Diversity of functional genes for methanotrophs in sediments associated with gas hydrates and hydrocarbon seeps in the Gulf of Mexico

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Abstract

Methanotrophs are ubiquitous in soil, fresh water and the open ocean, but have not been well characterized in deep-sea hydrocarbon seeps and gas hydrates, where methane is unusually abundant. Here we report the presence of new functional genes for the aerobic oxidation of methane by methanotrophs in marine sediments associated with gas hydrates and hydrocarbon seeps in the Gulf of Mexico. Samples were collected from two hydrate locations (GC185 and GC234): one hydrocarbon-seep location at a brine pool (GC233) and one background-marine location about 1.2 miles north of the brine pool (NBP). Community DNA was extracted from each location to establish clone libraries for the *pmoA* functional gene using a PCR-based cloning approach. Three hundred and ninety clones were screened by sequencing and 46 operational taxonomic units were obtained. Eight operational taxonomic units were present in every sample; one of them was predominant and accounted for 22.8–25.3% of each clone library. Principal-component analysis indicated that samples GC185 and GC234 were closely related and, along with GC233, were significantly different from NBP. These results indicate that methanotrophic communities may be similarly impacted by hydrocarbons at the gas-hydrate and seep sites, and can be distinguished from methanotrophic communities in the normal marine sediment. Furthermore, cluster analysis showed that 84.8% of operational taxonomic units from all samples formed distinct clusters, which could not be grouped with any published *pmoA* sequences, indicating that a considerable number of novel methanotrophic species may exist in the Gulf of Mexico.

Introduction

Methane cycling is an important biogeochemical process in cold seeps and gas hydrates (Valentine *et al.*, 2001; Zhang *et al.*, 2002; Dickens, 2003; Milkov *et al.*, 2003; Joye *et al.*, 2004; Sassen *et al.*, 2004; Milkov, 2005). Previous studies suggest that microbial activity in the water column may be governed by localized hydrocarbons released from hydrates or episodic mud volcanoes (LaRock *et al.*, 1994; Suess *et al.*, 1999). Studies on the rate of turnover in the water column of the Eel River Basin indicate that methanotrophs are active in areas where methane concentration is high, whereas active populations have not developed in areas characterized by low methane concentrations (Valentine *et al.*, 2001). Oxygen-demand studies at the Cascadia convergent margin also suggest that methane oxidation by methanotrophs is enhanced in methane plumes above hydrocarbon seeps and hydrate mounds (Suess *et al.*, 1999).

Most microbiological studies in gas-hydrate or hydrocarbon-seep sediments have focused on bacterial and archaeal communities performing anaerobic oxidation of methane (e.g. Hinrichs *et al.*, 1999; Boetius *et al.*, 2000; Pancost *et al.*, 2000; Lanoil *et al.*, 2001; Orphan *et al.*, 2001; Zhang *et al.*, 2002, 2003; Mills *et al.*, 2003; Joye *et al.*, 2004; Mills *et al.*, 2004). In the Gulf of Mexico (GOM), anaerobic oxidation of methane and related processes have been studied using both microbiological and biogeochemical approaches (Lanoil *et al.*, 2001; Zhang *et al.*, 2002, 2003, 2005; Mills *et al.*, 2003, 2004; Arvidson *et al.*, 2004; Formolo *et al.*, 2004; Joye *et al.*, 2004; Orcutt *et al.*, 2004; Sassen *et al.*, 2004; Orcutt *et al.*, 2005). The community structure of aerobic methanotrophs in gas-hydrate systems has received much less

attention. Recently, Inagaki *et al.* (2004) showed that aerobic methanotrophs co-occurred with anaerobic bacteria and archaea in close proximity at methane seeps of the Kuroshima Knoll of Japan. Our goal in this study was to compare the composition of methanotrophic communities associated with the gas hydrates and hydrocarbon seeps with that of methanotrophic communities in normal marine sediment in the GOM. The molecular diversity of *pmoA* was examined by performing PCR-based sequencing. The results indicated significant diversity of methanotrophs associated with different types of environments rich in organic carbon. To our knowledge, this is the first *pmoA*-guided assessment of methanotrophic communities affected by gas hydrates and hydrocarbon seeps in the GOM marine sediments.

