

Microbial diversity in surface sediments of the Xisha Trough, the South China Sea

Li Tao*, Wang Peng, Wang Pinxian

State Key Laboratory of Marine Geology, Tongji University, Shanghai 200092, China

Abstract: Microbial communities were obtained from the surface sediments of the Xisha Trough using the culture-independent technique. The characteristics of the 16S rDNA gene amplified from the sediments indicated that archaeal clones could be grouped into Euryarchaeota and Crenarchaeota, respectively. Two archaeal groups, Marine Crenarchaeotic Group I and Terrestrial Miscellaneous Euryarchaeotal Group, were the most dominant archaeal 16S rDNA gene components in the sediments. The remaining components were related to the members of Marine Benthic Group B, Marine Benthic Group A, Marine Benthic Group D, Novel Euryarchaeotic Group and C3. The bacterial clones exhibited greater diversity than the archaeal clones with the 16S rDNA gene sequences from the members of Proteobacteria, Planctomycetes, Actinobacteria, Firmicutes, Chloroflexi, Acidobacteria, candidate division OP8, Bacteroidetes/Chlorobi and Verrucomicrobia. Most of these lineages represented uncultured microorganisms. The result suggests that a vast amount of microbial resource in the surface sediments of the South China Sea has not been known.

Key Words: Xisha Trough; microbial diversity; 16S rDNA; sediment

Deep marine sediments were estimated to contain a huge amount of biomass equivalent to one-third of the Earth's total biomass and up to 65% of the global prokaryotic biomass^[1,2]. But the habitats exhibit very low microbial activities^[3]. Little has been known about how important role the habitats play in the global material cycles. Although deep-sea hydrothermal vents have recently become the most interesting area to study the prokaryotic populations and activities in the extreme environments, a lot of studies have also been developed on deep-sea sediments in order to survey a huge amount of unknown microbial resources. Remarkably, the Ocean Drilling Program (ODP), since 10 years ago, has ever organized several cruises to study the populations and distributions of bacteria and archaea in marine sediments around the margin of the Pacific Ocean, including the Japanese Sea^[4], the Woodlark Basin (Leg 180)^[5], the Nankai Trough (Leg 190)^[6], the Peru margin (Leg 201)^[7] and the Cascadia margin (Leg 146)^[7,8]. Additionally, sediments of the Okhotsk Sea^[9] and the Mediterranean Sea^[10] were studied either. More than 99% of subseafloor prokaryotes are difficult or even unable to be cultured owing to the limitation of recent technologies. Therefore, using 16S

rDNA-based molecular technologies is the unique effective way to assess the diversity of prokaryotes in subseafloor sediments.

The South China Sea, near to the West Pacific "warm pool", is one of the marginal seas around the Pacific Ocean. Two research groups have ever assessed the bacterial diversities in Nansha sediments. Their results showed that the components of bacterial communities were similar^[11], and yet they were different^[12] from the bacterial communities in the adjacent marine sediments. However, both groups failed to construct a clone library sufficient enough for the real bacterial community. In this study, using 16S rDNA-based technologies, we estimated the diversities of bacteria and archaea in surface sediments of the Xisha Trough. More than 200 clones of bacterial and archaeal 16S rDNA gene were selected randomly and used to analyze restriction fragment length polymorphisms (RFLP). Among them, a total of 29 bacterial phylotypes and 18 archaeal phylotypes were identified. The phylogenetic analysis represented that the bacterial population exhibited higher diversity than the archaeal population.

1 Materials and methods

1.1 Sample collection

Sediment core MD05-2902 was collected from 3697 m water depth at Xisha Trough (17°57.70'N, 114°57.33'E) between May 15 and June 8, 2005 (IMAGS 147). The surface sediment sample (~0.1 m below seafloor) was obtained by a clean sterilized plastic tube. The sample was preserved at -20°C aboard and stored at -80°C after being transported to the laboratory.

