Methylothermus subterraneus sp. nov., a moderately thermophilic methanotroph isolated from a terrestrial subsurface hot aquifer

Hisako Hirayama,¹ Yohey Suzuki,² Mariko Abe,¹ Masayuki Miyazaki,¹ Hiroko Makita,¹ Fumio Inagaki,³ Katsuyuki Uematsu⁴ and Ken Takai¹

Correspondence

Hisako Hirayama

hirayamah@jamstec.go.jp

¹Institute of Biogeosciences, Japan Agency for Marine-Earth Science & Technology (JAMSTEC), 2-15 Natsushima-cho, Yokosuka, 237-0061, Japan

²Institute for Geo-Resources & Environment, National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Higashi, Tsukuba, 305-8567, Japan

³Geomicrobiology Group, Kochi Institute for Core Sample Research, Japan Agency for Marine-Earth Science & Technology (JAMSTEC), Monobe B200, Nankoku, Kochi, 783-8502, Japan

⁴Department of Technical Services, Marine Works Japan Ltd, 2-16-32 Kamariyahigashi, Kanazawa-ku, Yokohama, 236-0042, Japan

A novel methane-oxidizing bacterium, strain HTM55^T, was isolated from subsurface hot aquifer water from a Japanese gold mine. Strain HTM55^T was a Gram-negative, aerobic, motile, coccoid bacterium with a single polar flagellum and the distinctive intracytoplasmic membrane arrangement of a type I methanotroph. Strain HTM55^T was a moderately thermophilic, obligate methanotroph that grew on methane and methanol at 37–65 °C (optimum 55–60 °C). The isolate grew at pH 5.2–7.5 (optimum 5.8–6.3) and with 0–1 % NaCl (optimum 0–0.3 %). The ribulose monophosphate pathway was operative for carbon assimilation. The DNA G + C content was 54.4 mol% and the major fatty acids were C_{16:0} (52.0 %) and C_{18:1ω7c} (34.8 %). Phylogenetic analysis of the 16S rRNA gene sequence indicated that strain HTM55^T was closely related to *Methylothermus thermalis* MYHT^T (99.2 % 16S rRNA gene sequence similarity), which is within the class *Gammaproteobacteria*. However, DNA–DNA relatedness between strain HTM55^T and *Methylothermus thermalis* MYHT^T was ≤39 %. On the basis of distinct phylogenetic, chemotaxonomic and physiological characteristics, strain HTM55^T represents a novel species of the genus *Methylothermus*, for which the name *Methylothermus subterraneus* sp. nov. is proposed. The type strain is HTM55^T (=JCM 13664^T=DSM 19750^T).

The existence of aerobic methanotrophs inhabiting extreme environments has been verified so far by cultivation experiments and direct detection of the methane monooxygenase genes specific to aerobic methanotrophs. Thermophilic methanotrophs have been isolated from such extreme environments and consist of the gammaproteobacterial (type I) genera *Methylothermus*, *Methylocaldum* and *Methylococcus* and the verrucomicrobial genus *Methylacidiphilum* (Bodrossy *et al.*, 1997; Bowman *et al.*, 1993; Eshinimaev *et al.*,

2004; Op den Camp *et al.*, 2009; Tsubota *et al.*, 2005). The genus *Methylothermus* represents the most thermophilic methanotrophs isolated so far. It was reported that *Methylothermus* sp. strain HB, isolated from Hungarian hot springs, grew at temperatures up to 72 °C (Bodrossy *et al.*, 1999). Unfortunately, strain HB was lost before it received a validly published name within the genus *Methylothermus*. The genus name *Methylothermus* was validly published by Tsubota *et al.* (2005), with strain MYHT^T as the type strain of the type species *Methylothermus thermalis* gen. nov., sp. nov. Although strains HB and MYHT^T seemed to belong to different species within the genus *Methylothermus*, on the basis of their low 16S rRNA gene sequence similarity (92.6 %), *Methylothermus thermalis* is currently the only species and strain MYHT^T is the only currently extant strain of the genus *Methylothermus*.

In a previous study by our laboratory, a 16S rRNA gene sequence that was closely related to the genus *Methylothermus*

Abbreviations: pMMO, particulate methane monooxygenase; RuMP, ribulose monophosphate; sMMO, soluble methane monooxygenase.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, particulate methane monooxygenase and 3-hexulose-6-phosphate synthase and 6-phospho-3-hexuloisomerase gene sequences of strain HTM55^T are AB536747, AB536748 and AB591834, respectively.

