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Methylomonas paludis sp. nov., the First Acid-Tolerant Member of the Genus *Methylomonas*, from an Acidic Wetland

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ABSTRACT

An aerobic methanotrophic bacterium was isolated from an acidic (pH 3.9) *Sphagnum* peat bog in north-eastern Russia and designated strain MG30^T. Cells of this strain are Gram-negative, pale-pink-pigmented, non-motile, thick rods that are covered by large polysaccharide capsules and contain an intracytoplasmic membrane system typical of type I methanotrophs. They possess a particulate methane monooxygenase enzyme (pMMO) and utilize only methane and methanol. Carbon is assimilated via the ribulose-monophosphate pathway; nitrogen is fixed via an oxygen-sensitive nitrogenase. Strain MG30^T grows in a pH range of 3.8-7.3 (optimum pH 5.8-6.4) and at temperatures between 8 and 30°C (optimum 20-25°C). The major cellular fatty acids are C16:1*ω5t,* C16:1*ω8c,* C16:1*ω7c,* and C14:0; the DNA G+C content is 48.5 mol%. The isolate belongs to the family *Methylococcaceae* of the class *Gammaproteobacteria* and displays 94.7-96.9% 16S rRNA gene sequence similarity to members of the genus *Methylomonas.* However, strain MG30^T differs from all taxonomically characterized members of this genus by the absence of motility, the ability to grow in acidic conditions, and low DNA G+C content. Therefore, we propose to classify this strain as a novel, acid-tolerant species of the genus *Methylomonas*, *Methylomonas paludis* sp. nov. Strain MG30^T (=DSM 24973^T = VKM B-2745^T) is the type strain.

Keywords: *Methylomonas paludis* sp. nov., acid-tolerant methanotroph, acidic wetlands.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the partial sequences of the *pmoA*, *mxaF* and *nifH* genes of *Methylomonas paludis* MG30^T are HE801216-HE801219, respectively.

The genus *Methylomonas* belongs to the class *Gammaproteobacteria*, the family *Methylococcaceae*, and encompasses strictly aerobic, obligate utilizers of C1 compounds with type I intracytoplasmic membranes (ICM) and the ribulose-monophosphate pathway of carbon assimilation (Whittenbury & Krieg, 1984; Bowman *et al*., 1993). The first described methanotrophic bacterium, which was isolated and named as '*Bacillus methanicus*' by Söhngen in 1906, represents the type species of the genus *Methylomonas*, *M. methanica* (Whittenbury & Krieg, 1984). Besides *M. methanica*, this genus includes four other validly described species, *M. aurantiaca*, *M. fodinarum* (Bowman *et al*., 1990), *M. scandinavica* (Kalyuzhnaya *et al*., 1999), and *M. koyamae* (Ogiso *et al.*, 2012). Members of these species are Gram-negative, rod-shaped or coccobacillary cells that produce pink or orange carotenoid pigments, are motile by means of a single flagellum, and grow between pH 5.0 and 9.0, with an optimum at pH 6.5 to 7.0.

Representatives of the genus *Methylomonas* are especially abundant in various semi-neutral or slightly alkaline environments, such as rice paddies, water-saturated soils, volcanic areas, fresh and marine waters, lakes and sediments (Auman *et al*., 2000; Auman & Lidstrom, 2002; Hutchens *et al*., 2004; Lin *et al*., 2004; Lüke *et al*., 2010; Dianou *et al.*, 2012). Recently, however, some evidence for the presence of *Methylomonas*-like bacteria in acidic boreal forest soils and *Sphagnum*-dominated wetlands was obtained in several cultivation-independent studies (Morris *et al.*, 2002; Jaatinen *et al.*, 2005; Chen *et al.*, 2008, Kip *et al*, 2011a). These findings were difficult to interpret until the ability to grow at pH below 5.0 was reported for *Methylomonas* sp. strain M5, which was isolated from an acidic *Sphagnum* peat bog in the Netherlands (Kip *et al*, 2011b). This isolate, however, was only partially characterized and was not described taxonomically.

In this paper, we report isolation of another representative of the genus *Methylomonas* from an acidic *Sphagnum*-dominated wetland in north-eastern Russia. We confirm the ability of this methanotroph to grow at pH below 5.0 but demonstrate its acid-tolerant rather than acidophilic nature, and describe this bacterium as a novel, acid-tolerant *Methylomonas* species.

