Diversity and activity of methanotrophic related bacteria in subsurface sediments of the Krishna–Godavari Basin, India

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The present study assesses the distribution, diversity and activity of aerobic methanotrophic related bacteria (MRB) dwelling in Krishna–Godavari (KG) basin, India. The counts of MRB ranged from non-detectable (ND) to 8.6×10^4 CFU gram dry weight of the sediment, with maximum counts at 24.2 m below seafloor. Greater methanotrophic bacterial abundance was at the surface/subsurface sediments of the core at station MD161-8 than at the bottom sediments. Identification of these isolates by 16S rDNA sequence analysis showed their taxonomic affiliation to *Alcanivorax, Methylophaga, Marinobacter, Joostella, Methylobacterium, Desulfovibrio* species and other uncultured bacterial clones. The isolates grew under optimum pH of 8, temperature of 28°C and salinity of 35, and on different carbon sources like yeast extract, D-glucose, ethanol and methanol. The addition of nitrogen sources like amino acids and yeast extract improved methanotrophic activity by the isolates. These results add to our understanding of MRB and their activity in modulating the emission of methane from gas hydrate-rich ecosystems like the KG basin.

Keywords: Gas hydrates, methanotrophs, river basin, subsurface sediments.

BACTERIA are ubiquitous in the subsurface environments and are known to play an important role in different biogeochemical processes. The decomposition of organic matter by microbial activity in marine sediments leads to methane (CH_4) accumulation[1]. The accumulation of methane in the form of gas hydrates acts as a major source of 'biogenic' methane generated by microbes. Methane being one of the most potent greenhouse gases, accounts for approximately 15% of the greenhouse effects[2]. Among the bacterial groups, methanotrophs have the unique ability to grow on methane as their sole carbon and energy source through which they regulate the methane flux to the atmosphere[3].

Methanotrophs, the bacteria which oxidize methane, consume up to 80% of the CH_4 produced in the freshwater and marine environments[4]. These bacteria also act as natural filters[5] and play an important role in global carbon, oxygen and nitrogen cycling[4]. Methanotrophs are also known to be widespread in nature, and they mostly reside in soil and aquatic habitats at the interface between the reduced zones of the environment[6] in the marine sediments[7], where methane production and oxidation may take place.

These bacteria can be obligate or facultative methanotrophs[8]. Based on their physiology and phylogeny, methanotrophs are broadly classified into three major groups, namely α-, β- and γ-Proteobacteria[9]. Besides, depending on the pathways that they utilize for carbon assimilation, they are classified as type-I and type-II methanotrophs. The type-I methanotrophs utilize the ribulose monophosphate (RuMP) pathway while the type-II methanotrophs use the serine pathway. The oxidation of methane is also reported to occur in thermoacidophilic bacterium belonging to the phylum Verrucomicrobia[10]. Also, studies with methane hydrate-bearing deep marine sediments in a forearc basin indicated domains with bacterial clones representing Bacteroidetes, Planctomycetes, Actinobacteria, Proteobacteria and green non-sulphur groups[11]. Considering this large variation in microbial population structures within the hydrate-bearing sediments, the present study assesses the diversity of methanotrophic related bacteria (MRB) and/or methanotrophs in the subsurface sediments of the Krishna–Godavari (KG) basin in the Bay of Bengal, where rich deposits of gas hydrates are found. The work attempts to look into the methanotrophic group of organisms present in these sediments, their characteristics and identity. This would improve our understanding of the ecology of gas hydrate-rich ecosystems. It also explores the activity of MRB and/or methanotrophs in regulating the level of CH_4 gas emission to the atmosphere.

Materials and methods

Study area and sampling

The KG basin located in the middle of the eastern continental margin of India, covers an area of 50,000 sq. km.

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The study site MD161-8 in the KG basin is at 15°51.8624′N lat. and 81°50.0692′E long., on the crest of a mount where fault/fractures are associated with gas migration features such as acoustic chimneys, acoustic voids and acoustic turbid layer[12]. Pressure cores for the present study were collected using Calypso piston corer at a water depth of 1033 m during the cruise on-board *RV Marion Dufresne* (2007). The core was sub-sectioned on-board for ten depths between 0.2 and 24.2 m below sea floor (mbsf). The sub-samples were used for microbiological studies on the distribution, diversity and activity of aerobic methane oxidizing bacteria (MOB). The sediments of the core are characterized by a total organic carbon content between 1% and 2%, methane hydrates and authigenic carbonate layers. The site MD161-8 chosen for this study has one of the richest gas hydrate accumulations reported in the KG basin.

Total counts

The sediment dilution for total counts (TC) was prepared by suspending ~1 g of uniformly mixed sediment sample in 9 ml of autoclaved and 0.22 µm filtered sea water. After thorough mixing, 2 ml of the dilution was withdrawn and fixed with hexamine buffered formalin to a final concentration of 2%. TC was determined following the acridine orange direct count method[13].

Enumeration of methanotrophic related bacteria

The enumeration of MRB was carried out in nitrate–mineral–salts (NMS) medium[14]. The medium was prepared in 50% sea water and was solidified by the addition of 1.8% (w/v) agar. A 10^{-1} dilution was prepared by suspending ~1 g of uniformly mixed sediment subsample in 9 ml of sterile sea water. A 0.1 ml aliquot of the dilution was spread-plated on NMS agar plates and incubated in anaerobic bags purged with air : methane in the ratio of 30 : 70, and clamped to avoid gas leakage. The plates were incubated at 28 ± 2°C and observed for growth at 3–7 days interval over a period of 3–4 weeks. Control plates incubated without methane were maintained to detect colonies of non-methane oxidizing bacteria. The colonies that formed were then enumerated and expressed as colony forming units (CFU) after normalizing for gram dry weight of the sediment (g dw^{-1}).