Materials and methods

Sampling sites

Samples were collected from four locations in the Grand Canyon leasing block (27°44.8′N and 91°13.3′W) in the GOM (Fig. 1). The water depths at these locations were about 540 m. The Johnson Sea-Link research submersible was used for this study. Two samples were collected from sites GC185 and GC234, where gas hydrate occurred. A third sample was from site GC233, which did not have gas hydrate but had a high flux of hydrocarbon gases (Sassen *et al.*, 1999). This sample was collected about 50 m from a brine pool at GC233. A fourth sample was from normal marine sediment at a location about 1.2 miles north of the brine pool (NBP). All samples used in this study were collected from push cores at a depth of 0–5 cm. At GC185 and GC234, the sediment contained decomposing nodules of gas hydrate, was stained with crude oil, and smelled of hydrogen sulfide. The invertebrate community at GC234 consisted of tubeworms, mussels and clams (Fisher *et al.*, 2000). The macroorganisms were avoided during push-core collection of the sediment. Neither bacterial mats nor the invertebrate community was found at the NBP.

DNA extraction and purification

DNA was extracted from the sediment samples (5 g) using a modified procedure (Zhou *et al.*, 1996) and an UltraClean Mega Prep Soil DNA kit (Mo Bio Laboratory Inc., Solana Beach, CA) and was purified using the Wizard DNA Clean-up System (Promega, Madison, WI). Purified DNA was used for further PCR amplification.

PCR amplification and cloning

PCR products of 472 bp were amplified in a 9700 Thermal Cycler (Perkin-Elmer, Wellesley, MA) using primer pair a189F (GGNGACTGGGACTTCTGG) and mb661R (CCG GMGCAACGTCYTTACC). This primer was selected because it only targets *pmoA* and not *amoA* (Costello & Lidstrom, 1999). The constituents of the 20 μL PCR reaction have been described previously (Yan *et al.*, 2003), except that the bovine serum albumin was not used in this study. The PCR parameters were 30 s at 80 °C and 2 min at 94 °C, followed by 30 cycles of amplification with each cycle consisting of 30 s at 94 °C, 30 s at 55 °C, and 40 s at 72 °C. The last cycle, however, was 5 min at 72 °C.

The 20 μL PCR reaction was replicated five times for each sample in order to reduce the potential for artifact (Qiu *et al.*, 2001). The PCR products from the five replicates were combined and run on electrophoresis gel containing 0.8%

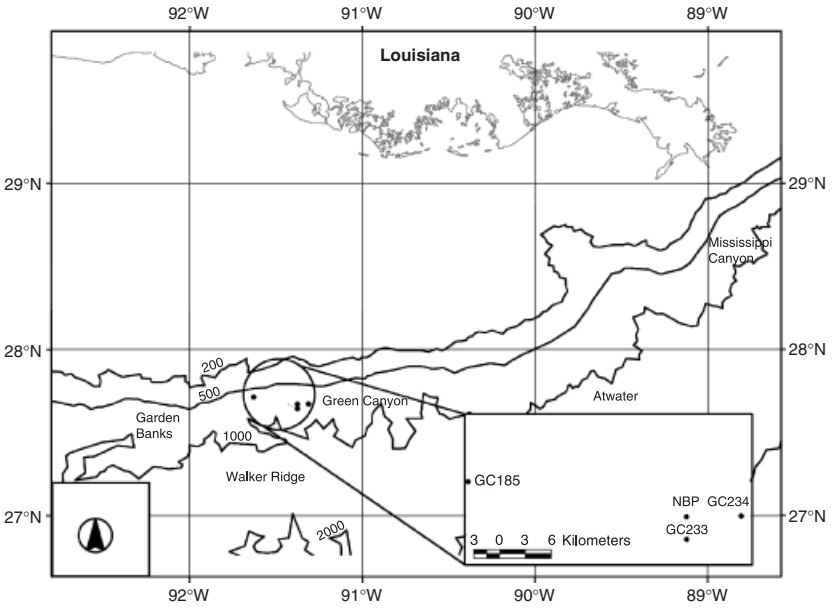

Fig. 1. Locations of gas-hydrate sites (GC185 and GC234) and brine-pool site (GC233) in the Gulf of Mexico. Site NBP was about 1.2 miles north of the brine pool at site GC233.