Strain MG30^T was isolated from a sample collected in July, 2010 from 5-10 cm below the surface of *Sphagnum* peat (pH 3.9) on the bank of the bog lake Germanovskoe, island Valaam, Karelia, north-eastern Russia (61^{o}22'N, 31^{o}07'E). A methanotrophic enrichment culture was obtained using liquid medium M2 containing (in grams per litre distilled water) KNO_3, 0.25; KH_2PO_4, 0.1; $MgSO_4 \times 7H_2O$, 0.05; $CaCl_2 \times 2H_2O$, 0.01; and NaCl, 0.02, with the addition of 0.1% (by volume) of a trace elements stock solution containing (in grams per litre) EDTA, 5; $FeSO_4 \times 7H_2O$, 2; $ZnSO_4 \times 7H_2O$, 0.1; $MnCl_2 \times 4H_2O$, 0.03; $CoCl_2 \times 6H_2O$, 0.2; $CuCl_2 \times 5H_2O$, 0.1; $NiCl_2 \times 6H_2O$, 0.02, and Na_2MoO_4, 0.03. The medium pH was 5.5. A whole-cell hybridization with type I methanotroph-specific probe set M84 + M705 (Eller *et al*., 2001) revealed some cells of gammaproteobacterial methanotrophs in this enrichment culture. These cells represented thick rods that were arranged in pairs or in short chains. An aliquot of the respective cell suspension was spread plated onto the agar medium M2 and the plates were incubated at 20°C in desiccators under a methane/air (30 : 70) gas mixture. The colonies that developed on these plates after 3 weeks of incubation were again screened for the presence of type I methanotrophs by means of whole-cell hybridization. The selected colonies were picked and purified by successive re-streaking until a methanotrophic bacterium, designated strain MG30^T, was obtained in a pure culture.

The isolate was maintained on agar medium M2 and in liquid cultures. For growth in liquid media, 500 ml screw-cap serum bottles were used with a headspace/ liquid space ratio of 4:1. After inoculation, the bottles were sealed with silicone rubber septa, and methane was added aseptically using a syringe equipped with a disposable filter (0.22 µm) to achieve a 10-20% mixing ratio in the headspace. Bottles were incubated on a rotary shaker (120 rpm) at 20°C. Culture purity was ensured by examination under phase-contrast and electron microscopy and by plating on media containing the following different organic substrates: 10-fold diluted Luria–Bertani agar (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl) or M2 agar medium amended with 0.1% (w/v) glucose. The culture was considered to be pure when only one cell type was observed under light microscopy and no growth on nutrient rich media was observed.

Morphological observations and cell-size measurements were made with a Zeiss Axioplan 2 microscope and Axiovision 4.2 software (Zeiss). Cell morphology and the presence of cysts were examined by using batch cultures grown to the early-exponential, late-exponential and stationary growth phases. For electron microscopy, cells of exponentially growing cultures were collected by centrifugation and pre-fixed with 1.5% (w/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 1 h at 4 °C and then fixed in 1% (w/v) OsO4 in the same buffer for 4 h at 20 °C. After dehydration in an ethanol series, samples were embedded in Epon 812 epoxy resin. Thin sections were cut on an LKB-2128 Ultrotome, stained with 3% (w/v) uranyl acetate in 70% (v/v) ethanol and then post-stained with lead citrate (Reynolds, 1963) at 20°C for 4–5 min. Specimen samples were examined with a JEM-100B transmission electron microscope at an 80 kV accelerating voltage. To test the presence of flagella, cell suspensions from the cultures of strain MG30^T were dried onto grids and treated with 1% (w/v) phosphotungstic acid solution. Negatively stained cells were examined with a JEM-100C electron microscope at an 80 kV accelerating voltage.

Physiological tests were performed in liquid medium M2 with methane. Growth of strain MG30^T was monitored by measuring OD_{600} for 2 weeks under a variety of conditions, including temperatures of 4-37^{o}C, pH 3.0-8.0 and NaCl concentrations of 0-5.0 % (wt/vol). Variations in the pH were achieved by mixing 0.1M solutions of H_3PO_4, KH_2PO_4, and K_2HPO_4. Heat resistance was tested by heating cell suspensions at 50, 60, 70 or 80 °C for 10 min, followed by plating onto agar medium M2 and incubation at 20°C for 2 weeks. Desiccation resistance was assessed according to Whittenbury *et al*. (1970)**.** The range of potential growth substrates was examined using 0.05% (w/v) concentrations of the following carbon sources: ethanol, formate, glucose, acetate, pyruvate, malate, succinate and citrate. The ability of strain MG30^T to grow on methanol was tested in liquid medium M2 containing 0.01–5% (v/v) methanol. Nitrogen sources were tested by replacing KNO_3 in M2 with 0.05% (w/v) NH_4Cl, $NaNO_2$, urea, formamide, methylamine, glutamine, glycine, alanine, peptone, casamino acids or yeast extract. For N_2-

fixation experiments, a nitrate-free medium was used. Growth was examined after 1 month of incubation.