Characterization of the bacterial isolates

Bacterial colonies representing different morphological types were chosen from 11 different depths (Table 1). The selected bacterial colonies formed on NMS agar plates were examined for purity by microscopic observations. The isolates were examined for Gram-stain reaction and tested for motility, catalase, oxidase and urease[15]. Besides, their potential to utilize different carbon and nitrogen substrates was also tested[16]. The different carbon sources tested at a final concentration of 0.1% were D-glucose, yeast extract, urea, ethanol, methanol and formamide in the presence of potassium nitrate as the sole source of nitrogen. The isolates were also tested for their ability to utilize different nitrogen sources like L-leucine, L-tryptophan and L-methionine in the presence of methane as the carbon source, wherein potassium nitrate was excluded from the NMS medium composition. All carbon and nitrogen sources were filter sterilized by passing through a 0.22 µm filter prior to their addition in the autoclaved NMS medium. Growth was confirmed for carbon source utilization by the presence of colonies in NMS agar medium and by comparing the results with the negative controls. Physiological tests to determine the growth of organisms at different pH (5, 7 and 8), temperature (4°C, 28°C and 40°C) and salinity (0, 18 and 35) were carried out in NMS agar medium. Besides, tolerance of these isolates to 0.01% sodium azide (NaN_3), 0.01% sodium dodecyl sulphate (SDS) and 0.001% malachite green[16] was assessed in NMS agar slants.

Molecular identification of bacterial isolates by 16S rDNA analysis

Extraction of DNA from bacterial isolates: Genomic DNA was extracted from bacterial cultures grown in NMS broth at 28° ± 2°C (ref. 17). Briefly, the steps involved the harvesting of bacterial biomass by centrifugation, washing of the pellet with phosphate buffered saline, lysing of the bacterial cells using SDS, purification using phenol–chloroform–isoamyl alcohol mixture (25 : 24 : 1) and precipitation of DNA using ice cold absolute ethanol. The precipitated DNA was harvested by centrifugation and then washed with 70% ethanol before suspending the pellet in TE buffer (pH 8). The concentration and purity of DNA were checked using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Amplification of DNA by polymerase chain reaction: The amplification and sequencing of 16S rRNA gene was carried out using universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (3′-TTCAGCATTGTTCCATYGGCAT-5′). Amplification of the 16S rRNA gene was carried out with PCR master mix (Genie, Bangalore) in a final reaction mixture of 50 µl consisting of 0.5 µM of each primer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 1U *Taq* DNA polymerase and 0.05–1 µg of DNA template. The amplification was done for 25 cycles in a thermal cycler (Eppendroff, Germany) with temperature profiles of 5 min at 95°C, 1 min at 95°C, 60 sec at 50°C, 2 min at 72°C and final extension for 10 min at

72°C (ref. 18). The PCR amplified products were analysed by electrophoresis in 1% agarose gel and stained with 10 μg ml^{-1} ethidium bromide for 15 min in the dark. The DNA bands were visualized under UV and the images were captured using the gel documentation and analysis system (Bio-Rad, USA).

DNA sequencing and phylogenetic analysis: The amplified PCR products were purified prior to sequencing according to the manufacturer's guidelines, using the GenElute^{TM} PCR Cleanup Kit (Sigma, USA). The purified PCR products were sequenced using the ABI 3130 genetic analyser (geneOmbio Technologies Pvt Ltd, India). The phylogenetic analysis of the sequences was carried out as described in the literature[19,20].

In vitro experiment on methane oxidation by bacterial isolates

The bacterial isolates designated GH-02 to GH-12 were grown in enriched NMS broth at 28 ± 2°C. When the cell density reached an OD_{600} of 0.8–1.0, the cells were harvested by centrifugation at 5009 *g* for 10 min at 4°C (Sigma 3-18K). Cell pellets were washed with autoclaved and 0.22 μm filtered 50% sea water and used for the inoculation of experimental tubes. The bacterial isolates were checked for their ability to oxidize methane in the NMS broth medium with and without potassium nitrate. The different nitrogen sources used in NMS broth in the absence of added potassium nitrate were L-tryptophan, L-methionine, L-leucine and yeast extract in final concentrations of 0.1%. The experiment was conducted in 20 ml gas-tight vials containing 10 ml of NMS broth and 0.1 ml of the respective culture as the inoculum. The vials were sealed with autoclaved butyl-rubber stoppers and aluminium crimp-caps. This was followed by flushing of 2 ml of methane in the headspace as the only carbon source. The vials were shaken during incubation at 28 ± 2°C to avoid diffusion-controlled gradients. Uninoculated sterile medium in triplicates was used as control. Methane utilization by isolates was ascertained by calculating the level of methane in the headspace[1,21,22] after correcting for the corresponding controls at the end of the 28 days incubation period. Any change in methane levels that occurred in the control during incubation was assumed to be due to the leakage of gas from the vials.

In vitro experiment on methane oxidation by indigenous microbial communities

The oxidation rate of methane by indigenous microbial communities associated with the subsurface sediments was conducted in triplicate with sediment slurries. The sediment slurry was prepared aseptically in 9 ml of NMS broth (10^{-1} dilution), by suspending ~1 g of uniformly mixed sediment in a 20 ml gas-tight vial. In the case of control, the sediments were heat-killed prior to their addition in 9 ml of NMS broth. The vials were sealed with butyl-rubber stoppers and aluminium crimp-caps. This was followed by flushing of 2 ml of methane in the headspace. The vials were incubated at 28 ± 2°C for 5 days. At the end of incubation, 1 ml of the headspace gas was sampled for GC analysis[1]. The amount of methane oxidized was calculated by subtracting the initial values of methane in the headspace from the final values obtained after incubation.

Results

Total bacteria and methanotrophic related bacteria in sediments

The abundance of total bacteria in the sediment subsamples ranged from 1.9 to 3.0 × 10^9 cells g dw^{-1} with maximum value at 0.2 mbsf. It showed a decreasing trend with fluctuating values between depths. On the other hand, the abundance of MRB in these sediments ranged from non-detectable to 3.4 × 10^5 CFU g dw^{-1}. Maximum abundance was recorded at 0.2, 1.2 and 24.2 mbsf (Figure 1).

