Bangladesh J Microbiol, Volume 38, Number 2, December 2021, pp 63-71

Original Article

Two Novel Thermotolerant Methane Oxidizers from a Tropical Natural Gas Field in Bangladesh

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Aerobic thermotolerant methane oxidizers utilize methane as a sole carbon and energy source, and predominantly they are associated with the phylum *Proteobacteria*. Here we present two further new strains (HGS-45: coccus-shaped and HGF-47: rod-shaped and vibrioid) of thermotolerant obligate proteobacterial methanotrophic bacteria, which were isolated from an abandoned tropical natural gas field wet soil sample in the northeast of Bangladesh. Strains are Gram-negative, nonmotile, and capable of growth on methane and methanol as their energy sources. Isolates are thermotolerant and could grow up to 52ºC, optimally at 42ºC, but show no growth at 55 or 15ºC. Based on 16S rRNA gene sequence analyses and phylogenetically, HGS-45 is most closely related to the obligate Type Ib methanotroph *Methylococcus capsulatus* of the family *Methylococcaceae*, whereas HGF-47 is affiliated to Type IIa methanotroph *Methylocystis* sp. of the family *Methylocystaceae* and possesses Type II intracytoplasmic membrane (ICM) systems. Genes of particulate methane monooxygenase (pMMO) and the methanol dehydrogenase (MDH) were detected by PCR. Southern-blot analyses of genomic DNA from both strains were positive, implying the aerobic biological oxidation process from methane to methanol by the pMMO. Each strain presumably represents a novel species. Furthermore, both strains will increase our knowledge of thermotolerant methanotrophic proteobacterial diversity, cohabitation, and their participation to global carbon cycles as well as signifying biological methane sinks in the terrestrial natural gas field ecosystems.

Key Words: thermotolerant, proteobacteria, methanotrophic.

Introduction

Methane, which plays a key role in the global carbon cycle, is about 34 times more potent than CO_2 as a greenhouse gas and contributes a huge potential global climatic problem[1]. Abiogenic methane from Terrestrial geological sources (e.g. through seeps, rejected natural gas well-fields, gas venting or degassing of spring water) promote considerably large amounts of this important climate gas to the atmosphere[2]. Several terrestrial gas fields are situated in the north-east of Bangladesh. Some of the gas fields were formed in blowouts during the drilling, and became rejected because of irregularly high gas pressure or different technical reasons[3]. However, geological methane is still continously flowing out from these abandoned gas fields. Gas leakages due to accidents in the oil and gas sector can release large amounts of methane within short periods of time[4]. Such fields in the terrestrial tropical area may enhance the activity and structure of microorganisms in addition to methane eating bugs, and may have concequences of Earth's methane budget. Despite the fact that methane oxidizing bacteria (MOB) or methanotrophs are an environmentally important group of bacteria due to their critical role in reduction of methane emissions to the atmosphere from different ecosystems. These microorganisms have been reported to be ubiquitous in nature and isolated from a wide variety of thermal and non-thermal habitats. This group can be distinguished from other microorganisms by their unique capability of utilizing methane as sole carbon and energy source. They contain a unique multicomponent enzyme system called MMO (methane monooxygenase) that is classified into the two distinct types as a pMMO (particulate) and a sMMO (soluble). Until the present, research on methanotrophs have particularly emphasized on low temperature environments, and more than 60 type strains as cultivated representatives of mesophilic, psychrophilic or psychrotolerant growth at neutral pH were validly reported[5,6]. Currently, thermotolerant methane oxidizers with optimal growth temperatures between 30 and 44º C (a temperature range of 20–50º C) have been reported in the phyla of *Proteobacteria* (seven species: *Methylomarinum vadi*, *Methylococcus capsulatus*, *Mcc. chroococcus*, *Methylocaldum gracile*, *Mca. tepidum*, *Mca. marinum*, and *Methylocystis* sp. strain Se48) and *Verrucomicrobia* (two species: *Methyloacidiphilum cyclopophantes* and *Mac. tartarophylax*)[6-14]. Moreover, key functional genes (*pmoA*, encoding the pMMO; *mmoX*: encoding the sMMO, and *mxaF*: encoding the methanol dehydrogenase, MDH) were commonly applied for detecting aerobic methanotrophs in the various habitats[15,16].

