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METHODS

Molecular biologic techniques applied to the microbial prospecting of oil and gas in the Ban 876 gas and oil field in China

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Abstract Currently, molecular biologic techniques achieve a great development in studies of soil samples. The objective of this research is to improve methods for microbial prospecting of oil and gas by applying culture-independent techniques to soil sampled from above a known oil and gas field. Firstly, the community structure of soil bacteria above the Ban 876 Gas and Oil Field was analyzed based on 16S rRNA gene clone libraries. The soil bacteria communities were consistently different along the depth; however, *Chloroflexi* and *Gemmatimonadetes* were predominant and methanotrophs were minor in both bacteria libraries (DGS1 and DGS2). Secondly, the numbers of methane-oxidizing bacteria, quantified using a culture-dependent procedure and culture-independent group-specific real-time PCR (RT-PCR), respectively, were inconsistent with a quantify variance of one or two orders of magnitude. Special emphasis was given to the counting advantages of RT-PCR based on the methanotrophic *pmoA* gene. Finally, the diversity and distribution of methanotrophic communities in the soil samples were analyzed by constructing clone libraries of functional gene. All 508-bp inserts in clones phylogenetically belonged to the methanotrophic *pmoA* gene with similarities from 83% to 100%. However, most of the similarities were below 96%. Five clone libraries of methanotrophs clearly showed that the anomalous methanotrophs (*Methylosinus* and *Methylocystis*) occupy the studied area.

Keywords Community structure · Gas and oil field · Methanotrophic *pmoA* gene · Real-time PCR · Clone library

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Introduction

The microbial prospecting of oil and gas (MPOG) is a surface oil exploration technology that has been used for approximately seven decades, ever since the possibility of using methane-oxidizing bacteria for gas exploration was considered (Mogilewskii 1938). MPOG is based on the theory that various light hydrocarbons escape and rise to the surface of the earth from subterranean oil and gas deposits (Klusman and Saeed 1996; Saunders et al. 1999; Thrasher et al. 1996), and such hydrocarbons have a marked effect on the soil above these deposits. Conditions are then created which are favourable for the development of highly specialized bacterial populations. The detection of the anomalous distribution and activity of specialized bacterial populations in soil samples can be used to forecast the existence of oil and gas deposits. Until now, applications of MPOG technology have been based on culture-dependent microbial approaches involving the isolation and enumeration of hydrocarbon-oxidizing bacteria that inhabit soil samples and has gained some application attention

(Wagner et al. 1998, 2002). However, uncultivated microorganisms represent up to 99% of those naturally occurring in the environment (Ward et al. 1990; Singleton et al. 2001; Orphan et al. 2000). Therefore, the results of culture-dependent MPOG techniques do not give a clear picture of complete hydrocarbon-oxidizing bacterial communities and accurate numbers of hydrocarbon-oxidizing bacteria within a natural environment. With the development of science and technology, numerous culture-independent molecular biology techniques are now available to overcome the disadvantages of the culture-dependent MPOG technology.

Molecular techniques related with 16S ribosomal DNA (RNA) have been proven effective as a basis for understanding the microbial diversity in environmental communities. The cloning and sequencing of 16S rDNA is sufficient for the identification of the microorganisms present in a given habitat and for the discovery of previously unknown diversity (Hugenholtz et al. 1998). These techniques were also applied to investigate microbial communities in the formation water of the produced water of oil fields (Kaster et al. 2009; Lysnes et al. 2009). However, analyzing microbial communities in soil samples in oil and gas fields for MPOG has seen less study. Knowledge of the relationship between the indigenous microbial communities and MPOG will be important for the success of MPOG.

Hydrocarbon-oxidizing bacterial anomalies have been considered indicators of the existence of oil and gas deposits (Sealy et al. 1974a, 1974b). Among hydrocarbon-oxidizing bacteria, methane-oxidizing bacteria are usually predominant in soil samples above oil and gas reservoirs because the dominating composition of light hydrocarbons in oil and gas reservoirs is methane (Jones et al. 2000). In order to improve MPOG technology, this study investigates culture-independent molecular approaches using phylogenetic and functional gene probes to analyze the diversity and activity of methanotrophs and to detect the presence and abundance of methanotrophs taken directly from environmental samples without cultivation. Methanotrophs aerobes utilize methane, not only as their sole energy source, but also for carbon assimilation (Hanson and Hanson 1996). The functional genes of methanotrophs include *pmoA*, *mmox* and *maxF*, which encode different enzymes of particulate methane monooxygenase, soluble methane monooxygenase and methanol dehydrogenase, respectively (Horz et al. 2001). Since the *pmoA* gene can be found in all cultured methanotrophs except *Methylocella* spp., it acts as the optimum target gene, which is widely used to assess methanotrophs in soil samples (Holmes et al. 1999; Mohanty et al. 2006).

The most-probable-number (MPN) procedure has traditionally been applied to determine the numbers of colony-forming units (CFUs) of methanotrophs in soil samples. The real-time polymerase chain reaction (RT-PCR) is now being widely used to detect and quantify various target microorganisms without experimental cultivation (Dionisi et al. 2003; Skovhus et al. 2004; He et al. 2007). Articles regarding the quantitative analysis of methanotrophs have also been published (Kolb et al. 2003; Zheng et al. 2008). Culture-based microbial techniques are valuable in order to gain information and understand the physiologic and metabolic characteristics of isolated methanotrophs. However, isolated methanotrophs present in soil samples cannot represent the entire methanotrophic community. Investigations of sequence-based *pmoA* phylogeny, which are consistent with 16S rRNA-based phylogeny, were successfully performed to study the composition of the methanotrophs community (Murrell et al. 1998; Mohanty et al. 2006).

The main objectives of this study were to quantity and analyze methanotrophs in the aforementioned soil samples within a known gas and oil field using culture-independent molecular approaches. It was proven that these approaches are more advantageous in MPOG than the previously applied, culture-dependent techniques.

