Methylothermus thermalis gen. nov., sp. nov., a novel moderately thermophilic obligate methanotroph from a hot spring in Japan

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A novel moderately thermophilic methanotroph, strain MYHT^T, was isolated from a hot spring in Japan. The isolate grew on methane or methanol at 37–67 °C, and optimally at 57–59 °C. It was found to be a Gram-negative aerobe, with colourless colonies of non-motile coccoid cells, possessing type I intracytoplasmic membranes and regularly arranged surface layers of linear (*p*2) symmetry. Strain MYHT^T expressed only the particulate methane monooxygenase and employed the ribulose monophosphate pathway for formaldehyde assimilation. It is a neutrophilic and halotolerant organism capable of growth at pH 6.5–7.5 (optimum pH 6.8) and in up to 3 % NaCl (optimum 0.5–1 % NaCl). Phylogenetic analysis based on 16S rRNA gene sequence analysis indicated that strain MYHT^T is most closely related to the thermophilic undescribed methanotroph '*Methylothermus*' HB (91 % identity) and the novel halophilic methanotroph *Methylohalobius crimeensis* 10Ki^T (90 % identity). Comparative sequence analysis of particulate methane monooxygenase (*pmoA*) genes also confirmed the clustering of strain MYHT^T with '*Methylothermus*' HB and *Methylohalobius crimeensis* 10Ki^T (98 and 92 % derived amino acid sequence identity, respectively). The DNA G + C content was 62.5 mol%. The major cellular fatty acids were C_{16:0} (37.2 %) and C_{18:1ω9c} (35.2 %) and the major polar lipids were phosphatidylethanolamine and phosphatidylglycerol. The major ubiquinone was Q-8. On the basis of comparative phenotypic and genotypic characteristics, a new genus and species, *Methylothermus thermalis* gen. nov., sp. nov., is proposed, with MYHT^T as the type strain (= VKM B-2345^T = IPOD FERM P-19714^T).

INTRODUCTION

Obligate methanotrophs are a highly specialized group of bacteria that utilize methane and methanol as sole carbon and energy sources. They are widespread in nature and inhabit soils, wetlands, fresh and marine waters, lakes and sediments, being mostly neutrophilic and mesophilic

(Hanson & Hanson, 1996). Nevertheless, in the past decade, investigations of extreme environments with high and low pH, temperatures or salinities have led to the discovery of a variety of extremophilic and extremotolerant methanotrophs (Murrell *et al.*, 1998; Trotsenko & Khmelenina, 2002).

The solubility of CH₄ and O₂ in aqueous solution drops with increasing temperature, thus limiting growth of methanotrophic bacteria. However, gas solubility in natural waters of low ionic strength (< 100 mM) decreases by only one-third when the temperature increases from 30 to 60 °C. This explains the existence of methanotrophs in various habitats with elevated temperatures. The earliest described heat-tolerant methanotroph was *Methylococcus capsulatus*, which grows at temperatures up to 50 °C (Foster & Davis, 1966; Whittenbury *et al.*, 1970). Several other moderately thermophilic and thermotolerant species, including *Methylococcus thermophilus*, '*Methylococcus ucrainicus*', *Methylocaldum*

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Abbreviations: ICM, intracytoplasmic membrane; PLFA, phospholipid fatty acid; pMMO and sMMO, particulate and soluble methane monooxygenase, respectively; RuMP, ribulose monophosphate.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and partial sequence of the *pmoA* gene of *Methylothermus thermalis* strain MYHT^T are AY829009 and AY829010, respectively.

A figure showing the oxidation and assimilation of ¹⁴C-methane by cells of strain MYHT^T is available as supplementary material in IJSEM Online.

szegediense, *Methylocaldum tepidum* and *Methylocaldum gracile*, were subsequently described (Malashenko *et al.*, 1975; Malashenko, 1976; Bodrossy *et al.*, 1995, 1997, 1999; Eshinimaev *et al.*, 2004).

Very recently, a representative of a novel group of truly thermophilic methanotrophs, strain HB, was isolated from Hungarian and Japanese hot springs (Bodrossy *et al.*, 1999), with temperature limits for growth between 40 and 70 °C and an optimum at 55–62 °C. Strain HB was closely related to the known thermotolerant *Methylococcus* and *Methylocaldum* species, as indicated by 16S rRNA and particulate methane monooxygenase (*pmoA*) gene sequence analyses. Strain HB represented a new genus, and the informal name '*Methylothermus*' was proposed for this methanotroph (Bodrossy *et al.*, 1999). However, no comparative studies of the phenotypic features of strain HB with those of known thermophilic and thermotolerant methanotrophs have been performed. Moreover, this organism is no longer extant (K. L. Kovács, personal communication), which prompted our attempts to isolate and characterize thermophilic methanotrophs from a Japanese hot spring. Herein, we describe a novel moderately thermophilic methanotrophic strain.

METHODS

Sampling. Sediments from a hot spring located in Hyogo, Japan were collected in 15 ml plastic tubes during September 2001, and kept at 4 °C before use.