A supplementary figure and two supplementary tables are available with the online version of this paper.

was identified in subsurface geothermal aquifer water (71.5 °C) in the Hishikari gold mine, Japan (Inagaki *et al.*, 2003). Further culture-independent analyses of microbial mats found on the tunnel floor in the gold mine, where the geothermal water (69 °C) was naturally discharging through the cracks of the basement rocks, demonstrated the predominance of methanotrophs of the genus *Methylothermus* in the mat community (Hirayama *et al.*, 2005b). On the basis of these results, cultivation experiments were attempted by targeting a variety of metabolic pathways and several novel thermophilic chemolithoautotrophs have been isolated and characterized (Hirayama *et al.*, 2005a; Takai *et al.*, 2002, 2003). In this report, we describe a novel thermophilic methanotroph, strain HTM55^T, from the gold mine microbial communities. Based on the taxonomic characterization, this report proposes that strain HTM55^T represents a novel species of the genus *Methylothermus*.

Sample mixtures of hot water and microbial mats were taken from the discharge point (69 °C) of the subsurface aquifer water in the Hishikari gold mine, Kagoshima, Japan. The site description has previously been reported in detail (Hirayama *et al.*, 2005b). For cultivation, Mmj medium was prepared by adding 15 µM CuSO₄ to mj medium [containing 1⁻: 0.5 g NaHCO₃, 0.25 g NH₄Cl, 0.25 g NaNO₃, 3.0 g NaCl, 14 mg K₂HPO₄, 80 mg CaCl₂, 0.34 g MgSO₄·7H₂O, 0.42 g MgCl₂·6H₂O, 33 mg KCl, 0.05 mg NiCl₂·6H₂O, 0.05 mg Na₂SeO₃·5H₂O, 0.01 mg Na₂WO₄, 2 mg Fe(NH₄)₂(SO₄)₂·6H₂O, 1 ml trace mineral solution (Balch *et al.*, 1979) and 1 ml vitamin mixture (Balch *et al.*, 1979)]. Enrichments were started by inoculating 3 ml Mmj medium with 1 ml sample in 15 ml tubes. The headspace gas was composed of CH₄/N₂/CO₂/O₂ (40:40:15:5; 200 kPa). The final pH of Mmj medium with the headspace gas was approximately pH 6.2. The tubes were incubated for 2 weeks at 55, 60 or 70 °C.

Methane-oxidizing coccoid cells grew only in the tube incubated at 55 °C. The enriched culture was purified by a serial dilution-to-extinction technique, which was repeated at least four times before the culture was identified as being pure. The following is a brief description of the technique. An active culture was diluted 1:10 in Mmj medium nine times consecutively. Each tube was incubated under the same conditions as for the enrichment. The most dilute tube indicating cell growth was further diluted and incubated in the same manner. Finally, a 1:2 dilution was conducted to obtain a pure culture that theoretically originated from a single cell. The purity of the culture was verified by successful direct sequencing of the partial 16S rRNA gene at least three times using independently grown cultures. Growth of the isolate on multicarbon compounds was examined repeatedly but was not observed, indicating that there were no heterotrophic contaminants in the culture. The isolate was designated strain HTM55^T. Strain HTM55^T was maintained and examined afterwards in Mmj medium supplemented with a reduced CuSO₄ concentration (1 µM) because of an obvious inhibitory effect of CuSO₄ at concentrations above 4 µM. All cultivations were

in liquid Mmj medium with the same headspace gas composition as the enrichment, unless otherwise mentioned. Colony formation was examined twice on Mmj agar with incubation in a gas-tight chamber containing CH₄/N₂/CO₂/O₂ (40:45:5:10; 100 kPa); however, colony formation was not observed after 3 weeks. Therefore, the ability of strain HTM55^T to form colonies has not yet been demonstrated.

Cell morphology was determined by phase-contrast microscopy with an Olympus BX51 microscope and by electron microscopy with a JEOL JEM-1210 electron microscope at 120 kV. Cells of strain HTM55^T in exponential and early stationary phase were coccoid, 0.8–1.4 µm in diameter and motile with a single polar flagellum (Fig. 1a, b). Electron microscopy of negatively stained cells revealed a lot of flagella removed from the cells, with only a few of the cells connected to the flagellum. It is unclear whether the flagella fell out actively or whether they were removed passively by physical impact during cultivation or sample preparation. No cyst-like cells were evident in the culture after 1 month of storage at growth temperatures. A bundle of type I intracytoplasmic membranes was observed in the ultrathin sections of the cells (Fig. 1c, d). Most of the cells contained intracellular inclusions, seen as low-electron density granules, probably comprising poly-β-hydroxybutyrate (Fig. 1b, c).

