biodegradation and bioremediation agents (Higgins et al., 1980; Oremland et al., 1994; Bodrossy et al., 1995). Methanotrophic bacteria are widespread in natural habitats and are found in soils, wetlands and sediments as well as in fresh, saline and alkaline waters (Whittenbury et al., 1970; Hanson & Hanson, 1996).

Analysis of methanotrophic populations in Lake Washington revealed that a broad diversity of methanotrophic species is present (Costello & Lidstrom, 1999; Auman et al., 2000; Auman & Lidstrom, 2002); they may represent up to 30 % of the total bacterial population in the site (Costello et al., 2002). Pure cultures of both type I and type II methanotrophs have been isolated from the site and have been shown to be related closely to known groups of methanotrophic bacteria belonging to the genera Methylophanas, Methylobacter, Methylosinus and Methylocystis (Auman et al., 2000). According to culture-independent analysis of the methylo trophic community in Lake Washington, one of these phylotypes, isolate LW14T, represented one of the dominant groups in this site (Kalyuzhnaya et al., 2004). On the basis of phylogenetic analysis, isolate LW14T was classified as a member of the genus Methylobacter, with a 16S rRNA gene sequence similarity level of about 95 % to representatives of the genus Methylobacter (Auman et al., 2000). In a later publication, a novel genus, Methylosarcina, was proposed to accommodate two novel methanotrophic isolates, from soils, that possess divergent 16S rRNA genes and peculiar morphological and biochemical features (Wise et al., 2001). Close relatedness of the 16S rRNA gene sequence from isolate LW14T to the sequences of the newly described Methylosarcina species (96 % similarity) prompted us to conduct a taxonomic analysis of isolate LW14T. Isolate LW14T, characterized here, is the first example of a Methylosarcina species from an aquatic environment.
Strain LW14T was grown at 30°C in 0.25 or 1 l flasks containing 25 or 100 ml NMS minimal medium (Whittenbury et al., 1970), respectively. Cultures were shaken (at 200 r.p.m.) for 3–5 days under a methane/air atmosphere (1:1). Cultures were maintained on solid NMS medium containing 1.5% (w/v) Difco agar under a methane/air atmosphere (1:1) for 3–6 days. For long-term storage, 100 μl DMSO was added to 1 ml exponentially growing liquid culture and the mixed suspension was stored at −80°C. The following strains were used as controls: *Methylosarcina fibrata* DSM 13736T and *Methylosarcina quisquiliarum* DSM 13737T [obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany], ‘*Methylobacter bovis*’ VKM B-2112, ‘*Methylobacter chroococcum*’ VKM B-2114 and ‘*Methylobacter vinelandii*’ VKM B-2113 [obtained from the All-Russian Collection of Micro-organisms (VKM), Pushchino, Russia] and *Methylomicrobium album* ACM 3314T (Lidstrom laboratory collection). The cultures were grown under methane/air in the NMS medium described above.

