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Original Article

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Detection of Thermoacidophiles from Yellowstone Hot Spring

Tajul Islam[1, 2,*], Ruben Michael Ceballos[3], and Lise Øvreås[1]

[1]*Department of Biological Sciences, University of Bergen, Thormøhlensgate 53 B, PO box, 7803, 5006 Bergen, Norway,* [2]*Bergen Katedralskole, Kong Oscars gate 36, 5017 Bergen, Norway,* [3]*University of Arkansas, Fayetteville, AR 72701, USA*

Aerobic methanotrophic bacteria maintain an unrivalled capability of utilizing methane as their sole carbon and energy source and have been retrieved from various environments. Phylogenetically, true aerobic thermoacidophilic methane oxidizers capable of growing below pH 3 have hitherto been associated only with the phylum *Verrucomicrobia*. In this report, the initial detection of a moderately thermoacidophilic *Methylococcus*-like Type Ib methanotroph of the class *Gammaproteobacteria* from an acidic thermal spring (50ºC and pH 2.8) in the Yellowstone National Park, USA is presented. The isolate, termed YT-MC, was identified in a methane enrichment (55ºC), which may represent a novel strain in the family *Methylococcaceae* Type Ib. The existence of this bacterium in the enrichments was demonstrated by the detection of *pmoA* gene, Southern blotting technique, phase-contrast, and electron microscopy. The coccus-typed cells showed tubular membranes instead of intracytoplasmic membrane systems (ICM). The soluble methane monooxygenase (sMMO) was not detected by PCR, indicating that the biotransformation of methane to methanol is oxidized by the particulate methane monooxygenase (pMMO). Moreover, YT-MC performs in a formerly undiscovered active biological methane sink in geothermal acidic environments, magnifying our knowledge of its ecological role in methane cycling, diversity, and coexistence of aerobic methanotrophy. Furthermore, the present study also reports the isolation and identification of an alphaproteobacterial heterotroph (strain YT-AC) and a verrucomicrobial methanotroph (strain YT-VM) from the same environment.

Keywords: Yellowstone, thermal spring, thermoacidophilic, methanotrophs, pMMO

Introduction

Microorganisms in thermal systems play a significant role for biogeochemical cycling particularly with reducing flows of terrestrial natural greenhouse gases, which contributes a direct impact on Earth's climate-related regulations. Currently methane acts as a greenhouse gas thirty-four times as strong as CO_2 and contributes to influence the global atmosphere[1,2]. At high pressure and temperature of the underground reservoirs, the geological methane is produced through catalytic reactions or thermogenic decomposition of buried organic matter. In geothermal acidic habitats, a part of volcanic gases and hydrothermal solutions are emitted through degassing of spring water, gas venting, seeps, and mud volcanoes[3,4]. The flow and formation of acidic geothermal gases (methane, hydrogen sulphide and other natural gases) vary significantly over time[5]. Microbes can convert hydrogen sulphide (H_2S) into sulfuric acid (H_2SO_4) by oxidation, resulting in values of low pH. Moreover, this low pH can also be affected by seasonal rainfall[6].

Especially, aerobic methane oxidizers represent an environmentally significant group due to their key actively operating role in the mitigation of methane emissions to the atmosphere from thermal ecosystems. But these microbes are distinguished from other microbes by their unique capability of using methane as their sole carbon and energy source and for maintaining a multicomponent enzyme system that comprises of two forms: a particulate membrane-bound enzyme (pMMO) and a cytoplasmic soluble membrane-free enzyme (sMMO)[7,8]. According to molecular diversity and taxonomically the aerobic methanotrophs are so far included into two phyla namely *Pseudomonadota* (synonym *Proteobacteria*[10]) and *Verrucomicrobia*. Besides these, recent studies reported a new phylum of the Gram-negative methane oxidizing bacteria (MOB) named *Candidatus* Mycobacterium methanotrophicum in the phylum *Actinobacteria* (a member of Gram-positive bacteria), which was enriched and cultivated from an extremely acidic cave microbial ecosystem[11]. The reported aerobic gammaproteobacterial methanotrophs are presently reclassified into six distinct families, and they are largely comprised of neutrophilic, psychrotolerant, mesophilic, thermotolerant, and moderately thermophilic organisms (growth at pH 3.1" 9 and at temperatures between 5 and 60ºC)[12-17], whereas the phylum *Verrucomicrobia* contains three thermoacidophilic strains of the genus *Methylacidiphilum* (growth temperature range: 37 to 65 ºC and pH range: 0.8 to 6.0) are recovered from acidic geothermally heated soils and a hot spring[18-20].

In contrast to acid-tolerant or acidophilic moderately thermophilic methane oxidizers (T_{max} < 60 ºC), our understanding of culturable

Corresponding author:
Tajul Islam, Department of Biological Sciences, University of Bergen, Thormøhlensgate 53 B, PO box, 7803, 5006 Bergen, Norway, Email: tajisl@hotmail.no

thermoacidophilic aerobic methanotrophs (i.e. growth at pH < 4) is still restricted. None of them were recovered or detected from acidic thermal habitats yet. Although, several species of the acidophilic or acid-tolerant methanotrophs (mesophilic, psychrotolerant) of the phylum *Pseudomonadota* have been described (Table S1). A high diversity of gammaproteobacterial methanotrophs, distantly related to *Methylocaldum-Metylococcus* genera were detected by the *pmoA* gene libraries from volcanic soils (pH 5.8) at the geothermal field of Favara Grande (Italy). Furthermore, alphaproteobacterial species belonging to the genus *Methylocystis* were isolated from enrichment cultures[21]. These organisms grew at a pH range of 3.5 to 8 and at temperatures between 18 and 45 °C. Recently, the first recovered acid-tolerant (at a pH range 4.2"7.5) moderately thermophilic gammaproteobacterial strains BFH1 and BFH2 were recovered from tropical topsoil with methane seeps habitat in Bangladesh and most probably denote a novel Type Ib methanotrophic genus of the family *Methylococcaceae*. Both strains made a cluster with the genus *Methylocaldum* as the closest described relatives[14].