low-melting agarose gel. This gave a single clear band, suggesting high purity of the PCR product. The band was excised and purified using the QIA Quick Gel Extraction Kit (QIAGEN, Inc., Valencia, CA). Recovered DNA after purification was precipitated by adding 5 μL of 3 M NaAC (pH 5.2) and 250μL of 100% ethanol. The precipitants were then collected as pellets by centrifugation at 14 000 ***g*** for 30 min and washed with 70% ethanol. They were left at room temperature for 10 min and then resuspended in 6 μL of H_2O. Two microliters of the purified PCR products were ligated with PCR2.1 TOPO vector (Invitrogen Life technologies, Carlsbad, CA) to produce a 10 μL ligation solution. From the solution, 2 μL were transferred to 50 μL of *Escherichia coli* competent cells, according to the manufacturer's instructions.

Sequencing and phylogenetic analysis

Ninety-two to 103 colonies were randomly picked for each sample and were screened for *pmoA* inserts. The inserts were amplified in a 30 μL PCR reaction using TA primer pairs specific to the vector (Qiu *et al.*, 2001). The amplified inserts were then purified using the MultiScreen PCR plate (Millipore, Bedford, MA). One microliter of the recovered template (20 μL) was used in a 5 μL sequencing reaction, which contained 1 μL of the template, 1 μL of a fivefold-strength sequence buffer, 1 μL of ABI BigDye® Terminator v.3.1 (Applied Biosystems, Foster City, CA), 3 pmol of a189F primer, and water. Sequencing was conducted using a 3700 DNA analyzer (Perkin-Elmer).

Sequences were edited using the SEQUENCHER program (v.4.0, Gene Codes, Ann Arbor, MI). The edited sequences were then blasted and compared with *pmoA* genes published in the database. Sequences from this study and reference sequences from GenBank were assembled and aligned using BIOEDIT (Hall, 1999) and CLUSTALX1.81 software. A phylogenetic tree and distance matrix were obtained using MEGA3 software based on the neighbor-joining method. The nucleic acid sequence accession numbers in GenBank are DQ514617-DQ514651 and DQ518566-DQ518575.

Determination of the operational taxonomic units (OTUs) is important for performing diversity studies using environmental sequences. In order to eliminate errors due to editing, five individual clones were used for statistical analysis. Each clone was sequenced five times. The results showed that there was less than 2% error in sequence-editing between replicate sequences. Therefore, sequences with 98% identity were clustered into one OTU.

Data analysis

Principal-component analysis (PCA) was performed using SYSTAT 10 software (SPSS, Inc., Chicago, IL). Using clone library data, samples from different sites were grouped or separated based on the patterns of OTUs shared by all samples and the distinct OTUs from each sample. In this study, each OTU represented a percentage of the total clones from each sample.

Results

Analysis of pmoA clone library

Three hundred and ninety clones were sequenced for the four samples studied. Among these, 99 clones were from GC233, 92 from GC185, 103 from GC234, and 96 from NBP (Table 1). Sequences were aligned, and similarities between any two sequences were calculated using CLUSTALX and MEGA software (Thompson *et al.*, 1994; Kumar *et al.*, 2001). Rarefaction analysis was performed to estimate the diversity within each sample. The results indicated that the number of unique clones reached saturation from 30 clones for GC234 to about 50 clones for GC233. Therefore, the OTUs (below) were expected to represent the community composition of methanotrophs in each sample.

Forty-six OTUs were obtained: 26 from GC233, 23 from GC185, 24 from GC234, and 24 from NBP. Each site also contained four to six unique OTUs that were not shared by any other site (Table 1). However, the frequency of occurrence was low for these clones.

Pairwise comparisons (Table 2) showed that 30–44% of the total OTUs and 59–84% of the total clones recovered from each site were shared with those of another site. The two gas-hydrate sites, GC185 and GC234, had the highest

Table 1. Characteristics of *pmoA* gene clones from marine sediments at gas hydrate (GC 185, GC 234), hydrocarbon seep (GC 233), and normal marine site (NBP) in the Gulf of Mexico

	GC 233	GC 185	GC 234	NBP
Total clones screened	99	92	103	96
No. of OTUs*	26	23	24	24
Unique OTUs†	6	4	4	4
Percentage of OTUs in total clones screened	26.3	25.0	23.3	25.0

*OTUs based on the similarities between sequences; sequences having or above 98% identity were defined as one OTU.
†OTU present in a particular sample only.