For enzymic studies, methane-grown cells of strain MG30^T were harvested by centrifugation (15000×g, 30 min) and resuspended in 0.05 M Tris-HCl buffer (pH 7.5). Cells were disrupted by ultrasonic treatment (6×30 sec), centrifuged at 15000×g for 40 min to remove unbroken cells and the resulting supernatant was used for enzyme assays. The protein content of extracts was determined by the modified method of Lowry (Schacterle, Pollack, 1973). The following enzymes were assayed spectrophotometrically at 30ºC in cell-free extracts using earlier published methods: methanol dehydrogenase EC 1.1.99.8 (Anthony, Zatman, 1964); formate dehydrogenase EC 1.2.1.2 with NAD^+ (Johnson, Quayle, 1964); NADH- or NADPH-dependent hydroxypyruvate reductase EC 1.1.1.29 and serine-glyoxylate aminotransferase EC 2.6.1.45 (Blackmore, Quayle, 1970); glucose-6-phosphate dehydrogenase EC 1.1.1.49 (Kornberg, Horecker, 1955); 6-phosphofructokinase (PFK) EC 2.7.1.11 (van Dijken, Quayle, 1977); pyrophosphate-dependent PFK EC 2.7.1.90 (Beschastny *et al*., 1992); 3-hexulose-6-phosphate synthase EC 4.1.2.43 (Ferenci *et al*., 1974). These enzyme assays were conducted using a Shimadzu UV-1700 spectrophotometer (Japan). Specific activity of ribulose bisphosphate carboxylase EC 4.1.1.49 was assayed radiometrically in 0.5 ml of the following reaction mixture: 100 mM Tris-HCl buffer (pH 7.6), 10 mM $MgCl_2$, 12 mM $NaH^{14}CO_3$ (1.0 μCi/μmol), 0.5 mM ribulose-1,5-bisphosphate (RuBP), and cell-free extract (0.1 to 0.5 mg protein). After pre-incubation at 30ºC for 2 min, the reaction was initiated by addition of $NaH^{14}CO_3$. Aliquots (0.05 ml) were withdrawn, placed on glass-fiber paper (GF/F) pieces (1x1 cm) and immediately fixed by addition of 0.05 ml of 6 N HCl to remove $^{14}CO_2$ excess. RuBP-dependent incorporation of radioactivity into acid-stable products was determined using a liquid scintillation spectrometer LS 6500 Multipurpose scintillation counter (Backman Coulter, USA).

For fatty acid analyses, cells were grown on liquid mineral medium M2 with methane and harvested in the late exponential growth phase. Fatty acid analysis was performed as described for *Methylocystis heyeri* (Dedysh *et al*., 2007). The DNA base composition of strain MG30^T was

determined by thermal denaturation using a Unicam SP1800 spectrophotometer (UK) at a heating rate of 0.5°C min^{-1}. The mol % G+C value was calculated according to Owen *et al.* (1969). The DNA of *Escherichia coli* K-12 was used as the standard. PCR-mediated amplification of the 16S rRNA gene was performed using primers 9f and 1492r and reaction conditions described by Weisburg *et al*. (1991). Partial fragments of the *pmoA* (active-site polypeptide of particulate methane monooxygenase (pMMO), *mxaF* (large subunit of methanol dehydrogenase), and *nifH* (dinitrogenase reductase) genes were amplified using the primers and the reaction conditions described by Holmes *et al*. (1995), McDonald & Murrell (1997), and Dedysh *et al*. (2004), respectively. Phylogenetic analyses were carried out using the ARB program package (Ludwig *et al.*, 2004). The trees were constructed using distance-based (neighbor-joining), maximum-likelihood (DNAml), and maximum-parsimony methods. The significance levels of interior branch points obtained in neighbor-joining analysis were determined by bootstrap analysis (1000 data re-samplings) using PHYLIP (Felsenstein, 1989).

Isolate MG30^T was represented by Gram-negative and non-motile rods (1.0-1.5 μм wide by 1.0-4.0 μм long). Cells often appeared in pairs or in long chains and produced large polysaccharide capsules up to 3.0–5.0 μm thick (Fig. 1a). Two-week-old colonies of strain MG30^T were round, 4-6 mm in diameter, slimy, pale-pink with an entire edge and a smooth surface. Liquid culture of novel isolate displayed white turbidity. Formation of a surface pellicle in static liquid cultures was not observed.

Electron microscopy analysis of ultrathin sections of cells of strain MG30^T showed a typical Gram-negative structure of the cell wall and the presence of intracytoplasmic membranes (ICM), arranged as stacks of vesicular disks (Fig. 1b). This ICM arrangement is characteristic of type I methanotrophs. No flagella were revealed in specimens of negatively stained cells of this bacterium. Strain MG30^T did not survive heat or desiccation, and no exospores or cysts were observed regardless of cell age or culture conditions.

Methane and methanol were the only substrates utilized by strain MG30^T. Methanol supported growth only when used at concentrations below 2% (v/v); the most active growth

occurred at a methanol concentration of 0.25% (v/v). No growth was observed on multicarbon compounds. Nitrate, ammonium chloride, glutamine, casamino acids, peptone and yeast extract were used as sources of nitrogen. Strain MG30^T was also capable of slow growth in liquid nitrogen-free medium under microaerobic conditions (1.0–2.0% O_2 in flask headspace). The *nifH* gene fragment from this bacterium was most closely related (91% nucleotide sequence identity and 99% derived amino acid sequence identity) to the corresponding gene fragment from *Methylomonas methanica*.

Strain MG30^T grew in the pH range 3.8-7.3, with the optimum at pH 5.8-6.4 (Fig. 2). Given that the ability to grow at pH below 5.0 has not yet been reported for any of the taxonomically characterized representatives of the *Methylomonas*, our novel isolate represents the first acid-tolerant member in this genus. However, it is clearly less acidophilic than the previously described alphaproteobacterial methanotrophs from *Sphagnum*-dominated peatlands, i.e. *Methylocella palustris*, *Methylocapsa acidiphila* and *Methyloferula stellata*, which show optimal growth at pH 5.0-5.5 (Dedysh *et al*. 2000, 2002; Vorobev *et al*., 2011).

