Methanomethylovorans thermophila sp. nov., a thermophilic, methylotrophic methanogen from an anaerobic reactor fed with methanol

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A novel thermophilic, obligately methylotrophic, methanogenic archaeon, strain L2FAWT, was isolated from a thermophilic laboratory-scale upflow anaerobic sludge blanket reactor fed with methanol as the carbon and energy source. Cells of strain L2FAWT were non-motile, irregular cocci, 0.7–1.5 μm in diameter and usually occurred singly (sometimes forming clusters of two or four cells). The cells stained Gram-negative and lysed immediately in 0.1% (w/v) SDS. Growth was inhibited by chloramphenicol and tetracycline, but not by penicillin, bacitracin, spectinomycin, vancomycin or kanamycin. Methanol and mono-, di- and trimethylamine were used as substrates, but H2/CO2, formate, acetate, propanol, dimethyl sulfide and methanethiol were not. The temperature range for growth was 42–58°C, with an optimum at 50°C. The fastest growth was observed at a salinity below 100 mM NaCl; no growth occurred above 300 mM NaCl. The optimal pH for growth was 6.5; growth was observed from pH 5 to pH 7.5. The G+C content of the genomic DNA was 37.6 mol%. Analysis of the 16S rRNA gene sequence and the partial methyl-CoM reductase gene sequence revealed that the organism was phylogenetically closely related to Methanomethylovorans hollandica DMS1T (98% similarity for the 16S rRNA gene sequence and 91% similarity for the methyl-CoM reductase gene sequence). The DNA–DNA relatedness between L2FAWT and Methanomethylovorans hollandica DMS1T was 46%. On the basis of these results, strain L2FAWT (= DSM 17232T = ATCC BAA-1173T) represents the type strain of a novel species, for which the name Methanomethylovorans thermophila sp. nov. is proposed.

The utilization of methylated substrates among methanogenic archaea is restricted almost exclusively to members of the family Methanosarcinaceae. The only exceptions are the members of the genus Methanosphaera, which can convert methanol only in the presence of H2. The family Methanosarcinaceae comprises eight genera: Methanococcoides, Methanohalobium, Methanohalophilus, Methanolobus, Methanomethylovorans, Methanimicrococcus, Methanosalsum and Methanosarcina. The genus Methanosarcina includes the most versatile species of all the methanogens capable of using H2/CO2, acetate and methylated compounds (methanol, methylated amines and, in some cases, methylated sulfides) (Boone et al., 1993). The only member of Methanimicrococcus, Methanimicrococcus blatticola (originally named ‘Methanomicrococcus blatticola’), resembles Methanosphaera species in that it can utilize methanol (or methylated amines) only by complete reduction in the presence of H2 (Sprenger et al., 2000). The members of the other six genera are all obligate methylotrophs, utilizing methanol, methylated amines and, in some cases, methylated sulfides. Species of the genus Methanomethylovorans are typical freshwater isolates (having low salt tolerance), whereas the other obligate methylotrophs are generally regarded as halophiles (Garcia et al., 2000).

The genus Methanomethylovorans, described in 1999, represents a group of freshwater species that can utilize methanol, methylated amines, dimethyl sulfide and methanethiol for methanogenesis (Lomans et al., 1999). Methanomethylovorans hollandica DMS1T was isolated from a eutrophic freshwater pond sediment in the Netherlands (Lomans et al., 1999) and represents the only Methanomethylovorans species with a validly published name.
**Methanomethylovorans** sp. strain ZB was isolated from Baldegger Lake in Switzerland (Simankova et al., 2003), where the temperature is only 5–6°C throughout the year. The third strain, ‘Methanomethylovorans victoriae’ strain TM, was isolated from the sediment of the eutrophic Lake Victoria in Mwanza, East Africa (Muyodi, 2000). Methanomethylovorans strains have also been detected in samples from rice-field soils (Lueders et al., 2001), oil-contaminated groundwater (Watanabe et al., 2002), freshwater sediments (Lomans et al., 2001), sludge from an anaerobic baffled reactor treating industrial dye waste (Plumb et al., 2001) and in a bioreactor treating dichloropropane-contaminated wastewater (Schlotelburg et al., 2002). The three Methanomethylovorans strains described were all enriched on either trimethylamine or dimethyl sulfide and are all mesophilic (growth above 40°C was not obtained). In this paper, the isolation and characterization of a novel thermophilic strain, L2FAWT, from thermophilic sludge of an anaerobic bioreactor are described. It is proposed that this strain represents a novel methylotrophic species.