Table 2. Pairwise comparison demonstrating the genetic overlap of *pmoA* genes between two sites*

Site	GC 185	GC 234	NBP
GC 233	14/40 (143/191)	13/39 (164/202)	15/39 (155/195)
GC 185		16/36 (163/195)	12/40 (109/185)
GC 233			12/40 (147/199)

*Each pairwise comparison shows the number of OTUs common to both sites vs. the total number of OTUs of both sites. Numbers in parentheses indicate the clones common to both sites vs. total clones of the two sites.

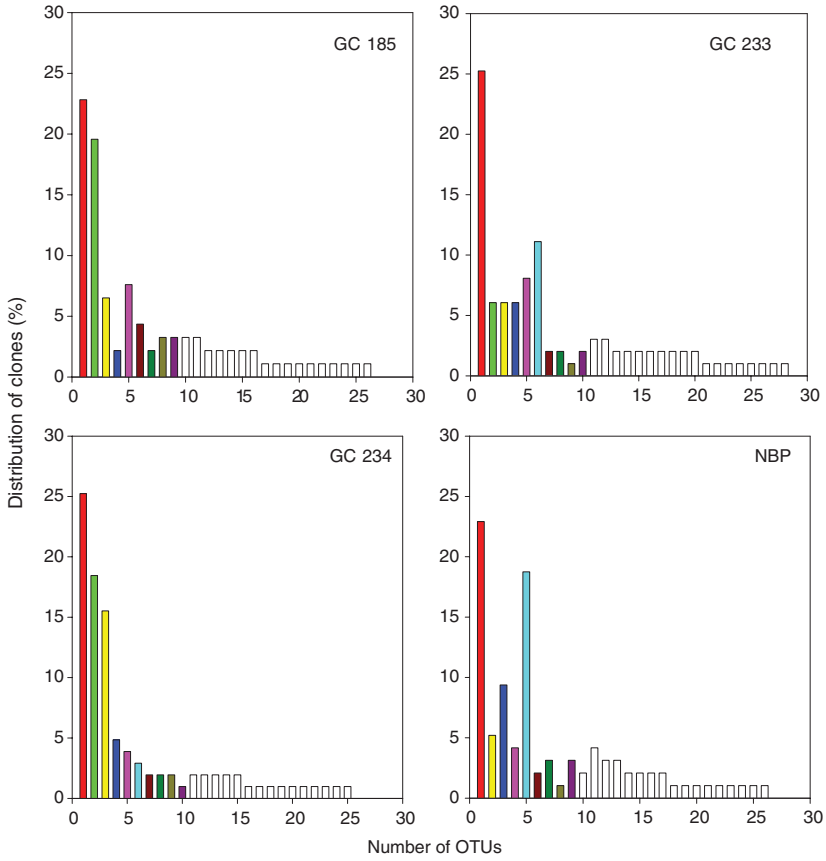

Fig. 2. The clone distribution and overlap among samples for *pmoA* gene sequences. Each color represents a unique sequence and the same color shared between samples represents the same sequence (or OTU). The white bars represent unique sequences observed only in one sample or sequences shared by only two samples. The bar graphs are based on percentage of the clone number of each unique sequence accounted for by the total number of clones examined in each sample. Red, OTU GC233-B04; green, OTU GC185-F06; cyan, OTU GC233-E01.

percentages of similarity in terms of OTUs and clones. The background-site NBP shared the lowest percentages of OTUs and clones with the two gas-hydrate sites. The hydrocarbon-seep site GC233 shared intermediate similarities in OTUs and clones with NBP and GC185 or GC234.

The distribution of the OTUs was different from sample to sample (Fig. 2). Eight OTUs (represented by GC233-C12, GC233-D01, GC233-B12, GC233-F02, GC233-H05, GC233-A07, GC233-B06 and GC233-B04) were observed in all samples. OTU GC233-B04 was predominant in all samples and accounted for 22.8–25.3% of total OTUs in each sample. OTU GC185-F06 was the second most abundant clone in GC185 (19.6%) and GC234 (18.5%) and much less abundant (6.1%) in GC233. By contrast, OTU GC233-E01 was the second most abundant clone in GC233 (11.1%) and NBP (18.8%) and much less abundant (2.9%) in GC234.

