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

Hiroyuki Iguchi, Hiroya Yurimoto and Yasuyoshi Sakai

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-8502, Japan.

Author for correspondence: Yasuyoshi Sakai. Tel: +81 75 753 6385. Fax: +81 75 753 6454. E-mail: ysakai@kais.kyoto-u.ac.jp

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.

1 We propose a new genus, *Methylovulum* gen. nov., and the new species *Methylovulum*
2 *miyakonense* sp. nov. for the strain.
3
4 Soils from the forest in Hyogo, Japan (35º28' N, 134º33' E) were collected in
5 July 2006. The soil sample was combined with 5 ml nitrate mineral salt (NMS) medium
6 (Whittenbury *et al.*, 1970) at pH 6.8 in a 25 ml vial. The vial was sealed with a butyl
7 rubber cap and with a crimped aluminum seal, after which 5 ml methane was added to
8 achieve a 20% (v/v) atmospheric concentration. The vial was incubated at 28ºC with
9 shaking. Turbid enrichment cultures were sub-cultured in fresh NMS medium. The
10 enrichment culture was serially diluted and spread onto NMS agar plates. The plates
11 were incubated for 2 weeks at 28ºC in a jar filled with a methane/air mixture. A colony
12 was inoculated to liquid NMS medium with methane and 0.01% tryptone (Bacto
13 Tryptone, Becton, Dickinson and Company), and cultivated. The cultivation in liquid
14 medium and single colony isolation from agar plates were repeated until a single colony
15 morphotype was obtained. Analysis of cell uniformity by light microscopy, and the
16 absence of growth on Luria-Bertani (LB) agar (1% tryptone, 0.5% yeast extract, 1%
17 NaCl) or tryptic soy agar (Becton, Dickinson and Company) were used as criteria for
18 assessment of culture purity. Since the addition of tryptone stimulated the growth of
19 the isolate, the isolate was usually cultivated in liquid NMS medium with methane and
20 0.01% tryptone, and was subcultuerd at 2-4 week intervals.
21 Morphological observations were performed using phase-contrast microscopy.
22 Cyst formation was observed by the method of Vela & Wyss (1964). Cell fixation and
23 observation using electron microscopy were performed at Tokai Electron Microscopy
24 Analysis (Aichi, Japan). Exponentially growing cells were fixed with 2%
25 glutataraldehyde and 2% para-formaldehyde in 0.1 M phosphate buffer (pH 7.4). After

1 cells were washed with 0.1 M phosphate buffer, they were subjected to a secondary
2 fixation with 2% osmium tetroxide in 0.1 M phosphate buffer. Cells were then
3 dehydrated with 50%, 70%, 90% and 100% ethanol. The cells were embedded in Quetol
4 812 (Nisshin EM) / methyl oxirane (1:3), and polymerized. Sections of 70 nm were cut on
5 LKB 2088 ultrotome V (LKB-Produkter AB), stained with uranyl acetate and lead stain
6 solution (Sigma-Aldrich), and viewed by JEM-1200EX transmission electron microscopy
7 (JEOL) operating at 80 kV.

8 Analysis of the ability to utilize various carbon sources was tested in liquid
9 NMS medium. Methanol and formaldehyde were added at 10-50 mM, and formamide
10 and ethanol were added at 0.1% (v/v). The following compounds were added to the
11 medium at concentrations of 0.1% (w/v): methylamine, formate, glucose, sucrose,
12 galactose, lactose, fructose, citrate, succinate, pyruvate, acetate and tryptone.
13 Utilization of various nitrogen sources was tested in liquid NMS medium in which
14 KNO_3 was replaced by one of the following compounds at 0.05% (w/v); NH_4Cl, $NaNO_2$,
15 urea, peptone, tryptone, yeast extract, casamino acid, glycine, alanine, lysine, arginine,
16 glutamate, glutamine, aspartate, asparagine, tryptophan, methionine, threonine,
17 cysteine and histidine. The temperature range for growth was tested in liquid NMS
18 medium at 5, 10, 15, 20, 24, 28, 32, 34 and 37 °C. The effect of pH was tested at 28 °C in
19 liquid NMS medium of which pH was adjusted by phosphate buffer (6.0-7.5) or
20 citrate/phosphate buffer (5.0-6.5) at concentrations of 10 and 25 mM. Growth was also
21 checked in liquid NMS medium without using any buffer, but using HCl or NaOH to
22 adjust pH (5.0-8.0). To determine the optimum salt concentrations, NaCl was added to
23 liquid NMS medium at concentrations of 0.1-0.5%.