Two key functional molecular gene markers like *pmoA* (encoding a subunit of the particulate methane monooxygenase, pMMO) and *mmoX* (encoding a subunit of the soluble methane monooxygenase, sMMO) were often applied for detecting and diversity analysis of C_1-utilizing bacteria in various environments[22]. Research on methanotrophs has primarily emphasized on low or moderate temperature habitats and until now about 21 acidophilic or acid-tolerant proteobacterial strains have been described[23] (Table S1). Despite that isolating of moderately thermoacidophilic methane consuming bacteria from extreme environments (less than pH 3) is completely a challenging approach. Except thermoacidophilic verrucomicrobial methanotrophs (growth pH range 0.8-6.0), no true moderately thermoacidophilic methane oxidizers of the phylum *Pseudomonadota* have so far been reported or isolated.

For this study, a methane-consuming mixed culture was cultivated from an acidic thermal spring in Yellowstone National Park, USA. The culture was mostly made up of two thermoacidophilic obligate methylotrophs and a heterotroph. Using PCR-sequencing and Southern hybridization techniques, we were able to detect the key methane oxidation enzyme of a moderately thermoacidophilic *Methylococcus*-like methanotroph, which possibly belongs to the family *Methylococcaceae* Type Ib of the phylum *Pseudomonadota,* and thus expands *Methylococcaceae* Type Ib diversity. Additionally, we have successfully identified a moderately thermophile of heterotrophic alphaproteobacterial species and a likely new species of the verrucomicrobial methanotrophic genus *Methylacidiphilum*.

Materials and Methods

Sampling, enrichments, and growth conditions

A mixed environmental sample of sediment and water was collected in April 2009 from an acidic thermal spring outflow, which is positioned in the Norris geyser basin of the Yellowstone National Park, WY, USA (44°43'34.0" N, 110°42'15.0" W, 2303 meters above sea level). Using a handheld multi-meter (Oakton, USA), pH was measured as 2.8 and and *in situ* temperature of the spring sediment was 52.3ºC. For cultivation of moderately thermoacidophilic methane oxidation bacteria, the primary enrichment (1st transfer) was implemented in a 120 mL sterile serum flask as follows: 3 ml water sediment slurry was added to 20 mL of low-salt mineral medium supplemented with NH_4Cl[14,24] (0.1 $g \cdot L^{-1}$; LMA), the pH was adjusted to 3.0 with 1M HCl, the flask was closed with a butyl rubber stopper with an aluminium crimp seal, a mixture of 80% methane (purity 99.5%, Yara Praxair, Oslo, Norway) and 20% air aseptically was added through a syringe in the headspace. Eventually, the flask was shaken at 125 rpm for 7 days at 55 ºC while being kept in the dark. Furthermore, the primary enrichment was also elaborated with a nitrate-free low-salt mineral medium (M3)[20]. Vitamins or Cerium chloride ($CeCl_3$; Rare Earth Elements, REE) were not added to both media during cultivation.

Cultivation of thermoacidophilic strains and electron microscopy

The obtained primary enrichments from LMA with methane was further enriched by transferring up to five times in the same culture conditions. In addition, the subsequent enrichments were also performed with methanol. The 5th transfer was used for serial dilutions and 100 µL of each dilution (10^{-5} and 10^{-6}) were spread onto LMA agar plates (20 $g \cdot L^{-1}$, Difco) and R2A agar (Reasoner's 2A[25]) plates for identification of thermoacidophilic heterotrophs. The pH of the agar plates were adjusted at ~3.0 using 1M HCl. The LMA plates were incubated for five weeks at 55ºC in jars filled with methane and no extra air was supplemented, whereas plates of R2A agar were incubated without adding methane. Observed colonies were picked and re-streaked onto fresh plates and re-incubated at 55 ºC with same culture conditions. The 5th transfer (termed as S1Y) was also evaluated by PCR-DGGE[20,26] (16S rRNA gene; Fig. S1), phase-contrast (Fig. S2) and transmission electron microscopy as well as growth without methane. The morphological studies of the enrichment cultures and strains were accomplished by phase-contrast microscopy (Nikon, Eclipse E400 microscope) and JEOL-1230 electron microscopy[20].

PCR, Southern blot hybridization, and phylogenetic Analyses

Isolated genomic DNA (DNA kit, Sigma-Aldrich) of 1st and 3rd transfers were subjected to PCR in efforts to detect proteobacterial methanotrophic functional genes like *pmoA and mmoX* (Table S2), which was performed using a Veriti 96 well Thermal Cycler (Applied Biosystems) and Dynazyme™, a ~High-fidelity DNA polymerase (Finnzymes). The protocols of PCR and sequencing were followed as previously depicted[14]. Furthermore, only detected amplified fragments of the gene *pmoA* was cloned using a TOPO-TA Cloning Kit (Invitrogen). Southern blotting technique was applied for the detection of pMMO and sMMO. Genomic

