Microbial community structure and diversity in deep-sea hydrothermal vent sediments along the Eastern Lau Spreading Centre

WEI Manman¹, ZHANG Rubing³, WANG Yuguang¹, JI Houguo¹, ZHENG Jia¹,

CHEN Xinhua⁴, ZHOU Hongbo^{1,2*}

¹ School of Minerals Processing and Bioengineering, Central South University, Changsha 410083, China

² Key Laboratory of Biometallurgy, Ministry of Education, Changsha 410083, China

³ Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao 266101, China

⁴ Third Institute of Oceanography State, Oceanic Administration, Xiamen 361005, China

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Abstract

The aim of this study is to investigate microbial structures and diversities in five active hydrothermal fields' sediments along the Eastern Lau Spreading Centre (ELSC) in the Lau Basin (southwest Pacific). Microbial communities were surveyed by denatured gradient gel electrophoresis (DGGE) and clone library analysis of 16S rRNA genes. The differences in microbial community structures among sediment samples from the five deep-sea hydrothermal sites were revealed by DGGE profiles. Cluster analysis of DGGE profiles separated the five hydrothermal samples into two groups. Four different 16S rRNA gene clone libraries, representing two selected hydrothermal samples (19-4TVG8 and 19-4TVG11), were constructed. Twenty-three and 32 phylotypes were identified from 166 and 160 bacterial clones respectively, including *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Nitrospirae* and *Planctomycetes*. The phylum *Proteobacteria* is dominant in both bacterial libraries with a predominance of Gamma-*Proteobacteria*. A total of 31 and 25 phylotypes were obtained from 160 and 130 archaeal clones respectively, including Miscellaneous Crenarchaeotic Group, Marine Group I and III, Marine Benthic Group E, Terrestrial Hot Spring Crenarchaeota and Deep-sea Hydrothermal Vent Euryarchaeota. These results show a variety of clones related to those involved in sulfur cycling, suggesting that the cycling and utilization of sulfur compounds may extensively occur in the Lau Basin deep-sea hydrothermal ecosystem.

Key words: microbial diversity, deep-sea hydrothermal vent, DGGE, 16S rRNA gene clone libraries

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1 Introduction

Microbial populations thriving in deep-sea hydrothermal vents have been extensively investigated since the discovery of these environments and their vast associated biota (Van Dover, 2000). The environment is characterized by steep environmental gradients of temperature, pH, redox potential and metal compounds (McCollom and Shock, 1997). It is generally regarded that such an environment provides diversity niches for microbial communities and has been also a major source of novel microbes with unique physicochemical characteristics. In the previous studies, direct isolation of microorganisms as well as the use of molecular techniques has revealed the presences of several different physiological and phylogenetic groups of prokaryotes in the extreme marine environment (Jeamthong, 2000). Although numerous novel lineages of uncultivated bacteria and archaea inhabited in deep-sea hydrothermal fields have been successfully detected (Takai et al., 2003; Schrenk et

al., 2003), a large number of unknown microbial resources still need to be identified and explored in other deep-sea hydrothermal fields.

The ELSC including the Valu Fa Ridge (VFR) is an about 400 km long spreading centre associated with the opening of the Lau Basin, a young back-arc basin with active spreading ridges and off-axis seamount volcanoes, behind the Tonga zone. This field displays unique geochemical and biologic characteristics with high concentrations of metal sulfides and elemental sulfur relative to hydrothermal vent systems of the East Pacific Rise (EPR) and other well-studied vent sites in the Pacific Ocean (Fouquet et al., 1991; Michael and Seewald, 2007; Herzig and Hannington, 1995). A variety of potential electron donors (sulfides, organic C, CH₄, and Fe) and electron acceptors (O₂, SO₄²⁻, and CO₂) have been identified in these environments, potentially supporting the growth of diverse heterotrophic and autotrophic prokaryotes. Additionally,

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Corresponding author, E-mail: zhouhb@mail.csu.edu.cn

the formation of this back-arc basin is a relatively recent geological event and the basin has been hydrothermally active for only a short time. The ELSC was chosen as a representative because it provides diverse microbial niches due to steep physical and chemical gradients. These attributes make the Lau Basin the best active hydrothermal vent sites to study prokaryotic phylogenetic and metabolic diversity (Michael and Seewald, 2007). The information about the distribution, diversity, and composition of the microbial communities at Lau Basin deep-sea hydrothermal vents was relatively little, with only several short comments published (Michael and Seewald, 2007). Thus a thorough investigation on prokaryotic communities in this hydrothermal vent system is quite necessary.