24 For enzyme assays cells grown on methane were suspended in 20 mM Tris-HCl
25 buffer (pH 7.5) containing 5 mM $MgCl_2$, 10% glycerol and 1 mM dithiothreitol, and were

1 disrupted by sonication. After the sample was centrifuged at 5,000 x *g* for 30 min, the
2 resulting supernatant was subjected to the following assays. The activity of
3 3-hexulose-6-phosphate synthase was measured by the disappearance of formaldehyde
4 (Kato, 1990). The activity of 6-phospho-3-hexuloisomerase was assessed as described by
5 Arfman *et al.* (1990).

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

14 For PCR amplification the following primer sets were used; 27f-1492r for the
15 16S rRNA gene (Weisburg *et al.*, 1991), A189-mb661 for the *pmoA* gene (Holmes *et al.*,
16 1995; Costello & Lidstrom, 1999), mmoXA-mmoXB for the *mmoX* gene (Auman *et al.*,
17 2000) and PolF-PolR for the *nifH* gene (Poly *et al.*, 2001). PCR reactions were performed
18 with Ex Taq polymerase (Takara Bio) using 30 cycles of 97°C for 30 s, 55°C for 30 s and
19 72°C for 30 s (*pmoA* and *nifH*) or 90 s (16S rRNA and *mmoX*). The products were
20 sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit and ABI 3130 genetic
21 analyzer (Applied Biosystems). The 16S rRNA gene sequences (positions 28-1491,
22 according to the International Union of Biochemistry nomenclature for *Escherichia coli*
23 16S rRNA), the deduced amino acid sequences of the *pmoA* gene (169 amino acids) and
24 the deduced amino acid sequence of the *mmoX* gene (411 amino acids), respectively,
25 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 °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 *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 $16:1\omega7c$ and $16:1\omega5t$ 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

1 the type I methanotrophs. In the phylogenetic tree of the 16S rRNA gene, strain HT12^T
2 was clustering between the groups *Methylosoma*, *Crenothrix* and *Methylobacter* (Fig. 2).
3 The sequence similarities to the closest strains were 93.1% to *Methylosoma difficile* LC
4 2^T and 92.6% to *Methylobacter tundripaludum* SV96^T. The *pmoA* gene sequence of
5 strain HT12^T was closely related to that of *Methylosoma difficile* LC 2^T (93% deduced
6 amino acid sequence identity), *Methylomicrobium japanense* NI^T (93% identity) and
7 *Methylobacter* sp. BB5.1 (92% identity). Strain HT12^T formed a distinct branch from
8 these related strains in the *pmoA* phylogenetic tree (Fig. S1). The phylogeny of the
9 *pmoA* gene is largely consistent with that of the 16S rRNA gene, although strain HT12^T
10 was separate from *Methylosoma difficile* LC 2^T in the *pmoA* phylogenetic tree. The
11 deduced *mmoX* amino acid sequence between strain HT12^T and the related species of
12 *Methylomonas* differed by 3-5 % (Fig. S2). The DNA G + C content of strain HT12^T was
13 49.3 mol% (Table 1).
14 The comparative sequence analysis of the 16S rRNA gene and the *pmoA* gene
15 showed that strain HT12^T was most closely related to the genus *Methylosoma* and
16 *Methylobacter*. However, the 7% sequence differences are too large to classify strain
17 HT12^T within these genera, and strain HT12^T distinctly situated in the phylogenetic
18 trees (Fig. 1 and S1). The fatty acids profile (Table 2) and the sMMO expression also
19 exhibit the novelty of strain HT12 in type I methanotrophs. Therefore we propose that
20 strain HT12^T represents a novel genus and a novel species.
21
22 **Description of *Methylovulum* gen. nov.**
23 *Methylovulum* (Me.thy.lo'vu.lum. N.L. neut. n. *methyl* the methyl group; N.L.
24 neut. n. *ovulum*, small egg; N.L. neut. n. *Methylovulum*, small methyl-using egg).
25 Cells are Gram-negative, aerobic, non-motile, coccoids or short-rods shaped. No