The temperature range for growth of strain MG30^T was 8-30ºC, with the optimum at 20-25ºC. No growth occurred at 34 and 37°C. Strain MG30^T was highly sensitive to NaCl; its growth was completely inhibited at NaCl concentrations above 0.1% (w/v). The specific growth rate of this methanotroph under optimal growth conditions was 0.04-0.05 h^{-1} (equivalent to a doubling time of 13.8-17.3 h). The $OD_{600}{}^{max}$ in a culture grown under the optimum conditions reached 1.0-1.2 after 3 days of incubation.

The activities of the enzymes potentially involved in the oxidation of methanol and in primary C_1-assimilation were assayed in extracts of methane-grown cells of strain MG30^T (Table 1). This bacterium possessed a classic, PQQ-containing methanol dehydrogenase, which required alkaline pH and NH^{4+} ions for its activation. Methanol dehydrogenase was active with PMS as an artificial electron acceptor. A key enzyme of the ribulose monophosphate (RuMP) cycle, 3-hexulose-6-phosphate synthase, was detected in cell extracts of strain MG30^T suggesting that the RuMP cycle is involved in formaldehyde assimilation in this bacterium. In addition, activities of

carbon metabolism enzymes, such as glucose-6-phosphate dehydrogenase and pyrophosphate-dependent PFK, were revealed in strain MG30^T (Table 1). Activity of ATP-dependent 6-phosphofructose kinase (PFK) was not detected. The presence of pyrophosphate-dependent PFK and absence ATP-PFK is characteristic of many methanotrops. Activity of the ribulose-1,5-bisphosphate carboxylase, a key enzyme of the Calvin cycle, as well as hydroxypyruvate reductase and serine-glyoxylate aminotransferase, the serine pathway-specific enzymes, were not detected. Apparently, the RuMP cycle is the basic pathway for C1-assimilation in methanotrops belonging to the genus *Methylomonas.*

The PLFA (polar lipid derived fatty acids) profile in cells of strains MG30^T was characterized by the predominance of C16:1 fatty acids, which is typical for PLFA profiles in type I methanotrophs (Bowman *et al*., 1991; Bowman *et al*., 1993). The major components of this profile were C16:1*ω5t*, C16:1*ω8c*, C16:1*ω7c*, and C14:0 fatty acids (Table 2). In general, the PLFA profile in strain MG30^T was quite similar to those reported for taxonomically characterized representatives of the genus *Methylomonas* (Bowman *et al*., 1993; Ogiso *et al.*, 2012). The only PLFA feature that distinguishes strain MG30^T from other *Methylomonas* species is the high content of C16:1*ω5t* (~35% of total fatty acids).

Comparative sequence analysis of the 16S rRNA gene showed that strain MG30^T belongs to the family *Methylococcaceae* of the class *Gammaproteobacteria* and displays 94.7-96.9% 16S rRNA gene sequence similarity to members of the genus *Methylomonas* (Fig. 3). Highest 16S rRNA gene sequence similarity (97.7%) was observed between strain MG30^T and taxonomically uncharacterized *Methylomonas* sp. strain M5, which was also isolated from a *Sphagnum*-dominated wetland in the Netherlands (Kip *et al*., 2011b). By contrast to other characterized *Methylomonas* spp., strain MG30^T had relatively low G+C content of 48.5 mol% (Table 3).

Phylogenetic analysis based on fragments of the *pmoA* gene, which encodes the β-subunit of pMMO, revealed that strain MG30^T displays only 86.8-88.9% nucleotide sequence identity (or 92.9-95.0% derived amino acid sequence identity) to *pmoA* gene fragments from other members

of the genus *Methylomonas* (Fig. 4). The PmoA identity with other methanotrophic representatives of the family *Methylococcaceae* ranged between 73.4 and 89.3%.

No *mmoX* gene encoding a subunit of soluble MMO was detected in strain MG30^T using any of three widely used PCR primer combinations: mmoXA/mmoXD (Auman *et al*., 2001), mmoX1/mmoX2 (Miguez *et al*., 1997), and mmoX206f/mmoX886r (Hutchens *et al*., 2004). DNA from *Methylocella silvestris* BL2^T was used as a positive control in all three PCR reactions. The colorimetric naphthalene oxidation test (Graham *et al.*, 1992) for sMMO activity in cells of strain MG30^T grown on Cu-free medium was also negative. The results suggest that sMMO is not present in strain MG30^T.

In summary, cell morphology and physiology as well as the 16S rRNA and *pmoA* gene phylogenies characterize strain MG30^T as a member of the genus *Methylomonas.* However, our novel isolate differs from all taxonomically characterized members of this genus by the absence of motility, the ability to grow in acidic conditions, and low DNA G+C content (Table 3). We propose to classify strain MG30^T as a novel, acid-tolerant species of the genus *Methylomonas*, *Methylomonas paludis* sp. nov. Taxonomically uncharacterized *Methylomonas* sp. strain M5 is phylogenetically related to *M. paludis* MG30^T sp. nov. but, according to the data reported by Kip *et al*. (2011b), is more acidophilic, possesses sMMO and differs with regard to its PLFA composition. This suggests that, most likely, strain M5 is a member of another, as-yet-uncharacterized species of the genus *Methylomonas*.