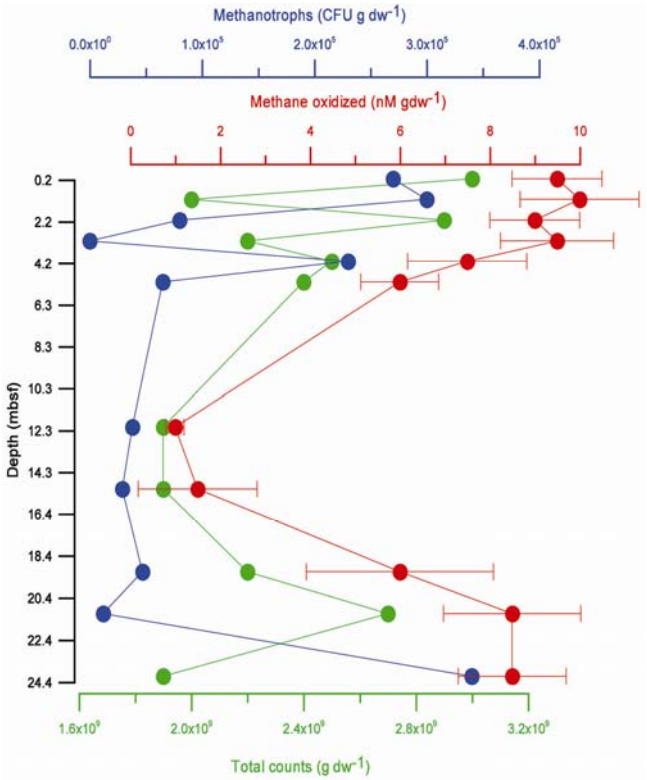

Figure 1. Depth profile of bacterial abundance, methanotrophs and methane oxidation at subsurface sediments of the Krishna–Godavari Basin. Each data point represents values normalized for gram dry weight of the sediment (g dw^{-1}). The error bars represent the average value of triplicates ± standard deviation.

Characteristics of the isolates

The bacterial isolates with the ability to oxidize methane were all Gram-negative rods, motile and positive for cytochrome oxidase and catalase enzymes. Out of the 11 isolates studied, 91% was positive for urease. The test for tolerance against NaN_3, SDS and malachite green indicated that all the isolates were sensitive to SDS and malachite green, with 27% of the isolates tolerant to NaN_3. Physiological response of the isolates to different pH, temperature and salinity showed growth for all isolates at pH 5, 7 and 8, with optimum growth at pH 8 (Table 1). Among the different temperatures of 4°C, 28°C and 40°C, growth was evident only at 28°C during a week of incubation. No growth was evident for any of the test isolates either at 4°C or at 40°C. Tests for tolerance to different salinity showed growth of isolates at salinity of 0, 18 and 35 with optimum growth at the latter. Growth on different carbon sources showed that yeast extract and methanol supported efficient growth of all isolates. With other carbon sources like glucose, ethanol and formamide, growth was evident with only 82%, 64% and 9% of isolates respectively. Use of amino acids l-tryptophan, L-leucine and L-methionine, and yeast extract as nitrogen sources in NMS medium supported growth of all the bacterial isolates (Table 1).

Phylogenetic identification of methanotrophic related bacterial species

Phylogenetic analysis of 11 bacterial isolates with the capacity to oxidize methane aerobically showed that they belonged to two major phyla: (i) Proteobacteria (73%) and (ii) Bacteroidetes (9%). Remaining 18% of the isolates belonged to the uncultured bacterial clones. Proteobacteria represented by α-, γ- and δ-Proteobacteria constituting 18%, 46% and 9% respectively. Of the total culturable fraction, the dominant genera were represented by *Methylobacterium* (18%), *Methylophaga* (18%), and an uncultured bacterial clone (18%) (Figure 2). The other genera represented by *Alcanivorax*, *Desulfovibrio*, *Marinobacter*, *Joostella*, and an uncultured bacterial clone constituted 45% of the total culturable fraction. Dominant genus among the phylum Bacteroidetes was represented by an uncultured bacterial clone and phylum Proteobacteria by *Methylobacterium* and *Methylophaga*. Isolates GH-02 and GH-03 resembled *Methylobacterium* sp. with sequence similarity of 92.42% and 85.28% respectively. On the other hand, isolates GH-04 and GH-05 resembled *Methylophaga* sp. with sequence similarity of 99.91% and 98.96% respectively. Isolates GH-06, GH-07, GH-10 and GH-12 formed clusters with *Marinobacter*, *Alcanivorax*, *Joostella* and *Desulfovibrio*, with maximum sequence similarity of 96.32%, 99.67%, 99.61% and 82.71% respectively. Whereas isolates GH-08, GH-09 and GH-11 showed 99%, 98% and 99% homology to the 16S rRNA gene sequences of the uncultivated bacterial clones respectively (Figure 2).

Methanotrophic activity (oxidation of methane) by bacterial isolates

The oxidation of methane by the bacterial isolates ranged from 0.026 ± 0.004 to 0.097 ± 0.003 µM in NMS broth, with potassium nitrate as the sole source of nitrogen after 28 days. The maximum oxidation of methane in the NMS broth was at 0.097 ± 0.003 µM with isolate GH-02. On the other hand, the oxidation of methane with different amino acids and yeast extract as nitrogen source was also observed with different bacterial strains. Maximum oxidation was by isolate GH-02 at 1.05 ± 0.105 µM with L-tryptophan. Least oxidation of methane was recorded with bacterial isolates in the presence of L-methionine. The oxidation of methane with added methionine ranged from 0.014 ± 0.001 to 0.056 ± 0.006 µM with isolate GH-07 showing maximum methane oxidation rate and isolate GH-09 showing minimum oxidation rate (Table 2).