Growth conditions. For the enrichment and isolation of methanotrophs, a basal ammonium nitrate mineral salt (ANMS) medium was used, containing (g l⁻¹): KNO₃, 0.25; NH₄Cl, 0.25; KH₂PO₄, 0.13; Na₂HPO₄·12H₂O, 0.358; MgSO₄·7H₂O, 0.4; CaCl₂, 0.1; ferric-EDTA, 0.00019; and Na₂MoO₄·6H₂O, 0.00013. To this medium was added 0.5 ml l⁻¹ of a stock solution of trace elements, containing (g l⁻¹): CuSO₄·5H₂O, 0.2; FeSO₄·7H₂O, 0.5; ZnSO₄·7H₂O, 0.4; H₃BO₃, 0.015; CoCl₂·6H₂O, 0.05; disodium EDTA, 0.25; MnCl₂·4H₂O, 0.02; and NiCl₂·6H₂O, 0.01. The pH of the medium was adjusted to 6.8 with NaOH. In addition, 2.5 ml l⁻¹ of a filter-sterilized vitamin stock solution [mg l⁻¹: thiamine hydrochloride, 10; nicotinic acid, 20; pyridoxamine, 10; *p*-aminobenzoic acid, 10; riboflavin, 20; biotin, 1; and cyanocobalamin (vitamin B₁₂), 1] was added to media used for the enrichment, which was performed at 59 °C in 50 ml ANMS medium in 250 ml conical flasks. The gas phase of the flasks was replaced by a methane/air mixture (1:1) every 3–4 days. The cultures were shaken at 100 r.p.m. (Clim-O-Shaker).

Isolation of methanotrophs. The mineral composition of the growth medium was optimized, based on an analysis of the methane consumption activities. Rates of ¹⁴C-methane oxidation and assimilation by cells incubated in basal ANMS medium and in two- or fourfold-concentrated media, and also in ANMS medium containing various concentrations of trace elements, were estimated by using the method described previously (Sokolov & Trotsenko, 1995). Since the rate of methane oxidation was higher at elevated concentrations of the trace elements (two- or fourfold) and both processes (methane oxidation and methane assimilation) were inhibited by CaCl₂, the medium was modified slightly and then used for pure culture isolation. The modified (M) medium contained (g l⁻¹): NH₄Cl, 0.5; KH₂PO₄, 0.13; Na₂HPO₄·12H₂O, 0.358; MgSO₄·7H₂O, 0.5; CaCl₂, 0.02; ferric-EDTA, 0.0038; Na₂MoO₄·6H₂O, 0.00026;

and 1 ml l⁻¹ of a stock solution of trace elements containing (g l⁻¹): CuSO₄·5H₂O, 0.2; FeSO₄·7H₂O, 0.5; ZnSO₄·7H₂O, 0.4; H₃BO₃, 0.015; CoCl₂·6H₂O, 0.05; disodium EDTA, 0.25; MnCl₂·4H₂O, 0.02; and NiCl₂·6H₂O, 0.01. M medium was not supplemented with vitamin solution.

After two or three passages of the enrichment in liquid ANMS and then in M medium, serial dilutions were spread onto plates containing a mixture of 80 % M medium and 20 % autoclaved culture fluid from the initial enrichment, and 2.0 % (w/v) Bacto agar (Difco). The plates were incubated for 1–2 weeks at 53 °C in anaerobic jars filled with a methane/air (1:1) gas mixture. Individual colonies were restreaked onto fresh plates and reincubated. The resulting single colonies were checked for purity by light microscopy and placed into liquid M medium. Pure cultures were routinely maintained at 53 °C on solidified M medium. Absence of growth on various organic solid media and cell uniformity tested by light and electron microscopy were used as criteria for culture purity.

Electron microscopy. Ultrathin sections of exponentially grown cells and negatively stained preparations were prepared and examined using a JEOL JEM 100B electron microscope, as described previously (Khmelina *et al.*, 1999).

Utilizable carbon and nitrogen sources. The ability of the isolate to use various organic compounds as a carbon source was tested in liquid M medium supplemented with autoclaved or filter-sterilized substrates (methylamine, formate, formaldehyde, formamide, acetate, pyruvate, citrate, malate, succinate, D-glucose, D-xylose, D-arabinose, maltose, sucrose, mannitol, ethanol, glycerol and yeast extract) at a final concentration of 0.5 g l⁻¹. The ability of the isolate to grow on methanol was tested using a liquid mineral medium containing 0.05–1 % (v/v) methanol. Nitrogen sources were tested by using agar medium in which NH₄Cl was replaced by one of the following compounds at a final concentration of 0.05 % (w/v): KNO₃, NaNO₂, (NH₄)₂SO₄, methylamine, dimethylamine, urea, formamide, glycine, L-alanine, L-lysine, L-arginine, L-glutamate, L-glutamine, L-asparagine, L-lysine, L-aspartate, L-tryptophan, L-methionine, L-threonine, L-cysteine, L-histidine, Tris, disodium EDTA, Casamino acids and yeast extract.