1.2 Methods

1.2.1 DNA extraction and purification

Bulk DNA was extracted directly from the sediment sample by using the Zhou protocol with modifications^[13]. The DNA extraction procedure involved resuspending 5 g sediment sample (wet weight) in 13.5 mL extraction buffer (100 mmol/L Tris-HCl, 100 mmol/L EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 mol/L NaCl, 1% CTAB) and 100 µL protease K. The suspension was incubated at 37°C for 30 min and shaken at 225 r/min. Then, 1.5 mL 20% SDS was added and the mixture was mixed and incubated at 65°C for 2 h. Subsequently, samples were centrifuged for 20 min at 10000 r/min and the supernatants were removed to a new microcentrifuge tube. The DNA was precipitated by adding 1 volume of phenol: chloroform: isoamyl alcohol (25:24:1), followed by 1 volume of chloroform extraction. The DNA was then precipitated by adding 1 volume of isopropanol. Finally, the DNA was washed with 70% cold ethanol, air dried and resuspended in 100 µL sterile water. The DNA solution was purified by using QIAquick PCR purification kit (QIAGEN).

1.2.2 Construction and sequence analysis of 16S rDNA libraries

In order to amplify 16S rDNA gene, the oligonucleotide primers used were bacterial primers, Eubac27F (AGAGTTT-GATCMTGGCTCAG) and Eubac1492R (GGTTACCTTGT-TACGACT), and archaeal primers, Arch21F (TTCCGGTTG-ATCCYGCCGGA) and Arch958R (YCCGGCGT-TGAMT-CCAATT). 35 cycles of amplification of bacterial 16S rDNA gene were performed at 94°C denaturation for 1 min, at 55°C annealing for 1 min and at 72°C elongation for 1.5 min. Touch-down PCR amplification of archaeal 16S rDNA gene was used as follows: 10 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min; the annealing temperature decreased by 0.2°C after each cycle, followed by 30 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 1 min.

The PCR products were cloned into the pMD-18T Vector (TaKaRa). Primer sets were used to reamplify rDNA inserts from individual clones to generate template DNA for RFLP analysis by using *MspI* (Fermentas). Clones were group into operational taxonomic units (OTU), and plasmid DNA isolated from single representatives of each OTU was sequenced.

1.2.3 Phylogenetic analysis

The sequences were examined for chimeric sequences by

using the CHECK-CHIMERA program from RDP II (ribosomal database project), and the available sequences were aligned by BLASTN program (www.ncbi.nlm.nih.gov/BLAST). After aligning sequences by ClustalX (Version 1.8), the phylogenetic trees were constructed by the PAUP software (Version 4.0b10)^[14]. The phylogenetic relationships were determined by the Neighbor-Joining algorithm with Juke-Cantor corrections.

1.2.4 Accession number of nucleotide sequences

The sequences have been deposited in the GenBank database under accession numbers EU048590–EU048636.

2 Results

Bacterial and archaeal libraries of 16S rDNA were comprised of 119 bacterial clones and 124 archaeal clones, and 29 bacterial and 18 archaeal phylotypes (less than 98% sequence similarity) were identified. Several diversity indexes were calculated by using the SPADE software^[15] as follows: species richness was estimated to be 32 ± 3 for bacteria and 22 ± 5 for archaea by using the ACE-1 algorithm (95% confidence interval); Shannon index was assessed to be 3.087 ± 0.068 for bacteria and 2.312 ± 0.085 for archaea by using the maximum likelihood algorithm. It was obvious that the bacterial population exhibited greater diversity than the archaeal population. The coverage values of bacterial and archaeal libraries were up to 0.95 and 0.96, respectively, indicating that the clone libraries were sufficient to assess the diversities of prokaryotes. The estimation of the species richness exhibited that less than 1/5 of bacterial and 1/3 of archaeal phylotypes were left out of the libraries.