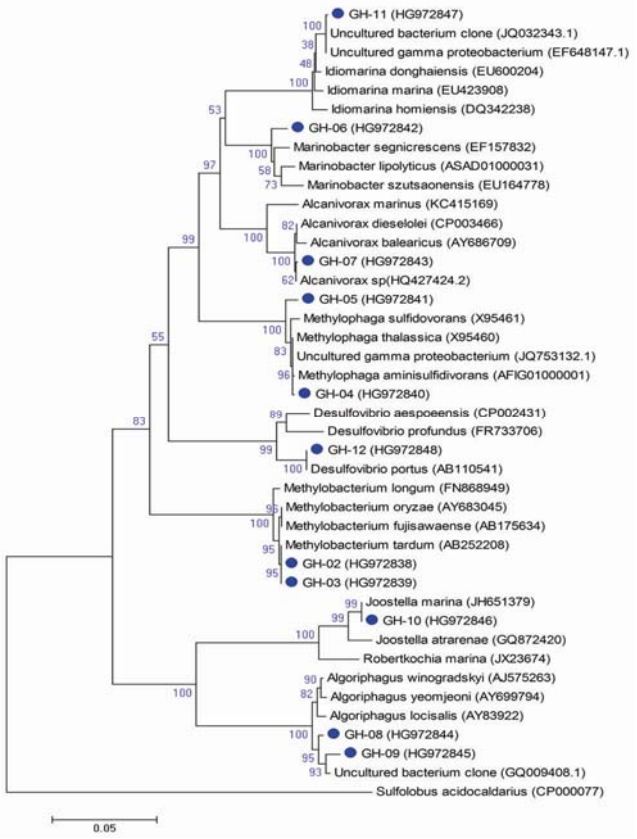

Figure 2. Neighbour-joining phylogenetic tree of bacterial isolates constructed using MEGA 4.01 software. The bootstrap values were calculated as percentage of 1000 replicates. The bar indicates 0.05 substitutions per site. The sequence from *Sulfolobus acidocaldarius* was taken as outgroup. Figures in brackets indicate the accession numbers and the isolates labelled with markers refer to the test isolates.

Table 1. Characteristics of the bacterial isolates from Krishna–Godavari basin sediments with the potential to oxidize methane

Isolate	GH-02	GH-03	GH-04	GH-05	GH-06	GH-07	GH-08	GH-09	GH-10	GH-11	GH-12
Sample	2.2	3.2	0.2	1.2	4.2	5.2	12.2	15.2	19.2	21.2	24.2
Depth (mbsf)											
Gram stain (±)	–	–	–	–	–	–	–	–	–	–	–
Motility	+	+	+	+	+	+	+	+	–	+	+
Biochemical reactions											
Oxidase	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	–	+	+	+	+	+	+
Substrate utilization											
(a) Carbon source (0.1%)											
D-Glucose	+	+	–	+	+	+	+	+	–	+	+
Yeast extract	+	+	+	+	+	+	+	+	+	+	+
Ethanol	–	+	+	+	+	+	+	–	–	–	+
Methanol	+	+	+	+	+	+	+	+	+	+	+
Formamide	–	+	–	–	–	–	–	–	–	–	–
(b) Nitrogen source (0.1%)											
L-tryptophan	+	+	+	+	+	+	+	+	+	+	+
L-methionine	+	+	+	+	+	+	+	+	+	+	+
L-leucine	+	+	+	+	+	+	+	+	+	+	+
Yeast extract	+	+	+	+	+	+	+	+	+	+	+
Physiological tests											
(a) pH											
5.0	+	+	+	+	+	+	+	+	+	+	+
7.0	+	+	+	+	+	+	+	+	+	+	+
8.0	+	+	+	+	+	+	+	+	+	+	+
(b) Temperature (°C)											
4	–	–	–	–	–	–	+	+	–	–	–
28	+	+	+	+	+	+	+	+	+	+	+
40	–	–	–	–	–	+	–	–	–	–	–
(c) Salinity											
0	+	+	+	+	+	+	+	+	+	+	+
18	+	+	+	+	+	+	+	+	+	+	+
35	+	+	+	+	+	+	+	+	–	+	+
Tolerance tests (0.01%)											
(d) Malachite green	–	–	–	–	–	–	–	–	–	–	–
(e) NaN_3	+	–	–	+	–	–	–	–	–	+	–
(f) SDS	–	–	–	–	–	–	–	–	–	–	–

Mbsf, Metres below sea floor.

Oxidation rates of methane by sediment-associated microbial communities

The oxidation rate of methane by bacterial communities associated with subsurface sediments was higher at the surface than at the subsurface. It ranged from 1 ± 0.4 to 10 ± 1.32 nM g dw^{-1} after 5 days incubation. The maximum oxidation of 10 ± 1.32 nM g dw^{-1} was at 1.2 mbsf and minimum oxidation of 1 ± 0.4 nM g dw^{-1} was at 12.2 mbsf (Figure 1). The controls had negligible oxidation rates of methane.

Discussion

Methanotrophs are distributed in a wide variety of natural environments, including marine water and sediments[23]. Their distribution within the bacterial groups was once thought to be limited to 11 genera of Proteobacteria[8]. However, newly discovered representatives of bacteria belonging to the phylum Verrucomicrobia have been reported to possess methane-oxidizing potential[24]. Besides, some of the organisms that utilize the serine cycle for carbon assimilation are identified as facultative methanotrophs[10]. Therefore, it is possible that more number of organisms with the potential to oxidize methane persist in the environment and the observation of methanotrophs or MRB in the subsurface may account for their methane oxidizing activity.

