BACTERIAL AND ARCHAEAL DIVERSITY IN ABYSSAL COLD SEEP SEDIMENTS FROM THE DONGSHA AREA OF THE SOUTH CHINA SEA

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ABSTRACT
Microbial diversity of an active cold seep from the Dongsha area of South China Sea was characterized by collecting a 5.5-m core and by using 16S rRNA gene analysis. DNA was extracted from three vertical zones (top, middle and bottom) of the core and the 16S rRNA gene was amplified by polymerase chain reaction (PCR), cloned and sequenced. Six clone libraries were constructed with a total of 395 sequences. Pore water chemistry suggested a coupled reaction between sulfate reduction and methane oxidation. Phylogenetic analysis indicated presence of sulfate-reducing bacteria and anaerobic methane-oxidizing archaea. The archaeal diversity was high at the surface, but decreased sharply with increased depth. Marine Benthic Group (MBG)-D and halobacteriales were the dominant groups at the surface sediments, and the MBG-B group became predominated with increased depth, reaching 38.9% and 62.5% in the middle and bottom sediments, respectively. Similarly, the bacterial diversity was higher at the top and decreased with depth. Proteobacteria was the dominant group in the top of the core, but became minor deeper within the sediments. With increased depth, Chloroflexi and JS1 became predominant groups and reached up to 78.8% and 70.3%, respectively. Overall, our results suggested that microbial diversity in South China Sea cold seep was high and community structure and diversity changed greatly with depth, in response to changes in the physical and chemical conditions of the sediments.

Keywords: microbial diversity, cold seep, gas hydrates, South China Sea

1. INTRODUCTION
The South China Sea (SCS) is one of the marginal seas around the Pacific Ocean. Tectonically, it is a passive margin setting grading into the SCS Basin and it abuts on the accretionary wedge formed offshore with the south-western Taiwan Island. During the past decades, a number of geophysical and geological cruises were performed in this area, and old and/or active methane seepages were found in the Dongsha area of the SCS[1]. These cruises also showed the presence of gas hydrates, and gas hydrate samples were obtained by drilling in the Shenhu area of the northern SCS in 2007.

A few studies examined microbial communities in the SCS. The results showed that archaeal and bacterial diversities of the SCS sediments were similar to those in other deep-sea sediments[2,3]. However, little is known about the microbial community composition and its correlation with geochemical conditions in cold seep sediments in the SCS. This knowledge is of great importance for understanding the biogeochemical processes in

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globe cold seep ecosystems. The objective of this study was to study microbial communities in sediments of a gravity piston core (Core DSH-1) collected from an abyssal methane seepage area in the northern SCS.

2. MATERIALS AND METHODS

2.1 STUDY AREA AND SAMPLES

The study area is located at the end of the Formosa Canyon (FC) in the South-Western Taiwan Basin in the northern SCS. The FC has developed along the northwest part of the LRTF [Luzon-Ryukyu Transform Fault \(^7, 8\)]. The abyssal plain in and adjacent to the FC, with a water depth ranging from 2900 m on flanks to 3400 m at the center of the FC channel (Fig. 1), is covered by sediments characterized by silty clay interbedded with turbidities.

Since the first evidence of cold seeps was discovered by the Chinese research vessel “Haiyang 4”, this area was named as “Haiyang 4” area by the “SONNE” 177 cruise (SO177), a cruise of the cooperative research project between Chinese and German leading marine research institutions, i.e. the Guangzhou Marine Geological Survey and Leibniz-Institut für Meereswissenschaften Kiel \(^9, 10\).

In summer 2006, a 5.55-m sediment gravity piston core DSH-1 was collected from the water depth of 3016 m during the “Haiyang 4” cruise.. Lithologically, Core DSH-1 is composed of silty clay interbedded with turbidite layers. Onboard geochemical analyses showed a sharp increase of methane concentration from 2.1 to 20.4 μM at a depth of about 400 cmbsf at Core DSH-1 \(^11\).

2.2 METHODS

Core DSH-1 was immediately dissected into 50 cm sections onboard as soon as it was retrieved. The first 5 cm of each section was cut for microbial analyses. Approximately 1 cm\(^3\) sediments were taken from the top of the 5-cm sections with a syringe and added into a sterilized bottle with 9 ml filtered (0.22-μm) seawater. A 4% (v/v) formaldehyde solution was added to the bottle to fix the cells and the bottle was stored at 4 °C until cell counting. Then the 5-cm sections of sediments were stored in liquid nitrogen for microbial molecular work.