2.1 Analysis of the bacterial library

Except 2 unidentified phylotypes, the bacterial library fell into 8 groups (Fig. 1). Most of the sequences were affiliated to Proteobacteria (30.5% of the bacterial library), Planctomycetes (20.3% of the bacterial library), Actinobacteria (3.4% of the bacterial library), Firmicutes (15.3% of the bacterial library) and Chloroflexi (8.5% of the bacterial library). A small partition belonged to Acidobacteria (3.4% of the bacterial library), candidate division OP8 (2.5% of the bacterial library), Bacteroidetes/Chlorovi and Verrucomicrobia (each accounting for 1.7% of the bacterial library).

Proteobacteria identified 2 distinct subdivisions: Alphaproteobacteria and Deltaproteobacteria, which accounted for 11% and 19.5% of the bacterial library, respectively. Alphaproteobacteria were grouped with both uncultured clones and cultured representatives. Two of the uncultured phylogenetic close relatives were isolated from West Pacific “warm pool” sediments (92%–96% similarity) and one from Antarctic ice-covered lake water (91% similarity). The cultured representatives originated from *Stella humosa* (91% similarity), endosymbiont of *Inanidrilus makropetalos* (90% similarity) and an unidentified member of the family Rhodospirillaceae

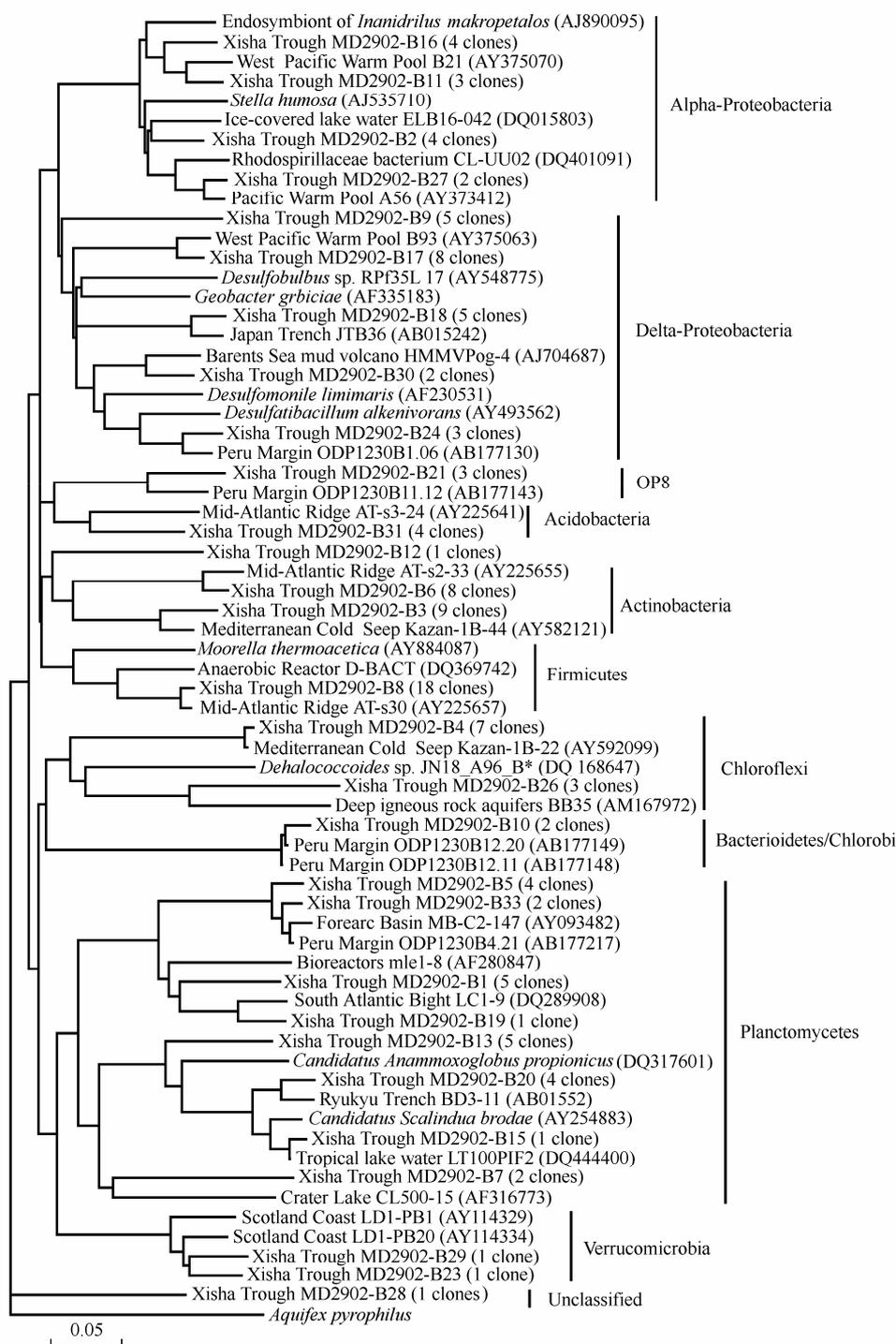


Fig. 1 Phylogenetic relationships of the environmental clones derived from surface sediments of the Xisha Trough as determined by neighbor-joining analysis on 16S rRNA gene sequences by using PAUP* 4.0, Ver. 10

The clones from this study were indicated by MD2902-Bxx (2902 = core number, B = bacterial clone, xx = clone number). The scale bar represents 0.05 substitution per nucleotide position.

(93% similarity), respectively. Sequences belonging to Deltaproteobacteria were phylogenetically closely related to clones isolated from deep marine sediments, including the West Pacific “warm pool”, the Japanese Trough, the Barents

Sea cold seep and the Peru margin. Cultured representatives originated from genera *Desulfobulbus*, *Geobacter*, *Desulfomonile* and *Desulfatibacillum* (83%–89% similarity).