In the present study, several bacterial genera that have not been previously reported to oxidize methane are now shown to do so. The abundance of these bacteria in

Table 2. Oxidation of methane by bacterial isolates in nitrate mineral salt medium in the presence of different nitrogenous substrates

	Methane oxidized (μM)				
Isolate	Potassium nitrate (0.1%)	Tryptophan (0.1%)	Yeast extract (0.1%)	Leucine (0.1%)	Methionine (0.1%)
GH-02	**0.097** ± 0.003	**1.050** ± 0.105	**0.366** ± 0.017	**0.434** ± 0.035	**0.036** ± 0.010
GH-03	**0.092** ± 0.011	**0.983** ± 0.113	**0.857** ± 0.037	**0.246** ± 0.012	**0.036** ± 0.007
GH-04	**0.088** ± 0.006	**0.272** ± 0.014	**0.120** ± 0.011	**0.008** ± 0.006	**0.034** ± 0.007
GH-05	**0.068** ± 0.009	**0.930** ± 0.034	**0.568** ± 0.007	**0.283** ± 0.007	**0.034** ± 0.013
GH-06	**0.054** ± 0.007	**0.193** ± 0.006	**0.176** ± 0.030	**0.365** ± 0.009	**0.036** ± 0.011
GH-07	**0.057** ± 0.008	**0.577** ± 0.057	**0.067** ± 0.014	**0.815** ± 0.020	**0.056** ± 0.006
GH-08	**0.071** ± 0.007	**0.193** ± 0.010	**0.185** ± 0.018	**0.398** ± 0.007	**0.022** ± 0.008
GH-09	**0.057** ± 0.002	**0.218** ± 0.026	**0.344** ± 0.011	**0.098** ± 0.009	**0.014** ± 0.001
GH-10	**0.081** ± 0.004	**0.134** ± 0.018	**0.258** ± 0.003	**0.120** ± 0.010	**0.020** ± 0.010
GH-11	**0.031** ± 0.011	**0.070** ± 0.006	**0.081** ± 0.009	**0.059** ± 0.009	**0.015** ± 0.007
GH-12	**0.026** ± 0.004	**0.062** ± 0.010	**0.098** ± 0.015	**0.092** ± 0.010	**0.025** ± 0.006

Values in bold are average of triplicate readings and ± values refers to their standard deviation.

this study constituted 0.01% of the total bacterial abundance and was 10^4–10^5 CFU g dw^{-1}. The present results corroborate with the methanotrophic counts reported[25] for diverse soils and sediments in the range of 10^4–10^6 CFU g dw^{-1}. However, the distribution of bacteria in the methane hydrate-bearing sediment cores could be distinct from those in hydrate-free cores. The gas hydrate-bearing sites harbour members of the uncultured JS1 group as the dominant representatives often constituting >50% of the representative clones[26], while for sites lacking hydrates, the phylum Chloroflexi represents nearly 80% of the clones[26]. Thus, microbial community composition of methane hydrate-bearing sediments varies considerably from that of hydrate-free sites. In our study, the phylogenetic analysis of the cultured representatives of bacteria from the methane-bearing KG basin sediments indicated that they fall into different taxonomic groups with majority of the bacteria belonging to the Proteobacterial group. The present results agree with the report[8] where the methanotrophs are classified into different groups, namely α-, β- and γ-Proteobacteria. The γ-Proteobacterial members differ from α-Proteobacterial members in their metabolism by using the RuMP pathway for carbon assimilation (type-I) instead of the serine pathway (type-II). The α-Proteobacterial members GH-02 and GH-03 in the present study could be assigned to members of the order Rhizobiales and most closely affiliated with *Methylobacterium* sp. In the present study, the characteristic features of *Methylobacterium*[23] were observed with isolates GH-02 and GH-03. Both the isolates oxidized methane and showed growth with D-glucose and methanol as carbon source. The isolates also differed from the earlier reports[23] in their inability to grow with ethanol and yeast extract as carbon sources. The addition of nitrogen sources in the NMS medium accelerated the methane oxidizing activity of isolate GH-02 at rates higher than all other bacterial isolates that were studied. Besides, both the isolates expressed high tolerance to salinity, which was earlier reported to be inhibited at 1% NaCl concentrations[27] and showed growth at pH 8 in contrast to the inhabitants of freshwater environments which show preference for pH 7 (ref. 28). Thus, the presence of isolates GH-02 and GH-03 in the subsurface sediments could help in the recycling of organic carbon and in regulating the emission of methane. The results of this study indicate that they belong to facultative methane utilizing bacteria[27] and differ from that of type-II methanotrophs consisting of the genera *Methylosinus* and *Methylocystis*[8].

On the other hand, isolates GH-04 and GH-05 represent the γ-Proteobacteria belonging to members of the order Thiotrichales and most closely affiliated with *Methylophaga* sp. Its occurrence has also been reported from a wide variety of environments, including coastal marine waters[29,30]. The presence of methylotrophs like *Methylophaga* in this study may suggest their active role in the metabolism of C1 carbon substrates in the KG basin sediments. These isolates oxidize methane at the expense of different carbon and nitrogen sources. Likewise, the ability of these organisms to utilize different carbon substrates like methanol and methylamine has also been reported earlier[30]. However, the isolates GH-04 and GH-05 differed from each other, wherein the former was intolerant to NaN_3 and incapable of growing on D-glucose, whereas the latter was tolerant to NaN_3 and capable of growing on D-glucose. The results suggest the flexibility in their tolerance and metabolism of substrates. It could suggest that they belong to moderately halophilic marine methylotrophs[30] and differ from that of type-I methanotrophs consisting of the genera *Methylomonas*, *Methylobacter*, *Methylococcus* and *Methylomicrobium*[8].

