Methylovulum miyakonense gen. nov., sp. nov., a type I methanotroph isolated from forest soil.

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Type  Journal Article

Textversion  author
Methylovulum miyakonense gen. nov., sp. nov., a novel type I methanotroph from a forest soil in Japan

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Subject category: Proteobacteria.

Running title: Methylovulum miyakonense gen. nov., sp. nov.

Abbreviations: pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase; NMS, nitrate mineral salt.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, pmoA, mmoX and nifH sequences of strain HT12 are AB501287, AB501285, AB501286 and AB524080, respectively.
ABSTRACT

A novel methanotroph, strain HT12\textsuperscript{T}, was isolated from a forest soil in Japan. Strain HT12\textsuperscript{T} is a Gram-negative, aerobic, non-motile, coccoid, pale brown colored bacterium. The strain only grows on methane and methanol as the sole carbon and energy source. Cells grow at 5-34\textdegree C (optimum 24-32\textdegree C). The strain possesses both particulate and soluble methane monooxygenases, and employs the ribulose monophosphate pathway for formaldehyde assimilation. The major cellular fatty acids are 16:0 (46.9\%) and 14:0 (34.2\%), whereas unsaturated C\textsubscript{16} fatty acids which are signature for type I methanotrophs are absent. Comparative sequence analysis of the 16S rRNA gene showed that the most closely related strains are \textit{Methylosoma difficile} LC 2\textsuperscript{T} (93.1\% identity) and \textit{Methylobacter tundripaludum} SV96\textsuperscript{T} (92.6\% identity). Phylogenetic analysis of the \textit{pmoA} gene indicated that strain HT12\textsuperscript{T} represents a distinct branch, and that the \textit{pmoA} amino acid sequence displayed 7\% divergence from the closest species. The DNA G + C content is 49.3 mol\%. Therefore we propose that strain HT12\textsuperscript{T} represents a novel genus and species, \textit{Methylovulum miyakonense} gen. nov., sp. nov. Strain HT12\textsuperscript{T} (= NBRC 106162\textsuperscript{T} = DSM 23269\textsuperscript{T} = ATCC BAA-2070\textsuperscript{T}) is the type strain.
Methanotrophs are a group of aerobic bacteria that utilize methane as the sole carbon and energy sources. They inhabit soils, wetlands, sediments, fresh and marine waters, lakes and peat bogs, and work as biofilters to reduce methane emissions into the atmosphere (Hanson & Hanson, 1996).

Methanotrophs are divided into two major subgroups. The type I methanotrophs, which belong to $\gamma$-Proteobacteria comprise the genera Methylomonas, Methylobacter, Methylocaldum, Methyloplasm, Methylosarcina, Methylohalobius, Methylosphaera, Methylothermus, Methylosoma and Methylococcus. The type II methanotrophs, which belong to $\alpha$-Proteobacteria comprise the genera Methylosinus, Methylocystis, Methylocella and Methylocapsa. Recently methanotrophs which are not within the classical types of characteristics have been reported. Crenothrix polyspora and Clonothrix fusca, which form filaments consisting of groups of sheathed cells were considered to be type I methanotrophs from phylogenetic traits (Stoecker et al., 2006; Vigliotta et al., 2007). The extremely acidophilic methane-oxidizing bacteria isolated from geothermal vents belong to the phylum, Verrucomicrobia rather than Proteobacteria (Pol et al., 2007; Dunfield et al., 2007; Islam et al., 2008).

In upland soils the methane concentration is low at or below atmospheric levels, but the diverse methanotrophs are active in response to the changes of temperature, soil moisture and nitrogen availability (Knief et al., 2003; Kolb et al., 2005; Horz et al., 2005; Mohanty et al., 2006; Mohanty et al., 2007; Singh & Kashyap, 2007). Although the analysis using the molecular markers (i.e., 16S rRNA gene, $pmoA$ gene, $mmoX$ gene and PLFAs) can clarify the ecology of methanotrophs and even indicate the presence of new methanotrophs at the site, the research on physiology and biochemistry of isolated methanotrophs on site is significant to reveal their precise work. In this paper, we report on the isolation and the characterization of the methanotroph from a forest soil.
We propose a new genus, *Methylovulum* gen. nov., and the new species *Methylovulum miyakonense* sp. nov. for the strain.