Materials and methods

Site description and sample collection

Soil samples were collected from the Ban 876 Gas and Oil Field within the DaGang Area (39°32′N, 117°38′E), TianJin, China, in October 2008. The DaGang Area, located in the Bohai Sea coast, is alkaline land with an average pH of 7.5. The underground gas and oil reservoir, covering 5 km^2, is 2,200~2,300 m beneath the earth's surface with a 200-m-thick cap rock. The gas-bearing bed, being approximately 24-m thick, extends smoothly along the same plane surface (Figs. 1 and 2). The relative density of natural gas, mainly comprised of 90% methane, is 0.6364 in this reservoir, and the formation pressure of the reservoir is approximately 22.5 MPa. Two specific sites were selected as collection points of soil samples. The first, named A, is 100 m from the central gas producing well, and the second, named B, is 200 m away in opposite direction. Five soil samples retrieved from depths of 0.5, 1, 1.5, 2 and 2.5 m were collected from each sampling plot. Soil samples measuring 100 g each were transferred to pre-sterilized, plastic sampling bags and were stored at −20°C.

DNA extraction

According to the manufacture's protocol of the FastDNA Spin Kit for Soil (Qbiogen, Carlsbad, CA, USA),

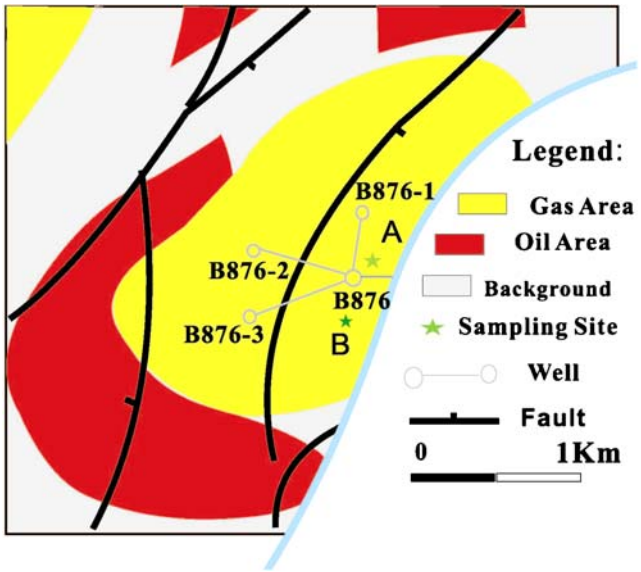

Fig. 1 Geological base of Ban876 gas and oil field. Ban876 Oil and Gas Field is a part of the Dagang Oil Field , Tianjin, China. Ban 876 Gas Storage Reservoir is located in the centre of northern Banqiao Oil and Gas Field

Genomic DNA was extracted from triplicate samples of 0.5 g of fresh soil samples that were thoroughly mixed before weighing. The final DNA density of each sample was formed by mixing three replicated DNA extractions and detected using a Nanodrop® ND-1000 UV–Vis Spectrophoto-meter (NanoDrop Technologies).

Enumeration of methane-oxidizing bacteria using a culture-dependent microbiologic method

The cell numbers of each soil sample of cultured methane-oxidizing bacteria were quantified by the MPN procedure (Dehority et al. 1989) accompanied by the Hungate roll-tube technique (Hungate 1969). One gramme of fresh soil sample was completely suspended in 9 ml of pre-sterilized water in order to prepare decimal dilutions ($10^{-1}$to 10^{-8}). A 0.5 ml aliquot of each dilution was injected into a Hungate tube containing 4.5 ml of modified soil containing a mineral salts medium (Atlas and Lawrence 1996) while the temperature of the soil medium was approximately 50° C. The tube was immediately rolled to cause the medium to solidify on the tube wall. Finally, 5 ml CH_4 was injected into the tube. All Hungate tubes were put into an incubator at 30°C for 9 days. After incubation, the colonies of methane-oxidizing bacteria were manually counted and used to quantify the CFU/g soil.

Quantitative analysis of methanotrophs using a culture-independent method

Real-time PCR amplification was carried out in an iCycle iQ5 thermo cycler (Bio-Rad). The reaction mixture was prepared according to the recommendations of the manufacturer of SYBR® Premix Ex Taq™ (Takara Bio, Otsu, Shiga, Japan). The forward and reverse primers of A189f