1 cysts are formed. Cells possess stacks of intracytoplasmic membranes, typical of type I
2 methanotrophs. Cells grow on methane and methanol as the single carbon source.
3 Methane is oxidized by pMMO and sMMO. C1-compounds are assimilated via the
4 ribulose monophosphate pathway. No atmospheric nitrogen fixation occurs. The major
5 cellular fatty acids are 16 : 0 and 14 : 0. DNA G + C content is 49 mol%. Phylogenetically,
6 it belongs to *γ-Proteobacteria* (type I methanotroph or *Methylococcaceae*), and the most
7 closely related genus is *Methylosoma*. The type species is *Methylovulum miyakonense*.
8

9 **Description of *Methylovulum miyakonense* sp. nov.**

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

12 This description is as for the genus with the following amendments. Cells are
13 coccoids or short rods, 1.5-2.5 μm in length and 1.0-2.0 μm in width. Optimum growth is
14 between 24-32°C and around pH 6.5. Cells are sensitive to NaCl above 0.2% (w/v). The
15 type strain is $HT12^T$ (= NBRC 106162^T = DSM 23269^T = ATCC BAA-2070^T).
16

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.

1 # REFERENCES

2

3 **Arfman, N., Bystrykh, L., Govorukhina, N. I. & Dijkhuizen, L.**
4 **(1990).** 3-Hexulose-6-phosphate synthase from thermotolerant methylotrophic
5 *Bacillus* C1. *Methods Enzymol* **188**, 391-397.

6 **Auman, A. J., Stolyar, S., Costello, A. M. & Lidstrom, M. E. (2000).**
7 Molecular characterization of methanotrophic isolates from freshwater lake sediment.
8 *Appl Environ Microbiol* **66**, 5259-5266.

9 **Bowman, J. P., Sly, L. I., Nichols, P. D. & Hayward, A. C. (1993).**
10 Revised taxonomy of the Methanotrophs: description of *Methylobacter* gen. nov.,
11 emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species,
12 and a proposal that the family *Methylococcaceae* includes only the group I
13 Methanotrophs. *Int J Syst Bacteriol* **43**, 735-753.

14 **Costello, A. M. & Lidstrom, M. E. (1999).** Molecular characterization of
15 functional and phylogenetic genes from natural populations of methanotrophs in lake
16 sediments. *Appl Environ Microbiol* **65**, 5066-5074.

17 **Dunfield, P. F., Yuryev, A., Senin, P., Smirnova, A. V., Stott, M. B.,**
18 **Hou, S., Ly, B., Saw, J. H., Zhou, Z., Ren, Y., Wang, J., Mountain, B. W.,**
19 **Crowe, M. A., Weatherby, T. M., Bodelier, P. L., Liesack, W., Feng, L., Wang,**
20 **L. & Alam, M. (2007).** Methane oxidation by an extremely acidophilic bacterium of
21 the phylum Verrucomicrobia. *Nature* **450**, 879-882.

22 **Ezaki, T., Saidi, S. M., Liu, S. L., Hashimoto, Y., Yamamoto, H. &**
23 **Yabuuchi, E. (1990).** Rapid procedure to determine the DNA base composition from
24 small amounts of gram-positive bacteria. *FEMS Microbiol Lett* **55**, 127-30.