For transmission electron microscopy, cells were washed with double-distilled water and fixed in modified Karnovsky’s fixative (2% paraformaldehyde, 2.5% gluteraldehyde, 8 mM CaCl₂ in 0.1 M cacodylate buffer, pH 7.4) for 2 h at 4°C. Samples were washed in cacodylate buffer and post-fixed in 1% osmium tetroxide in buffer for 2 h at room temperature. Following three 5 min washes, cells were embedded in 1.5% Noble agar (Difco) and dehydrated in a graded series of ethanol (35, 50, 70, 80, 90, 95, 100%). Blocks were then infiltrated at three concentrations of propylene oxide: Spurr’s resin (2:1; 1:1; 1:3, respectively), ending with 100% Spurr’s resin. Thin sections of samples were stained with 7% uranyl acetate and Reynolds’ lead citrate for 20 and 10 min each, respectively. Samples were viewed by using an 1200 Ex II transmission electron microscope (JEOL) operated at 80 kV. For scanning electron microscopy, cells were fixed as described above, washed three times for 5 min with double-distilled water, spotted onto plastic coverslips coated with 1% poly-L-lysine, dehydrated in a graded series of ethanol to 100%, then subjected to critical-point drying. Samples were sputter-coated with gold/palladium and viewed with a JSM 6300F scanning electron microscope (JEOL) at 15 kV. The microscopy revealed large, non-motile, coccobacillary cells, 0.7–1.0 μm in width and 1.0–1.2 μm in length (Fig. 1a, b, c). Cells usually occurred singly or in pairs, but sometimes formed chains of three to five cells (Fig. 1b, c). Typical cells had one distinct feature: a holdfast-like structure,
0.8–1.0 μm in length and 0.2–0.3 μm in width (Fig. 1a). Cells reproduced by binary fission and did not visibly form cysts or other resting bodies. Thin sectioning showed that cells had a Gram negative-type cell-wall structure, and internal membranes typical of type I methanotrophs (Fig. 1d).

The growth-substrate range was tested in liquid NMS medium supplemented with one of the following autoclaved or filter-sterilized substrates (0.05% w/v): methyleneamine, formate, dimethylsulfate, vanillin, choline, betaine, oxaloacetate, acetate, pyruvate, citrate, malate, succinate, glucose, maltose, fructose, succrose, manitol, ethanol, glycerol, serine, valine, alanine, cysteine, asparagine, arginine, aspartate, methionine, histidine, tryptophan and yeast extract. The ability to grow on methanol was tested at concentrations of 0.01–1% (v/v). Methane or methanol, but no other substrates tested, supported growth of isolate LW14T. In the case of methanol, maximal growth occurred when cells were grown at low concentrations (<0.1% methanol, v/v), with optimal growth occurring at 0.05% (v/v). Nitrogen sources were tested by using solid medium in which KNO3 had been replaced by one of the following compounds, at 0.05% (w/v): (NH4)2SO4, methylamine, urea, guanidine, glycine, serine, valine, alanine, cysteine, asparagine, arginine, aspartate, methionine, histidine, threonine, proline, glutamate, phenylalanine, tryptophan, peptone or yeast extract. For N2-fixation experiments, a nitrate-free medium was used. Isolate LW14T used nitrates, urea, serine, aspartate and glutamate as nitrogen sources, but was unable to grow in the absence of a fixed nitrogen source, suggesting that it cannot fix N2. The inability to detect the nifH gene, which encodes the Fe protein of nitrogenase, has been reported previously for this strain (Auman et al., 2001).

Heat resistance was tested by heating cell suspensions at 50, 60, 70 or 80°C for 10 min, then plating them onto solid medium and incubating them at optimal conditions for 2 weeks. Desiccation resistance was assessed according to Whittenbury et al. (1970). Sensitivity to antibiotics was examined by spreading appropriate dilutions of the culture onto solid medium and placing, on top of the cultures, paper discs (Difco) that contained one of the following antibiotics (μg ml−1): neomycin (30), kanamycin (30), ampicillin (10), penicillin (10), erythromycin (15), lincomycin (2), gentamicin (10), novobiocin (30), nalidixic acid (30), tetracycline (30), chloramphenicol (30) or streptomycin (10). Growth under methane was assessed after 2 weeks. The optimum pH and temperature were investigated in liquid medium. A mixture (0.05 M) of NaH2PO4/Na2HPO4 was used for buffering (pH 4.2–8.5).

After 5 days growth on agar plates under a methane/air atmosphere, colonies of isolate LW14T were white, 1–2 mm in diameter, circular, convex and smooth. In liquid cultures, cells tended to clump together, forming white flakes. The specific growth rate in liquid cultures under methane/air was 0.05 ± 0.003 h−1. The strain grew at temperatures ranging from 4 to 35°C, with optimal growth occurring at 28–30°C. Isolate LW14T was not resistant to heat, desiccation or freeze-drying under the conditions tested. Isolate LW14T was found to be catalase-positive, resistant to nalidixic acid, streptomycin, tetracycline, chloramphenicol, penicillin and ampicillin, and sensitive to erythromycin, novobiocin, neomycin, kanamycin, lincomycin and gentamicin, at the concentrations tested.