The frequency of other OTUs was low, and they were distributed differently in different samples. For example, OTUs GC185-B04, GC234-B07 and GC185-E01 were present only at GC234 and GC185. OTUs GC233-G11, GC233-G05 and GC185-F06 were present at GC185, GC234 and GC233 but not at NBP. OTUs GC233-A12, GC233-C05 and GC233-C10 were present at GC233 and NBP. Finally, OTUs GC233-B05, GC233-E01 and GC233-E06 were present at GC233, GC185 or GC234, and NBP.

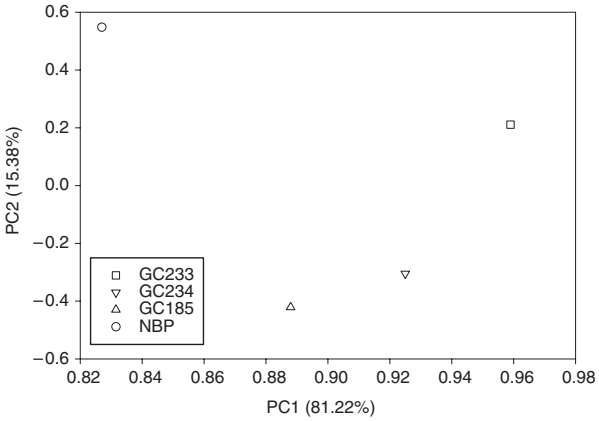

Fig. 3. Principal-component analysis based on the relative abundance of OTUs. The values in parentheses are percentages of the total variances of principal-component analysis derived from *pmoA* data.

PCA analysis indicated that GC185 and GC234 were clustered together (Fig. 3) and shared 91% similarity in term of genetic structure composition (note: similarity in

PCA analysis is a different concept from sequence similarity; see Materials and methods). GC233 was separated from GC185 and GC234 and shared 76–80% similarity with them. All three samples were separated from NBP and had low similarity (51–60%) with it.

Phylogenetic analysis

The unique 46 OTUs were blasted on the NCBI Website (http://www.ncbi.nlm.nih.gov/). The results indicated that 84.8% of the total OTUs and 93.1% of all 390 *pmoA* clones had no close relationship with any previously sequenced genes.

The phylogenetic tree was generated using the neighbor-joining method. Sequences of *pmoA* were divided into three clusters, and each cluster contained three groups (Fig. 4). In cluster I, group Ia accounted for 50% of the total OTUs and 75% of the total clones. Among the eight common OTUs distributed in all samples, six (GC233-B04, GC233-D01, GC233-C12, GC233-H05, GC233-B12 and GC233-A07) fell into group Ia. Group Ib included four OTUs (8.7% of total OTUs) and 16 clones (4.1% of total clones). In particular, OTU GC233-C10 occurred in all four samples. Group Ic included five OTUs and 25 clones. OTUs in cluster I had 72–81% similarity with the uncultured bacterium *pmoA* gene (AB064371).

In cluster II, group IIa consisted of one OTU (NBP-H10) and represented one clone, which had 90% similarity with *pmoA* gene AB064371 and 85% similarity with *pmoA* gene AB064376 (Fig. 4). Both genes are from a methane-biostimulated aquifer contaminated by trichloroethene (Baker *et al*., 2001). NBP-H10 was also similar to a *Methylobacter* species (AY007285) at the 86% level. Group IIb consisted of three OTUs, which represented clones from NBP (one clone), GC233 (two clones) and GC234 (one clone). They were 90% similar to *Bathymodiolus symbiont* pMMo-1 (AB062137) obtained from a deep-sea hydrothermal vent (http://wwwncbinlmnihgov/). Group IIc consisted of one OTU (GC233-F01) and represented one clone. It had 90.5% similarity with a *pmoA* gene (AB176935) from a methane-seep area at the Kuroshima Knoll in the southern Ryuku arc (Inagaki *et al*., 2004). OTU GC233-E01 represented 11 clones and 11.1% of the total OTUs from GC233, 18 clones and 18.8% of total OTUs from NBP, and three clones and 2.91% of total OTUs from GC234. GC233-E01 was not closely related to any published sequences.