Description of *Methylomonas paludis* sp. nov.

Methylomonas paludis (pa.lu'dis. L. gen. n. *paludis* of a swamp, of a marsh, of a bog). Gram-negative, non-motile rods, 1.0-1.5 μм wide by 1.0-4.0 μм long. Cells occur singly, in pairs or in short chains and are covered by large capsules. Reproduce by binary fission. Possess stacks of intracytoplasmic membranes typical of type I methanotrophs. No cysts are formed. Colonies are slimy, pale-pink with an entire edge and a smooth surface. Liquid cultures display homogeneous turbidity; no surface pellicle is formed. The temperature range for growth is 8-

30°C with the optimum at 20-25°C. Growth occurs between pH 3.8 and 7.3 with the optimum at pH 5.8-6.4. Methane and methanol are the only growth substrates. Methanol is utilized at concentrations below 2% (v/v); optimal growth occurs at 0.25% (v/v) CH_3OH. C1 compounds are assimilated via the ribulose monophosphate pathway. Growth factors are not required. NaCl inhibits growth at concentrations above 0.1 %. The predominant PLFAs are C16:1*ω5t*, C16:1*ω8c*, C16:1*ω7c*, and C14:0. Fixes atmospheric nitrogen; *nifH* gene present. The DNA G+C content of the type strain is 48.5 mol%. The type strain, MG30^T (=DSM 24973^T = VKM B-2745^T), was isolated from the acidic *Sphagnum* peat bog lake Germanovskoe, north-eastern Russia.

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Table 1. Enzyme activities in cell extracts of strain MG30^T grown on methane

Enzyme	**Cofactor(s)**	**Activity**, [nmol min^{-1} (mg protein)$^{-1}$]
Methanol dehydrogenase	PMS	66
Formate dehydrogenase	NAD^+	146
Hydroxypyruvate reductase	NADH NADPH	0 0
Serine-glyoxylate aminotransferase	NADH NADPH	0 0
Ribulose-1,5-bisphosphate carboxylase		0
Glucose-6-phosphate dehydrogenase	NAD^+ $NADP^+$	0 4.3
6-Phosphofructokinase	ATP PPi	0 94
3-Hexulose-6-phosphate synthase		56

Table 2. PLFA contents of strain MG30^T in comparison to other species of the genus *Methylomonas*. Major fatty acids are shown in bold.

Fatty acid	**Strain MG30^T**	***Methylomonas* spp.***
C14:0	**11.8**	**18.9-24.6**
iC15:0	-	0-2.5
aC15:0	-	0-2.4
C15:0	0.5	0-1.2
C16:1*ω8c*	**22.1**	**18.7-41.3**
C16:1*ω7c*	**13.9**	**4.35-15.3**
C16:1*ω6c*	5.0	4.5-13.3
C16:1*ω5c*	1.8	1.9-16.7
C16:1*ω5t*	**34.8**	**7.9-16.6**
C16:0	5.6	4.3-8.7
C17:1*ω7c*	-	0-0.7
C17:1*ω7t*	-	0-0.3
cyC17:0	-	0-2.1
C18:1*ω7c*	0.3	0.2-2.5
C18:1*ω5c*	-	0-1.7
C18:0	1.2	0-0.7
brC19:1	-	0-0.5
cyC19:0	-	0.2-0.4

* – summary of data for *Methylomonas methanica* ACM 3307^T, *Methylomonas fodinarium* ACM 3268^T, *Methylomonas aurantiaca* ACM 3406^T (from Bowman *et al*., 1993), and *Methylomonas koyamae* Fw12E-Y^T (from Ogiso *et al.*, 2012). Values are percentages of total fatty acids.

Table 3. Major characteristics that distinguish *Methylomonas paludis* sp. nov. from other described species of the genus *Methylomonas*: 1 - *Methylomonas paludis* sp. nov., 2 – *Methylomonas methanica* (data were taken from Bowman *et al*. 1993), 3 – *M. fodinarum* (data were taken from Bowman *et al*. 1990), 4 – *M. aurantiaca* (data were taken from Bowman *et al*. 1990), 5 – *M. scandinavica* (data were taken from Kalyuzhnaya *et al*. 1999), 6 – *M. koyamae* (data were taken from Ogiso *et al*. 2012).

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**
Cell shape	rods	rods	rods	rods	rod-like ovoids	rods
Cell size (μm)	1.0-1.5 × 1.0-4.0	0.5-1.0 × 1.0-2.0	0.7-1.0 × 0.8-1.2	0.5-0.8 × 0.8-1.5	0.6-0.8 × 1.5-1.7	0.8-1.1 × 1.2-2.5
Color of colonies	Pale-pink	Pink or red	Orange	Orange	Pink	Pink or orange
Motility	-	+	+	+	+	+
Chain formation	+	-	+	+	+	-
Capsule	+	+	+	+	+	ND
Temperature range (°C)	8 - 30	10 - 37	10 - 35	15 - 40	5 - 30	10 - 40
Temperature optimum (°C)	20 - 25	25 - 30	25	35	17	30
pH range	3.8 - 7.3	5.5 - 9.0	5.5 - 9.0	5.0 - 9.0	5.0 - 9.0	5.5 - 7.0
pH optimum	5.8 - 6.4	7.0	7.0	6.5	6.8 - 7.6	6.5
Tolerance to 1.0% NaCl	-	+	+	+	-	-
Growth at 37 ºC	-	+	-	+	-	+
N_2 fixation	+	+*	ND	ND	ND	ND
G+C content (mol%)	48.5	51 - 53	58 - 59	55 - 56	53.8	57.1

ND, not determined; * – based on *nifH* detection only.