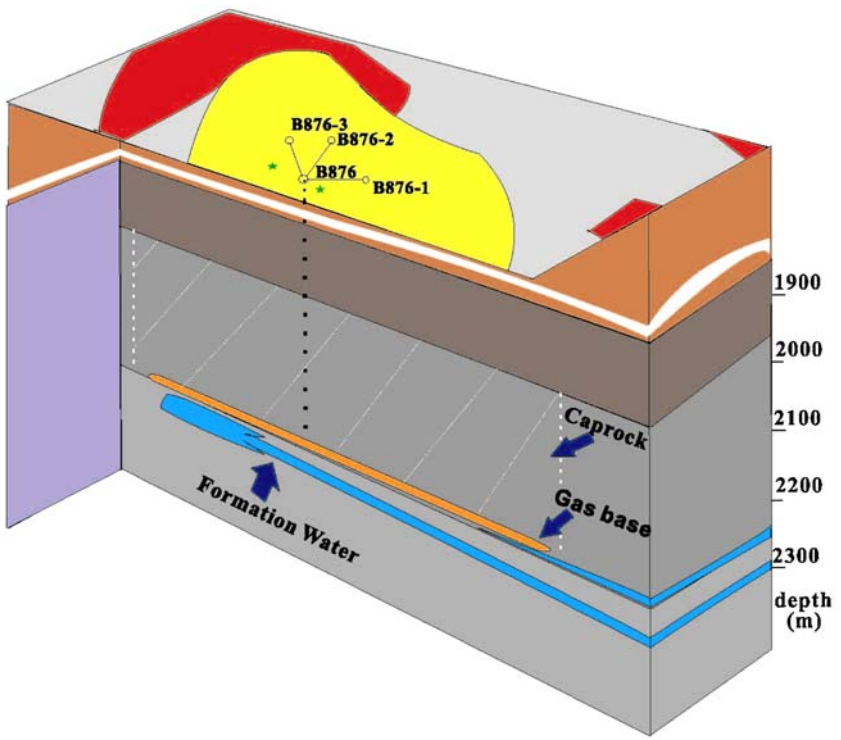

Fig. 2 Caprock's macrolithology of Ban 876 gas and oil field

(5′-GGNGACTGGGACTTCT GG-3′) (Holmes et al. 1999) and mb661R (5′-CCGGMG CAACGTCYTTACC-3′) (Kolb et al. 2003), amplifying the fragment of the methanotrophic *pmoA* gene, were used to detect the *pmoA* gene copy numbers of methanotrophs. DNA, used as template, was added to each reaction mixture at a level of 1–10 ng. The real-time PCR programme for the amplification of target DNA fragments was performed as follows: mixtures were processed at 95°C for 3 min followed by 40 cycles of 1 min at 95°C, 30 s at 55°C and then plated and read at 83°C. iCycler software (version 1.0.1384.0 CR) was used to analyze the amplification data.

Standard curve of real-time PCR

Plasmids of the positive clone containing methanotrophic *pmoA* gene fragments were extracted using a MiniBEST Plasmid Purification Kit (TaKaRa). A Nanodrop® ND-1000 UV–Vis Spectrophoto-meter (NanoDrop Technologies) was used to determine the plasmid DNA concentrations, which were then used to calculate the copy numbers of the *pmoA* gene. Tenfold serial dilutions of one optimal plasmid DNA, acting as templates in triplicate, were used as a real-time PCR assay in order to prepare a standard curve for the enumeration of methanotrophs.

Amplification of 16S rRNA gene and methanotrophic *pmoA* gene

The 16S rDNA in the bulk DNA were amplified by the PCR-reacting system of 25 μl including a 2.5 μL of 10 × PCR buffer (Mg^{2+} plus), 1 U *Taq* DNA polymerase (TakaRa) and 10 pmol of each universal bacteria-specific primer, 27F (5′-AGAGTTTGATCCTGGCTCAG -3′) and 1492r (5′-CTACGGCTACCTTGTTACG A-3′). The thermal cycler programme involved an initial denaturation at 94°C for 5 min, 40 cycles of 94°C for 30 s, 56°C for 60 s, 72°C for 90 s and a final extension step of 72°C for 10 min. The amplified fragments were approximately 1,450 bp.

The *pmoA* gene fragments of soil DNA were amplified using primer sets A189f/mb661R to construct methanotrophic *pmoA* gene libraries. PCR reactions were performed with 10 pmol of each primer, 5 nmol of deoxyribonucleoside triphosphate, 2.5 μl of 10 × PCR buffer (Mg^{2+} plus), 1.5 U *Taq* DNA polymerase (TakaRa), approximately 10 ng of soil DNA and super purified H_2O, supplemented to a total reacting volume of 25 μl. A thermal cycle programme, involving a touchdown PCR strategy (Horz et al. 2005), was performed as follows: after 4 min of initial denaturation at 94°C was used to melt double-stranded DNA, a touchdown procedure was followed consisting of 1 minute at 94°C, 1 min at decreasing annealing temperatures of 62°C to 52°C during the first 16 cycles and 1 min at 72°C. Then, 20 cycles of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C were added. Finally, an extension step of 10 min at 72°C was carried out. The products of amplification were 508 bp.

Construction of 16S rRNA gene and methanotrophic *pmoA* gene libraries

The obtained PCR products were purified with an Agarose Gel DNA Purification Kit (TaKaRa Biotechnology, Dalian, China) and ligated into a PGEM-T Easy Vector (Promega, Madison, WI, USA) as introduced by the suppliers. The ligated products were transformed into *Escherichia coil JM 109* competent cells (TaKaRa Biotechnology, Dalian, China) using chemical transformation. 100 μl cells, incubated for 1 h and 30 min, were spread on LB plates containing ampicillin (100 μg/ml), IPTG (50 Mm) and X-Gal (80 μg/ml). One hundred putative clones (white) from each plate were transferred to another labelled LB plate with ampicillin (100 μg/ml). A re-amplification, with sets of vector-specific primers T7/SP6, was taken to determine positive clones. Minimum cells of putative clones were used as templates in reaction mixtures during the re-amplification procedure. PCR products of positive clones were subjected to amplified ribosomal DNA restriction analysis (ARDRA) (Lagacé et al. 2004) with *HaeIII* and *HhaI* (TaKaRa). Clones with identical ARDRA profiles were classified into one operational taxonomic unit (OTU). Representative clones, belonging to different OTUs, were selected for sequencing. Clone libraries were statistically evaluated by rarefaction analysis using software of Analytic Rarefaction 1.3 (http://www.uga.edu/strata/software/Software.html) in which the expected number of different ARDRA groups vs. the number of positive clones in each library were calculated.

Sequencing was performed on an ABI PRISM 3730 DNA sequencer (SinoGenoMax Co., Ltd., Beijing, China). The obtained sequences were manually checked and edited using DNAMAN version 5.2.2.0. The partial sequences were submitted to the GeneBank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.gov) employing the Basic Local Alignment Search Tool algorithm to roughly determine their phylogenetic affiliation. The sequences being the nearest neighbours of the submitted sequences were cited from the GeneBank in order to construct neighbour-joining trees using DNAMAN software, version 5.2.2.0.

Nucleotide sequence accession numbers

16S rRNA and Methanotrophic pmoA gene sequences, submitted to the GeneBank, have been assigned accession numbers: GU056042-GU056081 (DGS1), GU056082-GU056120 (DGS2), GU056121-GU056131 (DG0.5), GU056132-GU056139 (DG1), GU056140-GU056150

(DG1.5), GU056151-GU056157 (DG2), GU056158-GU056164 (DG2.5).