Soils from the forest in Hyogo, Japan (35°28’ N, 134°33’ E) were collected in July 2006. The soil sample was combined with 5 ml nitrate mineral salt (NMS) medium (Whittenbury *et al.*, 1970) at pH 6.8 in a 25 ml vial. The vial was sealed with a butyl rubber cap and with a crimped aluminum seal, after which 5 ml methane was added to achieve a 20% (v/v) atmospheric concentration. The vial was incubated at 28°C with shaking. Turbid enrichment cultures were sub-cultured in fresh NMS medium. The enrichment culture was serially diluted and spread onto NMS agar plates. The plates were incubated for 2 weeks at 28°C in a jar filled with a methane/air mixture. A colony was inoculated to liquid NMS medium with methane and 0.01% tryptone (Bacto Tryptone, Becton, Dickinson and Company), and cultivated. The cultivation in liquid medium and single colony isolation from agar plates were repeated until a single colony morphotype was obtained. Analysis of cell uniformity by light microscopy, and the absence of growth on Luria-Bertani (LB) agar (1% tryptone, 0.5% yeast extract, 1% NaCl) or tryptic soy agar (Becton, Dickinson and Company) were used as criteria for assessment of culture purity. Since the addition of tryptone stimulated the growth of the isolate, the isolate was usually cultivated in liquid NMS medium with methane and 0.01% tryptone, and was subcultured at 2-4 week intervals.

Morphological observations were performed using phase-contrast microscopy. Cyst formation was observed by the method of Vela & Wyss (1964). Cell fixation and observation using electron microscopy were performed at Tokai Electron Microscopy Analysis (Aichi, Japan). Exponentially growing cells were fixed with 2% glutaraldehyde and 2% para-formaldehyde in 0.1 M phosphate buffer (pH 7.4). After
cells were washed with 0.1 M phosphate buffer, they were subjected to a secondary
fixation with 2% osmium tetroxide in 0.1 M phosphate buffer. Cells were then
dehydrated with 50%, 70%, 90% and 100% ethanol. The cells were embedded in Quetol
812 (Nisshin EM) / methyl oxirane (1:3), and polymerized. Sections of 70 nm were cut on
LKB 2088 ultrotome V (LKB-Produkter AB), stained with uranyl acetate and lead stain
solution (Sigma-Aldrich), and viewed by JEM-1200EX transmission electron microscopy
(JEOL) operating at 80 kV.

Analysis of the ability to utilize various carbon sources was tested in liquid
NMS medium. Methanol and formaldehyde were added at 10-50 mM, and formamide
and ethanol were added at 0.1% (v/v). The following compounds were added to the
medium at concentrations of 0.1% (w/v): methylamine, formate, glucose, sucrose,
galactose, lactose, fructose, citrate, succinate, pyruvate, acetate and tryptone.
Utilization of various nitrogen sources was tested in liquid NMS medium in which
KNO₃ was replaced by one of the following compounds at 0.05% (w/v): NH₄Cl, NaNO₂,
urea, peptone, tryptone, yeast extract, casamino acid, glycine, alanine, lysine, arginine,
glutamate, glutamine, aspartate, asparagine, tryptophan, methionine, threonine,
cysteine and histidine. The temperature range for growth was tested in liquid NMS
medium at 5, 10, 15, 20, 24, 28, 32, 34 and 37 °C. The effect of pH was tested at 28 °C in
liquid NMS medium of which pH was adjusted by phosphate buffer (6.0-7.5) or
citrate/phosphate buffer (5.0-6.5) at concentrations of 10 and 25 mM. Growth was also
checked in liquid NMS medium without using any buffer, but using HCl or NaOH to
adjust pH (5.0-8.0). To determine the optimum salt concentrations, NaCl was added to
liquid NMS medium at concentrations of 0.1-0.5%.