Strain L2FAWT was isolated from a methanol-degrading enrichment culture, which was obtained from a thermophilic anaerobic reactor (Paulo et al., 2002) and routinely maintained in our laboratory for about 2 years by means of successive transfers on methanol. The bicarbonate-buffered mineral medium used in this study was prepared as described by Stams et al. (1993). The gas phase consisted of N2/CO2 (80 : 20, v/v) at a pressure of 170 kPa. Substrates were added from 1 M stock solutions to give a final concentration of 25 mM, unless indicated otherwise. The enrichment culture consisted of only two morphologically distinct micro-organisms: irregular cocci (the predominant form) and short rods. Attempts to isolate the methanogenic cocci from this enrichment culture by using different techniques (Petri dishes, roll tubes, deep agar and soft agar) were not successful. The rod-shaped contaminant was not eliminated by serial dilution up to 10−8, even after the addition of vancomycin. A modification was made to the deep-agar technique: the agar medium was kept liquefied after inoculation. Visible colonies developed after 2 weeks incubation at 55°C. The well-separated colonies were picked by using hypodermic needles after cooling of the agar to room temperature; dilution series were used for further purification. The strain isolated from the highest dilution (10−8) was designated strain L2FAWT.

Colonies of strain L2FAWT grown in deep liquefied agar were smooth, milk-white, spherical and reached a diameter of 1 mm after 2 weeks incubation at 55°C. The cells of strain L2FAWT were non-motile, irregular cocci 0.7–1.5 μm in diameter; they occurred mainly singly but sometimes in clusters consisting of two to four cells (Fig. 1a). Big aggregates, which are typical of Methanosarcina species, were rarely observed. At the end of the exponential phase, cells became bigger and eventually lysed (Fig. 1b). During active methanogenesis on methanol, the culture broth gradually turned from light yellow to a greenish colour, which, upon exposure to air, became reddish-brown. This observation indicates that strain L2FAWT produced reduced forms of corrinoids ([Co-III]-B12), which were oxidized to red corrinoids ([Co-III]-B12) with oxygen, as observed for Methanosarcina barkeri (Mazumder et al., 1987).

Cells of L2FAWT stained Gram-negative. They lysed immediately upon the addition of SDS at a concentration of 0.1% (w/v) or Triton X-100 at a concentration of 4% (w/v), but resisted osmotic stress in demineralized water for 20 min, indicating that a proteinaceous cell wall was present (Boone & Whitman, 1988). Growth of strain L2FAWT was inhibited by the addition of chloramphenicol (100 μg ml−1) or tetracycline (100 μg ml−1), but not by penicillin (2 mg ml−1), bacitracin (10 μg ml−1), spectinomycin (100 μg ml−1), vancomycin (100 μg ml−1) or kanamycin (100 μg ml−1).

Strain L2FAWT could use only methanol, trimethylamine, dimethylamine and methylamine for growth. H2 was produced in trace amounts (80–160 Pa) during growth on methanol. Growth was not supported by H2/CO2 (headspace, 80 : 20; 170 kPa), CO/CO2/N2 (headspace, 20 : 20 : 60;...
170 kPa), formate, 2-propanol or acetate; none of these was co-utilized together with methanol. The strain did not utilize a mixture of acetate and hydrogen. The addition of hydrogen in the headspace (170 kPa) resulted in slower growth on methanol. When methanol-grown cultures were transferred into trimethylamine-containing medium, a lag phase of about 3 days was observed. The lag phase became shorter with subsequent transfers on trimethylamine. The same thing was observed when trimethylamine-grown cultures were transferred back to methanol-containing media. An increased lag phase was not observed after culture adapted to one methylated amine was transferred to medium containing a different methylated amine. These results confirm previous findings, i.e. that the enzymes involved in degradation of methanol and methylated amines are different and have to be induced (King, 1984). Dimethyl sulfide and methanethiol were also tested, but no growth of strain L2FAWT was observed at the concentrations used (2 mM and 200 μM for dimethyl sulfide and methanethiol, respectively). These two substances also could not be co-metabolized during growth on methanol (5 mM) or trimethylamine (5 mM).