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

1 Methanol Utilizers,1st edn, pp. 23-84. Edited by Murrell, J. C. & Dalton, H. New York:
2 Plenum Press.
3 **Hanson, R. S. & Hanson, T. E. (1996).** Methanotrophic bacteria. *Microbiol*
4 *Rev* **60,** 439–471.
5 **Holmes, A. J., Costello, A., Lidstrom, M. E. & Murrell, J. C. (1995).**
6 Evidence that particulate methane monooxygenase and ammonia monooxygenase may
7 be evolutionarily related. *FEMS Microbiol Lett* **132,** 203-208.
8 **Horz, H. P., Rich, V., Avrahami, S. & Bohannan, B. J. (2005).**
9 Methane-oxidizing bacteria in a California upland grassland soil: diversity and
10 response to simulated global change. *Appl Environ Microbiol* **71,** 2642-2652.
11 **Islam, T., Jensen, S., Reigstad, L. J., Larsen, O. & Birkeland, N. K.**
12 **(2008).** Methane oxidation at 55 degrees C and pH 2 by a thermoacidophilic bacterium
13 belonging to the Verrucomicrobia phylum. *Proc Natl Acad Sci U S A* **105,** 300-304.
14 **Katayama-Fujimura, Y., Komatsu, Y., Kuraishi, H. & Kaneko, T.**
15 **(1984).** Estimation of DNA base composition by high performance liquid
16 chromatography of its nuclease P1 hydrolysate. *Agric Biol Chem* **48,** 3169-3172.
17 **Kato, N. (1990).** 3-Hexulose-6-phosphate synthase from *Mycobacterium*
18 *gastri* MB19. *Methods Enzymol* **188,** 397-401.
19 **Knief, C., Lipski, A. & Dunfield, P. F. (2003).** Diversity and activity of
20 methanotrophic bacteria in different upland soils. *Appl Environ Microbiol* **69,**
21 6703-6714.
22 **Kolb, S., Knief, C., Dunfield, P. F. & Conrad, R. (2005).** Abundance
23 and activity of uncultured methanotrophic bacteria involved in the consumption of
24 atmospheric methane in two forest soils. *Environ Microbiol* **7,** 1150-1161.
25 **Mohanty, S. R., Bodelier, P. L., Floris, V. & Conrad, R. (2006).**

1 Differential effects of nitrogenous fertilizers on methane-consuming microbes in rice
2 field and forest soils. *Appl Environ Microbiol* **72**, 1346-1354.
3 **Mohanty, S. R., Bodelier, P. L. & Conrad, R. (2007).** Effect of
4 temperature on composition of the methanotrophic community in rice field and forest
5 soil. *FEMS Microbiol Ecol* **62**, 24-31.
6 **Pol, A., Heijmans, K., Harhangi, H. R., Tedesco, D., Jetten, M. S. &**
7 **OpdenCamp, H. J. M. (2007).** Methanotrophy below pH 1 by a new Verrucomicrobia
8 species. *Nature* **450**, 874-878.
9 **Poly, F., Monrozier, L. J. & Bally, R. (2001).** Improvement in the RFLP
10 procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in
11 soil. *Res Microbiol* **152**, 95-103.
12 **Rahalkar, M., Bussmann, I. & Schink, B. (2006).** *Methylosoma difficile*
13 gen. nov., sp. nov., a novel methanotroph enriched by gradient cultivation from littoral
14 sediment of Lake Constance. *Int J Syst Evol Microbiol* **57**, 1073-1080.
15 **Singh, J. S. & Kashyap, A. K. (2007).** Contrasting pattern of
16 methanotrophs in dry tropical forest soils: effect of soil nitrogen, carbon and moisture.
17 *Microbiol Res* **162**, 276-283.
18 **Stoecker, K., Bendinger, B., Schöning, B., Nielsen, P. H., Nielsen, J.**
19 **L., Baranyi, C., Toenshoff, E. R., Daims, H. & Wagner, M. (2006).** Cohn's
20 *Crenothrix* is a filamentous methane oxidizer with an unusual methane monooxygenase.
21 *Proc Natl Acad Sci USA* **103**, 2363-2367.
22 **Vela, G. R. & Wyss, O. (1964).** Improved stain for visualization of
23 azotobacter encystment. *J Bacteriol* **87**, 476-477.
24 **Vigliotta, G., Nutricati, E., Carata, E., Tredici, S. M., DeStefano, M.,**
25 **Pontieri, P., Massardo, D. R., Prati, M. V., DeBellis, L. & Alifano, P. (2007).**

1 *Clonothrix fusca* Roze 1896, a filamentous, sheathed, methanotrophic
2 gamma-proteobacterium. *Appl Environ Microbiol* **73**, 3556-3565.