In cluster III, group IIIa contained all published genes (Fig. 4). Group IIIb consisted of two OTUs (GC185-E03 and NBP-B03), and each OTU represented one clone. GC185-E03 had 91.3% similarity to *pmoA* gene AB176940, and NBP-B03 had 72.0% similarity to *pmoA* gene AB176939; both genes were from the methane-seep area at the Kuroshima Knoll in the southern Ryuku arc (Inagaki *et al*., 2004). Group IIIc had five OTUs (10.9%) and represented 12 clones (3.1%) from all samples. These OTUs had low frequency and 71–74% similarity to uncultured bacterium *pmoA* gene AB176939 and were distantly related (66% similarity) to the closest known sequences in the *Gamma-proteobacteria* (Fig. 4).

Discussion

Previous studies have shown that the *pmoA* gene encoding particulate methane monooxygenase of methanotrophic bacteria is highly similar to the *amoA* gene encoding ammonia monooxygenase in ammonia-oxidizing bacteria. (Bedard & Knowles, 1989; Ensign *et al*., 1993; Bourne *et al*., 2001). In this study, the primer pair 189F/661R (Baker *et al*., 2001) was used, which targets the *pmoA* gene only. The PCR-based method could cause artifacts during gene amplification, such as an overestimate of community diversity. Selection of a low concentration of DNA, a low number of cycles and a long elongation time can avoid this problem (Qiu *et al*., 2001). In this study, the optimal PCR condition was $5 \times 20\,\mu L$ reactions and 30 cycles, which minimized artifacts during PCR.

Methanotrophs are classified into two major groups (type I and type II) on the basis of membrane structure, pathways of carbon assimilation, lipid biomarker profile, and 16S ribosomal RNA gene (Anthony, 1982; Nichols *et al*., 1985; Ringelberg *et al*., 1989; Sundh *et al*., 1995; Hanson & Hanson, 1996; Bourne *et al*., 2000; Murrell & Radajewski, 2000). Most of the known marine methanotrophs belong to type I (Sieburth *et al*., 1987; Lidstrom, 1988; Kimura *et al*., 1999). A type II methanotroph was found to be most abundant in water columns above sediments in the Black Sea (Gal'chenko *et al*., 1988). Recently, Carini *et al*. (2005) and Lin *et al*. (2005) reported both type I and type II methanotrophs in a saline and alkaline lake, with type I being more abundant than type II across a depth profile.

The primer pair (189F/661R) used in this study was able to target both type I and type II methanotrophs (Bourne *et al*., 2001). All our sequences, however, belonged to the type I methanotroph in the *Gamma*-subdivision of the *Proteobacteria*. Whereas some OTUs were observed at all sites, others were specific for each site (Table 1). The results showed that 85% of OTUs (clusters I, IIIb and IIIc, and OTU GC233-E01) had 71–81% similarity to known sequences. Methanotrophs such as *Methylobacter* species, *Methylocaldum tepidum* and *Methylococcus capsulatus* are distinguished at the genus level when their *pmoA* nucleotide sequences have 71–85% similarities. On the basis of this criterion and the phylogenetic tree (Fig. 4), about 85% of our OTUs may represent novel species.

Seven of the 46 OTUs were closely related to known sequences; however, each of the seven OTUs contained only