Total sequences belonging to Actinobacteria, Acidobacteria,

candidate division OP8, Bacterioidetes/Chlorobi and Verrucomicrobia were related to uncultured clones retrieved from deep-sea sediments. One phylotype identified belonging to Acidobacteria was related to clones obtained from mid-Atlantic ridge hydrothermal sediments (86% similarity). Sequences belonging to Actinobacteria were closely related to clones retrieved from deep marine sediments of the mid-Atlantic ridge and the Mediterranean cold seep (93%–95% similarity). Uncultured representatives of both candidate division OP8 and Bacterioidetes/Chlorobi were retrieved from Peru margin deep-sea sediments (89%–98% similarity). Sequences belonging to Verrucomicrobia were related to clones retrieved from coastal sediments of Scotland (92% similarity).

Sequences belonging to Firmicutes showed distant relationships with an anaerobic thermophilic microorganism *Moorella thermoacetica* (82% similarity), and the closest phylogenetic relatives were obtained from hydrothermal sediments (98% similarity). Two phylotypes were composed of Chloflexi. Clone MD2902-B4 was identified to be the same as clone Kazan-1B-22 isolated from Mediterranean cold seep sediments (99% similarity), and distantly related to *Dehalococcoides* sp.

(79% similarity). Another phylotype was distantly related to clones obtained from deep igneous aquifers.

8 sequences belonged to Planctomycetes and were divided into 3 clusters. Sequences affiliated to Cluster I were closely related to uncultured clones isolated from a variety of marine sediments, including Peru margin deep-sea sediments, Forearc Basin methane hydrate-bearing deep marine sediments and South Atlantic shelf sediments (93%–97% similarity). Sequences affiliated to Cluster II were associated with members of the order *Planctomycetales* (85%–96% similarity). The sequence affiliated to Cluster III was distantly related to clones isolated from lake sediments.