3 **Wartiainen, I., Hestnes, A. G., McDonald, I. R. & Svenning, M. M.**
4 **(2006).** *Methylobacter tundripaludum* sp. nov., a methane-oxidizing bacterium from
5 Arctic wetland soil on the Svalbard islands, Norway (78 degrees N). *Int J Syst Evol*
6 *Microbiol* **56**, 109-113.

7 **Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991).**
8 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.

9 **Whittenbury, R., Phillips, K. C. & Wilkinson, J. F. (1970).**
10 Enrichment, isolation and some properties of methane-utilizing bacteria. *J Gen*
11 *Microbiol* **61**, 205-218.

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 : 1*ω*8*c*	19-41	-	12-19	-	-	-
16 : 1*ω*7*c*	8-15	56-58	14-20	11-46	60	-
16 : 1*ω*6*c*	5-13	4-5	6-14	0-12	15	-
16 : 1*ω*5*c*	2-6	6-8	6-7	0-9	-	-
16 : 1*ω*5*t*	8-17	10-11	6-28	0-6	-	-

16：0	4-9	8-9	11-18	34-56	8.5	46.9
16：1ω11*c*	ND	ND	ND	ND	2.44	-
16：0 3-OH	ND	ND	ND	ND	ND	8.00
17：1ω6*c*	-	-	-	-	ND	6.40

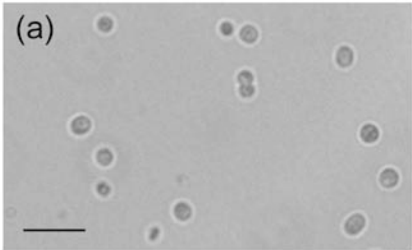
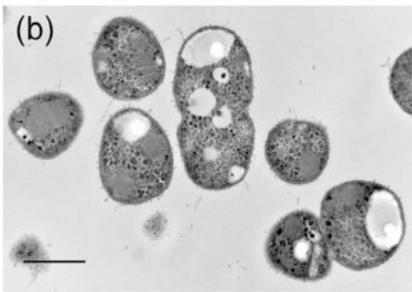
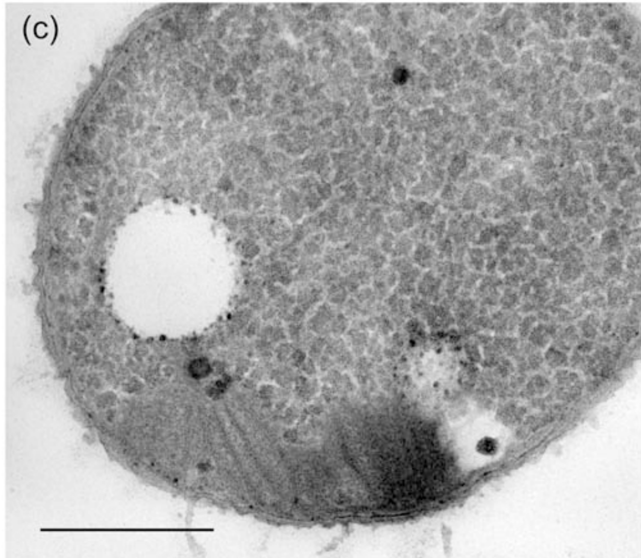
Figure legends

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)