Effect of pH, temperature and salinity on growth. The temperature range for growth was estimated by growing the isolate in liquid M medium at 26, 30, 37, 54, 57, 59, 62, 65, 67 and 69 °C. The effect of pH was investigated at an optimum temperature of 57 °C, in M medium supplemented with NaH₂PO₄/Na₂HPO₄ buffer of various pH values (4.2–8.5), at a final concentration of 0.025 M. To test the salt-dependence of growth, NaCl was added to M medium at 0.25, 0.5, 1, 2 or 3 % (w/v); 10 ml of the culture grown at pH 6.8 was used for inoculation. Aliquots were taken at 12–18 h intervals and the optical density at 600 nm (OD₆₀₀) was measured. The specific growth rate was calculated from increases in OD₆₀₀ during the exponential growth phase. To test the copper-dependence of ¹⁴CH₄ assimilation and oxidation rates, the cells were suspended in ANMS medium containing 0.4 µM CuSO₄·5H₂O, and additionally supplemented with CuSO₄·5H₂O (0, 0.5, 1, 2 and 4 µM). The cell suspensions were incubated at 57 °C and the radioactivity incorporated into the biomass or carbon dioxide was measured as described below.

Antibiotic sensitivity. To test for antibiotic sensitivity, the cells were spread onto plates of solidified M medium with discs (Difco) containing the following antibiotics (µg ml⁻¹): neomycin, 30; kanamycin, 30; ampicillin, 10; erythromycin, 15; lincomycin, 2; gentamicin, 10; novobiocin, 30; nalidixic acid, 30; or streptomycin, 10. Growth under methane was assessed after 1–2 weeks.

Enzyme assays. To determine soluble methane monooxygenase (sMMO) activity, naphthalene oxidation by 1-week-old colonies

grown on solid M medium without added copper was tested (Graham *et al.*, 1992). Particulate methane monooxygenase (pMMO) activity was measured as $^{14}\text{CH}_4$ consumption by whole cells (Sokolov & Trotsenko, 1995). For the preparation of cell-free extracts, exponentially grown cells were harvested by centrifugation at 6000 g for 30 min, and washed once in 50 mM Tris/HCl buffer (pH 7.5) containing 5 mM MgCl_2 . The cells were resuspended in the same buffer. Cells (0.5 g in 2 ml buffer) were sonicated three times for 30 s using an MSE sonifer (150 W, 20 kHz), in ice-cooled cuvettes. Cell debris was precipitated by centrifugation at 15 000 g for 30 min. The supernatant was used for enzyme assays, which were performed as described previously (Kalyuzhnaya *et al.*, 1999).

Cellular fatty acid profile and phospholipid analyses. For phospholipid analysis, exponentially grown cells at optimum temperature (59 °C) were harvested by centrifugation at 7000 g for 10 min. Phospholipids were determined according to the method described previously (Govorukhina & Trotsenko, 1989). Fatty acids and ubiquinones were analysed as described previously (Doronina *et al.*, 2003).

DNA isolation and DNA G+C content determination. DNA was isolated as described previously (Kalyuzhnaya *et al.*, 1999). DNA G+C content was determined by using the thermal denaturation method (Sambrook *et al.*, 1989).

Comparative sequence analysis. For PCR of the 16S rRNA gene, the universal bacterial primers 27f and 1492r (Giovannoni, 1991) were used. Amplification of a partial fragment of the *pmoA* gene (encoding the 27 kDa peptide of pMMO) was done using primers A189 and mb661r (Bourne *et al.*, 2001). Amplification of partial fragments of the *mmoX* gene (encoding the α -subunit of sMMO) was performed using primers according to PCR programmes described by Murrell *et al.* (1998). Amplification of partial fragments of the *nifH* (encoding the iron protein of the nitrogenase) and *cbbL* (encoding the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase) genes was performed as described by Dedysh *et al.* (2004) and Baxter *et al.* (2002), respectively. PCR was performed using a Hybaid Thermocycler, with a mixture containing TaqPol buffer (USB), 0.2 μg chromosomal DNA, 0.5 μM oligonucleotide primers, 0.5 mM each dNTP, 2.5 mM MgCl_2 and 2 U Taq polymerase. After denaturation at 95 °C for 2 min, the reaction mixture was subjected to 25 thermal cycles: 94 °C, 50 s; 50 s at annealing temperature (56 °C for *pmoA* gene-specific and 60 °C for 16S rRNA gene-specific primers); 72 °C, 50 s. At the final stage, the reaction mixture was incubated for 5 min at 72 °C. The size of the reaction products was checked on 1% (w/v) agarose gels. For sequencing, the reaction products were separated by electrophoresis in 1% low-melting point agarose (Gibco), and then the bands of corresponding size were excised and purified by using the agarase (Fermentas) digestion procedure, according to the manufacturer's protocol. The PCR products were sequenced by using a FemtoMol kit (Promega). Inferred polypeptide sequences of PmoA and 16S rRNA gene sequences were aligned manually with sequences retrieved from the GenBank database, using the CLUSTAL package of multiple alignment programs (Higgins & Sharp, 1988). Dendrograms were constructed using the TREECON programs package (version 1.3b) (Van de Peer & De Wachter, 1994).