Utilization of various energy and carbon sources other than methane was examined by adding one of the following substrates to Mmj medium: 0.05 % (w/v) formate, acetate, citrate, succinate, glucose, fructose, ribose, mannitol, methylamine, dimethylamine, methanol, ethanol and 2-propanol or 0.1 % (w/v) yeast extract and Casamino acids. Methane in the headspace of the tube was replaced with N₂ in these experiments. Strain HTM55^T was found to grow only on methane and methanol as the carbon and energy source. Growth on methanol was further tested at different concentrations (0.025–3 %, v/v). Strain HTM55^T grew at all methanol concentrations tested and the highest cell density was achieved at 0.5 % methanol. None of the other substrates supported growth. Nitrogen sources were examined by adding one of the following substrates (0.05 %, w/v) to Mmj medium instead of both NH₄Cl and NaNO₃: NH₄Cl, NaNO₃, NaNO₂, Tris, urea, formamide, methylamine, dimethylamine, Casamino acids, glycine, L-tryptophan, L-lysine, L-glutamic acid or L-aspartic acid. Strain HTM55^T grew well with NH₄Cl, NaNO₃, NaNO₂, Casamino acids and L-aspartic acid as the nitrogen source. Weak growth was observed with Tris, urea, L-lysine and L-glutamic acid. No growth was observed with any of the other nitrogen sources tested (formamide, methylamine, dimethylamine, glycine and L-tryptophan). In addition, no growth was found with N₂ in the headspace gas in the absence of any other nitrogen compounds. PCR experiments using two pairs of reported primers to amplify the *nifH* gene, which encodes the nitrogenase iron protein involved in nitrogen fixation, also did not generate plausible amplification products from strain HTM55^T DNA (Mehta *et al.*, 2003; Zehr & McReynolds, 1989). Three strains, *Methylococcus*

Fig. 1. Transmission electron micrographs of ultrathin sections of strain HTM55^T. ICM, intracytoplasmic membrane; IIG, intracellular inclusions of granules. Bars, 2 μm (a), 0.5 μm (b, c) and 0.1 μm (d).

capsulatus ATCC 19069^T, *Methylosinus sporium* DSM 17706^T and *Methylocella silvestris* DSM 15510^T, were used as positive controls in the *nifH* amplification.

Growth conditions for temperature, pH, NaCl concentration and Cu²⁺ concentration on methane were examined in Mmj medium and are summarized in Table 1. Strain HTM55^T grew at 37–65 °C (optimum 55–60 °C); no growth was observed below 33 °C or above 68 °C. The effects of pH and NaCl concentration on growth were tested at 55 °C. The pH of Mmj medium was adjusted with HCl or NaOH after the tube was sealed with headspace gas. Strain HTM55^T grew at pH 5.2–7.5 (optimum pH 5.8–6.3); no growth was observed at pH 5.0 or 8.0. Strain HTM55^T was moderately acidophilic compared to other thermophilic type I methanotrophs (Table 1). Strain HTM55^T grew with 0–1 % (w/v) NaCl (optimum 0–0.3 % NaCl); no growth was observed with 1.5 % NaCl. The effect of Cu²⁺ concentration on growth was tested under optimum growth conditions (55 °C, pH 6.3 and 0.3 % NaCl). Strain HTM55^T grew with 0–3 μM CuSO₄ and optimum growth was observed with 0.5 μM CuSO₄ (specific growth rate: 0.30 h⁻¹; doubling time: 2.3 h). The final cell yield was 2–7 × 10⁷ ml⁻¹ and the OD₆₆₀ was 0.07–0.25. CuSO₄ concentrations above 4 μM had an inhibitory effect on growth and the presence of 20 μM CuSO₄ completely inhibited the growth of strain HTM55^T. The growth rate and cell yield on methanol (0.5 %, v/v) were equal to those on methane.

The presence of soluble methane monooxygenase (sMMO) in strain HTM55^T was tested both by an enzyme activity

assay and PCR amplification. To measure sMMO activity, the naphthalene oxidation assay for a liquid culture (Csáki *et al.*, 2003) was performed with whole cells of strain HTM55^T grown without a Cu²⁺ supplement. The genes *mmoX*, *mmoY*, *mmoZ*, *mmoB* and *mmoC*, which encode sMMO, were examined by PCR amplification as described elsewhere (McDonald *et al.*, 1995; Miguez *et al.*, 1997). The presence of sMMO in strain HTM55^T was not proven by any of these methods. Meanwhile, the presence of particulate methane monooxygenase (pMMO) was confirmed by the identification of *pmoA* by PCR and sequencing experiments (see below).