For enzyme assays cells grown on methane were suspended in 20 mM Tris-HCl
buffer (pH 7.5) containing 5 mM MgCl₂, 10% glycerol and 1 mM dithiothreitol, and were
disrupted by sonication. After the sample was centrifuged at 5,000 x g for 30 min, the resulting supernatant was subjected to the following assays. The activity of 3-hexulose-6-phosphate synthase was measured by the disappearance of formaldehyde (Kato, 1990). The activity of 6-phospho-3-hexulosenisomerase was assessed as described by Arfman et al. (1990).

The cellular fatty acid analysis was performed at TechnoSuruga Laboratory (Shizuoka, Japan). Exponentially growing cells on methane in NMS medium were washed with 0.9% NaCl solution, freeze-dried and sent for the analysis. There, the cells were methylated and the methyl esters were subjected to gas chromatography. The fatty acids composition was determined by Sherlock Microbial Identification System (MIDI Inc.). The G+C content was analyzed at TechnoSuruga Laboratory. The DNA was extracted by the method of Ezaki et al. (1990). G+C content was measured by HPLC by the method of Katayama-Fujimura et al. (1984).

For PCR amplification the following primer sets were used: 27f-1492r for the 16S rRNA gene (Weisburg et al., 1991), A189-mb661 for the pmoa gene (Holmes et al., 1995; Costello & Lidstrom, 1999), mmoXA-mmoXB for the mmoX gene (Auman et al., 2000) and PolF-PolR for the nifH gene (Poly et al., 2001). PCR reactions were performed with Ex Taq polymerase (Takara Bio) using 30 cycles of 97°C for 30 s, 55°C for 30 s and 72°C for 30 s (pmoa and nifH) or 90 s (16S rRNA and mmoX). The products were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit and ABI 3130 genetic analyzer (Applied Biosystems). The 16S rRNA gene sequences (positions 28-1491, according to the International Union of Biochemistry nomenclature for Escherichia coli 16S rRNA), the deduced amino acid sequences of the pmoa gene (169 amino acids) and the deduced amino acid sequence of the mmoX gene (411 amino acids), respectively, were aligned with homologous sequences from the database using CLUSTAL W
program version 1.83 (http://clustalw.ddbj.nig.ac.jp/top-e.html). Phylogenetic trees were constructed by the neighbor-joining method with Kimura two-parameter model and were evaluated by bootstrap analysis based on 100 resampling replicates using CLUSTAL W program.

Strain HT12T was obtained from a forest soil sample collected in Hyogo prefecture, Japan, by the enrichment culture technique using methane as the sole carbon source. After several enrichments of the culture, strain HT12T was isolated as a single colony on an NMS agar plate using methane as the carbon source. The culture purity was verified by the failure to grow on LB agar and tryptic soy agar plates. This strain utilized methane in liquid NMS medium at a specific growth rate (µ) of 0.0093 h⁻¹ at the exponential phase.

Morphological and physiological characteristics of strain HT12T are summarized in Table 1. Two-week old colonies of strain HT12T were round, 1-2 mm in diameter, convex, pale brown in color and exhibited a smooth surface and an entire edge. As revealed by phase-contrast microscopy, the cells were non-motile, and coccoids or short rods that were 1.5-2.5 µm in length and 1.0-2.0 µm in width (Fig. 1a). Cysts were not observed on cells cultured in liquid or solid medium for 1 month. Electron microscopy analysis of ultrathin sections of cells revealed bundles of disk-shaped vesicles that are indicative of the typical intracytoplasmic membrane (ICM) of type I methanotrophs (Fig. 1b, c). The large inclusions of low electron density, presumably comprising poly-β-hydroxybutyrate granules, were also observed in the cells.

Strain HT12T only grew on methane and methanol (10-50 mM). None of the other carbon sources tested were utilized. Of the nitrogen sources tested, nitrate, NH₄Cl, glutamine and casamino acids were utilized. To lesser extent, cysteine, peptone and
tryptone were also utilized. No growth was observed in nitrogen-free medium although the \textit{nifH} gene was amplified by PCR. Strain HT12$^T$ is a mesophile that grows at temperatures ranging from 5 to 34 °C. Optimal growth was observed at 24-32 °C. Aggregation of cells was observed at temperatures below 20 °C. The strain was sensitive to NaCl, which inhibited growth at concentrations above 0.2%. Cells grew within a pH range of 6.0-7.5 when no additional buffers were used. When the medium was buffered, growth occurred at a pH of 6.0 and 6.5 (buffer at 10 mM), and at a pH of 6.5 (buffer at 25 mM).