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Strains Texas and Bath of the genus *Mcc. capsulatus* (growth optimum T_{opt} :37 ºC and T_{max}: 50º C) are demonstrated to be thermotolerant Type Ib methanotrophs of the family *Methylococcaceae*, and were isolated from sewage sludge and geothermally heated water, respectively. Only the strain Bath is the most well-studied, so far, of all methanotrophs in regard to physiology, molecular genetics and ecology[17]. Alphaproteobacterial methanotrophs are mostly mesophilic or psychrotolerant and mainly isolated from low-temperature ecosystems[18]. Untill now, only a single thermotolerant methanotrophic strain Se48, in the family *Methylocystaceae* was reported, and was recovered through cutivation efforts using a mud sample from a neutral thermal spring in the Transbaikal territory (Russia). This strain is a new species of the genus *Methylocystis* and is able to grow at temperature ranging from 15–53º C with optimum growth at 37º C[13]. Moreover, slightly acidophilic gammaproteobacterial methanotrophs (strains BFH1 and BFH2) were also reported from tropical topsoil (pH 5) by leakage of natural methane from a gas field area at Horipur in Bangladesh, but both strains are moderately thermophilic rather than thermotolerant methane oxidizers. They grow optimally at 51 to 55º C, and 16S rRNA gene phylogeny confirmed them in a well-separated group making a cluster together with the genus *Methylocaldum* as the closest relatives[19]. Previously, the presence of thermotolerant methane oxidizers was not reported from terrestrial gas well-field soils. In the view of the fact that we attemped to recover extant methane consuming thermotolerant bacteria. In this present study, we demonstrate the isolation and primary description of two novel strains retrieved from an abandoned tropical gas field wet soil samples through cultivation efforts. They are obligately thermotolerant aerobic methane oxidizers and belonging to the phylum *Proteobacteria*.

Materials and Methods

Sampling, Isolation and Cultivation Conditions

A soil sample (approximately 40 grams) was collected (20 cm below the ground surface) using a sterile Falcon tube from a rejected natural gas field in Sylhet, Bangladesh in July 2009. This exploded gas field (Sylhet-2) in Horipur is situated in a small village (at the side of Shikarkhan-Ultarpar road; 24°58’55.0"N 92°01’56.4"E, Figure S1) in the subdivisionJaintapur. The gas field was affected by blowouts during the drilling in 1962 and abandoned[3]. The pH and temperature of wet soil were measured by using a pH meter (MP220, Mettler Toledo) and a digital temperature sensor (Digitron, 2000T, Sifam Instruments, UK), respectively. On the day of sampling, the soil was frozen (–20º C). To prevent as much growth of mesophilic methanotrophs in the enrichment, we utilized 45º C as the incubation temperature. For producing the primary enrichment of thermotolerant methanotrophic populations, 3 grams of wet soil was inoculated directly into 20 mL of a low-salt mineral medium (LMM and pH 6.8) in a 120 mL serum vial[20]. The sterile serum vial was locked with a butyl rubber cap with an aluminium crimped seal and incubated under a mixture of methane-air (80-20%) atmosphere in the headspace (purity of methane, 99.5%, Yara Praxair, Oslo, Norway) at 45º C for three weeks without shaking. The gas mixture was replaced every 7 days. The primary enrichments became visibly turbid and were verified by phase-contrast microscopy (Eclipse E400 microscope, Nikon Corporation, Tokyo, Japan). Afterwards, 2 mL from the enrichment was transferred to fresh 20 mL LMM and incubated at the same growth conditions. After the 5th passages of subculturing at same culture conditions, cell suspensions were serially diluted and plated onto LMM containing gelrite solidified plates (20g L^{-1}, GelZan^{TM} CM, Sigma-Aldrich). The plates were incubated in a glass jar containing about 80% methane and 20% air at 45º C. After three weeks of incubation, colonies were picked and streaked onto new plates. Selected colonies were also examined by a phase-contrast microscope. Finally, picked colonies were used to make dilution series up to the order of 10^{-8} on to the microtiter plates. Both LMM and LMA were also used for regular cultivations with methane-air in the head space at 45º C[19]. Purity of both strains was evaluated by observation of a single colony growth on gelrite plates atmosphere without methane, on R2A agar plates[21], and repeated 16S rRNA gene sequencing analysis as well as using phase-contrast microscopy and electron microscopy.

Growth Conditions, Acetylene Inhibition Test and Naphthalene test

Various organic compounds, as a carbon source, were tested in liquid LMM supplemented with sterile multicarbon substrates (ethanol, acetate, pyruvate, succinate, methylamine, glucose, fructose, and yeast extract) at a concentration of 10 mM. Growth on methanol and formate was tested using LMM containing substrate concentrations of 0.1, 0.2, 0.25, and 0.5% (v/v). The tests of antibiotic susceptibility were evaluated a 10-day period incubation (10 µg mL^{-1}: ampicillin, tetracycline, streptomycin, erythromycin; 30 µg mL^{-1}: kanamycin and nalidixic acid). In addition, growth was also examined by replacing NH_4Cl and KNO_3 in both LMM and LMA in triplicate with N_2 from the air (20% air in the headspace) and methane. The generation time and the optical density (600 nm) were assessed. To verify the acetylene inhibition test, strains *M. capsulatus* Bath, *M. kamchatkense* Kam1 and *Methylococcace*ae Type Ib methanotroph strain BFH1 were used as positive controls under the same assay conditions[22,23]. 4% (v/v) acetylene was added in the headspace of three replicate bottles after incubation with methane and LMM. One bottle was used without adding acetylene as a control. The naphthalene-oxidation assay was performed for the verification of sMMO in LMM without copper[24]. *M. capsulatus* Bath and strain BFH1 were employed as positive control and as negative control of the assay, respectively[18]. The morphology of cells was determined using phase-contrast microscopy.