RESULTS AND DISCUSSION

Isolation of strain MYHT^T

Vitamins were required at the initial step of the methanotroph enrichment. Also, the enrichment grew better in liquid or on solidified ANMS media when culture fluid from the initial enrichment was added, implying possible

growth factor limitation. However, various colony types were obtained on solidified ANMS medium containing vitamins, and only heterotrophic contaminants remained after several transfers. Therefore, vitamins supporting the growth of both the methanotrophs and the heterotrophic satellites were excluded from the growth medium. Of the antibiotics tested, only erythromycin and nalidixic acid did not suppress growth of the methanotrophs, but slightly inhibited growth of heterotrophic contaminants. Thus, addition of these antibiotics to plates and improvement of the mineral composition of the medium favoured the isolation of a pure methanotrophic culture after several transfers.

Cultural and morphological characteristics

Small (1–2 mm in diameter), white, semi-transparent colonies with an entire edge and smooth surface were observed on agar plates after incubation at 53 °C for 1–2 weeks. Older colonies (1 month) became light brown and more rigid. The colonies grew faster and were relatively large (3 mm in diameter) when vitamins were added to the growth medium. As revealed by light microscopy, the cells grown in liquid culture in either exponential or stationary phase were mostly represented by coccoids of 0.6–0.8 μm in diameter (Fig. 1a). In contrast, the majority of cells grown on solid medium appeared as short rods, slightly varying in size (0.6–0.8 $\times$ 1.0–1.2 μm). The cells were non-motile, and multiplied by normal cell division. Electron microscopy analysis of ultrathin sections of cells showed a typical Gram-negative structure of the cell wall, and the presence of type I intracytoplasmic membrane (ICM) arranged as stacks of vesicular disks (Fig. 1b) that completely filled the cytoplasmic space. Glycogen inclusions and poly- β -hydroxybutyrate granules usually present in mesophilic methanotrophs were not observed. Also, the cells formed regularly arranged surface layers with p2 symmetry (Fig. 1c).

Physiological characteristics

Strain MYHT^T did not grow on the multicarbon substrates tested and used only methane and methanol as a carbon and energy source. Nitrate, ammonia, urea, tryptophan, lysine, glutamate, formamide and Tris were utilized as a nitrogen source. No growth was observed on nitrogen-free medium and the *nifH* gene was not detected with PCR analysis.

Maximal growth rate occurred at 57–59 °C. Strain MYHT^T was able to grow at 37 and 67 °C. Interestingly, the cells oxidized and assimilated methane even at 75 °C (see Supplementary Figure in IJSEM Online). The strain grew best at 57 °C in the presence of 0.5–1% NaCl (specific growth rate 0.1 h⁻¹ and generation time 7 h). The optimum NaCl concentration for growth at 65 °C was 0.5% (w/v). The isolate was capable of growth in up to 3% NaCl at 57 °C, but only in up to 1% NaCl at 65 °C. The pH range for growth was 6.5–7.5. Cells oxidized methane most actively in the presence of 0.9–1.4 μM CuSO_4 . Higher copper

Fig. 1. Cell morphology of strain MYHT^T. (a) Phase-contrast micrograph. (b) Ultrathin section of cell showing cell wall structure (CW) and the intracytoplasmic membrane (ICM) arrangement. (c) Highly magnified, negative-contrast image showing the surface layer with linear ($p2$) symmetry. Bars, 1 μm (a), 0.5 μm (b) and 0.1 μm (c).

concentrations (up to 2.4 μM) did not influence methane consumption.

Phospholipid fatty acid (PLFA) profiles

As can be seen from Table 1, strain MYHT^T contained specific fatty acids, consisting mainly of C_{16:0} (37.24 %) and C_{18:1 ω 9c} (35.16 %). The major polar lipids were phosphatidylethanolamine and phosphatidylglycerol. The major ubiquinone was Q-8.

Metabolic pattern

Cells grown under both copper-containing and copper-depleted conditions did not oxidize naphthalene, suggesting the absence of sMMO. Also, the *mmoX* gene was not detected by PCR using the respective primer set that is universal for the known *mmoX* genes of methanotrophs. Hence, pMMO activity appeared to be located in the ICM. Activities of the following enzymes involved in the sequential linear oxidation of CH₄ to CO₂ were found in cell-free extracts: methanol dehydrogenase, which is active with phenazine methosulphate as an artificial electron acceptor, and NAD⁺-dependent formate and formaldehyde dehydrogenases. Hexulosephosphate synthase activity was detected (Table 2), suggesting that strain MYHT^T assimilates methane carbon at the formaldehyde level via the ribulose monophosphate (RuMP) cycle. Neither hydroxypyruvate reductase (NADH) nor serine-glyoxylate aminotransferase activities were found, thus excluding the operation of the serine pathway. Also, no activity of the key enzyme of the Calvin cycle, ribulose-1,5-bisphosphate carboxylase/oxygenase, was found. The appropriate *cbbL* gene was not revealed by PCR. The complete tricarboxylic acid cycle does not operate in this organism because of

the absence of α -ketoglutarate dehydrogenase. Like many other methanotrophs, in strain MYHT^T the pyrophosphate-dependent, but not the ATP-dependent, 6-phosphofructokinase was operative. Also, two possibilities for the cleavage of hexosephosphates exist, via fructose-1,6-bisphosphate aldolase and 2-keto-3-deoxy-6-phosphogluconate aldolase. The presence of glutamine synthetase and glutamate synthase activities indicated the involvement of the glutamate cycle in ammonia assimilation by strain MYHT^T.

