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

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Subject category: *Proteobacteria*.

Runnning 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^T, was isolated from a forest soil in Japan. Strain HT12^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 5-34ºC (optimum 24-32ºC). 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_{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 *Methylosoma difficile* LC 2^T (93.1% identity) and *Methylobacter tundripaludum* SV96^T (92.6% identity). Phylogenetic analysis of the *pmoA* gene indicated that strain HT12^T represents a distinct branch, and that the *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^T represents a novel genus and species, *Methylovulum miyakonense* gen. nov., sp. nov. Strain HT12^T (= NBRC 106162^T = DSM 23269^T = ATCC BAA-2070^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 *γ-Proteobacteria* comprise the genera *Methylomonas*, *Methylobacter*, *Methylocaldum*, *Methylomicrobium*, *Methylosarcina*, *Mehylohalobius*, *Methylosphaera*, *Methylothermus*, *Methylosoma* and *Methylococcus*. The type II methanotrophs, which belong to *α-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 metahnotorphs 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 (35º28' N, 134º33' 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 28ºC 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 28ºC 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 subcultuerd 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% glutataraldehyde 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 KNO_3 was replaced by one of the following compounds at 0.05% (w/v); NH_4Cl, $NaNO_2$, 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 ºC. The effect of pH was tested at 28 ºC 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 $MgCl_2$, 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-hexuloisomerase 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 97ºC for 30 s, 55ºC for 30 s and 72ºC 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 HT12^T 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 HT12^T 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 HT12^T are summarized in Table 1. Two-week old colonies of strain HT12^T 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 HT12^T only grew on methane and methanol (10-50 mM). None of the other carbon sources tested were utilized. Of the nitrogen sources tested, nitrate, NH_4Cl, 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 *nifH* gene was amplified by PCR. Strain HT12^T is a mesophile that grows at temperatures ranging from 5 to 34 ºC. Optimal growth was observed at 24-32 ºC. Aggregation of cells was observed at temperatures below 20 ºC. 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 *pmoA* and *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 (*Methylomonas*, *Methylococcus* and *Methylomicrobium*). The activity of 3-hexulose-6-phosphate synthase and 6-phospho-3-hexuloisomerase 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 *Methylococcus* and *Methylomonas*, respectively, but no other methanotroph has both these fatty acids as major constituents. The fatty acids 16 : 1ω7*c* and 16 : 1ω5*t* are also predominant in many type I methanotrophs (Hanson & Hanson, 1996; Table 2). However, strain HT12^T did not contain these unsaturated C_{16} fatty acids.

Phylogenetic analysis of the 16S rRNA gene sequence and the partial *pmoA* and *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 HT12^T 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 2^T and 92.6% to *Methylobacter tundripaludum* SV96^T. The *pmoA* gene sequence of strain HT12^T was closely related to that of *Methylosoma difficile* LC 2^T (93% deduced amino acid sequence identity), *Methylomicrobium japanense* NI^T (93% identity) and *Methylobacter* sp. BB5.1 (92% identity). Strain HT12^T 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 HT12^T was separate from *Methylosoma difficile* LC 2^T in the *pmoA* phylogenetic tree. The deduced *mmoX* amino acid sequence between strain HT12^T and the related species of *Methylomonas* differed by 3-5 % (Fig. S2). The DNA G + C content of strain HT12^T was 49.3 mol% (Table 1).

The comparative sequence analysis of the 16S rRNA gene and the *pmoA* gene showed that strain HT12^T was most closely related to the genus *Methylosoma* and *Methylobacter*. However, the 7% sequence differences are too large to classify strain HT12^T within these genera, and strain HT12^T 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 HT12^T represents a novel genus and a novel species.

Description of *Methylovulum* gen. nov.

Methylovulum (Me.thy.lo'vu.lum. N.L. neut. n. *methyl* the methyl group; N.L. neut. n. *ovulum*, small egg; N.L. neut. n. *Methylovulum*, 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 HT12^T (= NBRC 106162^T = DSM 23269^T = ATCC BAA-2070^T).