To determine carbon fixation capabilities, a cell-free extract was prepared from a cell pellet (0.3–0.5 g, wet weight) harvested during the exponential growth phase. The cells were resuspended in 3 ml 0.1 M potassium phosphate buffer (pH 7.0) to assay for hexulose phosphate synthase and hydroxypyruvate reductase or 3 ml 0.1 M HEPES/NaOH (pH 8.3), 10 mM MgCl₂ buffer to assay for ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco). The cell suspension was sonicated for four periods of 30 s (150 W, 20 kHz) with ice-cooling and then centrifuged at 15 000 g for 10 min. The supernatant was used for the enzyme activity assays. For hexulose phosphate synthase in the ribulose monophosphate (RuMP) pathway, the colorimetric assay was carried out as described by Bodrossy *et al.* (1997), except for a minor change in the formaldehyde concentration in this study. The formaldehyde concentration in the enzyme reaction solution was reduced to one-tenth of the original method because the original concentration (67 mM) failed

Table 1. Characteristics of strain HTM55^T and physiologically and phylogenetically related gammaproteobacterial methanotrophs

Strains: 1, HTM55^T (this study); 2, *Methylothermus thermalis* MYHT^T (Tsubota *et al.*, 2005); 3, *Methylocaldum* (*Methylocaldum szegediense* OR2^T, strain O-12 and/or strain H-11; Bodrossy *et al.*, 1997; Eshinimaev *et al.*, 2004); 4, *Methylococcus thermophilus* (6 strains; Bowman *et al.*, 1993); 5, *Methylohalobius crimeensis* 10Ki^T and 4Kr (Heyer *et al.*, 2005). +, Positive; +/–, strain dependent; –, negative; ND, no data available.

Characteristic	1	2	3	4	5
Temperature for growth (°C)					
Range	37–65	37–67	30–62	37–55	15–42
Optimum	55–60	57–59	55	ND	30
pH for growth					
Range	5.2–7.5	6.5–7.5	6–8.5	5.5–9.0	6.5–7.5
Optimum	5.8–6.3	6.8	7.1–7.2	ND	7.0
NaCl for growth (%)					
Range	0–1	0–3	0–0.5	0–3	1.2–15
Optimum	0–0.3	0.5–1	0.1	ND	5.8–8.7
Activity of key enzyme in:					
RuMP pathway	+	+	+	+	+
Serine pathway	–	–	+	–	–
Calvin–Benson cycle	–	–	+	+	ND
Nitrogen fixation	–	–	–	+	–
Cyst formation	–	–	+/–	+	–
Motility	+	–	+	+	+
Intracellular inclusions of granules	+	–	–	+	+
Major fatty acids (%)					
C _{16:0}	52.0	37.2	63.7–65.0	39.7–47.0	22.8–23.0
C _{18:1ω7c}	34.8	–	–	–	51.9–60.5
C _{18:1ω9c}	–	35.2	–	–	–
C _{16:1}	–	–	11.9–13.3	–	–
C _{16:1ω7c}	–	–	–	17.2–45.9	–
DNA G + C content (mol%)	54.4 ± 0.4*	62.5	56.5–58.5	59–61	58.6–58.8

*The value is a mean of three determinations.

to lead to reasonable colour development in the following colorimetric assay developed by Nash (1953). Two negative controls were employed in the colorimetric assay: (1) a reaction without ribulose-5-phosphate; and (2) a reaction with the inactivated enzyme by heating at 96 °C for 15 min. Hydroxypyruvate reductase in the serine pathway was assayed as described by Large & Quayle (1963) and Rubisco in the Calvin–Benson cycle was assayed as described by Maeda *et al.* (2002).

Hexulose phosphate synthase activity was detected, indicating that strain HTM55^T assimilated carbon by the RuMP pathway. The presence of the RuMP pathway was further suggested by the presence of the *hps* and *phi* genes encoding 3-hexulose-6-phosphate synthase and 6-phospho-3-hexuloisomerase, respectively. The complete sequences of *hps* and *phi* from strain HTM55^T were determined on the basis of the results of the metagenomic analyses used for this gold mine environment (H. Takami, S. Nishi & A. Toyoda, unpublished data).

Neither activity of hydroxypyruvate reductase in the serine pathway nor activity of Rubisco in the Calvin–Benson cycle was detected. PCR amplification of *cbbL* encoding the large subunit of Rubisco was also attempted using three pairs of

primers as reported elsewhere (Alfreider *et al.*, 2003; Elsaied & Naganuma, 2001; Nanba *et al.*, 2004), but no plausible amplification products were generated.

In each experiment for metabolic enzymes and genes, *Methylococcus capsulatus* ATCC 19069^T, *Methylosinus sporium* DSM 17706^T, *Methylocella silvestris* DSM 15510^T or *Thiobacter subterraneus* JCM 12421^T were used as positive or negative controls. Representative metabolic characteristics of strain HTM55^T and those of physiologically and phylogenetically related gammaproteobacterial methanotrophs are summarized in Table 1.