Electron Microscopy, Fatty Acid Analysis and G+C Content Measurement

Exponentially grown cells (only HGF-47) were used to prepare ultrathin sections as described previously[22]. Fatty acid analysis was completed by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). Strains HGS-45 and HGF-47 were grown under optimal conditions and shipped to DSMZ, where the samples were processed further. The DNA base composition (G+C content, mol %) of the strain HGF-47 was determined by DSMZ.

PCR amplification and Southern blot hybridization of functional genes

Genomic DNA from both strains was extracted using GenElute Bacterial Genomic DNA kit (Sigma). The 16S rRNA genes, using the primer set 27f and 1492r, were amplified as described previously using a Veriti 96 well thermal cycler (Applied Biosystems; Table S1)[25]. The PCR products were purified and sequenced using the Big dye kit for automated DNA sequencers (ABI 3700 PE; Applied Biosystems). The genes encoding particulate methane monooxygenase (*pmoA)*, soluble methane monooxygenase (*mmoX*), and methanol dehydrogenease (*mxaF*) were also PCR amplified from genomic DNA using primers listed in PCR conditions, which was described previously[19] (Supplementary Table S1). Nucleotide (nt) sequences of the 16S rRNA gene were compared with available sequences in the GenBank database using the NCBI tools (Blastn). For further confirming of pMMO and sMMO, the Southern blotting hybridization technique was utilized. Genomic DNA from strains HGS-45 and HGF-47, *Methylococcaceae* Type Ib strain LS7-MC[26], *Methylococcaceae* Type Ib strain BFH1[19], *M. kamchatkense* Kam1[22] (as a negative control) and *M. capsulatus* Bath (as a positive control) was extracted (Supplementary Table S2). Then, DNA was digested with EcoRI and HindIII. Hybridization probes and the further process were followed as previously described.

Phylogenetic Analyses

16S rRNA gene (HGS-45: 1433 nt and HGF-47: 1385 nt) sequences were aligned using CLUSTAL W algorithm as implemented in the MEGA7 software package[27]. To make sure the phylogenetic affiliation, trees of the 16S rRNA gene and sequences were constructed using the Maximum-Likelihood (ML), Minimum-Evolution (ME) and Neighbor-Joining (NJ). The evolutionary distances were computed using the methods like the Jukes-Cantor method, Kimura 2-parameter and Maximum Composite Likelihood, which are also implemented in the MEGA7 software. The trees topology was decided by 1000 bootstrap replications. Nucleotide sequences determined in this study were deposited in the GenBank database. The accession numbers are as follows: for strain HGS-45: MZ267055 (16S rRNA) and for strain HGF-47: MZ267054 (16S rRNA).

Results and discussion

The pH and *in situ* temperature of the sample were 6.96 and 38.3° C, respectively. Inoculation at 45° C with wet soil and methane, after three weeks, microbial growth in the primary enrichments was observed (a density of about 10^8 cells mL^{-1}), and cells were confirmed by a phase-contrast microscope. In the primary and secondary enrichments, long rod-shaped cells with endospore (about 4 to 6 ìm) were observed, but they were not seen in the tertiary enrichments anymore. Two different colours of colonies (white: about 1 – 1.2 mm in diameter and light yellow and shiny: 1.3 – 1.5 in diameter) appeared on the gelrite plates. From growth of cells (selected colonies) with methane on the microtiter plates of dilution 10^{-8}, we were able to isolate two different strains, termed as HGS-45 and HGF-47, from a natural gas well-field wet soil. Strains did grow robust on methanol (0.2%) but no growth was seen with any multicarbon substrates, thus, demonstrating that strains are obligate aerobic methylotrophic bacteria and grow only on methane and methanol as the energy and carbon source. Heterotrophic contaminants were not observed on these media or gelrite plates. No growth was found without methane or methanol under aerobic conditions, and with methane or methanol under anaerobic conditions. Vitamins were not imperative for growth. They showed optimal growth on both LMM and LMA, and these media are generally 10 times diluted than NMS and AMS[28]. Strains could grow up to 52ºC, optimally between 40 and 42ºC, but did not grow at 55 or 15º C. The pH range of HGS-45 and HGF-47 was 6.0"8.5 (optimum 7.0) and 6.0"8.8 (optimum 6.7"7.3), respectively. The specific growth rates (ì) of HGS-45 and HGF-47 were determined to be 0.087 and 0.063, respectively. Growth did not occur by testing antibiotics. Cells utilized nitrate, ammonium or atmospheric N_2 as a nitrogen source. We observed that cells grew better with LMM containing KNO_3 rather than with LMA containing NH_4Cl. Acetylene acts an inhibitor that is generally used for completely preventing growth of methanotrophs[26,29]. Growth of both strains was impeded after adding 4% acetylene in the headspace. This observation can be compared to other aerobic methane oxidizers (both proteobacterial and verrucomicrobial methanotrophs), which indicates the presence of functional methane monooxygenase (MMO) enzyme systems[22,26].