Total microbial cells in the sediments were counted by using the method of acridine orange direct count (AODC) as previously described \(^12\).

Three sediment subsamples (DSH1, 5-10 cm depth at the top; DSH9, 400-405 cm depth in the middle, and DSH12, 550-555 cm depth at the bottom) were selected to correlate microbial community structure with these geochemical results. Community DNA was extracted from 0.5 to 1.0 g sediments by using UltraClean soil DNA extraction kit (MoBio, Solana Beach, Calif., USA) according to the manufacturer’s instructions.

The archaeal 16S rRNA gene was amplified with primers Arch21F (5’-TTC YGG TTG ATC CYG CCR GA-3’) and Arch958R (5’-YCC GGC GTT GAM TCC ATT T -3’) \(^13\). The bacterial 16S rRNA gene was amplified with primers Bac27F (5’-AGA GTT TGA TCM TGG CTC AG-3’) and Univ1492R (5’-CGG TTA C CT TGT TAC GAC TT-3’) \(^14\). A typical PCR mixture (25 μl volume) contained 1.5 mM MgCl\(_2\), 1×Tag buffer, each
deoxynucleoside triphosphate (dNTP) at a concentration of 200 μM, each primer at a concentration of 0.2 μM, 1.25 U of Taq DNA polymerase (Takara, Japan), and 0.5 to 1 μl of DNA extract. The following standard conditions were used for bacterial and archaeal 16S rRNA gene amplifications: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 and 54 °C (for bacteria and archaea, respectively) for 30 s, and extension at 72 °C for 2 and 1.5 min (for bacteria and archaea, respectively); and a final extension at 72 °C for 10 min. All PCRs were run in triplicate. PCR products were examined by electrophoresis on a 1% agarose gel.

PCR products were purified using a Gel Spin DNA purification kit (AXYGEN, USA), then ligated into the pGEM-T Easy Vector (Promega, USA) and transformed into Escherichia coli JM109 competent cells. Six clone libraries (three each for bacteria and archaea) were constructed. Recombinants were selected using LB indicator plates containing 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) with 100 μg/ml ampicillin and incubated overnight at 37 °C. Colonies were randomly selected and analyzed for the 16S rRNA gene inserts. Inserts were amplified using forward primer M13-RV (5’-CAG GAA ACA GCT ATG AC-3’) and reverse primer M13-47 (5’-GTT TTC CCA GTC AC GAC-3’). PCR reactions system was same as described above with the following PCR conditions: 94 °C for 10 min; 34 cycles of 94 °C for 30s, 52 °C for 30 s, 72 °C for 90s, with a final elongation step of 72 °C for 10 min. The randomly selected clones were sequenced (with primers Arch21F and Bac27F for archaea and bacteria, respectively) using with the BigDye Terminator version 3.1 chemistry (Applied Biosystems, Foster City, CA, USA). The 16S rRNA gene sequence was determined with an ABI 3730 automated sequencer. Nucleotide sequences were assembled and edited by using Sequencer v.4.8 (GeneCodes, Ann Arbor, MI).

The potential presence of chimeric sequences was examined with Bellerophon [15]. The secondary-structures of all obtained sequences were analyzed using the Vienna RNA Package [16]. Potential chimeric sequences were removed. The chimera-free sequences were blasted in the GenBank (http://www.ncbi.nlm.nih.gov). Operational taxonomic units (OTUs) were determined using DOTUR [17] with a 97% cutoff value. Based on dissimilar distance and pairwise comparisons, neighbor-joining phylogenetic trees were constructed from with the Jukes-Cantor distance model using the MEGA (molecular evolutionary genetics analysis) program, version 4.1. Bootstrap replications of 1000 were assessed. The sequences determined in this study were deposited in the GenBank database under accession numbers: GU475193-GU475255 and GU475256-GU475365 for archaea and bacteria, respectively.

The coverage (C) was derived from the equation C=1-(n/N), where n is the number of clones that occurred only once, and N is the total number of clones examined [18]. Rarefaction curves were calculated by the DOTUR program [17].

3. MAIN RESULTS

3.1. DOWN CORE VARIATION OF MICROBIAL ABUNDANCE

AODC results showed that the abundance decreased from 2.3 × 10^6 cells/g (wet weight) near the top to 0.5 × 10^6 cells/g at 200 cm depth, followed by an increase to the maximal value of 9.6 × 10^6 cells/g near the bottom. The trend corresponded to the methane concentration variation throughout the core, which was about 2.1 μM at the top of the core, and increased with depth, reached to > 20.4 μM at the bottom of the core (Fig. 2).