Apart from those isolates belonging to marine methylotrophs, several other bacterial members were also encountered. Isolates GH-07 representing the γ-Proteobacteria belonging to member of the order Oceanospirillales and

most closely affiliated with *Alcanivorax* sp. was detected in hydrate-bearing sediments of the KG basin. *Alcanivorax* has been known to play an important role in the biodegradation of petroleum hydrocarbon[31]. However, its importance in methane oxidation is unknown. In the present study, the isolate oxidized methane in the presence of different carbon and nitrogenous substrates. These findings suggest its importance as an ecological agent in decelerating the emission of methane. Also, the results of the present study show that isolate GH-07 is capable of utilizing different carbon substrates like ethanol, methanol, D-glucose and yeast extract and amino acids like L-tryptophan, L-leucine and L-methionine. Nonetheless, isolate GH-07 is intolerant to SDS, malachite green and NaN_3. These results indicate that isolate GH-07 is a variant of other reported strains of *Alcanivorax*, with efficient mechanisms of adaptations to distinctive subseafloor environments. Besides *Alcanivorax* sp. has been reported to sequester/oxidize Mn and Fe[32,33] and reduce nitrate[34], which indicates that it could take part in Fe, Mn and nitrite dependent marine methane oxidation[35,36].

Isolate GH-06 from the KG basin sediments, representing the γ-Proteobacteria belongs to an order Alteromonadales that is most closely affiliated with *Marinobacter* sp., a moderately halophilic, euryhaline cosmopolitan Fe^{2+}-oxidizing facultative chemoautotroph[37], with the ability to degrade hydrocarbons and reduce nitrate[38]. The isolate oxidized methane with L-leucine, L-methionine and L-tryptophan as nitrogen sources. It suggests the importance of the isolate in oxidizing methane in the KG basin sediments. Apart from amino acids, *Marinobacter* sp. also utilized organic acids like acetate, lactate and pyruvate for growth[39]. Thus, knowing the importance of iron in the cycling of methane and the iron-oxidizing capability of *Marinobacter* sp., we may speculate that oxidation of methane by this bacterium could be coupled to the cycling of iron in the KG basin sediments, with ferrous iron serving as the core element of the active site in soluble methane monooxygenase[40].

Another interesting isolate observed in the present study is GH-11, which represents an uncultured bacterium clone. The isolate oxidized methane in the presence of yeast extract and three different amino acids, namely L-leucine, L-methionine and L-tryptophan (Table 2). This bacterium has close affiliation with *Idiomarina* sp. belonging to the γ-Proteobacteria that has the ability to scavenge Fe(III) through siderophore production[41]. The isolate showed growth on different carbon sources like D-glucose, yeast extract and methanol, but failed to grow on ethanol and formamide. Nevertheless, the isolate was tolerant to NaN_3. These findings indicate their potential as methanotrophic related species with stringent adaptation to methane-bearing subsurface sediments and to ambient iron concentrations that are usually present in marine sediments.

Besides the α- and γ-Proteobacteria, isolate GH-12 representing the δ-Proteobacteria belongs to an order Desulfovibrionales and is most closely affiliated with *Desulfovibrio* sp. This species is known to oxidize methane anaerobically in small amounts while growing on lactate[42–44]. However, its potential to oxidize methane aerobically was previously unknown. In the present study, the oxidation of methane by isolate GH-12 occurred in the presence of yeast extract and L-leucine, while minimum oxidation occurred with L-methionine (Table 2). The isolate GH-12 utilized amino acids as a nitrogen source or an energy source, as has been described previously[45]. Further, its occurrence in the sediments of the KG basin could be important for the reduction of iron oxides[46]. The potential to reduce iron among *Desulfovibrio* sp. suggests its importance in the oxidation of methane coupled to the reduction of iron in the methane charged sediments of the KG basin.

Isolates GH-08 and GH-09, on the other hand, represent the uncultured bacterium clone. Both the isolates showed 97.77% and 97.46% homology to the 16S rRNA gene sequences of the cultivated bacteria representing Bacteriodetes of the order Sphingobacteriales, with taxonomic affiliation to the genus *Algoriphagus.* This genus is known to be oxidase-positive, able to tolerate 0%–10% salinity and to grow at 25°C and 40°C (ref. 47). The isolates GH-08 and GH-09 were equally efficient like that of *Algoriphagus* and exhibited good growth at salinity of 18 and temperatures of 4°C and 27°C. The bacterial members of genus *Algoriphagus* are not known to oxidize methane. Both the isolates GH-08 and GH-09 were highly efficient in oxidizing methane in the presence of different amino acids and yeast extract as nitrogen sources. Isolate GH-08 differed from isolate GH-09 in its ability to grow using ethanol as carbon source. These features signify their potential to grow under varying environmental conditions and also their ability to survive in the subsurface sediments using different carbon and nitrogenous substrates.

The other important isolate GH-10 representing the Bacteriodetes belongs to the order Flavobacteriales and is closely affiliated with *Joostella* sp. Though its ability to oxidize methane is unknown, in the present study, the isolate oxidized methane using yeast extract and amino acids as nitrogen sources. It showed growth with amino acids such as L-leucine, L-methionine and L-tryptophan and with carbon sources such as yeast extract and methanol. However, the isolate was less efficient in utilizing ethanol, formamide and D-glucose. Besides, this isolate showed positive results for oxidase, catalase and urease and grew at pH 5, 7 and 8 and at a temperature of 28°C. These results are in agreement with a previous report[48], where it has been shown based on biochemical and physiological tests that they are oxidase- and catalase-positive and capable of growth at 10–37°C and pH of 5.3–7.6.

Thus the results of the present study indicate that the KG basin sediments harbour growth of MRB that could play a vital role in controlling the emission of methane. Future works addressing the molecular physiology of the isolates would throw more light on the associated processes occurring in conjunction with methane oxidation in the KG basin sediments.