Strain L2FAWT was isolated using mineral medium prepared according to Stams (1993). Yeast extract (Difco) or peptone (0.2 %, w/v; BBL) slightly stimulated growth, but were not required. The temperature range for growth was 42–58 °C, with an optimum at 50 °C. Growth was not observed at 37 or 60 °C. Growth was optimal up to a NaCl concentration of 100 mM. Growth and methane formation were not observed at concentrations at or above 300 mM NaCl. Strain L2FAWT was able to grow at pH 5–0–7.5. The optimal pH was about 6.5. Remarkably, the growth rate of strain L2FAWT at pH 5.0 was still relatively high (55 % of the maximum at pH 6.5); this could be important in resistance to acidification caused by acetate formation by homoacetogens in anaerobic bioreactors (Yamaguchi et al., 1989). The addition of extra cobalt stimulated the growth of strain L2FAWT; the optimal concentration of cobalt in the mineral medium was between 0.5 and 2 μM. Further increases in cobalt concentration had inhibitory effects. Thus, strain L2FAWT is a thermophilic, slightly acidophilic, methylotrophic, freshwater methanogen. Under optimal conditions, the maximal specific growth rate is 0.05 h⁻¹.

For the phylogenetic analysis, a bead-beating- and phenol/ chloroform-based extraction method was used to extract DNA from strain L2FAWT (Zoetendal et al., 1998). The 16S rRNA gene was amplified with a GeneAmp PCR System 2400 thermocycler (Perkin-Elmer Cetus) using the primer set 7f and 1492r and Taq DNA polymerase (Invitrogen). The amplified fragment of the 16S rRNA gene was cloned into Escherichia coli JM109 (Invitrogen) using the pGEM-T Easy Vector System (Promega). Randomly selected recombinant clones were reamplified by PCRs with the vector-specific primers T7 and Sp6 (Promega) and sequenced using a sequencing kit (Amersham) and T7 and Sp6 IRD800-labelled sequencing primers (MWG-Biotech) (according to the manufacturer’s instructions). The DNA fragment of the methyl coenzyme M reductase (mcrA) gene was sequenced using the primer set ME1 [5’-GC(AC)ATGCA(AG)AT- (ACT)GG(AT)ATGTC-3’] and ME2 [5’-TCAT(GT)GC- (AG)TAGTT(AGT)GG(AG)TAGT-3’] as described previously (Hales et al., 1996). The partial 16S rRNA gene sequence (1363 bp) and the mcrA gene sequence (739 bp) obtained were compared with sequences deposited in GenBank, by using the BLAST search tool (Benson et al., 2004). On the basis of the sequence similarity of the 16S rRNA gene, the species most closely related to strain L2FAWT were Methanomethylovorans hollandica DMS1T (98 %), Methanomethylovorans sp. strain ZB (98 %) and ‘Methanomethylovorans victoriae’ TM (97 %). The following methanogenic species were also related to strain L2FAWT: Methanolobus oregonensis (94 %), Methanolobus taylorii (94 %), Methanosarcina lacustris (94 %), Methanosarcina mazei (93 %), Methanococoides burtonii (93 %) and ‘Methanohalophilus euhalobius’ (93 %). The mcrA gene sequence similarities with respect to Methanomethylovorans hollandica and Methanomethylovorans sp. ZB were 91 and 90 %, respectively. The deduced amino acid similarity between the two methyl-CoM reductases was 88 %. The values with respect to other species were below 82 %.