Genomic characteristics

The G + C DNA content of 62.5 mol% for strain MYHT^T is close to that found for type II and type X methanotrophs. The 16S rRNA gene database search showed that strain MYHT^T belongs to the class 'Gammaproteobacteria'. It is closely related to the thermophilic methanotroph 'Methylothermus' HB (91 % sequence identity, based on the comparison of 1442 available bases) and the novel halophilic methanotroph *Methylohalobius crimeensis* 10Ki^T (90 %). The other closest neighbours were the moderately thermophilic methanotrophs *Methylococcus thermophilus* ACM 3585^T and *Methylocaldum szegediense* OR2^T, and the thermotolerant *Methylococcus capsulatus* Bath (about 85–86 % 16S rRNA gene sequence identity). The phylogenetic position of strain MYHT^T within methanotrophs belonging to the class 'Gammaproteobacteria' is shown in Fig. 2.

Phylogenetic analysis based on partial *pmoA* gene sequences confirmed the clustering of strain MYHT^T with 'Methylothermus' strain HB (90 % nucleotide sequence identity and 98 % derived amino acid sequence identity) and *Methylohalobius crimeensis* 10Ki^T (83 and 92 %, respectively) (Fig. 3). In contrast, the partial *pmoA* gene sequence of

Table 1. Cellular fatty acid profiles of strain MYHT^T and the most closely related known methanotrophs

Strains: 1, *Methylococcus capsulatus* Bath (data from Bowman *et al.*, 1993); 2, *Methylococcus thermophilus* ACM 3585^T (Bowman *et al.*, 1993); 3, *Methylocaldum* sp. O-12 (Eshinimaev *et al.*, 2004); 4, *Methylohalobius crimeensis* 10Ki^T (Heyer *et al.*, 2005); 5, strain MYHT^T (this study). Values are given as a percentage of total fatty acids.

Fatty acid	1	2	3	4	5
C _{14:0}	0.8–0.62	1.5–1.9	1.97	2.45	1.24
C _{14:0} 2-OH					0.33
C _{15:0}	0–1.7	0–12.7	3.51	0.48	2.07
C _{15:0} 2-OH					0.17
C _{16:0}	22.5–22.9	39.7–47.0	63.67	23.02	37.24
C _{16:1}	12.4–55	17.2–56	11.90		
C _{16:1} ω7c				19.60	3.46
C _{16:0} 2-OH				0.93	8.41
C _{16:0} 3-OH			0.64		
9-O-Me-C _{16:0}			4.62		
C _{17:0}	0 to traces	0–1.4	0.68	0.29	2.52
C _{17:1}					0.19
i-C _{17:1}			0.34		
C _{17:1} ω6			0.43		
C _{17:1} ω8	0 to traces	0–0.4	0.26		
C _{17:cyc}	0–14.0	0–15.1	8.99	0.67	4.71
9-O-Me-C _{17:0}			0.60		
11-O-Me-C _{17:0}			0.60		
C _{18:0}	0–2.8	0.1–0.7	0.26	0.47	1.74
C _{18:1}	0–9.4	Traces to 3.4	0.17		
C _{18:1} ω7c				51.87	0.35
C _{18:1} ω9c					35.16
C _{19:0:cyc}	0.6–1.8	0–2.9			
C _{19:1:cyc}			1.37		2.41

strain MYHT^T exhibited no more than 77 % identity to the corresponding gene fragments (460 bp) of *Methylocaldum szegediense* OR2^T, *Methylocaldum gracile* VKM-14L^T and *Methylococcus capsulatus* Bath.

Given all the characteristic features of MYHT^T, we believe that this novel isolate should be classified as a type I thermophilic methanotroph. So far, information on true thermophilic methanotrophs is limited, with only a brief and non-formal taxonomic description of '*Methylothermus*' strain HB. Because the physiological, biochemical and molecular properties of '*Methylothermus*' strain HB were poorly investigated, a comparison of strain HB with strain MYHT^T is rather difficult. The lack of information was probably caused by the weak growth of '*Methylothermus*' strain HB, which did not exceed OD₅₄₀ 0.15, and therefore sufficient biomass could not be obtained (Bodrossy *et al.*, 1999). We have improved the growth of our isolate by optimization of the medium composition, by studying ¹⁴C-methane consumption and oxidation by cells. In contrast to '*Methylothermus*' strain HB, isolate MYHT^T

Table 2. Enzyme activities in cell extracts of strain MYHT^T

Activities are given as nmol min⁻¹ (mg protein)⁻¹. Abbreviation: PMS, phenazine methosulphate. There was no activity with any of the following enzymes and cofactors: formaldehyde dehydrogenase (PMS), ribulose-1,5-bisphosphate carboxylase/oxygenase, serine-glyoxylate transaminase, hydroxypyruvate reductase (NADH), α-ketoglutarate dehydrogenase (NAD), glutamate synthase (NADPH) and 6-phosphofructokinase (ATP).