1. Gonsalves, M.-J., Fernandes, C. E. G., Fernandes, S. O., Kirchman, D. L. and Loka Bharathi, P. A., Effects of composition of labile organic matter on biogenic production of methane in the coastal sediments of the Arabian Sea. *Environ. Monit. Assess*., 2011, **182**, 385–395.
2. Jang, I., Lee, S., Hong, J.-H. and Kang, H., Methane oxidation rates in forest soils and their controlling variables: a review and a case study in Korea. *Ecol. Res*., 2006, **21**, 849–854.
3. Kotelnikova, S., Microbial production and oxidation of methane in deep subsurface. *Earth Sci. Rev*., 2002, **58**, 367–395.
4. Carini, S., Bano, N., LeCleir, G. and Joye, S. B., Aerobic methane oxidation and methanotroph community composition during seasonal stratification in Mono Lake, California (USA). *Environ. Microbiol.,* 2005, **7**, 1127–1138.
5. Rahalkar, M. and Schink, B., Comparison of aerobic methanotrophic communities in littoral and profundal sediments of Lake Constance by a molecular approach. *Appl. Environ. Microbiol.,* 2007, **73**, 4389–4394.
6. Perry, J. J. and Staley, J. T., *Microbiology: Dynamics and Diversity*, Saunders College Publishing, New York, 1997.
7. Murrell, J. C., McDonald, I. R. and Bourne, D. G., Molecular methods for the study of methanotroph ecology. *FEMS. Microbiol. Ecol*., 1998, **27**, 103–114.
8. Hanson, R. S. and Hanson, T. E., Methanotrophic bacteria. *Microbiol. Rev*., 1996, **60**, 439–471.
9. Islam, T., Jensen, S., Reigstad, L. J., Larsen, Ø. and Birkeland, N.-K., Methane oxidation at 55°C and pH 2 by a thermoacidophilic bacterium belonging to the *Verrucomicrobia phylum*. *Proc. Natl. Acad. Sci. USA*, 2008, **105**, 300–304.
10. Semrau, J. D., DiSpirito, A. A. and Vuilleumier, S., Facultative methanotrophy: false leads, true results, and suggestions for future research. *FEMS Microbiol. Lett*., 2011, **323**, 1–12.
11. Reed, D. W., Fujita, Y., Delwiche, M. E., Blackwelder, D. B., Sheridan, P. P., Uchida, T. and Colwell, F. S., Microbial communities from methane hydrate-bearing deep marine sediments in a forearc basin. *Appl. Environ. Microbiol*., 2002, **68**, 3759–3770.
12. Mazumdar, A., João, H. M., Peketi, A., Dewangan, P., Kocherla, M., Joshi, R. K. and Ramprasad, T., Geochemical and geological constraints on the composition of marine sediment pore fluid: Possible link to gas hydrate deposits. *Mar. Pet. Geol*., 2012, **38**, 35–52.
13. Hobbie, J. E., Daley, R. J. and Jasper, S., Use of nuclepore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.,* 1977, **33**, 1225–1228.
14. Whittenbury, R., Phillip, K. C. and Wilkinson, J. F., Enrichment, isolation and some properties of methane utilizing bacteria. *J. Gen. Microbiol*., 1970, **61**, 205–218.
15. Gerhardt, P., Murray, R. G. E., Costilow, R. N., Nester, E. W., Wood, W. A., Krieg, N. R. and Phillips, G. B., *Manual of Methods for General Bacteriology*, American Society for Microbiology, Washington, DC, 1981.
16. Dianou, D. and Adachi, K., Characterization of methanotrophic bacteria isolated from a subtropical paddy field. *FEMS Microbiol. Lett*., 1999, **173**, 163–173.
17. Zeng, L. *et al.*, An effective method of DNA extraction for bioleaching bacteria from acid mine drainage. *Appl. Microbiol. Biotechnol.,* 2008, **79**, 881–888.
18. Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J. and Wade, W. G., Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl. Environ. Microbiol.,* 1998, **64**, 795–799.
19. Krishnamurthi, S., Bhattacharya, A., Mayilraj, S., Saha, P., Schumann, P. and Chakrabarti, T., Description of *Paenisporosarcina quisquiliarum* gen. nov., sp. nov., and reclassification of *Sporosarcina macmurdoensis* Reddy *et al.* (2003) as *Paenisporosarcina macmurdoensis* comb. nov. *Int. J. Syst. Evol. Microbiol.,* 2009, **59**, 1364–1370.
20. Sujith, P. P., Mourya, B. S., Krishnamurthi, S., Meena, R. M. and Loka Bharathi, P. A., Mobilization of manganese by basalt associated Mn(II) oxidizing bacteria from the Indian Ridge System. *Chemosphere*, 2014, **95**, 486–495.
21. Macy, J. M., Snellen, J. E. and Hungate, R. E., Use of syringe methods for anaerobiosis. *Am. J. Clin. Nutr.,* 1972, **25**, 1318–1323.
22. Parkes, R. J., Sass, H., Webster, G., Watkins, A. J., Weightman, A. J., O'Sullivan, L. A. and Cragg, B. A., Methods for studying methanogens and methanogenesis in marine sediments. In *Handbook of Hydrocarbon and Lipid Microbiology* (ed. Timmis, K. N.), Springer-Verlag, Berlin, 2010, pp. 3799–3826.
23. Aken, B. V., Peres, C. M., Doty, S. L., Yoon, J. M. and Schnoor, J. L., *Methylobacterium populi* sp. nov., a novel aerobic, pink-pigmented, facultatively methylotrophic, methane-utilizing bacterium isolated from poplar trees (Populus deltoidesnigra DN34). *Int. J. Syst. Evol. Microbiol*., 2004, **54**, 1191–1196.
24. Chistoserdova, L., Kalyuzhnaya, M. G. and Lidstrom, M. E., The expanding world of methylotrophic metabolism. *Annu. Rev. Microbiol*., 2009, **63**, 477–499.
25. Jugnia, L. B., Roy, R., Pacheco-Oliver, M. C., Miguez, C. B. and Greer, C. W., Production and consumption of methane in soil, peat and sediments from a hydro-electric reservoir (Robert-Bourassa) and lakes in the Canadian Taiga. In *Greenhouse Gas Emissions – Fluxes and Processes: Hydroelectric Reservoirs and Natural Environments* (eds Tremblay, A. *et al.*), Springer-Verlag, Berlin, 2005, pp. 441–466.
26. Inagaki, F. *et al.*, Biogeographical distribution and diversity of microbes in methane hydrate-bearing deep marine sediments on the Pacific Ocean Margin. *Proc. Natl. Acad. Sci. USA*, 2006, **103**, 2815–2820.
27. Trotsenko, Yu. A., Doronina, N. V., Li, Ts. D. and Reshetnikov, A. S., Moderately haloalkaliphilic aerobic methylobacteria. *Microbiology*, 2007, **76**, 293–305.
28. Aslam, Z., Lee, C. S., Kim, K. H., Im, W. T., Ten, L. N. and Lee, S. T., *Methylobacterium jeotgali* sp. nov., a nonpigmented, facultatively methylotrophic bacterium isolated from jeotgal, a traditional Korean fermented seafood. *Int. J. Syst. Evol. Microbiol*., 2007, **57**, 566–571.
29. Janvier, M., Frehel, C., Grimont, F. and Gasser, F., *Methylophaga marina* gen. nov., sp. nov. and *Methylophaga thalassica* sp. nov., marine methylotrophs. *Int. J. Syst. Bacteriol*., 1985, **35**, 131–139.
30. Neufeld, J. D., Schafer, H., Cox, M. J., Boden, R., McDonald, I. R. and Murrell, J. C., Stable-isotope probing implicates *Methylophaga* spp. and novel Gammaproteobacteria in marine methanol and methylamine metabolism. *ISME J*., 2007, **1**, 480–491.
31. Sabirova, J. S., Ferrer, M., Regenhardt, D., Timmis, K. N. and Golyshin, P. N., Proteomic insights into metabolic adaptations in *Alcanivorax borkumensis* induced by alkane utilization. *J. Bacteriol*., 2006, **188**, 3763–3773.
32. Hara, A., Baik, S. H., Syutsubo, K., Misawa, N., Smits, T. H., van Beilen, J. B. and Harayama, S., Cloning and functional analysis of *alkB* genes in *Alcanivorax borkumensis* SK2. *Environ. Microbiol*., 2004, **6**, 191–197.
33. Xuezheng, L., Aiguo, G. and Haowen, C., Isolation and phylogenetic analysis of cultivable manganese bacteria in sediments from the Arctic Ocean. *Acta Ecol. Sin*., 2008, **28**, 6364–6370.