3.2. MAIN RESULTS OF CLONE LIBRARY

A total of 213 archaeal 16S rRNA gene clone sequences (104, 77, and 32 for DSH1A, DSH9A, and DSH12A, respectively) and 182 bacterial 16S rRNA gene clone sequences (112, 33, and 37 for DSH1B, DSH9B, and DSH12B, respectively) were subjected to phylogenetic analyses. The numbers of the sampled clones represented 43.8%-100% coverage for the clone libraries. Rarefaction curves indicated that the number of OTUs for the archaeal library for the middle and bottom samples nearly reached saturation (Fig.3 A), but it was not enough for the surface sample, especially for the bacterial library (Fig. 3 B).
Archaeal diversity decreased rapidly with depth along the core (Fig. 3A), from fifty-four OTUs at the top to five and four OTUs at the middle and bottom, respectively. Bacterial diversity showed a similar trend with depth. In the top sediments, eight-five OTUs were observed, but the number of OTUs decreased to fifteen and ten for the middle and bottom sediment samples, respectively. Among the identified groups, MBG-D and halobacteria (21.2% each) were the predominant groups for archaea and *Proteobacteria* (25.9%) for bacteria for the top sediment sample. With depth, the MBG-B group became predominant, and reached 38.9% (middle) and 62.5% (bottom) for the middle and bottom samples, respectively. For bacteria, *Chloroflexi* and JS1 groups became dominant, and reached 78.8% and 70.3% for the middle and bottom samples, respectively.

**4. DISCUSSION**

**4.1. LOW MICROBIAL ABUNDANCE**

Microbial abundance in the DSH-1 core was lower than other SCS areas, which were typically around $10^7$ cells/g counted by AODC [4] and $10^9$ cells/g counted by fluorescence in situ hybridization (FISH) [5], and a few of other cold seep sediments,
such as Nile Deep Sea Fan, which was typically around $10^9$ cells/g counted by FISH \[19\]. But the microbial abundance in the DSH-1 core was a little higher than those sites ($< 1.5 \times 10^6$ cells/g sediments), which retrieved from the shallow water cold seep area far from the seep vent in the Dongsha area \[20\].

In addition, the depth-related variation of the microbial abundance showed the same trend as the methane concentration profile (Fig. 3). This positive correlation suggested that microbes seem to be sensitive to variations of methane concentration and they may be using methane as an energy source.

### 4.2 HIGH MICROBIAL DIVERSITY

High archaeal and bacterial diversity reflected in ten phylotypes of archaea and fifteen phylotypes of bacteria detected at the 5-10 cm depth in this core. In contrast, at the Qiongdongnan Basin sediments of the SCS, just two bacterial phylotypes and six archaeal phylotypes were detected \[4\]. In Xisha Trough at about 10 cmbsf, eight bacterial and seven archaeal phylotypes were detected \[8\]. At Shenhu \[3\] and Nansha \[21\] surface sediments, there were seven bacterial and six archaeal phylotypes were detected, respectively.

In western Pacific, like Nankai Trough cold seep surface sediments, only *Proteobacteria*, *Spirochaetaceae-Cytophaga*, gram-positive bacteria, an unknown group bacteria, MGI, and ANME archaea were detected \[23\]. In Sagami Bay, three bacterial and a few archaeal groups were found \[23\]. In Japan Trench, three phylotypes of bacteria, *Proteobacteria*, *Cytophaga*, and gram-positive bacteria, and MGI and some methanogenic archaea were detected \[24\]. At Florida Escarpment surface sediments, five phylotypes of bacteria and one group of *Methanosarcinales* and 3% *Crenarchaeota* were discovered \[25\]. In the Gulf of Mexico hydrocarbon seep, several bacterial phylotypes *Proteobacteria*, *Firmicutes* and *actinobacteria* and archaeal phylotypes *methanosarcinales* and *thermoplasmalae* were found \[26\].

We were unable to explain the differences at the present time, and could only roughly assume that the high microbial diversity in studied core might be controlled by much complex geochemical or micro-ecological conditions in the cold seep area of the northern SCS.