The sequences for the 16S rRNA and mcrA genes of strain L2FAWT and of other related methanogens were aligned by using CLUSTAL V (within the ARB package; Ludwig et al., 2004). Phylogenetic trees (Fig. 2) were constructed using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap values for the trees with the 16S rRNA and mcrA gene sequences were calculated using neighbour-joining analysis from 1000 replicate datasets and were re-evaluated by using SeqBoot and DNAPARS (within the PHYLIP package; Felsenstein, 1993). Whole genomic DNA of strain L2FAWT was isolated by using the method of Marmur (1961). The G+C content of the genomic DNA of strain L2FAWT (determined by thermal denaturation using a Pye Unicam SP 1800 spectrophotometer as described by Owen et al., 1969) was 37.6 mol%. The DNA–DNA relatedness (determined using the reassociation method described by De Ley et al., 1970) between Methanomethylovorans hollandica DMS1T and strain L2FAWT was 46 %.

Analysis of the 16S rRNA and mcrA gene sequences revealed that strain L2FAWT clustered with members of the genus Methanomethylovorans, showing the greatest level of similarity to Methanomethylovorans hollandica DMS1T. Like Methanomethylovorans hollandica DMS1T, strain L2FAWT is a freshwater species; a freshwater habitat is a key characteristic of members of the genus Methanomethylovorans and distinguishes them from other closely related genera, i.e. Methanolobus, Methanococoides, Methanohalobium and Methanohalophilus (Lomans et al., 1999). Strain L2FAWT differs from Methanomethylovorans hollandica DMS1T and other isolated Methanomethylovorans strains by its cell-wall composition, cell morphology, substrate spectrum, optimal temperature and genomic DNA G+C content (Table 1).
Besides using methanol and methylated amines, *Methanomethylovorans hollandiaca* DSM1\(^T\) and *Methanomethylovorans* sp. ZB can also utilize dimethyl sulfide and methanethiol for growth (Lomans et al., 1999; Simankova et al., 2003). Utilization of methanethiol and dimethyl sulfide was not reported for ‘*Methanomethylovorans victoriae*’ TM. Strain L2FAWT, however, cannot grow on dimethyl sulfide and methanethiol nor can it co-utilize them together with methanol or trimethylamine (Table 1). It is unlikely that strain L2FAWT lost its ability to degrade dimethyl sulfide and methanethiol because of long-term cultivation on methanol, since no methane was produced from the original enrichment culture or from the seed sludge of the upflow anaerobic sludge blanket (UASB) reactor when dimethyl sulfide or methanethiol was tested as a substrate (results not shown). Strain L2FAWT\(^T\) was unable to use dimethyl sulfide and methanethiol at lower concentrations (200 \(\mu\)M), even when other reducing agents such as cysteine or titanium citrate were used, or with complex medium (with addition of yeast extract and peptone, and supplemented with extra iron and cobalt). Consequently, it is clear that the lack of growth is not due to toxicity from dimethyl sulfide, methanethiol or sulphide, or a lack of essential nutrients.

The results of bootstrap analysis showed clear branching of strain L2FAWT\(^T\) from other *Methanomethylovorans* species and the genus *Methanosarcina*. DNA matrix distance analysis of the 16S rRNA gene showed that the mean distances from strain L2FAWT to all members of the genera *Methanolobus*, *Methanococcoides* and *Methanohalobium* were 0.066 \(\pm\) 0.006, 0.073 \(\pm\) 0.006 and 0.084 \(\pm\) 0.001, respectively. The value for strain L2FAWT with respect to members of the genus *Methanomethylovorans* was 0.022 \(\pm\) 0.001. Springer et al. (1995) suggested that, on the basis of phylogenetic analysis of the 16S rRNA and mcrA (b) gene sequences, showing the relationship between strain L2FAWT\(^T\) and members of the genera *Methanomethylovorans*, *Methanolobus*, *Methanosarcina* and *Methanococcoides*, constructed using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap values for both trees were calculated using neighbour-joining analysis after 1000 replicate datasets and re-evaluated by Seqboot and DNAPARS implemented in the PHYLIP package (Felsenstein, 1993). *Methanoseta concilii* is used as the outgroup. Bars, 10% sequence divergence.