The objectives of this study were to investigate the prokaryotic diversity in sediments from five deep-sea hydrothermal vents along the ELSC in the Lau Back Arc Basin and to analyze the interrelationship between environment and microbial diversity, which will generate data that could facilitate the isolation of novel microbes with unique functional characteristics.

2 Materials and methods

2.1 Site description and sample collection

Five sediment samples were collected from deep-sea hydrothermal vent fields along the ELSC during round-the-world ocean research of *Dayangyihao* in May 2007. As shown in Table 1, sample 19-4TVG8 was from the VFR at southern ELSC and the other four samples were collected from the main ELSC. Immediately after sampling, they were labeled and stored in sterile plastic tubes at -80°C until they were processed.

Sediment samples were analyzed for total nitrogen (TN), organic carbon and sulfate concentrations. Sediment temperature and pH were measured in situ. Pore water sulfate was measured by ion chromatography. Sediment organic carbon and total nitrogen content were measured in the laboratory with a PE 2400 Series II CHNS/O analyzer (Perkin Elmer, USA).

2.2 Microscopic counts

The total cell numbers in each sample were determined by direct microscopic counts, using DAPI (4', 6'-diamidino-2-phenylindole) fluorochrome staining technique. Sediment samples were placed on ice and sonicated to dislodge cells from sediment particles, and then were fixed with buffered polyformaldehyde at a final concentration of 1%. An aliquot of the fixed sediment slurry (10 μL) was diluted 1 000-fold in sterile PBS buffer (0.9 g NaCl, 15 mmol/L sodium phosphate buffer, pH 7.4), thoroughly shaken, and filtered through a black polycarbonate membrane. Staining with DAPI was performed for 20 min in the dark and cells were counted with an epifluorescence microscopy (Olympus BX-60 M). Each sample was measured in triplicate.

2.3 Genomic DNA extraction

Total community genomic DNA was extracted from 5 g of each sediment sample by the method of Zhou et al. (1996) with some modifications. The protocol encompassed grinding, three cycles of freezing and thawing, chemical lyses in a high-salt extraction buffer (1.5 mol/L NaCl) by heating of the suspension in the presence of sodium dodecyl sulfate (SDS) and cetyltrimethyl ammonium bromide (CTAB), and a proteinase K step. The obtained DNA was stored at -20°C and used as the template for further PCR amplification.

2.4 PCR amplification of 16S rRNA gene fragments

In order to assess the prokaryotic diversity of the sediment samples, DGGE and restriction fragment length polymorphism (RFLP) analysis of 16S rRNA gene were performed. For construction of clone libraries, *Bacteria*-specific primers 27F (5'-AGAGTTTGTATCCTGGCTCAG-3') and 1492R (5'-GGT-TACCTTGTTCACGACTT-3') and *Archaea*-specific 21F (5'-TTCC-GGTTGATCCYGCCGGA-3') and 958R (5'-YCCGGCGTTGAMTC-CAATT-3') were used. Each PCR mixture (50 μL) contained 2.5 mmol/L MgCl_2 , 0.2 mmol/L dNTP, 0.2 pmol/L each primer, 1 $\times$ PCR buffer (Fermentas), and 1 U of Taq DNA polymerase (Fermentas). An initial denaturation step of 5 min at 94°C was followed by 32 cycles of 94°C for 45 s, 55°C for 40 s, and 72°C for 2 min. The final extension step was 72°C for 10 min. PCR amplifications were purified with the Gel Extraction Kit (Bioteke Corporation, Beijing, China) in accordance with the manufacturer's instructions.