For analysis of the whole-cell fatty acids of strain HTM55^T, fatty acid methyl esters were prepared as described previously (Hirayama *et al.*, 2005a) using cells grown exponentially at 55 °C. Fatty acid methyl esters were extracted with hexane and analysed using a Finnigan TRACE DSQ GC-MS system (Thermo Fisher Scientific) equipped with a polar capillary column SP-2560 (Supelco, 100 m × 0.25 mm × 0.2 µm) under a helium flow of 1.8 ml min^{–1} and an oven temperature program increasing from 155 °C (1 min) to 175 °C at 1 °C min^{–1}. The major fatty acids of strain HTM55^T and related methanotrophs are summarized in Table 1 and the

detailed compositions are given in Supplementary Table S1 (available in IJSEM Online). The major fatty acids of strain HTM55^T were C_{16:0} (52.0 %) and C_{18:1ω7c} (11-*cis*-octadecenoic acid; 34.8 %). C_{16:0} is a major component in thermophilic and thermotolerant species of proteobacterial type I methanotrophs. Mesophilic strains of the genus *Methylohalobius*, the closest genus to *Methylothermus*, also indicate relatively high C_{16:0} ratios (Heyer *et al.*, 2005). Other type I methanotrophs belonging to the genera *Methylomonas*, *Methylobacter*, *Methylochromobium*, *Methylosarcina*, *Methylosphaera* and *Methylosoma* contain less than 20 % C_{16:0} fatty acids (Bowman *et al.*, 1993, 1995, 1997; Kalyuzhnaya *et al.*, 2005; Rahalkar *et al.*, 2007). It was revealed that both strain HTM55^T and *Methylothermus thermalis* MYHT^T possess C_{18:1} fatty acids as major components. Other studies showed that C_{18:1} fatty acids are, if contained, only minor components in most type I methanotrophs, including the thermophilic strains of the genera *Methylocaldum* and *Methylococcus* (Bowman *et al.*, 1993, 1995, 1997; Eshinimaev *et al.*, 2004; Kalyuzhnaya *et al.*, 2005; Rahalkar *et al.*, 2007). An exception is the mesophilic strains of *Methylohalobius*, which are reported to contain more than 50 % C_{18:1ω7c} (Heyer *et al.*, 2005). The thermotolerant *Methylohalobius* sp. IT-9 (Hirayama *et al.*, 2007) also contains 49.6 % C_{18:1} (H. Hirayama, T. Nunoura & K. Takai, unpublished data). The predominance of fatty acid C_{18:1} may be one of the characteristics commonly found in the phylogenetically related genera *Methylothermus* and *Methylohalobius*. Meanwhile, C_{18:1} fatty acids are generally abundant in the type II methanotrophs of the class *Alphaproteobacteria* (Bowman *et al.*, 1993; Dedysh *et al.*, 2007).

The G + C content of the genomic DNA was determined as described by Tamaoka & Komagata (1984). The G + C content of strain HTM55^T was 54.4 mol%, which is similar to the values of thermophilic members of the genus *Methylocaldum* (56.5–58.5 mol%) (Bodrossy *et al.*, 1997; Eshinimaev *et al.*, 2004). However, the G + C content of strain HTM55^T was far lower than that of *Methylothermus thermalis* MYHT^T (62.5 mol%; Table 1).

The nearly complete sequence (1444 bp) of the 16S rRNA gene from strain HTM55^T was amplified by PCR as described previously (Hirayama *et al.*, 2005b) and directly sequenced from both strands with a DNA sequencer (model 3130; Applied Biosystems). The 16S rRNA gene sequence was subjected to similarity analysis using the FASTA algorithm (<http://fasta.ddbj.nig.ac.jp/top-j.html>) and GENETYX-MAC version 14.0.5 software (Genetyx). The closest relatives of strain HTM55^T were the environmental clone HAUd-UB26, which had been obtained previously from the same gold mine environment (99.6 % 16S rRNA gene sequence similarity; Hirayama *et al.*, 2005b) and *Methylothermus thermalis* MYHT^T (99.2 %; Tsubota *et al.*, 2005). The results strongly indicated that strain HTM55^T belonged to the genus *Methylothermus*. Strain HTM55^T was moderately related to *Methylothermus* sp. HB (92.6 %; Bodrossy *et al.*, 1999) and the mesophilic and halophilic strain *Methylohalobius crimeensis* 10Ki^T (90.5 %; Heyer

et al., 2005). Members of other methanotrophic genera were only distantly related to strain HTM55^T (≤88.1 %).

For phylogenetic analysis, 16S rRNA gene sequences from type I and type X methanotrophic bacteria of the class *Gammaproteobacteria* were aligned using ARB software and corrected manually where necessary. Only unambiguously aligned nucleotide positions were used for the analysis. A phylogenetic tree was constructed using 835 sequence positions using the neighbour-joining algorithm in the SeaView version 4.0 package and evolutionary distances estimated with Kimura's correction. The phylogenetic tree indicated that strain HTM55^T belonged to a deeply branching and distinct lineage comprising the genera *Methylothermus* and *Methylohalobius* within the type I and type X methanotrophs of the class *Gammaproteobacteria* (Fig. 2).