DNA (only from 1st and 3rd transfer enrichments) were extracted from using GenElute Bacterial Genomic DNA kit (Sigma). Genomic DNA of *Mac kamchatkense* Kam1 was used as a negative control, whereas genomic DNA from *Methylococcus capsulatus* Bath, *Methylococcaceae* Type Ib strain LS7-MC[17] were used as positive controls. DNA was digested with EcoRI and HindIII. Radioactively labelled probes of *pmoA* and *momX* were used for hybridization and the further method were followed as previously described[20,27]. PmoA protein sequences (169 amino acids) and 16S rRNA gene sequences (486 nucleotides from a band of PCR-DGGE analysis) were compared with available sequences in the GenBank database using the NCBI tools (Blastp and Blastn). To carry out phylogenetic analysis, sequences were aligned using CLUSTAL W algorithm. Distances were processed and phylogenetic trees were composed using several methods (Neighbour Joining, Minimum-Evolution and Maximum Likelihood), and models (Kimura 2-parameter, Maximum composite likelihood, Tamura 3-parameter, Jukes-Cantor, and Dayhoff). These are performed in the software package of MEGA7[28]. The confidence of the trees was assessed by 1000 bootstrap replications. The partial sequences of the cloned gene *pmoA* (strain YT-MC), PCR-DGGE 16S rRNA gene sequences (strain YT-VM), and 16S rRNA gene sequences of the strain YT-AC have been deposited in GenBank under the accession numbers, OM372568, OM348580, and OM348537, respectively.

Results

The enrichments of moderately thermoacidophilic methane oxidizers were attained from an acidic thermal spring. The pH and *in situ* temperature were 2.8 and 53.4 °C, respectively. Two separate enrichments, LMA and M3 were established. After a week of incubation at 55 °C, the primary enrichment with LMA showed microbial growth of several morphotypes (mostly rod-shaped and coccus; a density of nearly 10^8 cells mL^{-1}) which were confirmed by phase-contrast microscopy, whereas a four-week incubation with a nitrate-free low-salt mineral medium M3 showed a weak turbidity of short rod- and long rod-shaped cells rather than coccus-type cells. However, as the interest was to isolate coccoid cells (designated YT-MC), further study was continued with only the LMA primary enrichments. Table 1 lists some traits of enrichment strain YT-MC in comparison to aerobic methanotrophs of the family *Methylococcaceae* Type Ib. Growth of coccoid and rod-shaped cells was observed also with methanol (3.5 µM). After three successive transfers with methane, the fraction of coccoid cells (appeared form of ellipsoid) was not higher in 4th and 5th transfer enrichments than the primary enrichment cultures. Absence of methane or methanol in the subsequent enrichment transfers (as negative controls), neither coccoid nor rod-shaped type cells were observed. No colonies were appeared in LMA agar plates with methane. But we were further able to recover a thermoacidophilic strain from a R2A agar

Table 1. *Major characteristics of the Methylococcus-type cells in the enrichment cultures and aerobic methanotrophs of the family Methylococcaceae Type Ib.*

Characteristic	1	2	3	4	5
Cell morphology	Coccoids	Coccoids	Coccoids	Coccoids	Rods-pleomorphic
Enrichment temp.(ºC)	Moderately Thermophilic (55)	Moderately Thermophilic (55)	Moderately Thermophilic (55)	Mesophilic/ Thermotolerant/ Thermophilic (42)	Thermophilic/ Thermotolerant (35"55)
pH of enrichments	3	7	6.7	6.5	7.0"7.2
Internal membranes	Tubular	Type I	Type I	Type I	Type I
Acidophilic condition	Thermoacidophilic	Neutrophilic	Acid-tolerant	Neutrophilic	Neutrophilic
Pigmentation	nd	White	White	Yellow	Brown/cream
Motility	“	“	“	“	+/”[a]
Growth on agar plates	“	“	“	+	+
Growth on gelrite plates	nd	+	+	+[b]	nd
pMMO	+	+	+	+	+
sMMO	“	“	“	+	“/+[c]
Growth on methanol (0.1%)	+	+	+	+	+/”
Isolation source (pH)	Acidic thermal Spring (2.8)	Alkaline thermal spring (8.82)	Tropical topsoil (5.0)	Hot spring and soil (6.0)	Manure, silage, marine, hot spring (6.0-7.0)

Strains: 1, The present study; 2, *Methylococcaceae* strain LS7-MC[17]; 3, *Methylococcaceae* strain BFH1[14]; 4, *Methylococcus capsulatus*[12]; 5, *Methylocaldum* spp.[32]; nd, not determined; +, positive results; –, negative results; Bold values show significant range of temperatures and pH of isolation sources among Type Ib methane oxidizers. [a]Only *Methylocaldum marinum* strain $S8^T$ showed motility. [b]*M. capsulatus* Bath did grow on tested gelrite plates in our lab (results were not shown). [c]possessed soluble methane monooxygenase gene[32].

plate (light round white colonies), which was denoted as strain YT-AC. This strain did not show any growth in the LMA or agar plates with methane but showed growth in the presence of methanol (0.1% v/v), acetate (18 mM), pyruvate (10 mM), glucose (10 mM), lactose (10 mM) and yeast extract (0.25% v/v) adjusted LMA to pH 3.0, indicating a heterotrophic acidophile.

Evidence for the detection of the key functional gene (*pmoA*) in the methane oxidation process of the enrichment cultures by PCR gave positive, whereas the gene *mmoX* was negative. Primers of the *pmoA* are generally used as diagnostic markers for Type Ib methanotrophs from different ecosystems[14,17,29]. But the primer sets did not work for the amplification of thermoacidophilic verrucomicrobial methanotrophs by PCR[18,20]. Southern blotting analysis of genomic DNA from enrichment cultures displayed positive signals with the *pmoA* probe, but the probe of *mmoX* provided no positive signals. This analysis reveals that methanotrophic isolate YT-MC does not incorporate the soluble form of MMO. This may clarify also that the aerobic methane oxidation in the enrichment cultures at pH 3 and 55ºC mainly operated by two different MOB.