Physiological properties of strains, compared to related thermotolerant proteobacterial methanotrophs, are presented in Table 1. Cells were Gram-negative, nonmotile, divided by binary division. Strain HGS-45 is coccus-shaped (Figure 1a), but strain HGF-47 is vibrioid-shaped and occurred individually. No aggregations of strain HGS-45 were seen, but some aggregations were observed for the strain HGF-47. TEM analysis of HGF-47 showed Gram-negative cell wall, and the presence of the Type II intracytoplasmic membrane (ICM) system appearing as paired of membranes aligned to the periphery (Figure 1c), which is observed in Type IIa methanotrophs (genus: *Methylocystis*) of

the family *Methylocystaceae*[30,31] (*Mcy. hyeri, Mcy. rosea*). Type II ICMs in *Methylocystis* species are responsible for methane oxidation. A large storage granule (most possibly poly-â-hydroxybutyrate), in most cells of strain HGF-47, has been observed in the analyses of TEM and scanning micrographs (Figure 1c, d).

Fatty acids profile comparison of both strains and other related thermotolerant species are presented in Table 2. The predominant fatty acids of strain HGS-45 were C16:1*ù7c* (30.82%) and C16:0 (49.34%) that accounting for 80% of the total amount of fatty acids. C16:0 is normally found in the genera *Methylococcus, Methylocaldum,* and *Methylococcaceae* strain BFH1 (thermotolerant and thermophilic: about 33-63%) of Type Ib methanotorphs of the family *Methylococcaceae*. Although C16:1*ù7c* is lacking in *Methylocaldum* spp., but 10-23% and 14% of it were found in *Methylococcus* sp. and strain BFH1, respectively[11,18]. About 31% of C16:1ω7*c* has not been previously reported in any described thermotolerant methanotrophs of Type Ib, whereas *Methylococcaceae* strain LS7-MC (thermophilic), *Methyloparacoccus murrellii* and *Methylomagnum ishizawai* contain more than 41% of it, and they were defined as moderately thermophilic and mesophilic methanotrophs, respectively[26,32,33]. The PLFA profiles of strain HGF-47 deviated significantly from mesophilic type IIa methanorophic species of the genus *Methylocystis* (Table 2). The essential components of strain HGF-47 were C18:1ω*7c* and C18:1ω*9c* fatty acids. These were also found in a thermotolerant methanotrophic *Methylocystis* strain Se48, which was recovered from a thermal spring[12] (Tsyrenzhapova et al., 2007). In the genera *Methylocystis* and *Methylosinus* contain predominantly C18:1ω*7c* and C18:1ω*8c* fatty acids. But our strain and Se48 lack of C18:1*ù8c*. Although, this fatty acid is common and specific marker to the genera *Methylosinus* and *Methylocystis*[12,34].

PCR amplification reactions of functional genes provided positive results except the gene *mmoX* of strain HGF-47 (Supplementary Table S1). In the naphthalene assay, strain HGS-45 and HGF-47 displayed a positive and a negative colorimetric response, respectively. Furthermore, the analysis of Southern blotting from genomic DNA showed positive signals with the *pmoA* and *mmoX* probes of strain HGS-45, concurrently the *mmoX* probe did not yield any positive signals of strain HGF-47 (Supplementary Table S2). These results assure that strain HGS-45 contains the soluble form of MMO, but strain HGF-47 does not have sMMO activity. The present of soluble methane monooxygenase enzyme in the strain HGS-45 was also verified using PCR amplification (Supplementary Table S1), Southern hybridization (Supplementary Table S2), and the naphthalene assay, indicating the significant conformity with the thermotolerant genus *Methylococcus*. This soluble form of MMO is widely found either in thermotolerant[7] or mesophilic MOB[18,33,35], but not in moderately thermophilic methane oxidizers[19,26] or mesophilic Type Ib methanotrophs[20]. These findings assure that strain HGS-45 is, most possibly, not a new species or subspecies of the moderately thermophilic Type Ib strains LS7-MC[26] (*Methylococcus*-like) or BFH1[19] (*Methylocaldum*-like) or other genera of the families *Methylomonadaceae* Type Ia,