Enzyme	Cofactor	Activity
Particulate methane monooxygenase*	–	5.0
Methanol dehydrogenase	PMS	28
Formaldehyde dehydrogenase	NAD	0.8
Formate dehydrogenase	PMS	8.6
	NAD	39.3
Hexulosephosphate synthase	–	97
6-Phosphofructokinase	PP _i	2.6
Fructose-1,6-bisphosphate aldolase	–	12
2-Keto-3-deoxy-6-phosphogluconate aldolase	–	1.7
Phosphoenolpyruvate carboxylase	–	0.5
Pyruvate carboxylase	–	1.9
Glutamate dehydrogenase	NADPH	1.6
Glutamine synthetase	ATP	3.8
Glutamate synthase	NADH	4

*Measured as ¹⁴CH₄ consumption by whole cells.

was able to grow up to an OD₆₀₀ value of 1.0, with a rather high growth rate ($\mu=0.1\text{ h}^{-1}$). Although the addition of vitamins stimulated the growth of both methanotrophs, these factors were excluded from the medium used for cultivation of strain MYHT^T, to prevent its possible contamination and subsequent loss of the culture.

In principle, the colony and cell morphologies of strain MYHT^T were similar to those of '*Methylothermus*' strain HB (Bodrossy *et al.*, 1999). Also, both organisms required the same level of CuSO₄ for growth and lacked sMMO. However, the upper temperature limit for growth (72 °C) and optimum values (62–65 °C) were somewhat higher for '*Methylothermus*' HB. Furthermore, the 16S rRNA gene similarity between strain MYHT^T and '*Methylothermus*' HB was low, indicating the affiliation of these strains at least to different species.

The metabolic pattern of strain MYHT^T was quite different from those of the extant thermophilic/tolerant type X methanotrophs belonging to the genera *Methylococcus* and *Methylocaldum*, but was similar to that of the extremely halophilic *Methylohalobius crimeensis* (Table 3). Obviously, strain MYHT^T uses only the RuMP cycle for carbon assimilation, whereas type X methanotrophs assimilate formaldehyde via both the RuMP and serine pathways, and are also able to grow autotrophically. These important differentiating properties allowed us to consider strain MYHT^T to be a type I methanotroph (Table 3).

Fig. 2. Phylogenetic tree showing the relationship of the 16S rRNA gene sequence of strain MYHT^T to those of some methanotrophs in the class 'Gammaproteobacteria'. The sequences were aligned using the CLUSTAL program; 1200 bp were used for the tree construction with the TREECON program (version 1.3b) using the neighbour-joining algorithm. Bootstrap values less than 50% are not shown. The tree was rooted using *Escherichia coli*. Bar, 0.1 substitutions per base position. GenBank accession numbers are given in parentheses.

The specificity of the cellular fatty acid composition, consisting primarily of C_{16:0} and C_{18:1ω9c} in equal proportions, clearly distinguishes strain MYHT^T from the other extant methanotrophs. Usually, C₁₆ fatty acids prevail in PLFA profiles of type I and type X methanotrophs, whereas

C₁₈ fatty acids occur in very small amounts. In contrast, C₁₈ fatty acids are predominant in type II methanotrophs. Only the newly isolated halophilic methanotroph *Methylohalobius crimeensis* belonging to the type I methanotrophs had a large proportion (51.87%) of C_{18:1ω7c} (Heyer *et al.*, 2005).

Fig. 3. Phylogenetic analysis of partial amino acid sequences encoded by the *pmoA* gene from some methanotrophs and the novel isolate MYHT^T. The partial AmoA protein sequence of the ammonia-oxidizing bacterium *Nitrosococcus oceanii* served as an outgroup. The tree was constructed with the TREECON program (version 1.3b) using the neighbour-joining algorithm. Bootstrap analysis from 100 replicates is shown (values less than 50% are not shown). Bar, 0.1 substitutions per amino acid position. GenBank accession numbers are given in parentheses.

Table 3. Major differentiating characteristics of the genera of thermotolerant and thermophilic methanotrophs

Genera: 1, *Methylococcus* (data from Bowman *et al.*, 1993); 2, *Methylocaldum* (Bodrossy *et al.*, 1997, 1999; Eshinimaev *et al.*, 2004); 3, *Methylohalobius* (Heyer *et al.*, 2005); 4, *Methylothermus* (this study, strain MYHT^T). Abbreviations: ND, not determined; RuBP, ribulose biphosphate.