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Table 1. Differentiation of strain HT12^T from other genera of type I methanotrophs

Data for reference genera are from Green (1992) (*Methylomonas* and *Methylobacter*), Bowman *et al.* (1993) (*Methylomonas*, *Methylobacter* and *Methylomicrobium*), Wartiainen *et al.* (2006) (*Methylobacter*) and Rahalkar *et al.* (2007) (*Methylosoma*).

Characteristic	***Methylomonas***	***Methylobacter***	***Methylomicrobium***	***Methylosoma***	**Strain HT12^T**
Cell morphology	Rods	Rods or cocci	Rods	Cocci, elliptical or rods	Cocci or short rods
Motility	+	Variable	+	-	-
Cyst formation	+	Variable	-	+	-
Pigmentation	Pink, white	Brown, yellow, pink	White	Pale pink	Pale brown
CH_4 oxidation	pMMO/sMMO	pMMO	pMMO/sMMO	pMMO	pMMO/sMMO
nifH gene	Variable	Variable	-	+	+
G + C content (mol%)	52-59	45-55	49-60	49.9	49.3

Table 2. Comparison of cellular fatty acids of strain HT12^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) (*Methylomonas*, *Methylobacter*, *Methylomicrobium* and *Methylococcus*) and Rahalkar *et al.* (2007) (*Methylosoma*). -, not detected (below detection limit); ND, not determined.

Fatty acid	***Methylomonas***	***Methylobacter***	***Methylomicrobium***	***Methylococcus***	***Methylosoma***	**Strain HT12^T**
12 : 0	ND	ND	ND	ND	2.74	-
14 : 0	19-25	7-10	1-2	1-6	8.55	34.2
15 : 0	0-1	0-4	-	0-13	0.79	2.97
16 : 1ω8*c*	19-41	-	12-19	-	-	-
16 : 1ω7*c*	8-15	56-58	14-20	11-46	60	-
16 : 1ω6*c*	5-13	4-5	6-14	0-12	15	-
16 : 1ω5*c*	2-6	6-8	6-7	0-9	-	-
16 : 1ω5*t*	8-17	10-11	6-28	0-6	-	-

16：0	4-9	8-9	11-18	34-56	8.5	46.9
16 : 1ω11c	ND	ND	ND	ND	2.44	-
16：0 3-OH	ND	ND	ND	ND	ND	8.00
17 : 1ω6c	-	-	-	-	ND	6.40

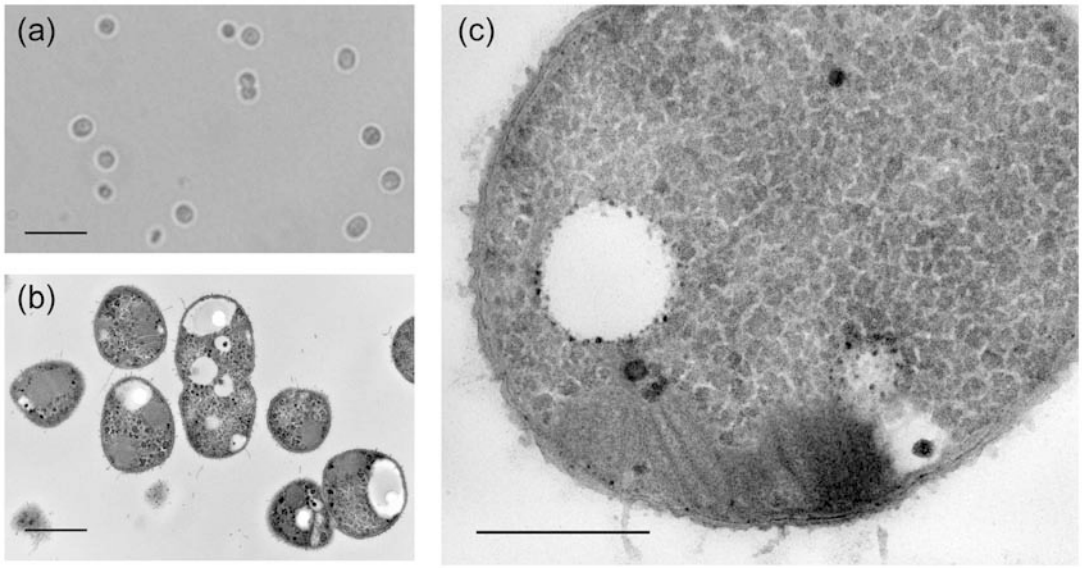
Figure legends

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 μm (a), 1 μm (b) and 0.5 μm (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.