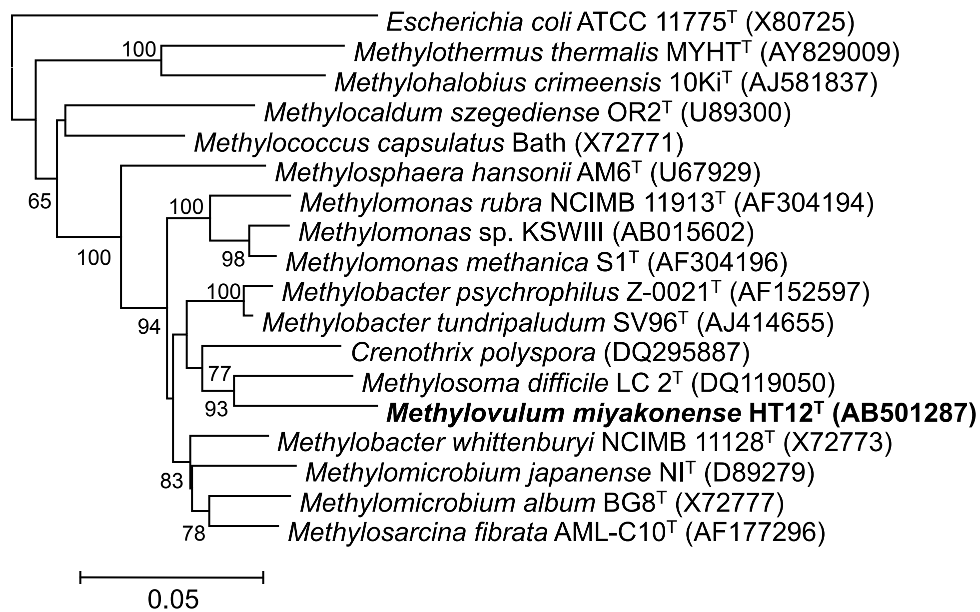


Fig. 2

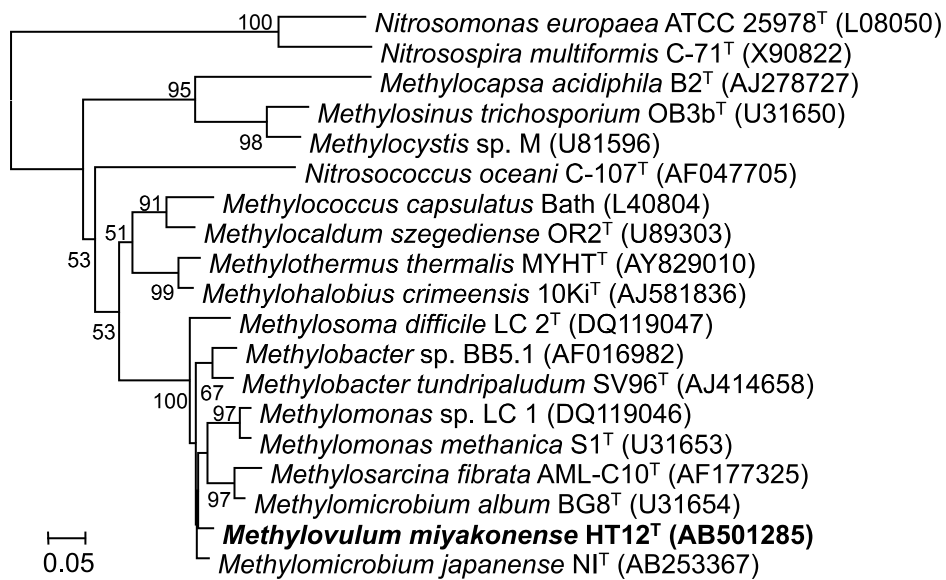


Fig. S1

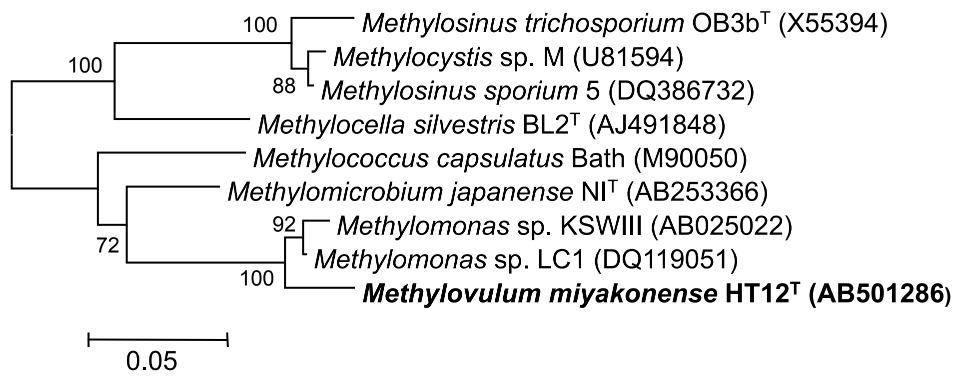


Fig. S2