Results

Methane-oxidizing bacterial and methanotrophic abundance

Methane-oxidizing, bacterial CFU/g soil and methanotrophic *pmoA* gene copy numbers, quantified using a MPN procedure and RT-PCR, respectively, are shown in Fig. 3. According to the culture-dependent method, the bacterial counts of methane-oxidizing bacteria in the samples of spot A decreased gradually (depth-on-depth) from 3.4×10^5 (0.5 m) to 2.7×10^3 (2.5 m). The counts in samples of spot B displayed the same tendency, decreasing from 4.6×10^5 (0.5 m) to 3.4×10^3 (2.5 m). According to the culture-independent molecular approach used for soil samples in spot A, the methanotrophic *pmoA* gene copy numbers (copies/g soil) initially fell from 6.78×10^5 (0.5 m) to 3.92×10^5 (1 m) and then grew to the highest copy numbers, 8.10×10^5, in a soil sample of 1.5 m, followed by a sharp decrease to the lowest copy numbers: 1.47×10^4 (2 m). Finally, in a soil sample of 2.5 m, the methanotrophic *pmoA* gene copy numbers rose slightly to 1.90×10^5. The highest methanotrophic *pmoA* gene copy numbers, 8.32×10^5, and the lowest number, 5.49×10^4, in soil samples of spot B, simultaneously appeared in samples of 1.5 m and 2 m, respectively. The results, based on RT-PCR, indicated methanotrophs inhabited soil samples from a depth of 1.5 m show the most active growth and metabolism. Therefore, the optimum sampling depth of sampling sites in subsequent investigations of MPOG should be determined based primarily on RT-PCR data.

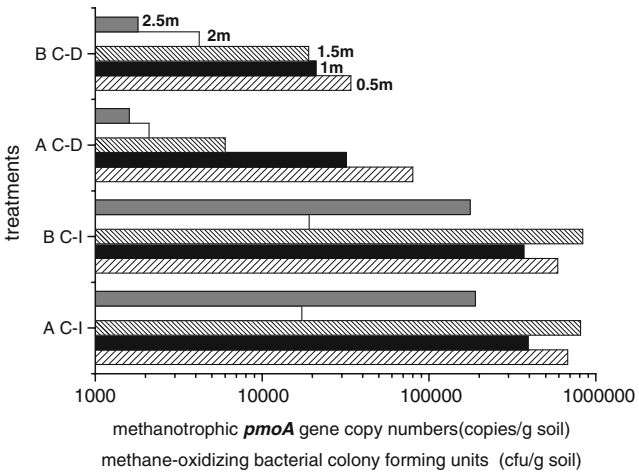

Fig. 3 Quantification of methane-oxidizing bacterial colony forming units (CFU/g soil) and methanotrophic *pmoA* gene copy numbers (copies/g soil) of soil samples collected from sampling sites A and B. C-D and C-I represent "culture-dependent" and "culture-independent," respectively. Numbers above the bars display the depths of samples

Analysis of 16S rRNA gene libraries

According to ARDRA of 300 positive clones, two bacteria libraries, DGS1 (soil sample of 1 m in sampling site A) and DGS2 (soil sample of 2 m in sampling site A), revealed diverse bacterial populations including 40 and 39 unique Phylotypes, respectively. Rarefaction curves of the two libraries tended to approach the saturation plateau indicating that 150 positive clones in each library could well cover the diversity of bacteria (Fig. 4). The closest bacteria affiliated with the DGS1 and DGS2 sequences were partially similar to each other; *Chloroflexi*, *Gemmatimondetes*, *Acidobacteria* and *Actinobacterium* could be found in the both libraries, and *Chloroflexi* (23% and 19%, respectively) and *Gemmatimondetes* (28% and 10%, respectively) were predominant in the both libraries. In addition, other dominant bacteria were *Rhodospirillaceae* and *Actinobacterium* covering 21% and 9%, respectively, in DGS1, and sulfur-oxidizing bacteria and *Acidobacteria* occupying 12% and 10%, respectively, in DGS2. The phylogenetic tree (Figs. 5 and 6) displays all of the nearest neighbours of the submitted sequences.

Phylogenetic analysis of *pmoA* gene clones

A total of 61 clones representing various OTUs were sequenced and subjected to phylogenetic analyses. All sequences were aligned with the GeneBank database and identified as *pmoA* gene sequences (508 bp). A phylogenetic analysis of methanotrophic *pmoA* genes, retrieved in this study, displayed clone sequences and were affiliated with *Methylosinus*, *Methylocystis*, *Methylomonas*, *Methylocaldum* and *Methylobacter* (Fig. 7). However, the majority of the sequences, in particular those belonging to deeper

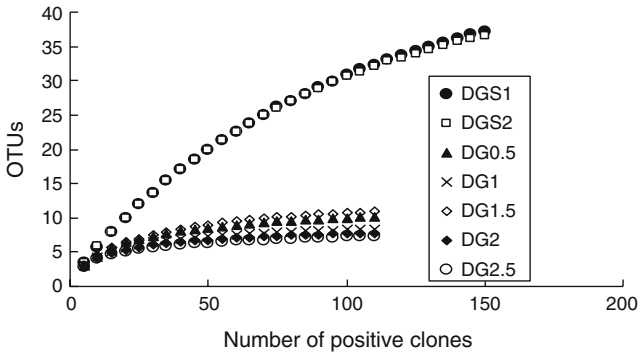

Fig. 4 Rarefaction analysis of 16S rRNA gene positive clones for clone libraries of DGS1, DGS2 and *pmoA* gene positive clones for clone libraries of DG0.5, DG1, DG1.5, DG2, DG2.5 using software of Analytic Rarefaction