(a)
(b)
(c)

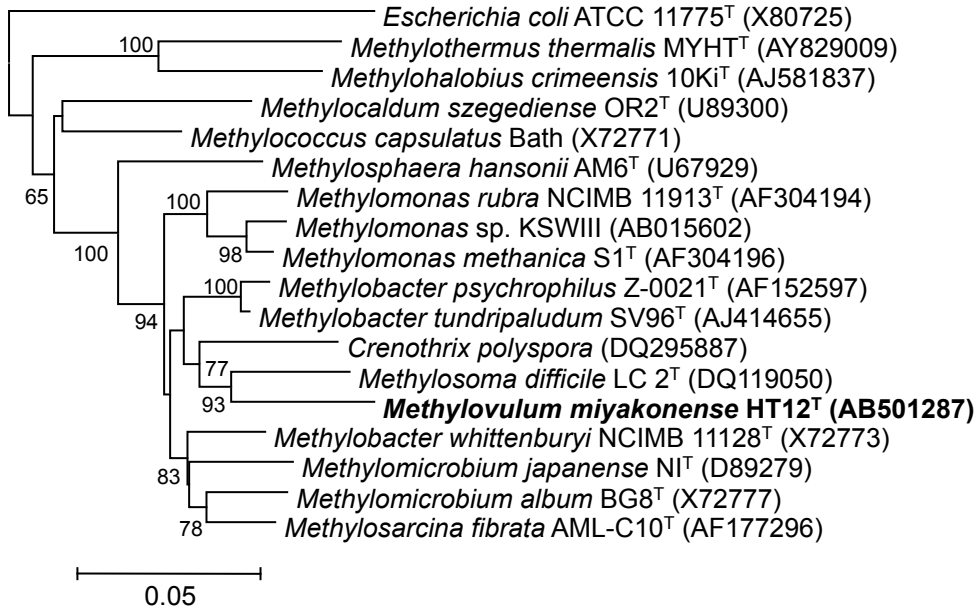

Escherichia coli ATCC 11775^{T} (X80725)
100
Methylothermus thermalis MYHT^{T} (AY829009)
Methylohalobius crimeensis 10Ki^{T} (AJ581837)
Methylocaldum szegediense OR2^{T} (U89300)
Methylococcus capsulatus Bath (X72771)
Methylosphaera hansonii AM6^{T} (U67929)
65
100
Methylomonas rubra NCIMB 11913^{T} (AF304194)
Methylomonas sp. KSWIII (AB015602)
100
98
Methylomonas methanica S1^{T} (AF304196)
100
Methylobacter psychrophilus Z-0021^{T} (AF152597)
94
Methylobacter tundripaludum SV96^{T} (AJ414655)
Crenothrix polyspora (DQ295887)
77
Methylosoma difficile LC 2^{T} (DQ119050)
93
***Methylovulum miyakonense* HT12^{T} (AB501287)**
Methylobacter whittenburyi NCIMB 11128^{T} (X72773)
83
Methylomicrobium japanense NI^{T} (D89279)
Methylomicrobium album BG8^{T} (X72777)
78
Methylosarcina fibrata AML-C10^{T} (AF177296)
0.05