Activities of enzymes characteristic of major methylo trophic pathways were tested. For cell-free extract preparation, LW14T cells grown under methane/air were pelleted by centrifugation at 5000 r.p.m. at 4°C, resuspended in 1 ml Tris (25 mM Tris/HCl, 10 mM EDTA, pH 7.2) or phosphate (25 mM KH2PO4/Na2HPO4, pH 7.2) buffer and disrupted by passage through a French pressure cell at 1·2 × 108 Pa. Cell extracts were centrifuged at 14 000 r.p.m. for 25 min at 4°C to remove cell debris. Hydroxypyruvate reductase and serine–glyoxylate aminotransferase were assayed as described by Goodwin (1990). Hexulose phosphate synthase was assayed as described by Shishkina et al. (1976). Phosphoribulokinase and ribulose bisphosphate carboxylase/oxygenase (RuBisCo) were assayed as described by Tabita (1980). Formate dehydrogenase was assayed as described by Chistoserdova et al. (2004). Methylene tetrahydrofolate dehydrogenase was assayed as described by Vorholt et al. (1998). The soluble methane monooxygenase (MMO) test was performed by using naphthalene oxidation (Bodrossy et al., 1995); soluble MMO activity in whole-cell suspensions was measured by using a fluorescence-based assay (Miller et al., 2002). Whilst the activity of particulate MMO was detected (Table 1), the tests for soluble MMO activity were negative, confirming previous studies (Auman et al., 2000). A high level of activity for hexulose phosphate synthase, the key enzyme of the ribulose monophosphate (RuMP) cycle for formaldehyde assimilation (Anthony, 1982), was detected (Table 1), whereas activities of key enzymes of the serine cycle (hydroxypyruvate reductase and serine–glyoxylate aminotransferase) or

**Table 1.** Enzyme activities in cell extract of isolate LW14T

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity [nmol min⁻¹ (mg protein)⁻¹]</th>
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</thead>
<tbody>
<tr>
<td>Particulate MMO</td>
<td>68 ± 4</td>
</tr>
<tr>
<td>Soluble MMO</td>
<td>ND</td>
</tr>
<tr>
<td>Hexulose phosphate synthase</td>
<td>120 ± 15</td>
</tr>
<tr>
<td>Hydroxypyruvate reductase</td>
<td>ND</td>
</tr>
<tr>
<td>Serine–glyoxylate aminotransferase</td>
<td>ND</td>
</tr>
<tr>
<td>RuBisCo</td>
<td>ND</td>
</tr>
<tr>
<td>Phosphoribulokinase</td>
<td>ND</td>
</tr>
<tr>
<td>Methylene tetrahydrofolate dehydrogenase</td>
<td>70 ± 5</td>
</tr>
<tr>
<td>Formate dehydrogenase</td>
<td>25 ± 5</td>
</tr>
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</table>
the ribulose bisphosphate cycle (RuBisCo and phosphoribulokinase) were not detected. High enzyme activity for methylene tetrahydrofolate dehydrogenase was detected, which is indicative of the presence of the H4F-linked pathway for C1 transfer (Vorholt et al., 1999). Although we were not able to test for the activities of key enzymes of the H4MPT pathway that has been shown to be widespread in methyloths (Vorholt et al., 1999), because of the lack of availability of the substrate, we have previously detected the presence of the mch, mtdB and fae genes, which code for key enzymes of the H4MPT-linked pathway (Vorholt et al., 1998, 1999; Hagemeier et al., 2000; Vorholt et al., 2000), via PCR amplification (Kalyuzhnaya et al., 2004).