To amplify the full sequence of *pmoA*, the following primers were developed on the basis of full sequences of the *pmoCAB* operons (encoding pMMO) from the GenBank database: *pmoC*-F2 5'-GGTCCGTTTCATGATYCTGCC-3' and *pmoB*-R1 5'-TCARGTCRWACCAGTGRAT-3', corresponding to positions 646–665 of *pmoC* and 139–157 of *pmoB*, respectively, of *Methylococcus capsulatus* Bath (GenBank accession no. U94337). Amplification was performed with *Ex Taq* polymerase (TaKaRa Bio) with the following 30-cycle programme; 96 °C for 25 s, 45 °C for 45 s and 72 °C for 90 s. The PCR fragment (approx. 1.3 kb) was directly sequenced from both strands and subjected to similarity analysis as described above. As summarized in Supplementary Table S2, the *pmoA* sequence of strain HTM55^T was very similar to the environmental *pmoA* clone *pmoU*-1, which was isolated from the same gold mine environment (99.0 % gene sequence similarity, 100 % amino acid sequence similarity; Hirayama *et al.*, 2005b), *Methylothermus thermalis* MYHT^T (98.7 % gene sequence similarity, 100 % amino acid sequence similarity) and *Methylothermus* sp. HB (90.6 % gene sequence similarity, 97.7 % amino acid sequence similarity). Strain HTM55^T was also related to *Methylohalobius crimeensis* 10Ki^T (83.0 % gene sequence similarity, 92.1 % amino acid sequence similarity). The alignment of the deduced partial *pmoA* sequences from various methanotrophs (nine genera of type I and one genus of type II) presented several distinctive residues that were shared with the genera *Methylothermus* and *Methylohalobius* (Supplementary Fig. S1).

The similarities between members of the genera *Methylothermus* and *Methylohalobius* in the 16S rRNA gene and *pmoA* sequences are consistent with the reports by Heyer *et al.* (2005) and Tsubota *et al.* (2005), suggesting that these two genera within the currently known gammaproteobacterial genera are closely related.

Because the 16S rRNA gene sequence of strain HTM55^T was quite similar to that of *Methylothermus thermalis* MYHT^T, genomic relatedness between these strains was examined by DNA–DNA hybridization (37 °C, 4 h) as described by Ezaki *et al.* (1989) using DNA from both strains as probes in reciprocal experiments. DNA–DNA relatedness between

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain HTM55^T among representative methanotrophs and the closest environmental clone of the class *Gammaproteobacteria*. Bootstrap values (>50%) based on 100 replicates are shown at branch nodes. *Methylosinus sporium* NCIMB 11126^T and *Methylocapsa acidiphila* B2^T, of the class *Alphaproteobacteria*, were used as an outgroup. Bar, 2 substitutions per 100 nt positions.

these strains was $\leq 39\%$. Generally, strains showing $<70\%$ DNA–DNA relatedness are recognized as representing separate species (Wayne *et al.*, 1987). According to this criterion, strain HTM55^T represents a novel species of the genus *Methylothermus*.

Other characteristics also distinguished strain HTM55^T from *Methylothermus thermalis* MYHT^T, as shown in Table 1. The difference in the DNA G + C content between both strains was as much as 8.1 mol%. Both strains possessed C_{18:1} fatty acids; however, the predominant fatty acid in strain HTM55^T was C_{18:1}ω7c (11-*cis*-octadecenoic acid) while that of *Methylothermus thermalis* MYHT^T is reported as C_{18:1}ω9c (9-*cis*-octadecenoic acid). The optimum growth temperatures of the two strains were similar. However, strain HTM55^T preferred more acidic conditions and lower salinity than *Methylothermus thermalis* MYHT^T; the optimum pH for strain HTM55^T (pH 5.8–6.3) was out of the range for *Methylothermus thermalis* MYHT^T (pH 6.5–7.5). The strains also differed in their abilities to utilize the nitrogen sources nitrite, aspartic acid, tryptophan and formamide; strain HTM55^T grew with nitrite and aspartic acid, but not with tryptophan and formamide, and vice versa for *Methylothermus thermalis* MYHT^T. Unlike *Methylothermus thermalis* MYHT^T, microscopy showed that cells of strain HTM55^T were motile, possessed a single flagellum and produced many intracellular inclusions (Fig. 1).

Considering these differences in morphological, physiological and chemotaxonomic characteristics between strain HTM55^T and *Methylothermus thermalis* MYHT^T, in addition to the phylogenetic analysis, it is evident that strain HTM55^T represents a novel species of the genus *Methylothermus*, for which the name *Methylothermus subterraneus* sp. nov. is proposed.