Fig. 2

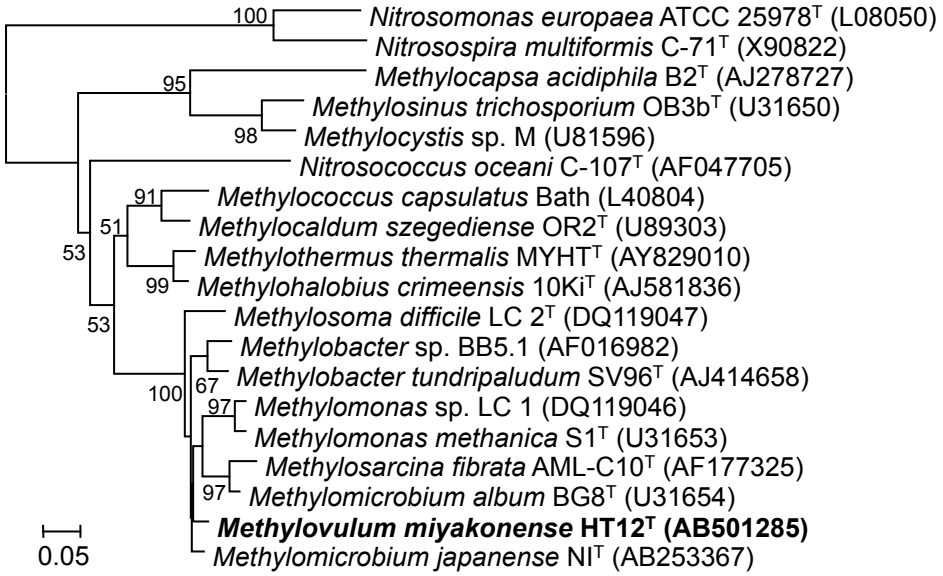

100
Nitrosomonas europaea ATCC 25978T (L08050)
Nitrosospira multiformis C-71T (X90822)
95
Methylocapsa acidiphila B2T (AJ278727)
Methylosinus trichosporium OB3bT (U31650)
98
Methylocystis sp. M (U81596)
Nitrosococcus oceani C-107T (AF047705)
91
Methylococcus capsulatus Bath (L40804)
51
Methylocaldum szegediense OR2T (U89303)
53
Methylothermus thermalis MYHTT (AY829010)
99
Methylohalobius crimeensis 10KiT (AJ581836)
53
Methylosoma difficile LC 2T (DQ119047)
Methylobacter sp. BB5.1 (AF016982)
Methylobacter tundripaludum SV96T (AJ414658)
100
67
97
Methylomonas sp. LC 1 (DQ119046)
Methylomonas methanica S1T (U31653)
Methylosarcina fibrata AML-C10T (AF177325)
97
Methylomicrobium album BG8T (U31654)
Methylovulum miyakonense HT12T (AB501285)
0.05
Methylomicrobium japanense NIT (AB253367)

Fig. S1

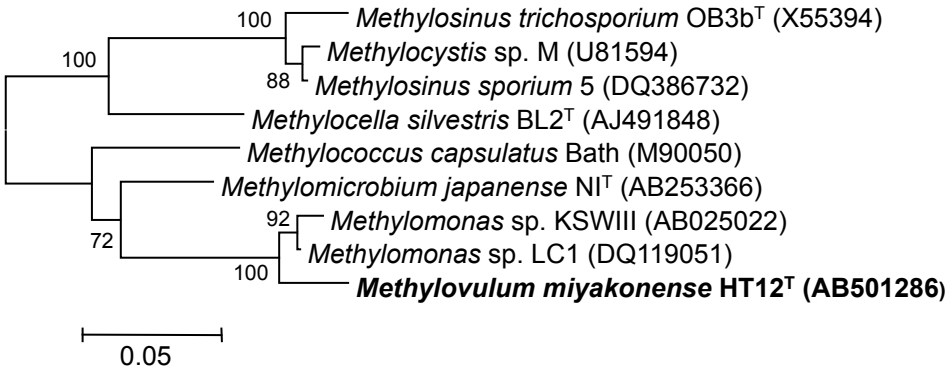

100
100
88
72
100
92
Methylosinus trichosporium OB3bT (X55394)
Methylocystis sp. M (U81594)
Methylosinus sporium 5 (DQ386732)
Methylocella silvestris BL2T (AJ491848)
Methylococcus capsulatus Bath (M90050)
Methylomicrobium japanense NIT (AB253366)
Methylomonas sp. KSWIII (AB025022)
Methylomonas sp. LC1 (DQ119051)
Methylovulum miyakonense HT12T (AB501286)
0.05

Fig. S2