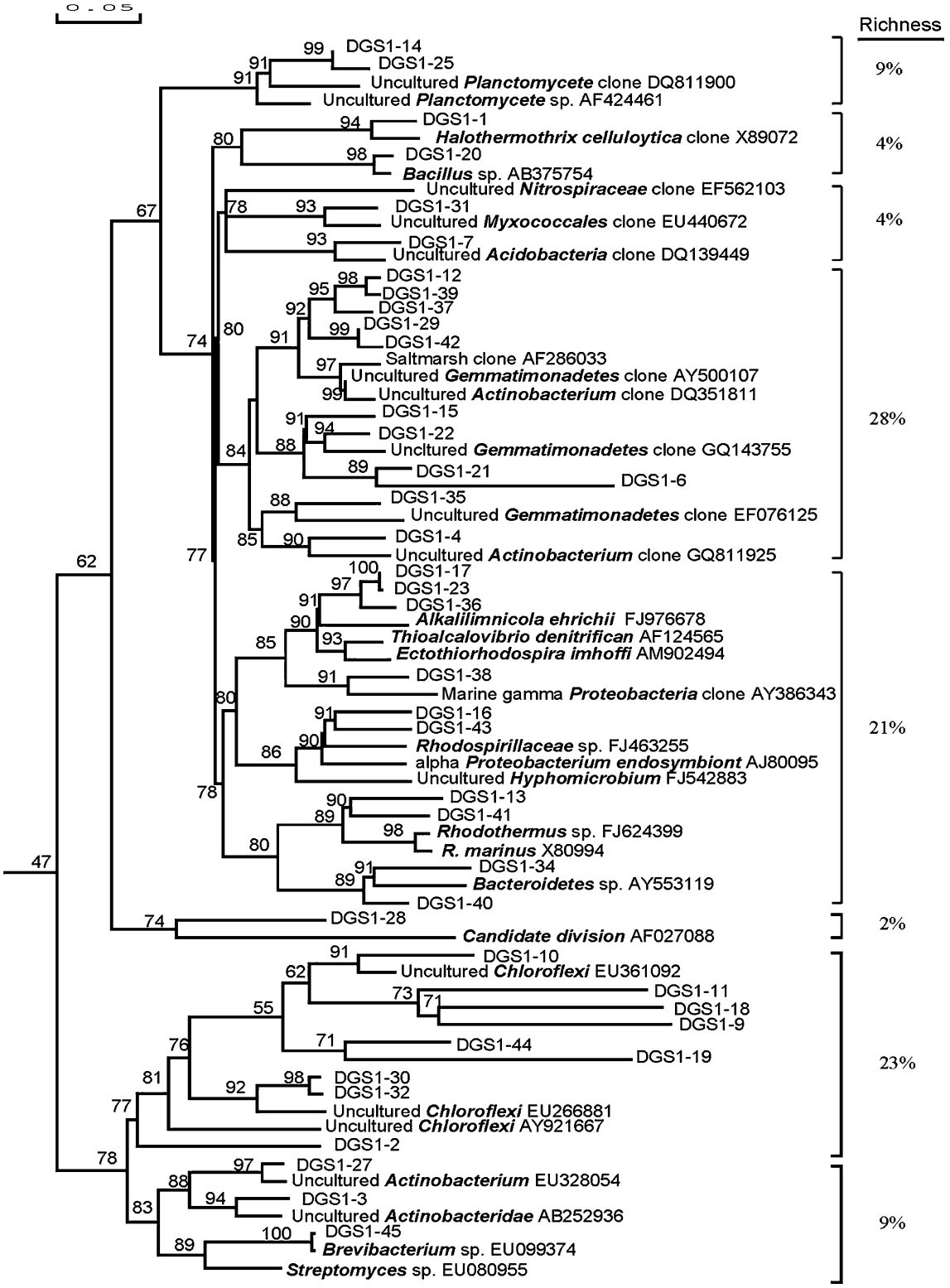

Fig. 5 Phylogenetic tree of bacteria 16S rDNA phylotypes of DGS1. The tree constructed was based on approximately 1450 bp 16S rRNA genes and their nearest clones retrieved from the GeneBank database. The percentages behind the branches represent the richness of each branch. Bootstrap values (>50%) are indicated at branch points. The scale bar represents 5% estimated sequence divergence