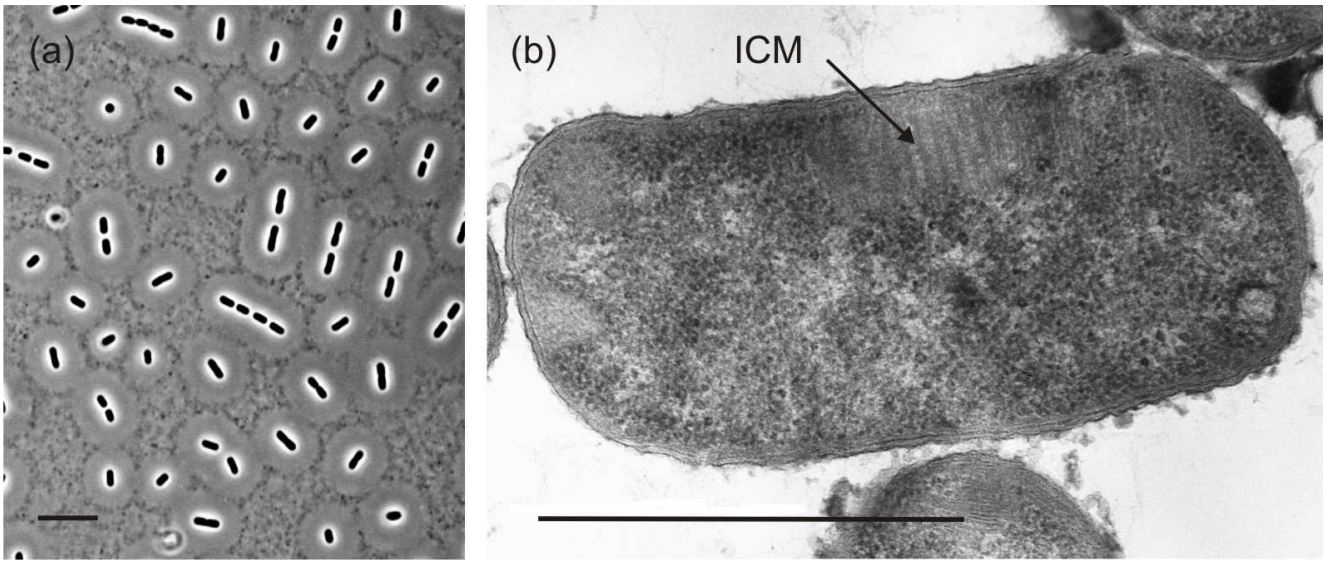


Figure 1.