Phospholipid fatty acid analyses were performed by Microbial Insights (http://www.microbe.com/). Lipids were recovered by using the modified Bligh–Dyer method (White et al., 1979). The membrane fatty acid profile determined for isolate LW14T is shown in Table 2: the major components were C16:1ω9c (33%), C16:1ω5t (26.9%) and C16:1ω7c (18%).

For DNA extraction and purification, cells (5 g wet weight) were resuspended in lysis buffer (10 mM NaCl, 20 mM Tris/HCl, pH 8.0; 1 mM EDTA; 100 mg proteinase K ml−1; 50 mg ribonuclease A ml−1; 0.5 % (w/v) SDS – all from Sigma) and incubated overnight. DNA was purified by phenol/chloroform extraction and ethanol precipitation (Sambrook et al., 1989). The DNA G+C content was determined by HPLC separation, performed in triplicate, as described by Tamaoka & Komagata (1984). DNA–DNA hybridization experiments were performed by using the 16S rRNA gene sequencing that of the sequence of the 16S rRNA gene showed 96 % similarity to that of the Methylosarcina species, but only 94–95 % similarity to that of the Methylobacter species. The results of the phylogenetic analyses performed by using the CLUSTAL W program (Higgins et al., 1996). Phylogenetic analyses were carried out by using the PHYLIP package (Felsenstein, 2003). Distance and parsimony methods were employed and 1000 bootstrap analyses were performed.

The DNA analysis revealed that the G+C content of the DNA is 53.3 ± 0.4 mol% (n = 3). The nearly complete sequence of the 16S rRNA gene showed 96 % similarity to that of the Methylosarcina species, but only 94–95 % similarity to that of the Methylobacter species. The results of the phylogenetic analyses performed by using the 16S rRNA gene sequences suggested that isolate LW14T was related most closely to the Methylosarcina species, with high bootstrap support (Fig. 2). DNA–DNA hybridization analysis revealed low levels of similarity with representatives of the genera Methylobacter, Methylosarcina and Methylophilum (<3 %), whilst the values for DNA–DNA hybridization with the two species of Methylosarcina – M. fibrata and M. quisquiliarum – were 17 and 25 %, respectively.

The genus Methylosarcina was proposed (Wise et al., 2001) to describe type I methanotrophs that diverge from representatives of previously described genera. The two novel species representing this genus, M. fibrata and M. quisquiliarum, were both isolated from soils and both possess the peculiar feature of having cells that assemble into irregularly shaped, retractile cell packets resembling sarcina-like clusters (Wise et al., 2001). Here, we characterize a novel freshwater-lake methanotrophic isolate, LW14T, and propose it to be the third representative of the genus Methylosarcina, on the basis of 16S rRNA gene sequencing and total DNA–DNA similarity. However, morphologically, isolate LW14T differs from the two previously described Methylosarcina species. First, we did not observe any sarcina-like structures in liquid or plate cultures of isolate LW14T, at any growth stage. Cells were observed singly or

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**Table 2. Major fatty acids of type I methanotrophic genera and isolate LW14T**