Characteristic	1	2	3	4
Cell shape	Coccioids	Rods/coccioids	Rods/coccioids	Coccioids
Motility	—	—	+	—
Resting stage	—	<i>Azotobacter</i> -like cysts	—	—
ICM arrangement	type I	type I	type I	type I
Growth parameters*:				
Temperature (°C)	20–55 (45)	30–61 (55)	15–42 (30)	37–67 (57–59)
pH	5.5–7.5 (6.5)	6–8 (6.5–7.2)	6.5–7.5	6.5–7.5 (6.8)
Salinity (% NaCl)	ND	0–2 (0.5)	1.5–15 (6–9)	0–3 (0.5–1)
CH ₄ oxidation	sMMO/pMMO	pMMO	pMMO	pMMO
C ₁ assimilation pathway	RuMP/RuBP	RuMP/RuBP	RuMP	RuMP
<i>cbbL</i> gene	+	+	—	—
<i>nifH</i> gene	+	+	—	—
Major PLFAs	C _{16:0} /C _{16:1}	C _{16:0} /C _{16:1}	C _{16:0} /C _{18:1}	C _{16:0} /C _{18:1}
G + C content (mol%)	62.5	58.5	58.7	62.5

*Optimum values are given in parentheses.

Analysis of the 16S rRNA and *pmoA* genes clearly showed that strain MYHT^T, together with ‘*Methylothermus*’ strain HB and *Methylohalobius crimeensis*, represent a deep-branching group of extremophilic methanotrophs, which might indicate their common origin and consequent divergence. Therefore, strain MYHT^T appears to be more closely related to the type I (mesophilic, halophilic methanotroph *Methylohalobius*) than to the type X (thermophilic/tolerant *Methylococcus* and *Methylocaldum*) methanotrophs.

Because the published data on the properties of ‘*Methylothermus*’ strain HB are not sufficient for a comprehensive comparison with those of strain MYHT^T, and also because strain HB is not available, we propose that strain MYHT^T represents a new genus, *Methylothermus* gen. nov. Based on the phenotypic and genotypic properties, and phylogeny, we propose that isolate MYHT^T should be classified as representing the type species of the genus *Methylothermus*, with the name *Methylothermus thermalis* sp. nov.

Description of *Methylothermus* gen. nov.

Methylothermus (Me.thy'lo.ther'mus. N.L. n. *methyl* the methyl group; N.L. masc. subst. from Gr. adj. *thermos* hot; *Methylothermus methyl-using thermotolerant organism*).

Cells are Gram-negative, non-motile coccioids, of 0.6–0.8 µm in diameter. Reproduce by binary division. Exospores, *Azotobacter*-type cysts or lipid cysts are not formed. Possess pMMO and type I ICM as stacks of membrane vesicles. Do not possess sMMO. Moderate thermophiles growing at 37–67 °C (optimum 57–59 °C). Obligate methanotrophs utilizing methane or methanol via the RuMP pathway.

Tricarboxylic acid cycle is deficient in α-ketoglutarate dehydrogenase. Major PLFAs are C_{16:0} and C_{18:1ω9c}. Major phospholipids are phosphatidylethanolamine and phosphatidylglycerol. Phylogenetically belong to the class ‘*Gammaproteobacteria*’ (type I methanotrophs). Habitats are hot springs. The type species is *Methylothermus thermalis*.

Description of *Methylothermus thermalis* sp. nov.

Methylothermus thermalis (ther'mal.is. N.L. masc. adj. *thermalis* pertaining to a hot spring).

Description is as for the genus with the following amendments. Grows on nitrate, ammonia, urea, tryptophan, lysine, glutamine, formamide and Tris as a nitrogen source. Is not able to fix atmospheric nitrogen. Prefers media containing 0.5–1 % NaCl and is capable of growth in 3 % NaCl. Neutrophile, growing at pH 6.5–7.5. DNA G + C content is 62.5 mol%. The type strain is MYHT^T (=VKM B-2345^T=IPOD FERM P-19714^T), which was isolated from a hot spring located in Hyogo, Japan.