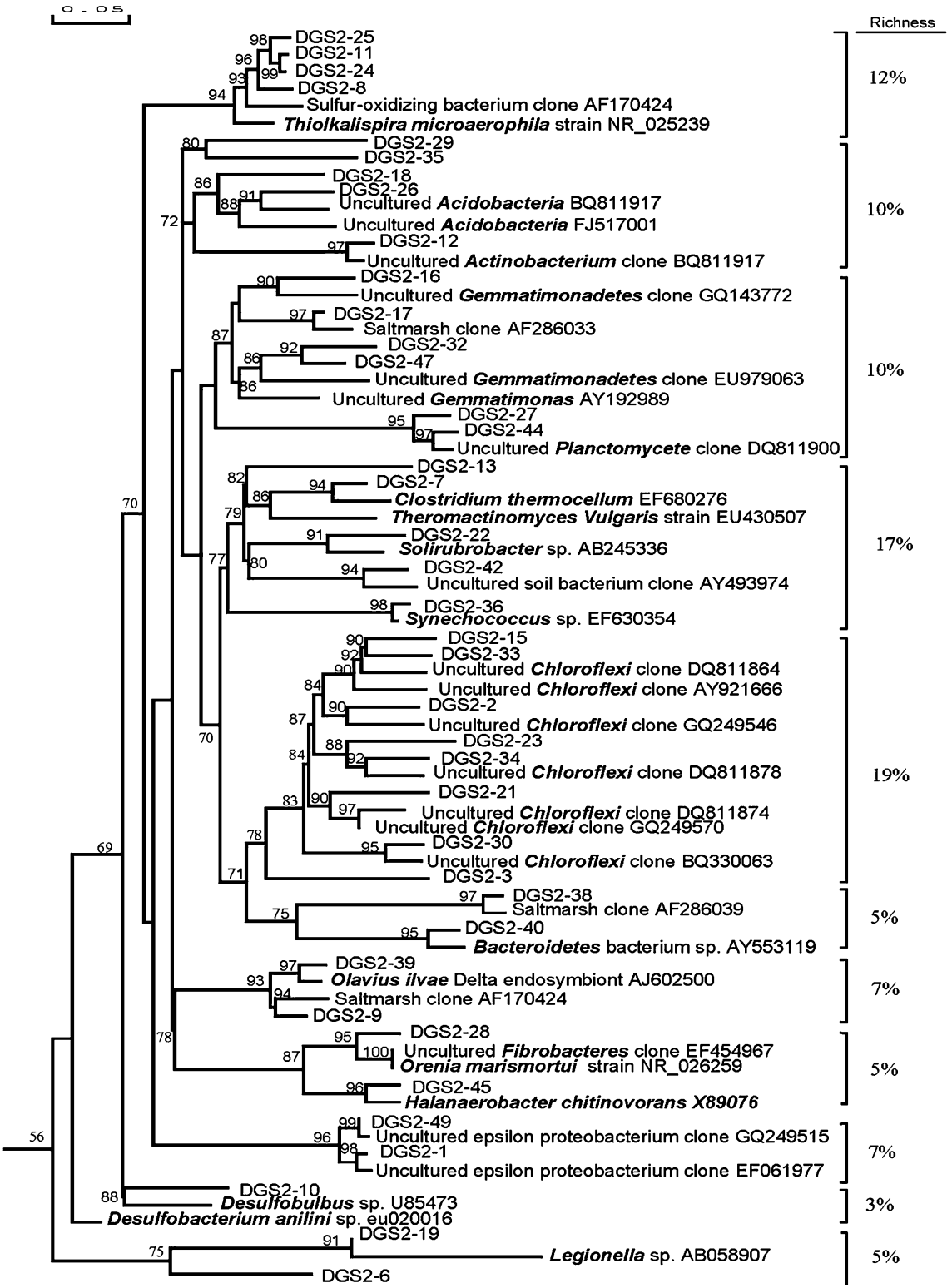

Fig. 6 Phylogenetic tree of bacteria 16S rDNA phylotypes of DGS2. The tree constructed was based on approximately 1450 bp 16S rDNA genes and their nearest clones retrieved from the GeneBank database. The percentages behind the branches represent the richness of each branch. Bootstrap values (>50%) are indicated at branch points. The scale bar represents 5% estimated sequence divergence

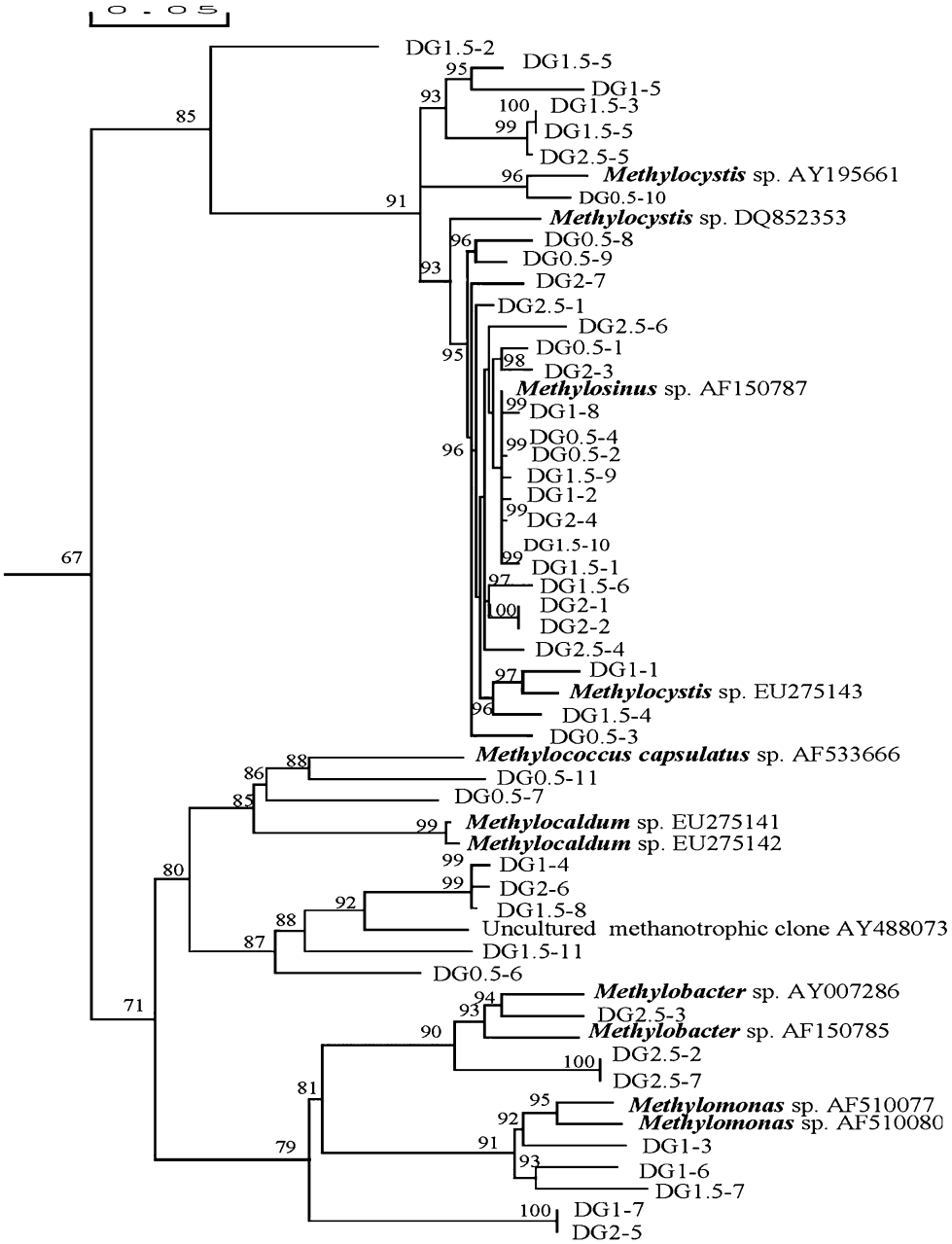

Fig. 7 Phylogenetic tree showing the relationships between representative clones of five libraries in this study and cultured methanotrophs. The tree constructed was based on 498~508 bp methanotrophic *pmoA* genes. Clones retrieved from the GeneBank database are indicated by bold-italics with their accession numbers. Bootstrap values (>50%) are indicated at branch points. The scale bar represents 5% estimated sequence divergence