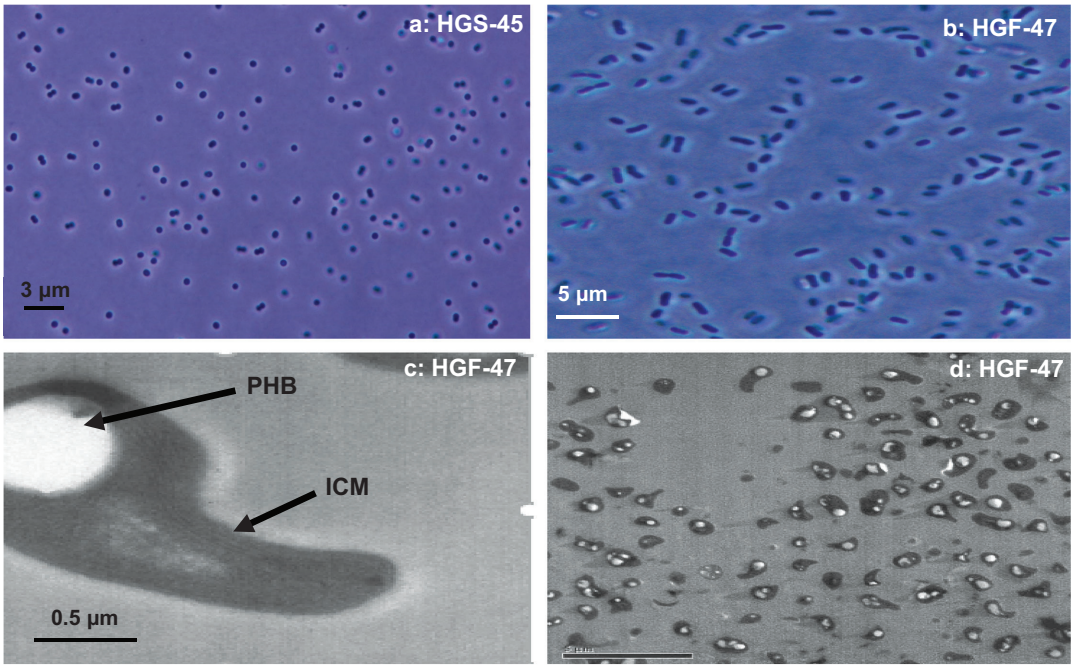

Fig. 1. *Cell morphology. (a) and (b) Phase-contrast micrographs of strains HGS-45 (a coccus and a diplococcus) and HGF-47 (rod and vibrioid) at 1000X magnification, respectively. Both strains were grown in LMM with methane for 5 days. (c) Transmission electron microscopy of ultra-thin sections of the strain HGF-47 and showing Type II intracytoplasmic membrane (ICM) systems and a poly-â-hydroxybutyrate intracellular granule (PHB). (d) A scanning electron micrograph (SEM) of the strain HGF-47. Scale bars: (a) 3 µm (b) 5 µm (c) 0,5 µm and (d) 5 µm.*

Methylococcaceae Type Ib and *Methylothermace* Type Ic within the order *Methylococcales*.

Using Blastn search of the 16S rRNA gene sequence, strain HGS-45 revealed a high sequence identity of 98.54 % to *Mcc. capsulatus* strain Texas (GenBank Accession No. NR_042183) and 98.47% to *Mcc. capsulatus* strain Bath complete genome (AE017282). The strain showed also 98.05 - 97.69% sequence identity to clones of uncultured bacteria from fractured water of a gold mine borehole in the USA (JX434221, JX434221, JX434214, JX434181, JX434258, and JX434207), naturally