Coccoid cells (i.e. strain YT-MC) were seen in the enrichment cultures. The cells appeared individually during observation by phase-contrast microscopy and electron microscopy (Fig. 1A, 1B and 1C). An ellipsoid structure emerged during cell division. Flagella were not observed. The isolate was non-motile and multiplied by binary fission. Divulged structures of tubular membranes were observed under TEM (TM; Fig. 1D). These tubular membranes were also seen in thermoacidophilic *Mac. infernorum* strain V4[19], whereas verrucomicrobial strain YT-VM revealed the presence of carboxysome-like membranes (CL), (Fig. 1E), which were also observed in *Mac. kamchatkense strain* kam1[20] and *Mac. fumariolicum* strain SolV[18], and other related strains of verrucomicrobial methanotrophs[30].

The detected *pmoA* gene (509 bp) analysis of the enrichment cultures, based on Blastn search, demonstrated maximum identities with *Methylococcus*-like cells from an alkaline thermal spring of Ethiopian Rift Valley (96.2% and 99.4% amino acid level) and a PCR-DGGE band of an uncultivated bacterium clone from a radioactive hot spring of Austria (94.1% and 98.4% amino acid level) having GenBank accession numbers, KP828775 and AM749116, respectively[17,31]. Further analysis of this gene exhibited lower homology (88.8 to 91.1%) to several uncultured bacterial clones from different geographical regions and ecosystems (KF901437, AF533666, JQ038175, KF836706,

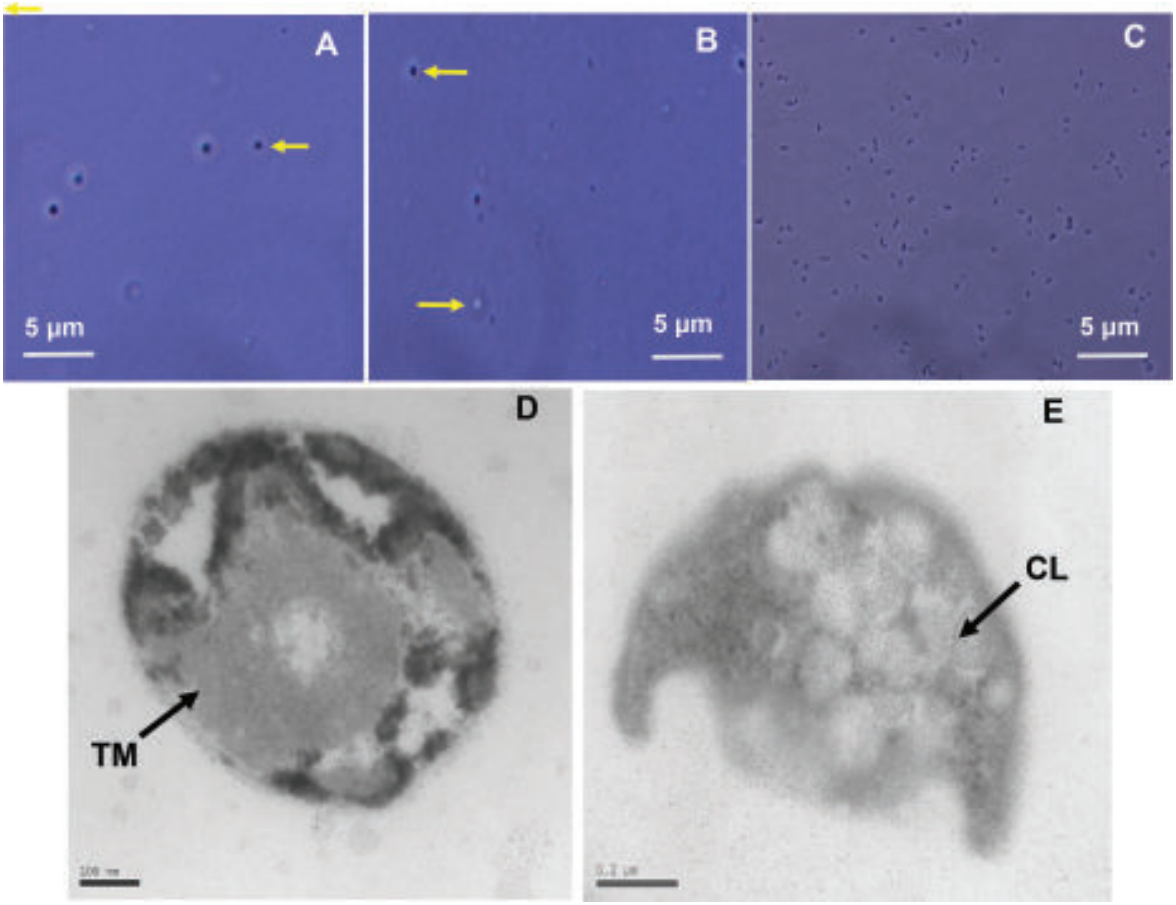

Fig. 1. *Morphology of the methanotrophic enrichments. (**A** and **B**), Phase-contrast photomicrographs of methane enrichments (1st and 3rd transfer enrichment cultures, respectively) grown in LMA at pH 3 and 55 °C for 7 days. (**C**), a phase-contrast image of YT-AC cells growth on methanol. (**D**), Transmission electron micrographs of the methanotrophic isolate YT-MC from 5th transfer and (**E**), a YT-VM cell. Ultrathin sections showing tubular membrane structures (TM) and extensive carboxysome-like structures (CL).*