![Phylogenetic trees of 16S rRNA (a) and mcrA (b) gene sequences](image-url)

*Fig. 2.* Phylogenetic trees of 16S rRNA (a) and mcrA (b) gene sequences, showing the relationship between strain L2FAWT\(^T\) and members of the genera *Methanomethylovorans*, *Methanolobus*, *Methanosarcina* and *Methanococcoides*, constructed using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap values for both trees were calculated using neighbour-joining analysis after 1000 replicate datasets and re-evaluated by SeqBoot and DNAPARS implemented in the PHYLIP package (Felsenstein, 1993). *Methanoseta concilii* is used as the outgroup. Bars, 10% sequence divergence.

Only a few thermophiles have been described within the family *Methanosarcinaceae*. Two thermophilic obligate methylotrophs, *Methanohalobium evestigatum* (Zhilina & Zavarzin, 1987) and *Methanosalsum zhilinae* (Mathrani et al., 1988), which is the only species in the genus, are halophiles. The first-described thermophilic species in this
Table 1. Characteristics of *Methanomethylovorans* strains and *Methanococcoides burtonii*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tr>
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<td>Cold lake</td>
<td>Eutrophic lake</td>
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<td>Clusters (2–4 cells)</td>
<td>Clusters (2–4 cells)</td>
<td>Single / clusters (up to four cells)</td>
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<tr>
<td>SDS sensitivity (w/v)</td>
<td>Lysed at 0.1% (50)</td>
<td>Not lysed at 1% (37)</td>
<td>NR</td>
<td>Not lysed at 1% (38)</td>
<td>Lysed at 0.1% (23–4)</td>
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<td>Temperature range (optimum) (ºC)</td>
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<td>12–40</td>
<td>NR</td>
<td>NR</td>
<td>6–30 (23–4)</td>
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<td>NaCl range (mM)</td>
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<td>NR</td>
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<td>34.4*</td>
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<td>NR</td>
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<td>+</td>
<td>+</td>
<td>NR</td>
<td>-</td>
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</table>

*Data from this study.*

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References


Felsenstein, J. (1993). PHYLIP (phylogeny inference package), version 3.5c. Distributed by the author, Department of Genome Sciences, University of Washington, Seattle, USA.


family, *Methanosarcina thermophila* (Zinder & Mah, 1979), which can utilize methanol, acetate and H₂/CO₂, is affiliated with other mesophilic species in the genus *Methanosarcina*. Two other thermophilic strains of *Methanosarcina* have been described that can utilize acetate and methanol but not H₂/CO₂ (Ollivier et al., 1984; Touzel et al., 1985). *Methanomethylovorans thermophila* strain L2FAWT, described here, is a thermophilic, obligately methylotrophic, freshwater species affiliated with other mesophilic species of the genus *Methanomethylovorans*.

Description of *Methanomethylovorans thermophila* sp. nov.

*Methanomethylovorans thermophila* (ther.mo.phi.la. Gr. adj. thermos hot; Gr. adj. philos loving; N.L. fem. adj. thermophila heat-loving).

Cells are irregular, non-motile and coccoid with a diameter of 0.7–1.5 µm. Cells occur singly and sometimes in clusters of two or four cells. Cells lyse in 0.1% (w/v) SDS. Cells stain Gram-negative. Temperature range for growth is from 42 to 58 ºC, with the optimum at 50 ºC. The type strain is slightly acidophilic (pH range 5–7.5, with an optimum at pH 6.5) and has a low salt tolerance (lower than 0.3 M NaCl). Methanol and methylated amines are the only catabolic and methanogenic substrates.

The type strain, strain L2FAWT (= DSM 17232T = ATCC BAA-1173T), was isolated from a UASB bioreactor treating paper-mill wastewater.