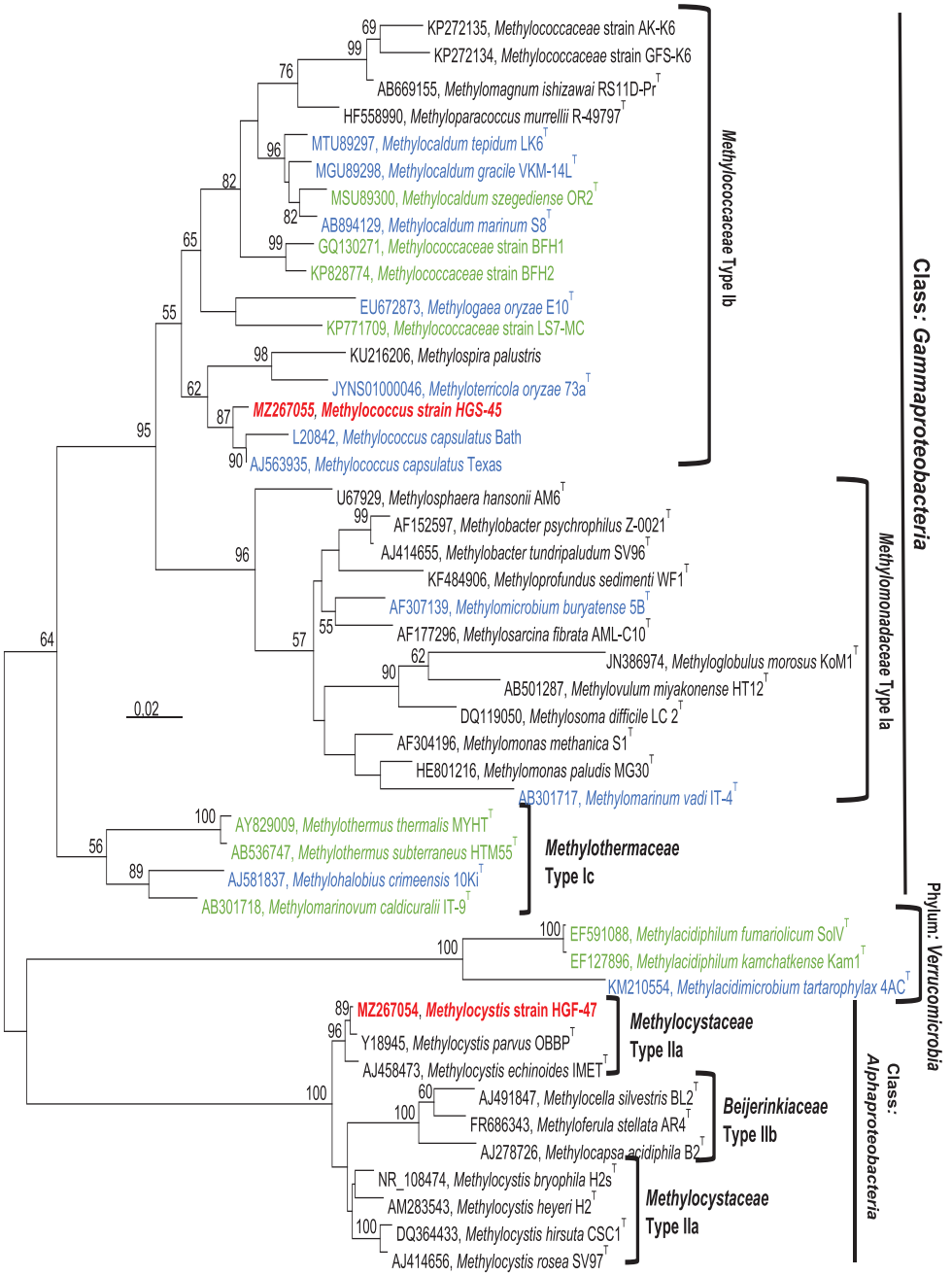

Fig. 2. *Maximum-Liklihood (ML) tree. A 16S rRNA gene-based phylogenetic tree of methanotrophic strains HGS-45 and HGF-47 (indicated in bold red) and representatives from the families Methylomonadaceae (Type Ia) and Methylococcaceae (Type Ib) of the class Gammaproteobacteria, and Methylocystaceae (Type IIa) of the class Alphaproteobacteria using MEGA7 software package. The tree is created with the Jukes-Cantor model and nodes supported by bootstrap values (percentages of 1000 data resamplings) e"50% are shown at each node. The scale bar denotes 0.02 changes per nucleotide position. The analysis involved 46 nucleotide sequences. There were a total of 1180 positions in the final dataset. Evolutionary analyses were accomplished in MEGA7[27]. Verrucomicrobial methanotrophs were used as an outgroup. Green: moderately thermophilic methanotrophs; blue: thermotolerant methanotrophs[6].*

composting sugarcane bagasse piles in Thiland[36], and fracture-derived groundwater in a deep gold mine of South Africa[37] (DQ088732). The high sequence identity of *Mcc. capsulatus* strains to HGS-45 suggests that they may have familiar physiology and metabolism features. Values of 16S rRNA gene sequence analysis by pairwise alignment of the strain HGS-45 and the cultured strains showed the most closest similarity with *Mcc. capsulatus* Texas (98.5%), *Methylococcaceae* strain BFH2 (93.7%), *Methyloterricola oryzae* 73a^T (93.5%), *Mcd. marinum* S8^T (93.4%), *Methylococcaceae* strain LS7-MC (93.2%) *Mcd. gracile* VKM-14L^T (92.9%), *Methylococcaceae* strain BFH1 (92.8%) *Mcd. szegediense* OR2^T (92.6%) (Supplementary Table S3). The phylogenetic analysis of 16S rRNA gene sequences using Neighbor-Joining tree revealed that strain HGS-45 was grouped in the genus *Methylococcus* of the family *Methylococcaceae* Type Ib (Figure 3). The same analysis situs was also established in the Minimum-Evolution (Supplementary Figure S2) and Neighbour-Joining (Supplementary Figure S3) trees, indicating that the16S rRNA phylogeny performed an uniform position between strain HGS-45 and other related Type Ib methane oxidizers of the famliy *Methylococcaceae.* Furthermore, it was also suggested that strain HGS-45 is not a member of other described methanotrophic genera in the families either *Methylomonadaceae* Type Ia or *Methylothermaceae* Type Ic of the class *Gammaproteobacteria*.