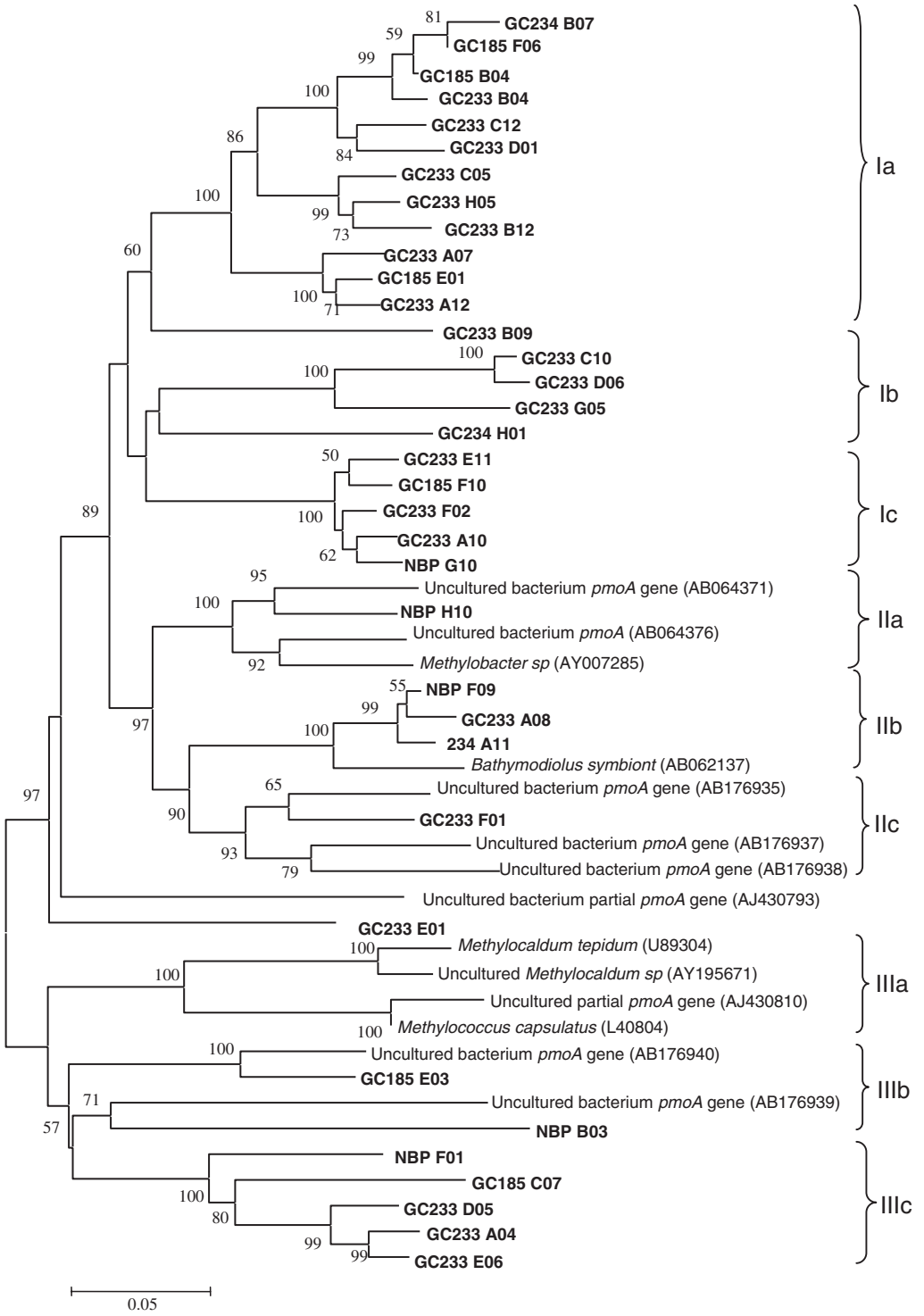

Fig. 4. Phylogenetic analysis of unique nucleotide sequences for *pmoA* from the Gulf of Mexico and reference sequences from the GenBank database. The scale bar represents 0.1 substitutions per nucleotide position. The tree was generated by a neighbor-joining algorithm under *p*-distant model, and 1000 bootstraps were performed. Bootstrap values of $< 50\%$ are not shown. Alignment gaps were processed by the pairwise deletion method in MEGA3 software. Only representative operational taxonomic units in cluster Ia are shown in this tree, in order to reduce the tree size. Sequences in bold are from the Gulf of Mexico; others are references from GenBank.

one clone, except for GC233-A08, which had two clones (Fig. 4). OTU GC233-F01 had three clones from GC233 and was related to sequence AB176935 (clusters IIc, Fig. 4) from the methane-seep area at the Kuroshima Knoll in the southern Ryuku arc in Japan (Inagaki *et al*., 2004). OTUs NBP-B03 and GC185-E03 (three clones each) were also related to sequences (AB176939 and AB176940, respectively) from the Ryuku arc in Japan (Inagaki *et al*., 2004), suggesting that these sequences may be globally distributed in methane-rich marine systems.

OTUs GC233-A08, GC234-A11 and NBP-F09 were 93–94% similar to *Bathymodiolus symbiont* pMMo-1 (AB062137) from the deep-sea hydrothermal vent in the Japan Sea (http://wwwncbinlmnihgov/). OTU NBP-H10 had 90% similarity to the uncultured bacterium *pmoA* gene (AB064371) from a methane-stimulated aquifer contaminated by trichloroethene in Kimtsu city in Japan (Baker *et al*., 2001). The observations above indicate that only a small portion of methanotrophs in the GOM are closely related to those of other deep-sea environments; the majority of methanotrophs detected in this study are novel. Some of the OTUs may be unique to the GOM environment.