34. Nakano, M., Okunishi, S., Tanaka, R. and Maeda, H., Denitrifying activity and homologous enzyme analysis of *Alcanivorax dieselolei* strain N1203. *Biocontrol. Sci.*, 2009, **14**, 97–105.
35. Beal, E. J., House, C. H. and Orphan, V. J., Manganese- and iron-dependent marine methane oxidation. *Science*, 2009, **325**, 184–187.
36. Ettwig, K. F. *et al.*, Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature*, 2010, **464**, 543–548.
37. Kaye, J. Z., Sylvan, J. B., Edwards, K. J. and Baross, J. A., *Halomonas* and *Marinobacter* ecotypes from hydrothermal vent, subseafloor and deep-sea environments. *FEMS Microbiol. Ecol.*, 2011, **75**, 123–133.
38. Gauthier, M. J., Lafay, B., Christen, R., Fernandez, L., Acquaviva, M., Bonin, P. and Bertrand, J. C., *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremely halotolerant, hydrocarbon degrading marine bacterium. *Int. J. Syst. Bacteriol.*, 1992, **42**, 568–576.
39. Handley, K. M., Héry, M. and Lloyd, J. R., Redox cycling of arsenic by the hydrothermal marine bacterium *Marinobacter santoriniensis*. *Environ. Microbiol.*, 2009, **11**, 1601–1611.
40. Merkx, M., Kopp, D. A., Sazinsky, M. H., Blazyk, J. L., Müller, J. and Lippard, S. J., Dioxygen activation and methane hydroxylation by soluble methane monooxygenase: a tale of two irons and three proteins. *Angew. Chem., Int. Ed. Engl.*, 2001, **40**, 2782–2807.
41. Hou, S. *et al.*, Genome sequence of the deep-sea γ-proteobacterium *Idiomarina loihiensis* reveals amino acid fermentation as a source of carbon and energy. *Proc. Natl. Acad. Sci. USA*, 2004, **101**, 18036–18041.
42. Sorokin, X., On the ability of sulfate-reducing bacteria to utilize methane for the reduction of sulfate. *Dokl. Akad. Nauk. SSSR, Ser. Biol.*, 1957, **115**, 816–818.
43. Davis, J. B. and Yarbrough, H. F., Anaerobic oxidation of hydrocarbons by *Desulfovibrio desulfuricans*. *Chem. Geol.*, 1966, **1**, 137–144.
44. Iversen, N., Interaktioner mellem fermenteringsprocesser og de terminale processer. Ph D thesis, Aarhus University, Denmark, 1984.
45. Stams, A. J. M., Hansen, T. A. and Skyring, G. W., Utilization of amino acids as energy substrates by two marine *Desulfovibrio* strains. *FEMS Microbiol. Lett.*, 1985, **31**, 11–15.
46. Coleman, M. L., Hedrick, D. B., Lovley, D. R., White, D. C. and Pye, K., Reduction of Fe(III) in sediments by sulfate-reducing bacteria. *Nature*, 1993, **361**, 436–438.
47. Krieg, N. R. *et al.*, *Bergey's Manual of Systematic Bacteriology*, Springer, New York, 2010, 2nd edn.
48. Quan, Z. X. *et al.*, *Joostella marina* gen. nov., sp. nov., a novel member of the family Flavobacteriaceae isolated from the East Sea. *Int. J. Syst. Evol. Microbiol.*, 2008, **58**, 1388–1392.

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