Strain HGF-47, analysis of the the 16S rRNA gene sequence using Blastn, showed also a relatively high sequence identity of 99% % to the *Methylocystis* species (AJ868421, AJ458508, JX505249, CP04433, MN511720, AF150805, NR_044946, NR_025544, HF558988, AF150790 and NR_025544)[30,38-40]. In addition, pairwise sequence alignment analysis of 16S rRNA gene sequences of strain HGF-47 revealed the most closest represented species like *Methylocystis parvus* OBBP^T (99.1%), *Methylocystis echinoides* IMET^T (98.7%), *Methylocystis hirsuta* CSC1^T (97.5%),, *Methylocystis rosea* SV97^T (97.3%), *Methylocystis heyeri* H2^T (97.1%), *Methylocystis bryophila* H2s^T (97.0%) (Supplimentary Table S3). This signifies that our strain possibly denotes a new species in the genus *Methylocystis* of the family *Methylocystaceae* IIa. The 16S rRNA gene trees of strain HGF-47 demonstrated the same topologies (Figure 2, S2 and S3), illustrating that this strain is not a member of other reported methanotrophic genera in families of *Beijerinkiaceae* (Phylum: *Alphaproteobacteria*) or *Methylococcaceae* Type Ib (Phylum: *Gammaproteobacteria*).

Phylogenetic analysis of the 16S rRNA gene of both strains, HGS-45 and HGF-47, uncovered that they are feasibly new species within the orders *Methylococcales* and Rhizobiales, respectively. Furthermore, this phylogenetic deduction was assisted by the physiological properties and cellular PLFA analyses (Table 1 and Table 2). The recovered isolates are thermotolerant and obligate methylotrophs, because they are capable of growing on either methane or methanol. Furhter both strains are not mesophilic or actual thermophilic, as they are not able to grow optimally at 30ºC or above 55ºC.

Table 1. *Comparison of the major characteristics of the strains HGS-45 and HGF-47 with other described aerobic Thermotolerant methanotrophs*

Characteristic	1	2	3	4	5	6	7	8
Cell morphology	Coccus	Coccus	Rod/pleomorphic	Rod/pleomorphic	Rods/coccus	Rods/coccus	Vibrioids	Rod/vibrioids
Temperature condition	Thermotolerant	Thermotolerant	Thermotolerant	Thermotolerant	Thermotolerant	Thermotolerant	Thermotolerant	Thermotolerant
Internal membranes	Type I	Type I	Type I	Type I	Type I	Type I	Type II	Type II
Poly-â-hydro xy-butyrate	“	“	“	“	“	“	+	+
Motility	“	“	+	+	“	“	“	“
Pigmentation	White	Light yellow	Light brown	Light brown	Brown	Brown	white	Light yellow
pMMO	+	+	+	+	+	+	+	+
sMMO	+	+	“	“	+	+	“	“
Growth on N-free medium	+	+	+	+	+	+	ND	+
Temperature range (opt.)	20-52 (40-42)	20-50 (37-42)	30-47 (42)	30-47 (42)	20-47 (36)	20-44 (37-43)	15-53 (37)	18-52 (40-42)
pH growth range (optimal)	6.0-8.5 (7.0)	5.5-7.5 (6.5)	5.5-7.5 (6.5)	5.5-7.5 (6.5)	6.0-8.0 (7.0)	4.5-8.1 (6.2-7.0)	5.0-7.5 (5.5-6.8)	6.0-8.8 (6.5"7.3)
Growth on methanol	+	+	“	“	+	+	+	+
G+C content (mol%)	nd	62.5	57.2	57.2	59.7	59.7	62	64.9
G+C content (mol%)[a]	59.4	60.3	56.7	56.7	58	59.7	nd	nd
Habitats	Gas field wet soil	Soil and thermal water	Soil and freshwater	Soil	Marine sediments	Marine sediments	Thermal spring mud	Gas field wet soil

Strains: *1*, This study (strain HGS-45); *2*, *Methylococcus capsulatus* (Bath)[8,12]; *3*, *Methylocaldum gracile* (VKM-14L^T)[11]; *4*, *Methylocaldum tepidum* (strain LK6^T)[11]; *5*, *Methylocaldum marinum* (S8^T)[7]; *6*, *Methylomarinum vadi* (IT-4^T)[10]; *7*, *Methylocystis* sp. (Se48)[13]; *8*, This study (strain HGF-47). +, positive results; –, negative results; nd, not determined. [a]16S rRNA, *pmoA*, and *mmoX* genes sequences were used for computing measurements of DNA G+C content (mol%).