The ecological patterns of methanotrophs have been observed in several studies. Using PCR-based clone library and quantitative competitive PCR, Inagaki *et al*. (2004) obtained the *pmoA* gene from a methane-seep area (MC), a reference area (MR) at a distance from a methane seep, and a gas-bubbling carbonate location (HC) in the Kurishima Knoll area in Japan. Their results showed that the number of *pmoA* gene copies was high in MC but low in HC, which suggested greater abundance of methane-oxidizing bacteria in the methane-seep area. Their phylogenetic analysis showed that *pmoA* genes were also diverse in the surface of MC. Carini *et al*. (2005) observed in a saline alkaline lake that rates of methane oxidation varied while the populations of methanotrophs remained stable; denaturing gradient gel electrophoresis profiling and *pmoA* gene analysis suggested that changes in methane-oxidation activity might correlate with changes in methanotroph community structure (Carini *et al*., 2005; Lin *et al*., 2005).

In this study, PCA analysis (Fig. 3) provided an insight into the spatial variability of community structure in the GOM. Based on analysis of OTUs, NBP was significantly separated from the other three samples, suggesting that the genetic composition was different between the normal marine (NBP) and the hydrocarbon-rich (GC233, GC185 and GC234) environments. Furthermore, OTU GC185-F06 (Fig. 3) was only observed in the hydrocarbon sites (19.6% of total clones for GC185, 18.5% for GC234 and 6.1% for GC233) and was predominant at GC185 and GC234. These results suggest that OTU GC185-F06 was likely to be specific to the hydrocarbon sites, perhaps as a direct response to the gas-hydrate or hydrocarbon-seep environment.

Gas hydrates are dynamic because their occurrence and distribution are affected by temperature, pressure and other environmental conditions (Buffett, 2000). Microbial communities may vary spatially and temporally due to changes in the physical and chemical conditions of the gas-hydrate or seep environment. In the GOM, gas hydrates occurred at GC185 and GC234 but not at GC233. Chemical measurements of sediment pore-water indicated that sulfate was depleted to zero below 2 cm at GC234 and still abundant (18–25 mM) below 5 cm at GC233 and NBP (Formolo, 2004). Sulfide levels, by contrast, were highest at GC234 (3–5 mM), intermediate at GC233 (1.4–2.1 mM), and lowest at NBP (< 0.01 mM) (Formolo, 2004). Such differences in chemistry may have contributed to the observed spatial variation in *pmoA* gene patterns at different locations.

Marine sediments associated with gas hydrates or hydrocarbon seeps are characterized by a rapid increase in consumption of oxygen with depth. For example, dissolved oxygen in pore-water at GC234 was reported to vary from about 200 μM at the surface to below the detection limit at 2–4 cm (W. Cai, University of Georgia; unpublished data). Methanotrophic DNA at GC234 was probably associated with anaerobic sediments because of a high level of production of hydrogen sulfide and depletion of oxygen in near-surface sediment (see above). This agrees with reports of methanotrophs from anoxic environments in freshwater lakes (Carini *et al*., 2005; Lin *et al*., 2005) or rice paddies (Eller & Frenzel, 2001). Such observations are consistent with laboratory findings, which demonstrate that methanotrophs survive during anaerobic starvation (Roslev & King, 1994, 1995). This leads to a caveat that *pmoA* sequences in this study may be from both resting and active cells because our clone libraries were constructed on the basis of DNA. Using RNA instead of DNA may allow us to determine whether *pmoA* gene sequences are actually from active cells of methanotrophs or not.

In summary, eight of the 46 OTUs were observed in all four samples. Six OTUs were specific to the gas-hydrate or hydrocarbon-seep environments. Community structures of methanotrophs in gas-hydrate environments were different from those in the normal marine sediment and the brine pool; the genetic similarity was high for the two gas-hydrate samples. By contrast, 84.8% of the total OTUs had no close relationship with any known methanothrophic species, suggesting that community structures of methanotrophs in the Gulf of Mexico may be significantly different from those of terrestrial, freshwater and other marine environments.

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