2.2 Analysis on archaeal library

17 phylotypes of the archaeal library were assigned to 7 groups that belonged to the phylum Euryarchaeota (63.2% of the archaeal library) and Crenarchaeota (19.8% of the archaeal library), and yet one phylotype was unidentified (17% of the archaeal library) (Fig. 2). 4 groups were composed of the phylum Crenarchaeota. Among them Marine Crenarchaeotic Group I (MG I) was the most dominant group (49.2% of the archaeal library), followed by Marine Benthic Group B

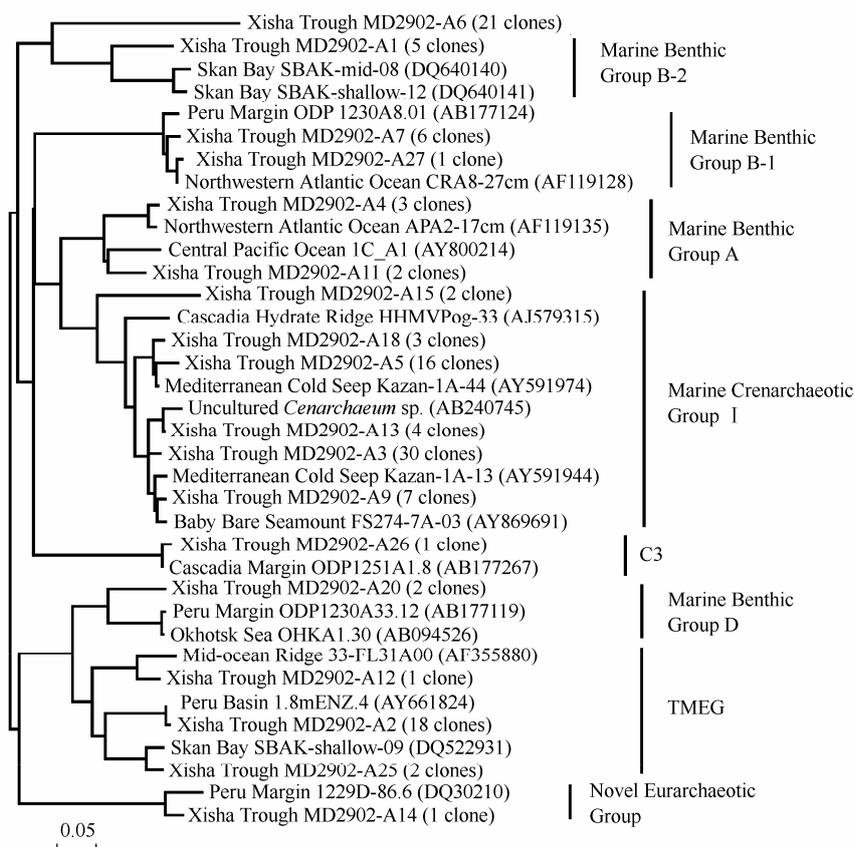


Fig. 2 Phylogenetic relationships of the representative environmental clones derived from surface sediments of the Xisha Trough as determined by neighbor-joining analysis on 16S rRNA gene sequences by using PAUP* 4.0, Ver. 10

TMEG: Terrestrial Miscellaneous Euryarchaeotal Group. The clones from this study were indicated by MD2902-Bxx (2902 = core number, B = bacterial clone, xx = clone number). The scale bar represents 0.05 substitution per nucleotide position.

(MBGB) (9.7% of the archaeal library), Marine Benthic Group A (MBGA) (4% of the archaeal library) and C3 (0.8% of the archaeal library). The phylum Euryarchaeota was divided into 3 groups. Most of the sequences belonged to Terrestrial Miscellaneous Euryarchaeotal Group (TMEG) (16.9% of the archaeal library), and a few were affiliated to Marine Benthic Group D (MBGD) (1.6% of the archaeal library) and Novel Euryarchaeotic Group (NEG) (0.8% of the archaeal library).