soil samples, had a relatively low similarity (83–96%) to the sequences of known isolated methanotrophs, which indicated that the majority of methanotrophs in the soil samples are uncultured bacteria.

Methanotrophic *pmoA* gene clone libraries

According to the profiles of the ARDRA, the distribution of methanotrophs, in the same depth of soil samples in spots A and B, were basically identical, and thus, five clone libraries (DG0.5, DG1, DG1.5, DG2 and DG2.5) based on the methanotrophic *pmoA* gene found in soil samples belonging to spot A were constructed as representatives. The clone libraries contained 11, 8, 11, 7 and 14 OTUs, respectively. Rarefaction curves of the five libraries indicate that number of positive clones in each library were sufficient (Table 1 and Fig. 4). The methanotrophic communities varied with the increasing depth of soil samples; *Methylosinus*-affiliated sequences appeared in all libraries and occupied certain dominant status of 76%, 28%, 59%, 87% and 38% in the DG0.5, DG1, DG1.5, DG2 and DG2.5 libraries, respectively. With the increasing depth of soil, *Methylocystis*-affiliated sequences in libraries rose initially from 11% (DG0.5) to 30% (DG1), reached 31% in the DG1.5 library, dropped to 4% in the DG2 library and disappeared in the DG2.5 library. However, *Methylobacter*-affiliated sequences were clearly detected in the DG2 library (58%). The methanotrophic diversity of libraries is

Table 1 Closest cultivated relatives of clones from five clone libraries

Library Positive clones	Phylotype[a]	Richness of library[b] (%)	Closest cultivated bacteria in GeneBank database	Similarity (%)
DG0.5 93	DG0.5-1	19	*Methylosinus* sp. AF150787	98
	DG0.5-2	16	*Methylosinus* sp. AF150787	99[c]
	DG0.5-3	26	*Methylosinus* sp. AF150787	95
	DG0.5-4	10	*Methylosinus* sp. AF150787	99[c]
	DG0.5-5	9	*Methylocystis* sp. AY195661	93
	DG0.5-6	10	*Methylococcus capsulatus* AF533666	85
	DG0.5-7	2	*Methylocaldum* sp. EU275141	85
	DG0.5-8	3	*Methylosinus* sp. AF150787	95
	DG0.5-9	1	*Methylosinus* sp. AF150787	96
	DG0.5-10	1	*Methylocystis* sp. AJ459046	96
	DG0.5-11	1	Uncultured type I methanotroph clone 0507-G20-37 EU275115	93
DG1 94	DG1-1	30	*Methylocystis* sp. EU275143	96
	DG1-2	27	*Methylosinus* sp. AF150787	99
	DG1-3	11	*Methylomonas* sp. AF510077	92
	DG1-4	14	Uncultured methanotrophic proteobacterium clone B33 AY488073	92
	DG1-5	9	*Methylosinus* sp. AF510081	91
	DG1-6	5	*Methylomonas* sp. AF510080	91
	DG1-7	2	*Methylobacter* sp.AF150785	83
	DG1-8	2	*Methylosinus* sp. AF150787	99
DG1.5 95	DG1.5-1	33	*Methylosinus* sp. AF150787	99
	DG1.5-2	13	*Methylocystis* sp. AF150791	83
	DG1.5-3	11	*Methylocystis* sp.DQ 852353	92
	DG1.5-4	17	*Methylosinus* sp.AF150787	96
	DG1.5-5	6	*Methylocystis* sp.DQ 852353	92
	DG1.5-6	5	*Methylosinus* sp.AF150787	97
	DG1.5-7	4	*Methylomonas* sp.AF510077	90
	DG1.5-8	5	Uncultured methanotrophic proteobacterium clone B77 AY488075	92
	DG1.5-9	2	*Methylosinus* sp. AF150787	99
	DG1.5-10	1	*Methylosinus* sp. AF150787	100
	DG1.5-11	1	*Methylocaldum* sp.EU275142	82
DG2 95	DG2-1	19	*Methylosinus* sp. AF150787	97
	DG2-2	39	*Methylosinus* sp. AF150787	97
	DG2-3	21	*Methylosinus* sp.AF150787	98
	DG2-4	6	*Methylosinus* sp.AF150787	99
	DG2-5	9	*Methylobacter* sp.AF150785	83
	DG2-6	4	Uncultured methanotrophic proteobacterium clone A19 AY488076	91
	DG2-7	2	*Methylosinus* sp. AF150787	96
DG2.5 93	DG2.5-1	28	*Methylosinus* sp. AF150787	93
	DG2.5-2	27	*Methylobacter* sp. AY007286	90
	DG2.5-3	30	*Methylobacter* sp. AY007286	94
	DG2.5-4	7	*Methylosinus* sp. AF150787	97
	DG2.5-5	4	*Methylocystis* sp. DQ852353	93
	DG2.5-6	3	*Methylosinus* sp. AF150787	96
	DG2.5-7	1	*Methylobacter* sp. AY007286	90

[a] Phylotype, each OUT in clone libraries represents one phylotype.