### 4.3 MAJOR GROUPS AND THEIR ROLES IN BIOGEOCHEMICAL PROCESSES

Archaeal group MBG-B was originally found at hydrothermal vent sites \[27\] and in present in a number of deep subsurface and hydrothermal vent sites \[27\]. Subsequently, this group was reported from deep sediments in hydrate zones at Nankai Trough, Peru continental shelf, and the sea of Okhotsk \[28-30\]. Inagaki et al (2006) showed that clone libraries from hydrate-bearing sites of the Cascadia and Peru Margins were dominated by MBG-B especially in the sulfate reducing zone in shallow sediments above gas hydrates. Thus, this group was proposed to play an important role in biogeochemical processes such as sulfate reduction and methane oxidation \[31\]. In this study, the percentage of the MBG-B increased with depth, and reached 38.9% and 62.5% in the middle and bottom of the core, respectively. Simultaneously, at these depths the chlorid ion showed a negative abnormity (less than seawater average value) and the methane concentration showed a sharp increase (Fig. 3). These microbial and the geochemical parameters illustrated that the geochemical environments was comparable to the gas hydrate sediments in Cascadia and Peru Margins \[32\]. There might be an upward methane diffusion from the sediments below.

Bacterial major groups were *Chloroflexi* and candidate division JS1. *Chloroflexi* was dominant in the middle sample and was a significant fraction in the bottom sample. This phylum has been recognized as a typical bacterial cluster containing a number of diverse environmental clones \[33\]. It is a deep-branching lineage of bacteria and can be divided into at least six major classes: *Anaerolineae*, *Caldilineae* (Subphylum I), ‘*Dehalococcoidetes*’ (Subphylum II), *Chloroflexi* (Subphylum III), a clone cluster called subphylum IV, and *Thermomicrobia* \[34\]. The 16S rRNA gene sequences obtained from marine sediments are frequently present within the classes *Anaerolineae*, *Caldilineae*, ‘*Dehalococcoidetes*’, and the unclassified subphylum IV \[29, 30, 32, 35, 36\]. Within
these classes, only a few representatives are obtained in culture, and thus characteristics about their metabolic functions remain limited [34]. However, based on relatedness of clone sequences to cultures, Inagaki et al (2006) inferred that closely related members of heterotrophic Chloroflexi living in marine sediments may grow syntrophically with hydrogenotrophic Chloroflexi species or other hydrogenotrophic microbes such as methanogens. Albeit speculative, the dominance of Chloroflexi clones in our study may be due to the presence of abundant methane at the investigated locations. Webster et al (2007) showed that Chloroflexi and candidate division JS1 seem to be well suited to life in the subsurface and are often associated with organic-rich sediments. JS1 was another predominant group of the bacterial libraries, especially at the bottom sample where there was abundant methane. This group has previously been found in methane-rich subsurface sediments where gas hydrates are present [32], suggesting that these bacteria may prefer sedimentary habitats with high concentrations of methane associated with hydrates [32]. This discovery suggests that some members of this division may be associated with methanogenic consortia and that others are adapted to or can tolerate high pressures [37]. However, representatives of this group have not been cultivated as yet, and thus their metabolic pathways remain unknown [34]. Only indirect evidence from stable-isotope probing exists suggesting that members of this clade are able to incorporate acetate and glucose (or glucose metabolite) under anaerobic, sulfate-reducing conditions [38].

Previous studies [32, 39] indicated that Chloroflexi and JS1 bacteria occur widely in organic-rich deep marine sediments. TOC in this area was low. But Chloroflexi and JS1 bacteria were abundant below 400 cm in this core. That might indicate that the presence of these two groups bacteria have no direct relation with TOC content. Microbial communities can be stratified in deep marine sediments, and surrounding geochemical and geological settings strongly affect the community structure.

As discussed above, although the metabolic functions of the MBG-B group of archaea, and Chloroflexi and JS1 of bacteria are largely unknown, the recognition of microbial populations that consistently occur in the presence of methane hydrates serves as a starting point for defining their ecological and biogeochemical significances [32]. So the retrievability of these uncharacterized phylogenetic clades in our study might further corroborate that metabolic functions of these microorganisms are related to methane seepage.

In conclusion, our data indicated a high microbial diversity in the cold seep at the northern SCS, especially in the surface sediments, in contrast with other areas of the SCS, and with other cold seeps worldwide. The major groups were consistent with those previously detected in various cold seep environments, including methane/organic-rich and/or putative gas hydrate-bearing marine sediments [32]. Some unique phylotypes were also present in sediments studied. Combined geochemical and microbial data suggested a coupled reaction of sulfate-reduction and methane oxidation at this methane seepage site.

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REFERENCES
located at the base of the Florida Escarpment. Extremophiles 2006. 10 (3):199-211.