Total sequences in the archaeal library were related to uncultured clones obtained from marine sediments. Two subdivisions of MBGB were identified: MBGB-1 and MBGB-2. Sequences belonging to MBGB-1 were closely related to clones obtained from deep marine sediments of the Peru margin and the northwestern Atlantic Ocean (96%–98% similarity). Sequences belonging to MBGB-2 were distantly related to clones obtained from Skan Bay shallow marine sediments (85% similarity). Sequences belonging to MBGA had relationships with clones obtained from ocean sediments of the northwestern Atlantic Ocean and the Central Pacific Ocean (89%–98% similarity). Sequences belonging to MG I were closely related to clones obtained from methane abundant or methane hydrate-bearing sediments, including the Cascadia hydrate ridge, the Mediterranean mud volcano and the Baby bare hydrate fluid (97%–98% similarity). Clone MD2902-A26 belonging to C3 was identified to be the same as clone ODP1251A1.8 isolated from Cascadia margin sediments (99% similarity). Sequence belonging to MBGD was associated with clones obtained from the deep marine sediments of the Cascadia margin and the Okhotsk Sea (both 87% similarity). Sequences belonging to TMEG were closely related to clones obtained from sediments of the mid-ocean ridge, the Peru Basin and the Skan Bay (92%–98% similarity). Sequence belonging to NEG was related to clones isolated from Peru margin sediments (85% similarity).

3 Discussion

The prokaryotic populations and activities in surface sediments are the greatest in seafloor environments, presumably reflecting the ceaseless input of fresh organic carbon which supplies substance and energy for the livings.

3.1 Bacterial diversity

Proteobacteria are a dominant component of the bacterial community in deep-sea sediments. Members of the group accounted for varied proportions to the bacterial library in near surface sediments with different origins. The percentages varied from the lowest 20% to the highest 95%, such as 95% in the Cascadia margin^[6], 78%–92% in the Japanese Trough^[17,18], 45%–63% in the Guaymas Basin^[19], 25% in site 1173 of the Nankai Trough^[6], 22% in site 1176 of the Nankai Trough (1 m beneath the seafloor (bsf))^[20], 38% in Peru Basin (6.7 m bsf)^[21], and 30.5% in this study. Therefore, we infer that the

percentage of Proteobacteria links little to the geographic location. Proteobacteria was identified to include the Alphaproteobacteria and Deltaproteobacteria subdivisions. Deltaproteobacteria occupied about 2/3. It was also the majority in Nansha surface sediments^[11]. However, our previous work indicated that 3 subdivisions of Proteobacteria were identified in Xisha Trough sediments at 6 m beneath the seafloor^[22]. We infer that the composition of Proteobacteria may be related to depth beneath the seafloor to some extent. Accordingly, lithological (*e.g.*, grain size, pore space, mineral composition and sedimentation rate) and geochemical (*e.g.*, pore water chemistry and methane content) characteristics of sediments may affect microbial habitats. Taking Okhotsk marine sediments as an example, Alphaproteobacteria and Deltaproteobacteria dominated in the mud layers, while Alphaproteobacteria and Gammaproteobacteria did in the volcano layers.

Sequences belonging to the Alphaproteobacteria subdivision have been detected in diverse marine environments^[23], including seawater, seafloor sediments and hydrothermal vent. Oxidation of reduced sulfur compounds may be a dominant type of metabolism. Most members of the Deltaproteobacteria subdivision are sulphate reducers and thus strictly anaerobic^[24]. In our case, all of the closest cultured representatives are in line with typical sulphate reducers. We inferred that sequences in this study most likely belonged to sulphate reducers. To sum up, members of the Proteobacteria group participate in the sulfur cycle, and under the anaerobic environment, abundant sulfur compounds supply energy resources for these chemolithotrophic or chemolithotrophic bacteria.