[b] Richness of library, the percentage of clone numbers of each OTU occupied the whole positive clones

[c] the values of similarity are same, but identities were gained from different numerical calculations (500/504; 503/504)

shown in Table 1. According to the analysis of methanotrophic *pmoA* gene clone libraries, detecting the anomalies of *Methylosinus* and *Methylocystis*, in particular the latter, enables them to be used as indicators for MPOG in the studied region. As a result, constructing methanotrophic *pmoA* gene clone libraries to analyze their diversity allows for the probability of improved success of using methanotrophs when applied to MPOG in other unknown areas.

Discussion

The important breakthroughs of this study were the application of culture-independent microbial molecular techniques for MPOG. Compared with traditional culture-dependent geo-microbial methods, the techniques used in this study are far more time-effective and advanced, and the obtained results were far more reliable and comprehensive.

Data reported in International Panel on Climate Change (2001) suggested that methanotrophs are active in upland soils in forests, grasslands and rice fields and account for 6% of the global atmospheric CH_4 sink. To a certain extent, they increase the activity of methanotrophs inhabiting upland soils and interfere with the application of MPOG. Therefore, soil samples, in most of cases of MPOG, were collected from a depth of 1 or 1.5 m (Wagner et al. 2002; Rasheed et al. 2008). However, the number of methanotrophs in soil samples of different depths is diverse. Targeted bacteria, regarded as indicators for MPOG, display their highest activity in optimum growing environments. In order to determine the optimum sampling depth in this study, soil samples from vertical depths of 0.5, 1, 1.5, 2 and 2.5 m, in the two sampling spots, were collected.

Bacteria detected from the soil samples at a depth of 1 and 2 m were primarily affiliated with *Chloroflexi*, *Gemmatimondetes*, *Acidobacteria*, *Actinobacterium* and *Rhodospirillaceae*, which were commonly found in samples associated with marine and alkaline environments (Miller et al. 2009; Li et al. 2009; Pearson and Rusch 2009). Appearance of these bacteria matched the geographical position and the characteristics of the sampling sites where they inhabit. However, there were few clones affiliated with the targeted bacteria, methanotrophs, which indicated that methanotrophs were inferior in the microbial communities.

In order to detect the methanotrophs and compare the advantages and disadvantages between traditional methods and molecular techniques for MPOG, bacterial counts of targeted bacteria were conducted using a culture-dependent MPN procedure and a culture-independent RT-PCR method, respectively. Results from the two methods displayed enormous discrepancies. The number of methane-oxidizing bacteria from the same samples, measured using a MPN procedure, are far fewer than those quantified by RT-PCR, with a quantify variance of one or two orders of magnitude. Additionally, the methane-oxidizing bacterial CFU/g soil, counted using the MPN procedure, decreased gradually (depth-on-depth) with the highest numbers in soil samples from 0.5 m, while methanotrophic *pmoA* gene copy numbers from soil samples of 0.5 to 2.5 m, enumerated using RT-PCR, initially decreased and then increased and decreased again; methanotrophs in soil samples of 1.5 m are the highest. Reasons for the discrepancies are: (1) Numerous studies have proven that we are currently aware of only a minor part of the diversity of microorganisms in nature (Amann et al. 1995; Muyzer et al. 1993). Therefore, a considerable number of methane-oxidizing bacteria are unable to grow during the culture-dependent MPN procedure. Thus, RT-PCR is able to amplify cultured and uncultured methanotrophs, which resulted in a large quantify variance between the two methods. (2) With increasing depth, simulating the inhabited environment of bacteria in a laboratory becomes more and more difficult. It is inevitable that the number of targeted bacteria, counted using a MPN procedure, would decrease gradually (depth- on-depth).

In this study, the phylogenetic analysis of the methanotrophic *pmoA* gene shows a clear picture of methanotrophic diversity with *Methylosinus*, *Methylocystis*, *Methylomonas*, *Methylocaldum* and *Methylobacter,* as found in soil samples. However, during a previous isolating process of methanotrophs for MPOG, it was difficult to culture the targeted bacteria because of their high specialization. Most methane oxidizers, isolated using culture-dependent geo-microbial methods, were from several other microorganisms (Wagner et al. 2002). In addition, most sequences have a low similarity (90–96%) with the sequences of cultured sequences, which further proved that methanotrophs are difficult to be cultured in experimental environments. In contrast to traditional isolating methods, the phylogenetic analysis of the methanotrophic *pmoA* gene, as used in this study, is more reliable for discovering the methanotrophic communities of investigated areas.

Methanotrophs exist in various environments with a diversify distribution of methanotrophic communities according to the geographical, compositional and physico-chemical differences of soils (Kolb et al. 2005; Mohanty et al. 2006). The construction of methanotrophic *pmoA* gene clone libraries help us unmask which genera of bacteria is dominant, which is minor in natural samples and which is valuable in finding bacterial anomalies and guides the application of MPOG. Five libraries, constructed in this study, show specific discrepancies of methanotrophic communities of different depth soil samples, which indicated that *Methylosinus* and *Methylocystis* should be analyzed further as the indicating bacteria for MPOG.

However, for other applications of MPOG, new methanotrophic *pmoA* gene clone libraries must be constructed in order to find anomalous methanotrophs, which act as indicators of MPOG.

In this study, we only chose methanotrophs as the subject to be investigated based on the fact that 90% of natural gas in the studied reservoir is comprised of methane. However, other hydrocarbon-oxidizing bacteria that use light hydrocarbons, namely ethane, propane and butane, representing inclusive substrates, were also studied in certain works regarding MPOG (Beghtel et al. 1987; Rasheed et al. 2008). According to previous articles, methanotrophs were widely used for MPOG (Baum et al. 1997; Wagner et al. 2002).

In conclusion, taking into account some disadvantages, culture-independent microbial molecular techniques based on the methanotrophic *pmoA* gene are a new progression in the application of MPOG. Culture-independent microbial molecular techniques will improve the accuracy rate of MPOG.

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