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

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An obligately methanotrophic bacterial strain, LW14^T, 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 monooxygenase (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 C_{16:0}18c, C_{16:1}ω7c and C_{16:1}ω5t 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 LW14^T is related most closely to representatives of the genus *Methylosarcina*. However, DNA–DNA hybridization analysis reveals only a distant relationship between isolate LW14^T and the previously described *Methylosarcina* species. On the basis of its phenotypic and genotypic characteristics, LW14^T represents a novel species of the genus *Methylosarcina*, for which the name *Methylosarcina lacus* sp. nov. is proposed, with LW14^T (= ATCC BAA-1047^T = JCM 13284^T) 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 *Methylobacter*, *Methylobacter*, *Methylobacter* and *Methylobacter* (Auman *et al.*, 2000). According to culture-independent analysis of the methylobacterial community in Lake Washington, one of these phylotypes, isolate LW14^T, represented one of the dominant groups in this site (Kalyuzhnaya *et al.*, 2004). On the basis of phylogenetic analysis, isolate LW14^T 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 LW14^T to the sequences of the newly described *Methylosarcina* species (96 % similarity) prompted us to conduct a taxonomic analysis of isolate LW14^T. Isolate LW14^T, characterized here, is the first example of a *Methylosarcina* species from an aquatic environment.

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Abbreviations: MMO, methane monooxygenase; RuMP, ribulose monophosphate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LW14^T is AY007296.

Strain LW14^T 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 13736^T and *Methylosarcina quisquiliarum* DSM 13737^T [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 3314^T (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% glutaraldehyde,

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,

Fig. 1. Scanning (a–c) and transmission (d) electron micrographs of LW14^T cells. (a) Individual cells with holdfast-like structures; (b, c) individual cells and pairs of ovoid or coccoid cells; (d) thin-sectioned cells showing type I cell-wall structure (CW), internal cytoplasmic membranes (ICM) and large inclusion bodies (IB). Bars, 1 µm.

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): methylamine, formate, dimethylsulfate, vanillin, choline, betaine, oxaloacetate, acetate, pyruvate, citrate, malate, succinate, glucose, maltose, fructose, sucrose, mannitol, 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 LW14^T. 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 LW14^T 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 LW14^T 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 LW14^T was not resistant to heat, desiccation or freeze-drying under the conditions tested. Isolate LW14^T 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, LW14^T 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 biphosphate 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 LW14^T

All activity determinations were carried out in triplicate. ND, Not detectable.

Enzyme	Activity [nmol min ⁻¹ (mg protein) ⁻¹]
Particulate MMO	68 ± 4
Soluble MMO	ND
Hexulose phosphate synthase	120 ± 15
Hydroxypyruvate reductase	ND
Serine-glyoxylate aminotransferase	ND
RuBisCo	ND
Phosphoribulokinase	ND
Methylene tetrahydrofolate dehydrogenase	70 ± 5
Formate dehydrogenase	25 ± 5

the ribulose biphosphate 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 methylotrophs (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 LW14^T is shown in Table 2: the major components were C_{16:1}ω8c (33%), C_{16:1}ω5t (26.9%) and C_{16:1}ω7c (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 competition procedure described by Johnson (1994). The following strains were tested: LW14^T, *Methylosarcina fibrata*, *Methylosarcina quisquiliarum*, ‘*Methylosarcina methanica*’, ‘*Methylobacter bovis*’, ‘*Methylobacter chroococcum*’

Table 2. Major fatty acids of type I methanotrophic genera and isolate LW14^T

Taxa: 1, *Methylomonas*; 2, *Methylobacter*; 3, *Methylochromobium*; 4, *Methylococcus*; 5, *Methylosarcina*; 6, LW14^T. NR, Not reported; ND, not detectable.