AF368374, AB113638 and MF106317). The additional analysis, which is based on pairwise nucleotide and partial-derived PmoA protein sequences, unveiled their close affinity with the described genera and species of Type Ib methanotrophs: 88.6% identity (99.4% at amino acid level) to *Mcc. capsulatus* Bath, 82.1–85.7% identity (92.9–94.5%) to *Methylococcaceae* strains AK-K6, GFS-K6, BFH1 and BFH2, 82.8% identity (93.3%) to *Methylomagnum ishizawai* RS11D-Pr^T, 81.6% identity (93.5%) to *Candidatus Methylospira mobilis,* 81.3% identity (95.3%) to *Methylogaea oryzae* E10^T, 80.7% identity (92.3%) to *Methylocaldum szegediense* OR2^T and 79.8% (94.1%) identity to *Methyloterricola oryzae* 73a^T (Table S4). Phylogenetic analysis of deduced *pmoA* amino acid sequences was assessed analysing 37 cultured and uncultured taxa occurring as acidophile, acid-tolerant, and environmental samples. The achieved tree was revealed that strain YT-MC was grouped along with *Methylococcaceae* strain LS7-MC, *Mcc. capsulatus* Bath, *Mcc. capsulatus* BL4, and uncultured bacteria clones from various ecosystems with well supported bootstrap values (Fig. 2). The related topology was also

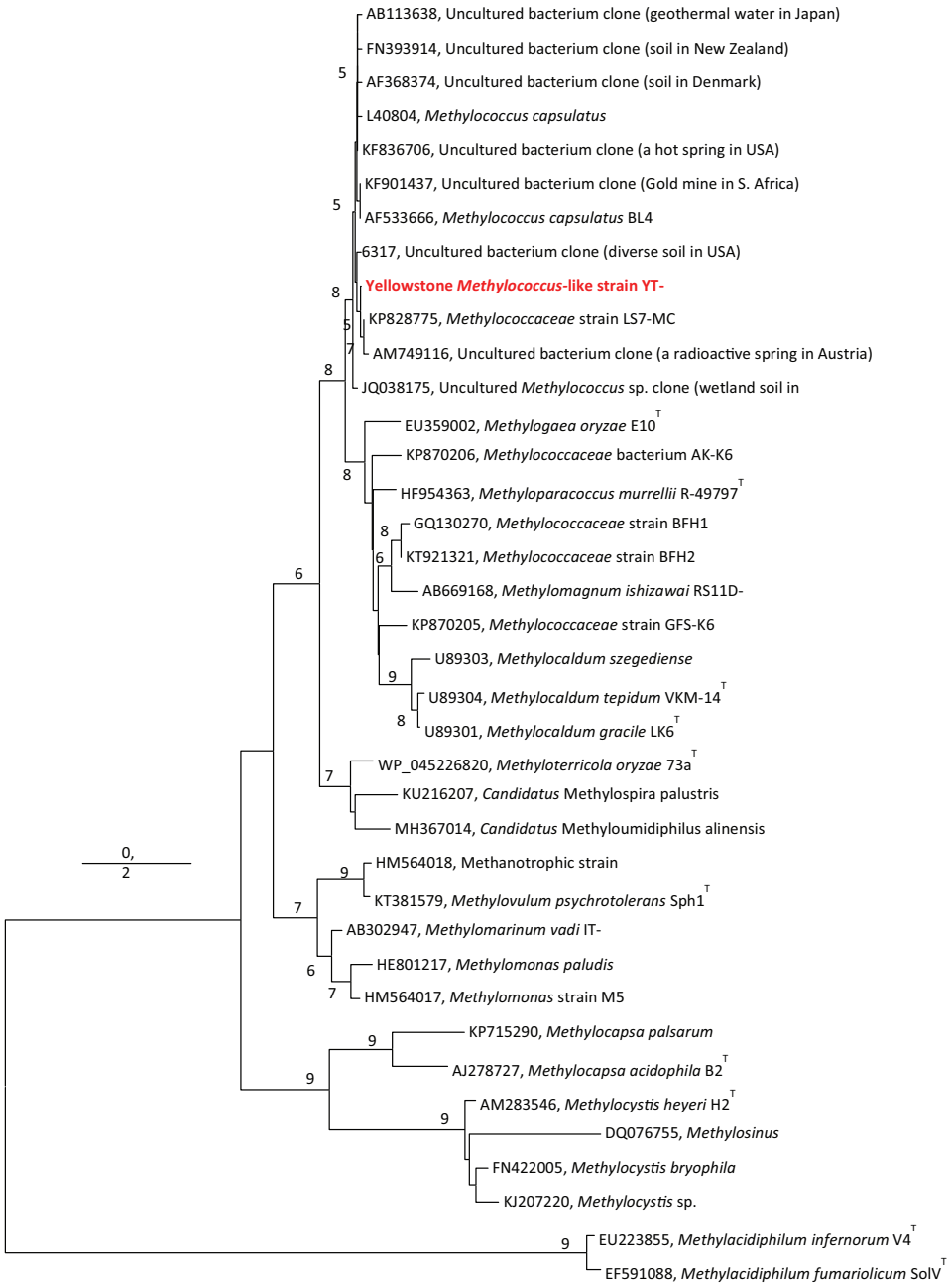

Fig. 2. *The phylogenetic tree (using the Neighbor-Joining method) that is based on derived deduced 167 PmoA amino acid sequences showing the position of strain YT-MC and other reported gammaproteobacterial methanotrophs[28]. Methylacidiphilum infernorum V4^T PmoA3 (EU223855) and, Methylacidiphilum fumariolicum SolV^T (EF591088), thermoacidophilic verrucomicrobial methanotrophs, were used as an outgroup.*

established employing Maximum-Likelihood (Fig. S3) and Minimum-Evolution (Fig. S4) phylogenetic trees, indicating that the PmoA-based trees yielded a steady placement within the family *Methylococcaceae* Type Ib methanotrophs.