Emended description of the genus *Methylothermus* Tsubota *et al.* 2005

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).

Gram-negative, motile or non-motile coccoids, 0.6–1.4 μm in diameter. Reproduction by normal cell division, with no resting stages (exospores, *Azotobacter*-type cysts and lipid cysts). Members possess pMMO and a typical type I intracytoplasmic membrane system, but not sMMO. Moderate thermophiles; grow at 37–67 °C (optimum 55–60 °C). Obligate methanotrophs; utilize methane or methanol via the RuMP pathway. The major fatty acids are C_{16:0} and C_{18:1}. Belongs to the class *Gammaproteobacteria* (type I methanotrophs); the closest genus is *Methylohalobius*. Habitats are terrestrial hot springs. The type species is *Methylothermus thermalis*.

Description of *Methylothermus subterraneus* sp. nov.

Methylothermus subterraneus (sub.ter.ra'ne.us. L. masc. adj. *subterraneus* under the earth, indicating the first isolation source).

Shows the following characteristics in addition to those given in the genus description. Motile coccoids, 0.8–1.4 μm in diameter. Grows at 37–65 °C (optimum 55–60 °C) and pH 5.2–7.5 (optimum pH 5.8–6.3). Grows well without NaCl (optimum 0–0.3% NaCl), but not with 1.5% NaCl. Utilizes methane and methanol via the RuMP pathway and grows on ammonia, nitrate, nitrite, Casamino acids, L-aspartic acid, L-lysine, L-glutamic acid, Tris and

urea as nitrogen sources. The major fatty acids are C_{16:0} and C_{18:1ω7c}.

The type strain, HTM55^T (=JCM 13664^T=DSM 19750^T), was isolated from subsurface hot aquifer water in the Hishikari gold mine, Kagoshima, Japan. The DNA G+C content of the type strain is 54.4 mol%.

Acknowledgements

We are grateful to Sumitomo Metal Mining Co. Ltd. for cooperation in this study and to Mr A. Tame for assistance with the electron micrography. We would like to thank Dr J. Tsubota for supplying us with a culture of *Methylothermus thermalis* MYHT^T.