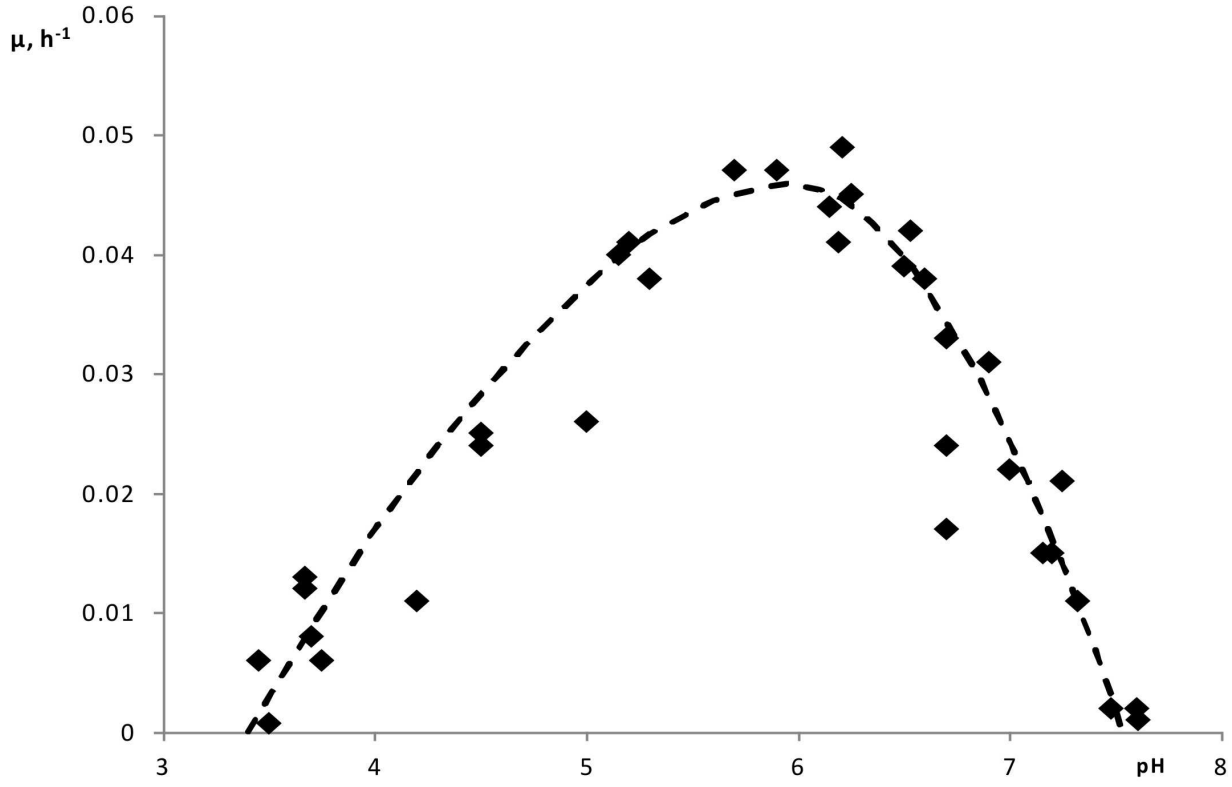
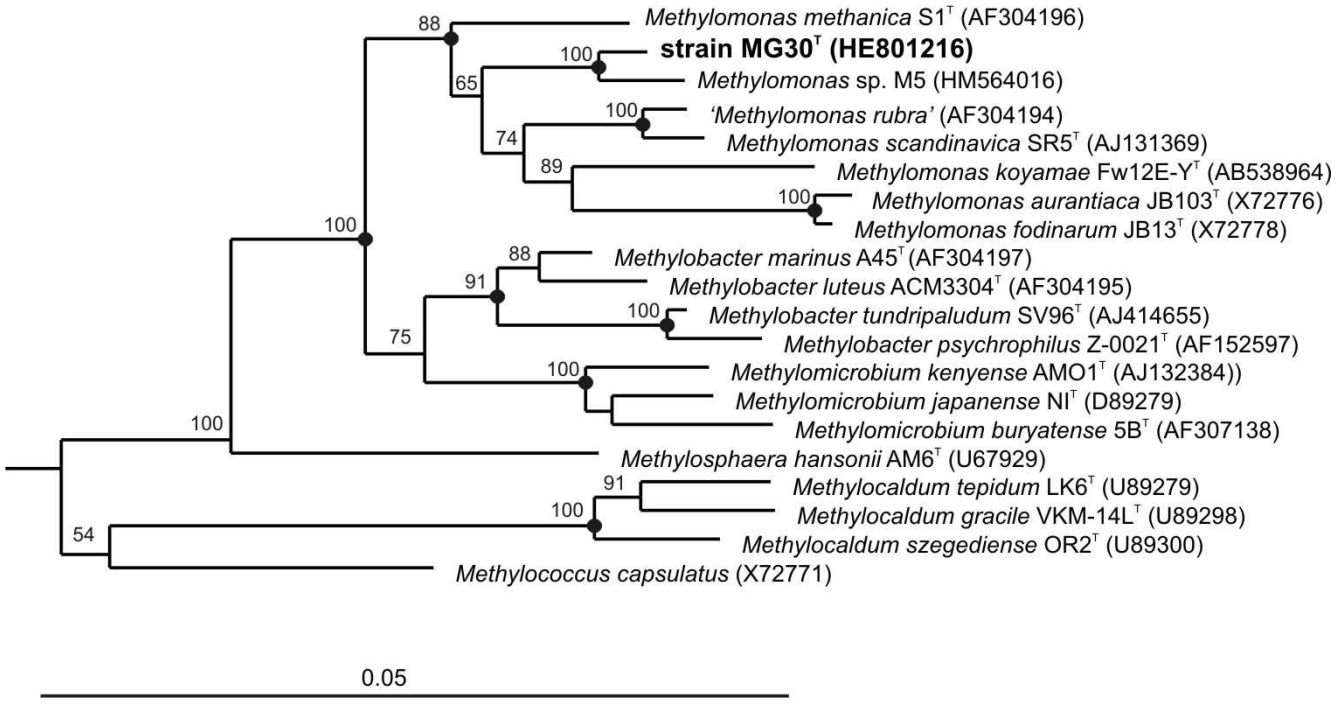