The 16S rRNA gene sequences of the strain YT-VM from PCR-DGGE analysis was acquired from the 5th transfer of the methane enrichment cultures. As a result of Blastn search, it shared highest significant similarity (98.8 to 99.0%) to uncultured bacterial clones from hot springs in the Yellowstone National Park (AY882820, AY882710, EU419170, and EU419168). Furthermore, the most related extant strains of verrucomicrobial thermoacidophilic methanotrophs were *Mac. kamchatkense* Kam1 (97.5%), *Mac. fumariolicum* SolV^{T} (97.3%), and *Mac. infernorum* V4^{T} (96.1%). Additional analysis showed 82.2 to 83.8% sequence similarity to the genus *Methylacidimicrobium*. The 16S rRNA gene phylogeny (Fig. 3), however, of strain YT-VM constituted a close association with the genus *Methylacidiphilum,* which was validated by highly bootstrap values (Fig. S5). This may clarify also that the aerobic methane oxidation in the enrichment cultures at pH 3 and 55 ^{o}C is mainly operated by two different MOB (i.e., a proteobacterial and a verrucomicrobial methane oxidizers).

The 16S rRNA gene sequences (1369 bp) of the heterotophic strain YT-AC were achieved by PCR, and Blastn search showed the highest similarity to clones of uncultured bacteria from geothermal acidic soils of New Zealand[32] and thermal spring sites of Yellowstone National Park (AF391980, AY882682, AY882795, AY882696, AY882695 AM749740, AY882793, AY882680, DQ834209). Moreover, pairwise sequence analysis of 16S rRNA gene of strain YT-AC with the closest reported relative exhibited 98.0% sequence similarity to moderately thermoacidophile bacterium *Acidicaldus organivorans* strain Y008^{T} (NR_042752; Table S6) from acidic hot spring (pH 2.7-3.7)[33]. The 16S rRNA phylogenetic trees for YT-AC, the Neighbor-Joining tree (Fig. 4) and the Maximum-Likelihood tree (Fig. S6) show the same topologies, suggesting that this bacterium may represent a novel thermoacidophilic species of the class *Alphaproteobacteria*.

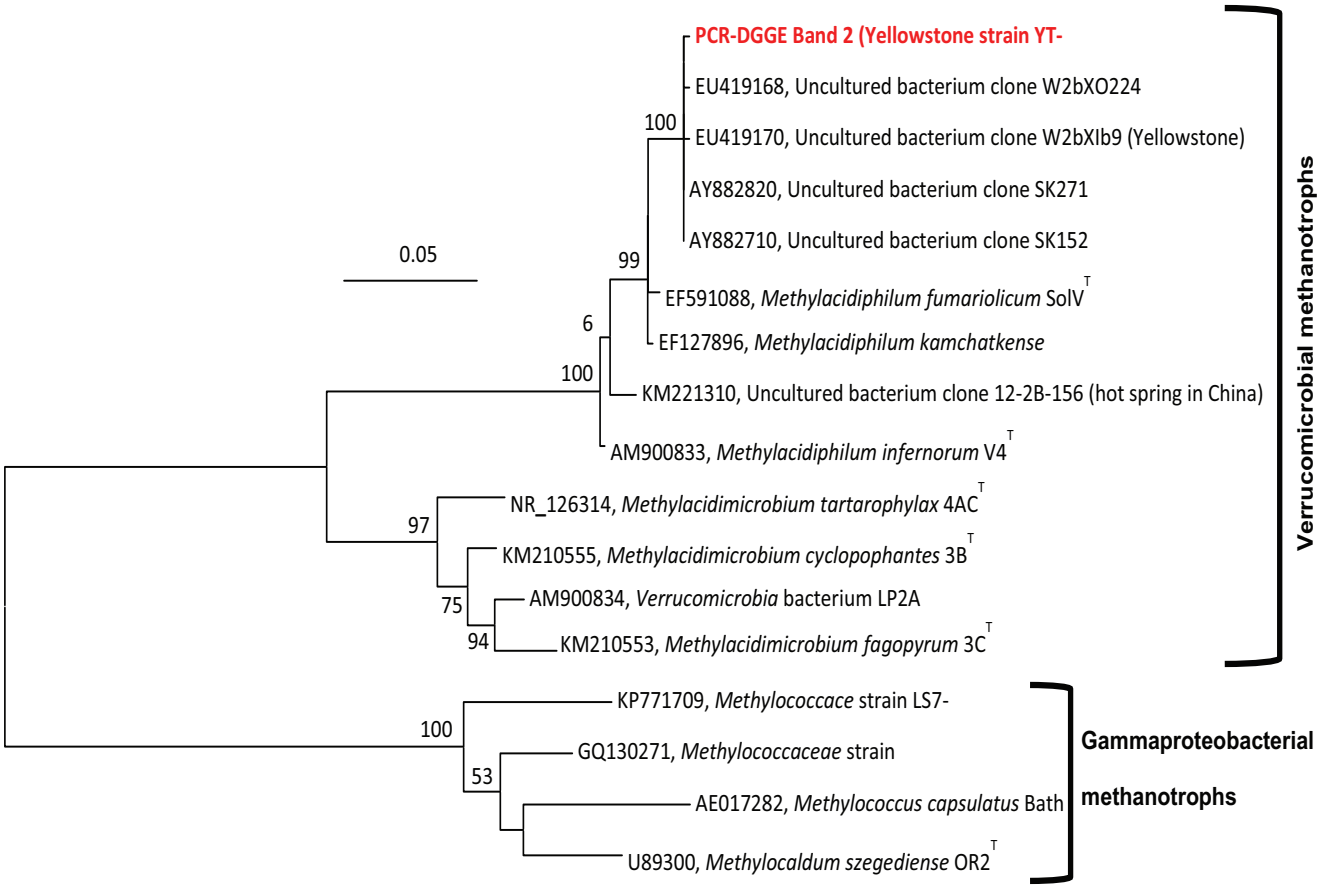

Fig. 3. *A 16S rRNA gene phylogeny (486 nucleotides; Neighbor-Joining method) of the detected strain YT-VM. The phylogenetic tree was inferred using the Neighbor-Joining method and the evolutionary distances were computed using the Kimura 2-parameter method[28].*