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REFERENCES

- Baxter, N. J., Hirt, R. P., Bodrossy, L., Kovacs, K. L., Embley, T. M., Prosser, J. I. & Murrell, J. C. (2002). The ribulose-1,5-bisphosphate carboxylase/oxygenase gene cluster of *Methylococcus capsulatus* (Bath). *Arch Microbiol* **177**, 279–289.
- Bodrossy, L., Murrell, J. C., Dalton, H., Kalman, M., Puskas, L. G. & Kovacs, K. L. (1995). Heat-tolerant methanotrophic bacteria from the hot-water effluent of a natural-gas field. *Appl Environ Microbiol* **61**, 3549–3555.
- Bodrossy, L., Holmes, E. M., Holmes, A. J., Kovacs, K. L. & Murrell, J. C. (1997). Analysis of 16S rRNA and methane monooxygenase gene sequences reveals a novel group of thermotolerant and thermophilic methanotrophs, *Methylocaldum* gen. nov. *Arch Microbiol* **168**, 493–503.
- Bodrossy, L., Kovács, K. L., McDonald, I. R. & Murrell, J. C. (1999). A novel thermophilic methane-oxidizing γ -Proteobacterium. *FEMS Microbiol Lett* **170**, 335–341.
- Bourne, D. G., McDonald, I. R. & Murrell, J. C. (2001). Comparison of *pmoA* PCR primer sets as tools for investigating methanotroph diversity in three Danish soils. *Appl Environ Microbiol* **67**, 3802–3809.
- Bowman, J. P., Sly, L. I., Nichols, P. D. & Hayward, A. C. (1993). Revised taxonomy of the methanotrophs: description of *Methylobacter* gen. nov., emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group I methanotrophs. *Int J Syst Bacteriol* **43**, 735–753.
- Dedysh, S. N., Ricke, P. & Liesack, W. (2004). NifH and NifD phylogenies: an evolutionary basis for understanding nitrogen fixation capabilities of methanotrophic bacteria. *Microbiology* **150**, 1301–1313.
- Doronina, N. V., Darmaeva, T. D. & Trotsenko, Y. A. (2003). *Methylophaga alcalica* sp. nov., a novel alkaliphilic and moderately halophilic, obligately methylotrophic bacterium from an East Mongolian saline soda lake. *Int J Syst Evol Microbiol* **53**, 223–229.
- Eshinimaev, B. Ts., Medvedkova, K. A., Khmelenina, V. N., Suzina, N. E., Osipov, G. A., Lysenko, A. M. & Trotsenko, Y. A. (2004). New thermophilic methanotrophs of the genus *Methylocaldum*. *Mikrobiologiya* **73**, 530–539 (in Russian).
- Foster, J. W. & Davis, R. H. (1966). A methane-dependent coccus, with notes on classification and nomenclature of obligate, methane-utilizing bacteria. *J Bacteriol* **91**, 1924–1931.
- Giovannoni, S. J. (1991). The polymerase chain reaction. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 177–203. Edited by E. Stackebrandt & M. Goodfellow. New York: Wiley.
- Govorukhina, N. I. & Trotsenko, Y. A. (1989). Phospholipid composition of methylotrophic bacteria. *Microbiology* (English translation of *Mikrobiologiya*) **58**, 318–323.
- Graham, D. W., Korich, D. G., LeBlanc, R. P., Sinclair, N. P. & Arnold, R. G. (1992). Applications of a colorimetric plate assay for soluble methane monooxygenase activity. *Appl Environ Microbiol* **58**, 2231–2236.
- Hanson, R. S. & Hanson, T. E. (1996). Methanotrophic bacteria. *Microbiol Rev* **60**, 439–471.
- Heyer, J., Berger, U., Hardt, M. & Dunfield, P. F. (2005). *Methylohalobius crimeensis* gen. nov., sp. nov., a moderately halophilic, methanotrophic bacterium isolated from hypersaline lakes of Crimea. *Int J Syst Evol Microbiol* **55**, 1817–1826.
- Higgins, D. G. & Sharp, P. M. (1988). CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. *Gene* **73**, 237–244.
- Kalyuzhnaya, M. G., Khmelenina, V. N., Kotelnikova, S., Holmquist, L., Pedersen, K. & Trotsenko, Y. A. (1999). *Methylomonas scandinavica* sp. nov., a new methanotrophic psychrotrophic bacterium isolated from deep igneous rock ground water of Sweden. *Syst Appl Microbiol* **22**, 565–572.
- Khmelenina, V. N., Kalyuzhnaya, M. G., Sakharovsky, V. G., Suzina, N. E., Trotsenko, Y. A. & Gottschalk, G. (1999). Osmoadaptation in halophilic and alkaliphilic methanotrophs. *Arch Microbiol* **172**, 321–329.
- Malashenko, Y. R. (1976). Isolation and characterization of new species (thermophilic and thermotolerant ones) of methane-utilizers. In *Microbial Growth on C1 Compounds*, pp. 293–300. Edited by H.G. Schlegel, G. Gottschalk & N. Pfennig. Gottingen: E. Goltze KG.
- Malashenko, Y. R., Romanovskaya, V. A., Bogachenko, V. N. & Shved, A. D. (1975). Thermophilic and thermotolerant methane-assimilating bacteria. *Mikrobiologiya* **44**, 855–862 (in Russian).
- Murrell, J. C., McDonald, I. R. & Bourne, D. G. (1998). Molecular methods for the study of methanotroph ecology. *FEMS Microbiol Ecol* **27**, 103–114.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sokolov, A. P. & Trotsenko, Y. A. (1995). Methane consumption in (hyper)saline habitats of Crimea (Ukraine). *FEMS Microbiol Ecol* **18**, 299–304.
- Trotsenko, Y. A. & Khmelenina, V. N. (2002). Biology of extremophilic and extremotolerant methanotrophs. *Arch Microbiol* **177**, 123–131.
- Van de Peer, Y. & De Wachter, R. (1994). TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput Appl Biosci* **10**, 569–570.
- Whittenbury, R., Phillips, K. C. & Wilkinson, J. F. (1970). Enrichment, isolation and some properties of methane-utilizing bacteria. *J Gen Microbiol* **61**, 205–218.