Fatty acid	1*	2*	3*	4*	5†	6
C _{12:0}	NR	NR	NR	NR	3.0±0.4	NR
C _{14:0}	22±3	9±2	1±1	<1	1.5±0.5	1.1
C _{16:1} ω8c	30±11	ND	16±3	ND	15±1	33.6
C _{16:1} ω7c	11±4	57±1	17±3	28±10	28±3	18.2
C _{16:1} ω6c	9±4	5±1	10±4	3±2	8±1	8.3
C _{16:1} ω5c	4±2	7±1	6±1	3±2	<1	ND
C _{16:1} ω5t	12±4	11±1	20±10	<1	25±5	26.9
C _{16:0}	7±2	8±1	15±3	44±8	19±1	9.9

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

†Data from Wise *et al.* (2001).

and *Methylochromobium album*. DNA (1 µg) from isolate LW14^T was labelled with [³H]dCTP (Amersham Biosciences) by using a nick translation kit (Roche). Competitor DNA was digested by using a combination of *Pst*I and *Hind*III restriction enzymes (New England Biolabs). DNA extracted from control strains (1 mg) was immobilized onto 0.22 µm Nytran N membranes (Schleicher & Schuell) and hybridized with the labelled LW14^T DNA [specific activity, 5 × 10⁶ d.p.m. (3 × 10⁸ Bq) µg⁻¹], as described by Sambrook *et al.* (1989). After hybridization, filters were counted in a liquid scintillation counter (Beckman) and the percentage of hybridization was calculated as described by Johnson (1994). Three independent tests were carried out for each pair of DNA preparations. Fragments of 16S rRNA genes were amplified by using the primers and protocols described by Lane (1991). DNA sequencing was carried out by using a BigDye 3.1 termination sequencing kit (Applied Biosystems). Analyses were performed by the Department of Biochemistry Sequencing Facility at the University of Washington, using an ABI 3700 high-throughput capillary DNA analyser. For phylogenetic analysis, DNA sequences were aligned 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 LW14^T 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 *Methylomonas*, *Methylobacter* and *Methylochromobium* (<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, LW14^T, 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 LW14^T differs from the two previously described *Methylosarcina* species. First, we did not observe any sarcina-like structures in liquid or plate cultures of isolate LW14^T, at any growth stage. Cells were observed singly or

Fig. 2. Phylogenetic tree showing the relationship of isolate LW14^T 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.

in pairs, and occasionally in chains of three to five cells. Additionally, LW14^T cells have a characteristic feature, namely the presence of a holdfast-like structure (Fig. 1) not previously seen in any known methanotrophic bacteria. The molecular content and physiological purpose of these holdfast-like structures remain unknown. Possibly they are involved in cell adhesion, and, if so, might play a role in the environmental fitness of bacteria associated with sediments. Physiologically, isolate LW14^T is a typical type I methanotroph capable of growth on methane and exhibiting poor growth on methanol. It utilizes particulate MMO for methane oxidation and does not possess soluble MMO. Formaldehyde is oxidized by the H₄MPT-linked oxidative pathway. An alternative H₄F-linked pathway may also participate in formaldehyde oxidation. Formaldehyde is assimilated via the RuMP cycle. In general, the metabolic-pathway pattern determined for isolate LW14^T is typical of known type I methanotrophs (Anthony, 1982; Vorholt *et al.*, 1999). The cellular fatty acid composition of strain LW14^T is comparable with previously published data for type I methanotrophs (Bowman *et al.*, 1995; Wise *et al.*, 2001), but it shows certain differences with respect to the known profiles. The most dominant fatty acid is C_{16:1}ω8c, which is typical of representatives of the genus *Methylomonas*, but, unlike *Methylomonas*, isolate LW14^T also possesses small amounts

of the fatty acid C_{14:0}. The second dominant fatty acid in the polar lipid composition of isolate LW14^T is C_{16:1}ω5t, which is characteristic of representatives of the genera *Methylosarcina* and *Methylobaculum*; however, levels of palmitic acid are lower in isolate LW14^T.

On the basis of the morphological features, which differ from those of described species 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 LW14^T 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. Colonies may be white. 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 4.0–7.0, with optimal growth at 28–30 °C and pH 5.5–6.5. Not resistant to heating, freezing or drying. Major fatty acids are C_{16:1}ω8c, C_{16:1}ω7c and C_{16:1}ω5t. The DNA G + C content is 53.3 ± 0.4 mol%.

The type strain, LW14^T (=ATCC BAA-1047^T=JCM 13284^T), was isolated from Lake Washington, Seattle, Washington, USA.

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