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1°</th>
<th>2°</th>
<th>3°</th>
<th>4°</th>
<th>5°</th>
<th>6°</th>
</tr>
</thead>
<tbody>
<tr>
<td>C12:0</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>3.0 ± 0.4</td>
<td>NR</td>
</tr>
<tr>
<td>C14:0</td>
<td>22 ± 3</td>
<td>9 ± 2</td>
<td>1 ± 1</td>
<td>&lt; 1</td>
<td>1.5 ± 0.5</td>
<td>1.1</td>
</tr>
<tr>
<td>C16:1ω8c</td>
<td>30 ± 11</td>
<td>ND</td>
<td>16 ± 3</td>
<td>ND</td>
<td>15 ± 1</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>C16:1ω7c</td>
<td>11 ± 4</td>
<td>57 ± 1</td>
<td>17 ± 3</td>
<td>28 ± 10</td>
<td>28 ± 3</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>C16:1ω6c</td>
<td>9 ± 4</td>
<td>5 ± 1</td>
<td>10 ± 4</td>
<td>3 ± 2</td>
<td>8 ± 1</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>C16:1ω5c</td>
<td>4 ± 2</td>
<td>7 ± 1</td>
<td>6 ± 1</td>
<td>3 ± 2</td>
<td>&lt; 1</td>
<td>ND</td>
</tr>
<tr>
<td>C16:1ω5t</td>
<td>12 ± 4</td>
<td>11 ± 1</td>
<td>20 ± 10</td>
<td>&lt; 1</td>
<td>25 ± 5</td>
<td>26 ± 9</td>
</tr>
<tr>
<td>C16:0</td>
<td>7 ± 2</td>
<td>8 ± 1</td>
<td>15 ± 3</td>
<td>44 ± 8</td>
<td>19 ± 1</td>
<td>9 ± 9</td>
</tr>
</tbody>
</table>

*Data from Bowman et al. (1993).
†Data from Wise et al. (2001).
in pairs, and occasionally in chains of three to five cells. Additionally, LW14T cells have a characteristic feature, in pairs, and occasionally in chains of three to five cells. LW14T to type I methanotrophic bacteria, based upon 16S rRNA

Fig. 2. Phylogenetic tree showing the relationship of isolate LW14T to type I methanotrophic bacteria, based upon 16S rRNA gene sequences. Strain numbers and GenBank accession numbers (in parentheses) are shown. Solid circles indicate bootstrap support above 90% and shaded circles indicate bootstrap support above 75%. Bar, 10% sequence divergence.

On the basis of the morphological features, which differ from those described of the genus Methylosarcina, the moderate degree of 16S rRNA gene sequence similarity (96%) and the overall low level of DNA–DNA similarity (25% or less), we propose that isolate LW14T belongs to a novel taxon at the species level, for which we propose the name Methylosarcina lacus sp. nov. As M. lacus possesses traits different from those described for the previously identified Methylosarcina species, an emended description of the genus Methylosarcina is presented here.

Emended description of the genus Methylosarcina

The genus description is as given by Wise et al. (2001), but with the following additions. Cells may not form sarcina-like aggregations. Cells may be coccobacillary, occurring singly or in pairs, sometimes forming chains of three to five cells. Cells may be non-motile, but form a holdfast-like structure. Cells may not form cysts or other resting bodies.

Description of Methylosarcina lacus sp. nov.

Methylosarcina lacus (la’cus. L. gen. n. lacus of a lake).

Cells are Gram-negative, non-motile coccobacilli, with a mean width and length of 0.7–1.0 and 1.0–1.2 μm, respectively. Reproduces by binary fission. Cells form holdfast-like structures that measure 0.8–1.0 μm in length and 0.2–0.3 μm in width. Type I intracytoplasmic membranes are present. Colonies grown on mineral salts medium under a methane/air atmosphere are white, uniform, 1–2 mm in diameter, circular, convex and smooth. Utilizes only methane and methanol via the RuMP cycle, and appears to lack soluble MMO. Nitrate, serine, aspartate and glutamate can be used as nitrogen sources. Catalase-positive, lacks the ability to fix nitrogen and grows within a temperature range of 4–35 °C and a pH range of 5.0–7.0, with optimal growth at 28–30 °C and pH 5.5–6.5. Not resistant to heating, freezing or drying. Major fatty acids are C16:1ω9c, C16:1ω7c and C16:1ω5t. The DNA G+C content is 53.3 ± 0.4 mol%.

The type strain, LW14T (=ATCC BAA-1047T = JCM 13284T), was isolated from Lake Washington, Seattle, Washington, USA.

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References