The \textit{pmoA} and \textit{mmoX} genes were amplified by PCR, and we also have cloned the complete sets of genes encoding the particulate methane monooxygenase (pMMO) and the soluble methane monooxygenase (sMMO) (Iguchi, H., Yurimoto, H. & Sakai, Y., unpublished results). The presence of both pMMO and sMMO in strain HT12$^T$ is a notable characteristic, since in type I methanotrophs, sMMOs have been identified within only three genera (\textit{Methylomonas}, \textit{Methylococcus} and \textit{Methylomicrobium}). The activity of 3-hexulose-6-phosphate synthase and 6-phospho-3-hexuloseisomerase were detected, which are the key enzymes in the ribulose monophosphate pathway.

Strain HT12$^T$ is characterized by a unique fatty acid profile compared with other type I methanotrophs (Table 2). The major fatty acids were 16:0 and 14:0. These fatty acids are the major types in the genera \textit{Methylococcus} and \textit{Methylomonas}, respectively, but no other methanotroph has both these fatty acids as major constituents. The fatty acids 16:1$^{\omega 7c}$ and 16:1$^{\omega 5t}$ are also predominant in many type I methanotrophs (Hanson & Hanson, 1996; Table 2). However, strain HT12$^T$ did not contain these unsaturated C16 fatty acids.

Phylogenetic analysis of the 16S rRNA gene sequence and the partial \textit{pmoA} and \textit{mmoX} gene sequences indicated that strain HT12$^T$ represents a new lineage within
the type I methanotrophs. In the phylogenetic tree of the 16S rRNA gene, strain HT12T was clustering between the groups *Methylosoma, Crenothrix* and *Methylobacter* (Fig. 2). The sequence similarities to the closest strains were 93.1% to *Methylosoma difficile* LC 2T and 92.6% to *Methylobacter tundripaludum* SV96T. The *pmoA* gene sequence of strain HT12T was closely related to that of *Methylosoma difficile* LC 2T (93% deduced amino acid sequence identity), *Methylomicrobium japonense* NIT (93% identity) and *Methylobacter* sp. BB5.1 (92% identity). Strain HT12T formed a distinct branch from these related strains in the *pmoA* phylogenetic tree (Fig. S1). The phylogeny of the *pmoA* gene is largely consistent with that of the 16S rRNA gene, although strain HT12T was separate from *Methylosoma difficile* LC 2T in the *pmoA* phylogenetic tree. The deduced *mmoX* amino acid sequence between strain HT12T and the related species of *Methylomonas* differed by 3-5% (Fig. S2). The DNA G + C content of strain HT12T was 49.3 mol% (Table 1).

The comparative sequence analysis of the 16S rRNA gene and the *pmoA* gene showed that strain HT12T was most closely related to the genus *Methylosoma* and *Methylobacter*. However, the 7% sequence differences are too large to classify strain HT12T within these genera, and strain HT12T distinctly situated in the phylogenetic trees (Fig. 1 and S1). The fatty acids profile (Table 2) and the sMMO expression also exhibit the novelty of strain HT12 in type I methanotrophs. Therefore we propose that strain HT12T represents a novel genus and a novel species.

**Description of Methylovlum gen. nov.**

*Methylovlum* (Me.thy.lo'vu.lum. N.L. neut. n. methyl the methyl group; N.L. neut. n. ovulum, small egg; N.L. neut. n. *Methylovlum*, small methyl-using egg).

Cells are Gram-negative, aerobic, non-motile, coccoids or short-rods shaped. No
cysts are formed. Cells possess stacks of intracytoplasmic membranes, typical of type I methanotrophs. Cells grow on methane and methanol as the single carbon source. Methane is oxidized by pMMO and sMMO. C1-compounds are assimilated via the ribulose monophosphate pathway. No atmospheric nitrogen fixation occurs. The major cellular fatty acids are 16:0 and 14:0. DNA G + C content is 49 mol%. Phylogenetically, it belongs to γ-Proteobacteria (type I methanotroph or Methylococcaceae), and the most closely related genus is Methylosoma. The type species is Methylovulum miyakonense.