Table 2. *Fatty acids comparison of the strains HGS-45, HGF-47 and other related aerobic thermotolerant methanotrophs.*

Fatty acids	1	2	3	4	5	6	7	8	9
C13:1	0.4								
C14:0	1.97	0.8-0.62							
C15:0	0.64	0-1.7							
C16:1ω*8c*						29.03			
C16:1ω7*c*	30.82	10.6-23.1				3.39		0.51	0.3-14.2
C16:1ω6*c*		3.9-12.3							
C16:1ω5*c*	9.22	3.2-9.0							
C16:1ω5t		1.8-6.0				2.75			
C16:0	49.34	33.5-56.0	43.4	nd	59.2	1.24		0.98	0.7-5.1
C16:1			45.9	nd	39.7				
9-o-Me-C16:0		0-14.0							
10-o-Me-C16:0						3.46			
C16:0 3OH	2.34								
C17:1ω*6c*		0-1.8							
C17:1ω*5c*	4.57								
11-o-Me-17:0		0-2.1							
C18:0	0.72							0.63	0-5.0
C18:1		0-6.5							
C18:1ω*9t*						14.67			
C18:1ω*8c*						31.98			52.9-73.6
C18:1ω7*c*		0-2.9				10.8	33	39.26	14.8-37.7
C18:1ω9*c*		0.6-1.8					60	58.45	
C19:0*cyc*								0.17	

Strains: *1*, This study (strain HGS-45); *2*, *Methylococcus capsulatus*[12]; *3*, *Methylocaldum gracile* (VKM-14L^T)[11]; *4*, *Methylocaldum tepidum* (LK6^T)[11]; *5*, *Methylocaldum marinum* (S8^T)[7]; *6*, *Methylocystis heyeri* (H2^T)[30]; *7*, *Methylocystis* sp. (Se48)[13]; *8*, This study (strain HGF-47); 9, *Methylosinus* and *Methylocystis*[11]. Values are given as a percentage of total fatty acids. Bold values show significant fatty acids. nd, not determined.

Conclusion

Mesophilic, psychrophilic and some moderately thermophilic aerobic methane oxidizing bacteria were validly described or reported. There are only a very few reported thermotolerant methane consuming bacteria with optimal growth temperature of 42 °C in the phyla *Proteobacteria* and *Verrucomicrobia*[5] (Figure 2). In this study, two more novel thermotolerant methanotrophic bacteria, strains HGS-45 and HGF-47 were recuperated from an abandoned tropical natural gas field wet soil samples in the north-east of Bangladesh. Both novel isolates show an obligate aerobic methylotrophic growth with methane and methanol as the sole carbon and energy sources and encompass the particulate methane monooxygenase (pMMO) in the methane oxidation process. In addtion, the strain HGS-45 also processes the soluble methane monooxygenase (sMMO) and methanol dehydrogenase (MDH). In consistent with the anyalysis of 16S rRNA gene and the detection of *pmoA* and *mxaF* together with biochemical tests, the strain HGS-45 is probably a new species in the genus *Methylococcus* of the family *Methylococcaceae* Type Ib methanotroph, whereas HGF-47 might also be a new species in the genus *Methylocystis* of the family *Methylocystaceae* Type IIa methanotroph. Both strains indicate a significant role in the previously unrecognized biological methane sink, proteobacterial thermotolerant diversity, and on the adaptation of this process to natural gas field environments. Moreover, this study may increase our perception about methane consuming bacteria in the terrestrial natural gas field territories, and future complete genome sequencing of the species may contribute insights into their adaptation, and ecophysiological significance as well as for feasible biotechnological application.

Supplementary Materials: Table S1: Primers for amplification of functional genes. Table S2: Results of Southern blot evaluation of radioactively labeled *pmoA* and *mmoX* probes. Table S3: Pairwise sequence alignment analysis of 16S rRNA gene sequences (https://www.ebi.ac.uk/Tools/psa/emboss_water/). Figure S1: A 16S rRNA gene phylogeny (Minimum Evolution). Figure S2: A 16S rRNA gene phylogeny (Maximum Likelihood).

Author Contributions: T. I. designed the experiments, T.I, M.H., N.T., M. A. H., H. M. R., and L. Ø. wrote, edited the manuscript and analysed the data. T. I. collected sample and performed the

isolation of strains and carried out morphological, physiological and phylogenetic analyses. L. Ø. contributed reagents/materials/ TEM/sequencing analysis/cultivation facilities. All authors read and approved the published version of the manuscript.

Acknowledgements:

The electron microscopy imaging was performed at the Molecular Imaging Center (MIC), Department of Biomedicine, University of Bergen. Norway.

Funding:

This research received no external funding.

Conflicts of Interest:

The authors declare that they have no conflict of interests.

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