Sequences related to Actinobacteria, Planctomycetes, Firmicutes and candidate division OP8 are detected frequently in seafloor sediments although not abundant. Planctomycetes are a dominant component next only to Proteobacteria in Xisha Trough sediments, and also comparatively abundant in Nansha sediments^[11]. But sequences related to the Planctomycetes group fail to dominate or even are rarely identified in marine sediments from the Pacific ocean margin, including the Nankai Trough^[6], the Japanese Trough^[17,18], the Peru margin^[7,21] and the Cascadia margin^[7]. Is the abundance of Planctomycetes a peculiarity of the bacterial community in sediments from the South China Sea? More evidences are needed to answer this query. The frequency of sequences related to Actinobacteria and Verromicrobia is rather low in deep-sea sediments. We suggest that most members of the 2 groups enter the sediments through the mixing progress of seawater and sediments. Core MD05-2902 contains multi-layers of turbid sediments. A great amount of sequences related to Actinobacteria were isolated from the turbid sediments at 6 m beneath the seafloor^[22].

Most members of Acidobacteria are unculturable and detected by molecular technologies. Sequences related to the Acidobacteria group are abundant in hydrothermal sedi-

ments^[24] and soils, following a geothermal heating event^[25], but rarely identified in deep-sea sediments. Most members are likely thermophilic. The discovery of one Acidobacteria-like sequence in Xiasha Trough sediments represents that the Acidobacteria group contains some indescribable cold-adapted organisms.

Chloroflexi dominates in organic-rich and hydrate-lacking sediments. The frequency of sequences related to Chloroflexi in nonhydrate is higher than in methane hydrate-bearing sediments^[7]. Accordingly, the presence of Chloroflexi can be used as the criterion of nonhydrate sediments. It will be informative for the survey of seafloor methane hydrate in the future.

3.2 Archaeal diversity

Both MG I (or Marine Crenarchaeotic Group, MCG) and TMEG were dominant components of the archaeal community in Xisha surface sediments. However, several typical benthic groups like MBGB, MBGA and MBGD accounted for small proportions. Sequences belonging to MG I are frequently detected in varied marine and territorial environments, and dominate in organic-rich and hydrate-lacking sediments^[7]. It has been reported that sequences belonging to MG I compose the whole archaeal community in sediments at one site of Pacific Ocean^[26], and account for 90% in Peru margin sediments. Sequences belonging to the TMEG group are distributed widely in multi-environment, including territorial, lake and marine sediments^[27,28]. All of the members of TMEG are uncultured. Little is known about the physiological characters. Maybe the TMEG group possesses miscellaneous metabolisms.

The MBGB (or Deep Sea Archaeal Group, DSAG) group was firstly identified in deep-sea hydrothermal sediments. Up to now, more and more MBGB-like sequences have been detected in deep marine sediments^[28]. Sequences related to MBGB are abundant in the sulphate reducing zone with methane upwelling from the bottom. The group dominated in methane hydrate-bearing sediments from site 1230 of the Peru margin and sites 1245 and 1251 of the Cascadia margin^[7]. The group probably plays an important role in sulphate reducing and methane oxidation^[29].

Our data suggest that both sulphate-reducing and methane-oxidation metabolisms dominate in the microbial community in surface sediments of Xisha Trough, and a large amount of methane is necessary. The bacterial library contains 2 bacterial groups whose members mostly live in seawater. Most members of the 2 groups enter the sediments through the turbid process.

Acknowledgements

We acknowledge the personnel of IMAGES 147 shipboard scientific parties for sampling. This study was supported by the Key Project of China National Programs for Fundamental

Research and Development (No. 2007CB815904), the National Natural Science Foundation of China (Nos. 40510487 and 40606032) and the Program for Young Excellent Talents in Tongji University, China (No. 2006KJ056).

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