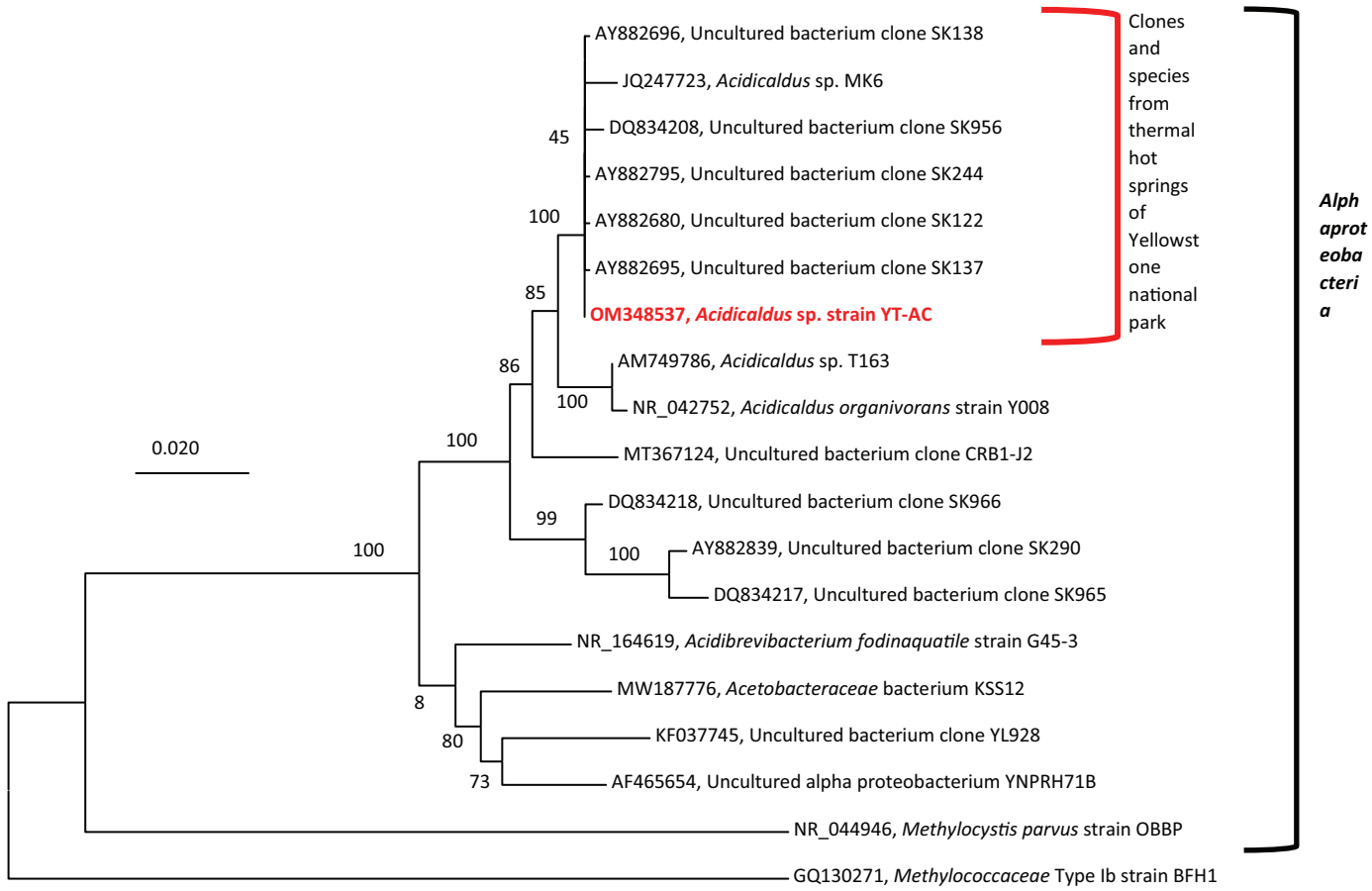

Figure 4: *A 16S rRNA phylogenetic tree (Neighbor-Joining method) of strain YT-AC. The evolutionary distances were computed using the Maximum Composite Likelihood method. The phylogenetic analysis was conducted on MEGA 7[28]. Methanotrophic bacteria of acid-tolerant moderately thermophilic Methylococcaceae Type Ib strain BFH1 (Gammaproteobacteria) and mesophilic Methylocystis parvus strain OBBP were included in the tree as an outgroup.*

Discussion

This study was to identify the *pmoA* gene in the isolates of Yellowstone National Park hot springs, demonstrating the existence of the thermoacidophilic proteobacterial *Methylococcaceae* Type Ib methanotrophs in an acidic thermal environment. This survey was mainly carried out in three phases. The first phase was methanotrophic cultivation on the suitable media (LMA and M3), continued further isolation process and the final phase for initial verification of thermoacidophilic *Methylococcus*-like cells by molecular detection using a functional marker gene like *pmoA*, Southern blotting analysis, phase-contrast and electron microscopy. Methanotrophic bacterial enrichments were established by inoculating water sediment slurry from an acidic thermal spring in the Yellowstone National Park. The methane oxidation capability by two isolates in the enrichments at pH 3 and at temperature 55 ºC were demonstrated, and a thermoacidophilic proteobacterial methanotroph as well as a verrucomicrobial methanotroph strain YT-VM of the genus *Methylacidiphilum* were prosperously detected. From the further isolation process, an alphaproteobacterial moderately thermophilic heterotroph was also recovered.