Description of Methylovulum miyakonense sp. nov.

Methylovulum miyakonense (mi.ya.ko.nen’se. N.L. neut. adj. miyakonense, of or belonging to Miyako, the ancient capital Kyoto)

This description is as for the genus with the following amendments. Cells are coccoids or short rods, 1.5-2.5 μm in length and 1.0-2.0 μm in width. Optimum growth is between 24-32°C and around pH 6.5. Cells are sensitive to NaCl above 0.2% (w/v). The type strain is HT12T (= NBRC 106162T = DSM 23269T = ATCC BAA-2070T).
ACKNOWLEDGMENT

This work was supported in part by Research Grant Programs for Natural Science from the Asahi Glass Foundation to Y.S. This work was also supported in part by a Grants-in-Aid for Scientific Research (B) 22380052 to Y.S., and Grants-in-Aid for Scientific Research (B) 22310046 to H.Y. from Japan Society for the Promotion of Science.
REFERENCES


Green, P. N. (1992). Taxonomy of methylotrophic bacteria. In Methane and


Table 1. Differentiation of strain HT12<sup>T</sup> from other genera of type I methanotrophs


<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>Methyloomas</em></th>
<th><em>Methylobacter</em></th>
<th><em>Methylomicrobium</em></th>
<th><em>Methylosoma</em></th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td><strong>HT12&lt;sup&gt;T&lt;/sup&gt;</strong></td>
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<tr>
<td>Cell morphology</td>
<td>Rods</td>
<td>Rods or cocci</td>
<td>Rods</td>
<td>Cocci, elliptical</td>
<td>Cocci or short or rods</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>Variable</td>
<td>+</td>
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<td>+</td>
<td>Variable</td>
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<tr>
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<td>Pale brown</td>
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<tr>
<td>CH&lt;sub&gt;4&lt;/sub&gt; oxidation</td>
<td>pMMO/sMMO</td>
<td>pMMO</td>
<td>pMMO/sMMO</td>
<td>pMMO</td>
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<td>45-55</td>
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Table 2. Comparison of cellular fatty acids of strain HT12\textsuperscript{T} with those in other type I methanotrophs

Values are percentages of the total fatty acids. Data for reference genera are from Bowman et al. (1993) (\textit{Methylomonas}, \textit{Methylobacter}, \textit{Methylomicrobium} and \textit{Methylococcus}) and Rahalkar et al. (2007) (\textit{Methylosoma}). -, not detected (below detection limit); ND, not determined.

<table>
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<th>Fatty acid</th>
<th>Methylomonas</th>
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<th>Methylomicrobium</th>
<th>Methylococcus</th>
<th>Methylosoma</th>
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**Figure legends**

**Fig. 1.** Cell morphology of strain HT12\(^T\). (a) Phase contrast micrograph of cells grown in liquid medium. (b, c) Electron micrographs of ultrathin section of cells. Bars, 5 \(\mu m\) (a), 1 \(\mu m\) (b) and 0.5 \(\mu m\) (c).

**Fig. 2.** Phylogenetic tree of 16S rRNA gene sequences showing the relationship of strain HT12\(^T\) to other type I methanotrophs. Bar, 0.05 changes per nucleotide sequence position. Bootstrap values less than 50% are not shown. GenBank accession numbers are given in parentheses.

**Fig. S1.** Phylogenetic tree of the derived amino acid sequences of \(pmoA\) gene from strain HT12\(^T\) and other methanotrophs. The AmoA sequences were used as the outgroup. Bar, 0.05 changes per amino acid sequence position. Bootstrap values less than 50% are not shown. GenBank accession numbers are given in parentheses.

**Fig. S2.** Phylogenetic tree of the derived amino acid sequences of \(mmoX\) gene from strain HT12\(^T\) and other methanotrophs. Bar, 0.05 changes per amino acid sequence position. Bootstrap values less than 50% are not shown. GenBank accession numbers are given in parentheses.
Fig. S1
Fig. S2