Figure 2.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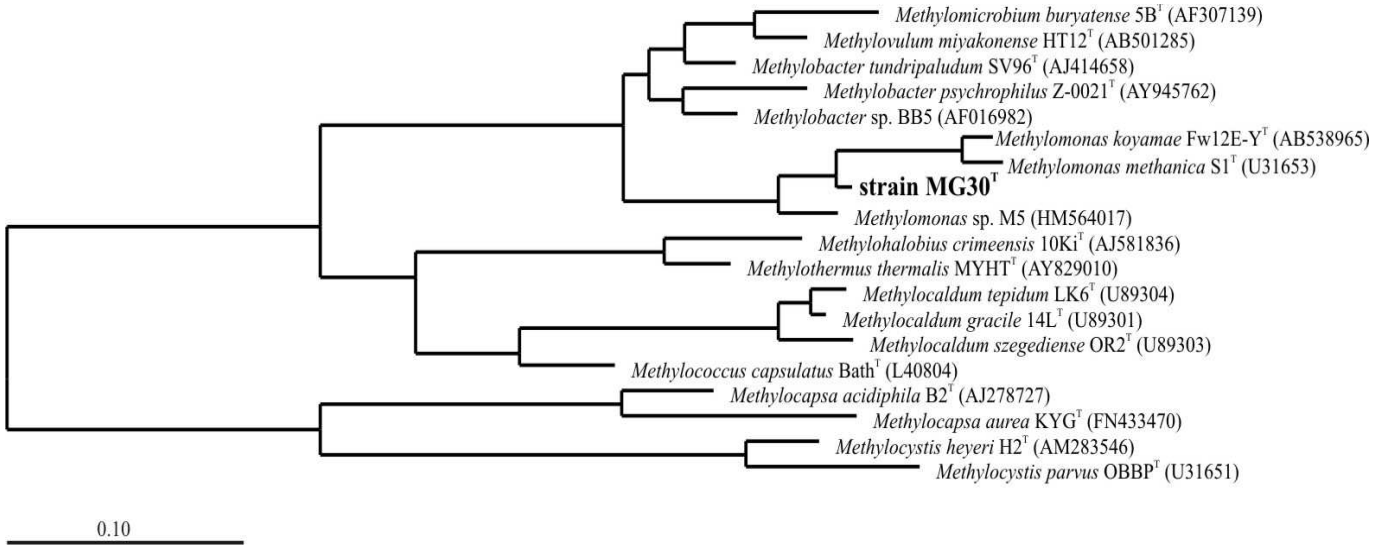
'Methylomonas rubra' (AF304194)
Methylomonas scandinavica SR5T (AJ131369)
Methylomonas koyamae Fw12E-YT (AB538964)
Methylomonas aurantiaca JB103T (X72776)
Methylomonas fodinarum JB13T (X72778)
Methylobacter marinus A45T (AF304197)
Methylobacter luteus ACM3304T (AF304195)
Methylobacter tundripaludum SV96T (AJ414655)
Methylobacter psychrophilus Z-0021T (AF152597)
Methylomicrobium kenyense AMO1T (AJ132384))
Methylomicrobium japanense NIT (D89279)
Methylomicrobium buryatense 5BT (AF307138)
Methylosphaera hansonii AM6T (U67929)
Methylocaldum tepidum LK6T (U89279)
Methylocaldum gracile VKM-14LT (U89298)
Methylocaldum szegediense OR2T (U89300)
Methylococcus capsulatus (X72771)
88
100
65
100
74
89
100
100
88
91
100
75
100
100
91
100
54
0.05

Figure 3.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Methylovulum miyakonense HT12^T (AB501285)
Methylobacter tundripaludum SV96^T (AJ414658)
Methylobacter psychrophilus Z-0021^T (AY945762)
Methylobacter sp. BB5 (AF016982)
Methylomonas koyamae Fw12E-Y^T (AB538965)
Methylomonas methanica S1^T (U31653)
strain MG30^T
Methylomonas sp. M5 (HM564017)
Methylohalobius crimeensis 10Ki^T (AJ581836)
Methylothermus thermalis MYHT^T (AY829010)
Methylocaldum tepidum LK6^T (U89304)
Methylocaldum gracile 14L^T (U89301)
Methylocaldum szegediense OR2^T (U89303)
Methylococcus capsulatus Bath^T (L40804)
Methylocapsa acidiphila B2^T (AJ278727)
Methylocapsa aurea KYG^T (FN433470)
Methylocystis heyeri H2^T (AM283546)
Methylocystis parvus OBBP^T (U31651)
0.10

Figure 4.

FIGURE CAPTIONS

Fig. 1. **(a)** Phase-contrast micrograph of cells of strain MG30^T grown in liquid mineral medium under methane for 5 days; bar, 10 μm. Cells are suspended in India ink to visualize large capsules around the cells. **(b)** Electron micrograph of ultrathin section of a cell of strain MG30^T; bar, 1 μm. ICM, intracytoplasmic membranes.

Fig. 2. Influence of medium pH on the growth of strain MG30^T. The specific growth rate (μ) was determined after 24 hours of incubation at a given pH value. The pH values were determined at the beginning and end of the incubation.

Fig. 3. 16S rRNA gene-based neighbor-joining tree showing the phylogenetic position of strain MG30^T in relation to other members of the family *Methylococcaceae*. The tree was calculated based on 1306 nt positions. Bootstrap values (percentages of 1000 data resamplings) >50% are shown. Black circles indicate that the corresponding nodes were also recovered in the maximum-likelihood and maximum-parsimony trees. The type II methanotrophs *Methyloferula stellata* AR4 (FR686343), *Methylocella silvestris* BL2 (AJ491847), *Methylocapsa acidiphila* B2 (AJ278726), *Methylosinus sporium* (Y18946), *Methylosinus trichosporium* OB3b (Y18947), and *Methylocystis parvus* (Y18945) were used as an outgroup. Bar, 0.05 substitutions per nucleotide position.

Fig. 4. Unrooted maximum-likelihood tree constructed based on 149 deduced amino acid sites of partial *pmoA* gene sequences, showing the position of strain MG30^T relative to other type I and type II methanotrophs. Bar, 0.1 substitutions per nucleotide position.