The gene *pmoA* was functionally applied to identify proteobacterial methanotrophs in various ecosystems. Detection of this gene in the enrichment cultures, which indicates presence of thermoacidophilic methanotrophs, maintaining the key methane oxidation process, and implying that proteobacterial Type Ib methanotrophs are more prevalent in acidic thermal habitats than previously anticipated. The absence of gene *mmoX*, suggested that cells in the enrichment cultures do not contain genes encoding the soluble methane monooxygenase. This gene is identified in the neutrophilic thermotolerant and mesophilic Type Ib (*Mcc. capsulatus*, *Methylocaldum marinum*[34] *and Methylomagnum ishizawai*[35]) and acidophilic alphaproteobacterial methanotrophs (the genus *Methylocella* and some species of the genus *Methylocystis*), but not in acid-tolerant moderately thermophilic bacteria[14] (*Methylococcaceae* Type Ib strains BFH1 and BFH2). Lacking sMMO in the subsequent enrichment cultures was also ensured by Southern blotting technique. This observation was also seen in the '*Methylothermus'* strain HB[36], *Methylocaldum* spp.[37], *Methylococcaceae* Type Ib strains (LS7-MC, BFH1, BFH2) and the verrucomicrobial methanotroph (*Mac. Kamchatkense* Kam 1). Moreover, these functional primers set do not amplify

verrucomicrobial methanotrophic DNA at annealing temperature of 55ºC. For the time being, none of moderately thermophilic methane oxidizers have been reported to exhibit both pMMO and sMMO enzymes system, most conceivably indicating that methanotrophic cytoplasmic sMMO does not exist in methanotrophic cells living above 55ºC.

Recovering of moderately thermoacidophile gamma-proteobacterial methane-oxidizing bacteria from low acidic thermal springs (pH 2 to 4) is still a demanding process. Not much is known regarding the identity, distribution, and community structure of these bacteria from such habitats. In particular, verrucomicrobial methanotrophs are highly acidophilic that depend on the presence of lanthanides (a group of rare earth elements, REEs) for optimal growth. Lanthanides act as indispensible cofactors for providing superior catalytic properties to pyrroloquinoline quinone (PQQ)-dependent methanol dehydrogenase (i.e. XoxF-type MDH)[38]. Therefore, it might have lacked certain essential elements in LMA, which did not afford to promote growth of strain YT-MC during the isolating procedures. Two isolated genera of aerobic methane oxidizers of the class *Gammaproteobacteria*, strain LS7-MC (the first moderately thermophilic Type Ib methanotroph, retrieved from an alkaline thermal spring sample from the Ethiopian Rift Valley)[17] and *Methylothermus"* strain HB (recovered from an underground hot spring arising from a natural gas field near Szentes, Hungary; the maximum recorded temperature range for growth at 40"72 °C, with an optimal temperature of 62–65 °C)[36] were described. Regretfully, both strains are no longer in existence.

Conclusion

In the enrichment cultures from Yellowstone National Park, we have detected an obligate moderately thermoacidophilic methanotroph belonging, most probably, to the family *Methylococcaceae* Type Ib of the phylum *Proteobacteria*. This bacterium appears to be *Methylococcus*-like methanotroph, which could only oxidize methane at pH 3 and at temperature 55ºC by using particulate methane monooxygenase (pMMO) enzyme system instead of soluble methane monooxygenase (sMMO). The isolate indicates also a previously unperceived biological methane sink, thrive to acidic terrestrial environments and methane oxidation diversity. Furthermore, detection of this isolate will expand our knowledge of acidophilic proteobacterial methanotroph ecology and its contribution to global carbon and nitrogen cycles. It is important to obtain a pure culture of this bacterium for further studies (i.e., genomic and biochemistry approaches), and can possibly a notable candidate for feasible biotechnological demands.

Will be included in separate section

Supplementary Materials : Table 1. Reported acid-tolerant and acidophilic aerobic methanotrophs within the phylum *Proteobacteria*. Table 2: PCR primers of this present study. Table 3. Results of Southern blot analysis of radioactively labeled *pmoA* and *mmoX* probes. Table 4. Pairwise sequence alignment analysis of *pmoA* gene and partial derived PmoA amino acid sequences. Table 5. Pairwise sequence alignment analysis of 16S rRNA gene sequences of the strain YT-VM and other related cultured and uncultured bacteria Table 6. Pairwise sequence alignment analysis of 16S rRNA gene sequences of *Acidicaldus* strain YT-AC and other related cultured and uncultured bacteria. Figure 1. Morphology of the methanotrophic enrichments (5^{th} enrichment cultures). Figure 2. PCR-DGGE of 16S rRNA gene analysis (5^{th} enrichment cultures). Figure 3. Phylogenetic tree of PmoA protein sequences (the strain YT-MC and other related acidophilic/acid-tolerant gammaproteobacterial methanotrophs) using the Maximum Likelihood method. Figure 4. Phylogenetic tree of PmoA protein sequences (the strain YT-MC and and other related acidophilic/acid-tolerant gammaproteobacterial methanotrophs) using the method of Minimum-Evolution. Figure 5. A 16S rRNA gene phylogeny (486 nucleotides; the Maximum Likelihood method) of the strain YT-VM. Figure 6. A 16S rRNA gene phylogeny (1369 nucleotides; Maximum Likelihood method) of the strain YT-AC.

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