References

- Alfreider, A., Vogt, C., Hoffmann, D. & Babel, W. (2003). Diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes from groundwater and aquifer microorganisms. *Microb Ecol* **45**, 317–328.
- Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R. & Wolfe, R. S. (1979). Methanogens: reevaluation of a unique biological group. *Microbiol Rev* **43**, 260–296.
- Bodrossy, L., Holmes, E. M., Holmes, A. J., Kovács, 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-oxidising γ -Proteobacterium. *FEMS Microbiol Lett* **170**, 335–341.
- 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.
- Bowman, J. P., Sly, L. I. & Stackebrandt, E. (1995). The phylogenetic position of the family *Methylococcaceae*. *Int J Syst Bacteriol* **45**, 182–185.
- Bowman, J. P., McCammon, S. A. & Skerratt, J. H. (1997). *Methylosphaera hansonii* gen. nov., sp. nov., a psychrophilic, group I methanotroph from Antarctic marine-salinity, meromictic lakes. *Microbiology* **143**, 1451–1459.
- Csáki, R., Bodrossy, L., Klem, J., Murrell, J. C. & Kovács, K. L. (2003). Genes involved in the copper-dependent regulation of soluble methane monooxygenase of *Methylococcus capsulatus* (Bath): cloning, sequencing and mutational analysis. *Microbiology* **149**, 1785–1795.
- Dedysh, S. N., Belova, S. E., Bodelier, P. L. E., Smirnova, K. V., Khmelenina, V. N., Chidthaisong, A., Trotsenko, Y. A., Liesack, W. & Dunfield, P. F. (2007). *Methylocystis heyeri* sp. nov., a novel type II methanotrophic bacterium possessing 'signature' fatty acids of type I methanotrophs. *Int J Syst Evol Microbiol* **57**, 472–479.
- Elsaied, H. & Naganuma, T. (2001). Phylogenetic diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes from deep-sea microorganisms. *Appl Environ Microbiol* **67**, 1751–1765.
- Eshinimaev, B. Ts., Medvedkova, K. A., Khmelenina, V. N., Suzina, N. E., Osipov, G. A., Lysenko, A. M. & Trotsenko, IuA. (2004). New thermophilic methanotrophs of the genus *Methylocaldum*. *Mikrobiologiya* **73**, 530–539 (in Russian).
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- 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.
- Hirayama, H., Takai, K., Inagaki, F., Nealson, K. H. & Horikoshi, K. (2005a). *Thiobacter subterraneus* gen. nov., sp. nov., an obligately chemolithoautotrophic, thermophilic, sulfur-oxidizing bacterium from a subsurface hot aquifer. *Int J Syst Evol Microbiol* **55**, 467–472.
- Hirayama, H., Takai, K., Inagaki, F., Yamato, Y., Suzuki, M., Nealson, K. H. & Horikoshi, K. (2005b). Bacterial community shift along a subsurface geothermal water stream in a Japanese gold mine. *Extremophiles* **9**, 169–184.
- Hirayama, H., Sunamura, M., Takai, K., Nunoura, T., Noguchi, T., Oida, H., Furushima, Y., Yamamoto, H., Oomori, T. & Horikoshi, K. (2007). Culture-dependent and -independent characterization of microbial communities associated with a shallow submarine hydrothermal system occurring within a coral reef off Taketomi Island, Japan. *Appl Environ Microbiol* **73**, 7642–7656.
- Inagaki, F., Takai, K., Hirayama, H., Yamato, Y., Nealson, K. H. & Horikoshi, K. (2003). Distribution and phylogenetic diversity of the subsurface microbial community in a Japanese epithermal gold mine. *Extremophiles* **7**, 307–317.
- Kalyuzhnaya, M. G., Stolyar, S. M., Auman, A. J., Lara, J. C., Lidstrom, M. E. & Chistoserdova, L. (2005). *Methylosarcina lacus* sp. nov., a methanotroph from Lake Washington, Seattle, USA, and emended description of the genus *Methylosarcina*. *Int J Syst Evol Microbiol* **55**, 2345–2350.
- Large, P. J. & Quayle, J. R. (1963). Microbial growth on C₁ compounds. 5. Enzyme activities in extracts of *Pseudomonas* AM1. *Biochem J* **87**, 386–396.
- Maeda, N., Kanai, T., Atomi, H. & Imanaka, T. (2002). The unique pentagonal structure of an archaeal Rubisco is essential for its high thermostability. *J Biol Chem* **277**, 31656–31662.
- McDonald, I. R., Kenna, E. M. & Murrell, J. C. (1995). Detection of methanotrophic bacteria in environmental samples with the PCR. *Appl Environ Microbiol* **61**, 116–121.
- Mehta, M. P., Butterfield, D. A. & Baross, J. A. (2003). Phylogenetic diversity of nitrogenase (*nifH*) genes in deep-sea and hydrothermal vent environments of the Juan de Fuca Ridge. *Appl Environ Microbiol* **69**, 960–970.
- Miguez, C. B., Bourque, D., Sealy, J. A., Greer, C. W. & Groleau, D. (1997). Detection and isolation of methanotrophic bacteria possessing soluble methane monooxygenase (sMMO) genes using the polymerase chain reaction (PCR). *Microb Ecol* **33**, 21–31.
- Nanba, K., King, G. M. & Dunfield, K. (2004). Analysis of facultative lithotroph distribution and diversity on volcanic deposits by use of the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase. *Appl Environ Microbiol* **70**, 2245–2253.
- Nash, T. (1953). The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem J* **55**, 416–421.
- Op den Camp, H. J. M., Islam, T., Stott, M. B., Harhangi, H. R., Hynes, A., Schouten, S., Jetten, M. S. M., Birkeland, N.-K., Pol, A. & Dunfield, P. F. (2009). Environmental, genomic and taxonomic perspectives on methanotrophic *Verrucomicrobia*. *Environ Microbiol Rep* **1**, 293–306.
- Rahalkar, M., Bussmann, I. & Schink, B. (2007). *Methylosoma difficle* gen. nov., sp. nov., a novel methanotroph enriched by gradient

cultivation from littoral sediment of Lake Constance. *Int J Syst Evol Microbiol* **57**, 1073–1080.

Takai, K., Hirayama, H., Sakihama, Y., Inagaki, F., Yamato, Y. & Horikoshi, K. (2002). Isolation and metabolic characteristics of previously uncultured members of the order *Aquificales* in a subsurface gold mine. *Appl Environ Microbiol* **68**, 3046–3054.

Takai, K., Kobayashi, H., Nealson, K. H. & Horikoshi, K. (2003). *Sulfurihydrogenibium subterraneum* gen. nov., sp. nov., from a subsurface hot aquifer. *Int J Syst Evol Microbiol* **53**, 823–827.

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.

Tsubota, J., Eshinimaev, B. Ts., Khmelenina, V. N. & Trotsenko, Y. A. (2005). *Methylothermus thermalis* gen. nov., sp. nov., a novel moderately thermophilic obligate methanotroph from a hot spring in Japan. *Int J Syst Evol Microbiol* **55**, 1877–1884.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Zehr, J. P. & McReynolds, L. A. (1989). Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. *Appl Environ Microbiol* **55**, 2522–2526.