For DGGE analysis, about 200-bp fragments of 16S rRNA genes were amplified by primers 341F-GC (5'-CGCCCCCGCG-CGCGGCGGGCGGGGCGGGGGCACGGGGGGCTACGGGAGG-CAGCAG-3') and 518R (5'-GTATTACCGCGGCTGCTGG-3') (Muyzer et al., 1996) for bacteria and primers PARCH 340F-GC (5'-CGCCCCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCCCTACGGGGYGASCAG-3') and PARCH 519R (5'-TTACCGC-GGCKGCTG-3') (Ovreas et al., 1997) for archaea, respectively. To increase the specificity of the amplification and reduce the formation of spurious by-products, a touchdown PCR was performed as follows: the annealing temperature was set to 65°C and decreased by 0.5°C at every cycle for 20 cycles, and then 15 additional cycles were performed. Denaturation was carried out at 94°C for 1 min, the annealing time was 1 min, and the primer extension was 72°C for 1 min. A final extension at 72°C for 10 min was performed.

2.5 DGGE analysis

DGGE was carried out in a CBS Scientific DGGE-2401 system (C.B.S. Scientific Company, Inc., Del Mar, CA, USA) by a previously described method with slight modifications (Muyzer et al., 1996). PCR products were resolved on polyacrylamide gels (8%, w/v) in 1 $\times$ TAE (20 mmol/L Tris-Cl, 10 mmol/L acetate, 0.5 mmol/L Na_2EDTA) using denaturing gradient from 40% to 55% (for bacteria) or 40% to 60% (for archaea) where 100% denaturant contained 7 mol/L urea and 40% formamide. Electrophoresis was carried out at a constant voltage of 200 V and a temperature of 60°C for 3–5 h. After electrophoresis, the gels were stained for 20 min with ethidium bromide and visualized on a UV gel documentation system and photographed.

2.6 Clone libraries and RFLP analysis

Four 16S rRNA gene clone libraries were constructed, one bacterial and one archaeal for hydrothermal sediment 19-4TVG8 and 19-4TVG11 respectively. For each library, the cleaned PCR products were linked with pGEM T-Vector (Promega, Madison, WI), transformed into *E. coli* DH5 α competent cells, and then positive clones were screened out through blue and white spot selection. About 160 white colonies for each library were randomly selected and stored on agar plates. Inserts were subsequently amplified from positive colonies with primers specific for the vector, M13F (5'-GTAAACGACGGCCAGT-3') and M13R (5'-CAGGAAACAGCTA TGAC-3'). The colony PCR products were digested separately with 2.5 U of *MspI* (Fermentas) and *Hin6I* (Fermentas) in a 20 μL reaction mixture as recommended by the manufacturer and

visualized on 3% (w/v) agarose gels stained with ethidium bromide. The resulting restriction digest patterns were compared and analyzed between samples.

2.7 Phylogenetic and cluster analysis

The DGGE fingerprints were analyzed using the Gel Pro Analyzer 3.1 (Media Cybernetics, MD, USA) for band detection, and then comparisons of microbial community compositions of five sediment samples were performed by cluster analysis using NTSYS-pc software package (Rohlf, 2002). Dendrograms were constructed by the unweighted pair group method with arithmetic mean (UPGMA) groupings with a similarity coefficient matrix. By comparing the size and number of digested fragments, clones with unique RFLP patterns were selected for sequencing. The sequences were firstly assembled, with vector trimming, and then compared with those in GenBank database by BLAST search to find closely related sequences. Sequences with being greater than and equaling to 97% similarity were assigned to the same phylotype. The sequences were aligned with their most similar species using Clustal X (version 1.8). The phylogenetic trees were constructed from evolutionary distances using the neighbor-joining method with bootstrap analysis for 1 000 replicates.

2.8 Diversity indices and statistical analysis

Rarefaction analysis was performed and diversity indices were calculated to analyze the microbial diversity within each clone library. The rarefaction curves were constructed using Analytic Rarefaction 1.3 (<http://www.uga.edu/strata/software/index.html>). The coverage of the libraries was calculated as defined by Good (1953), with the following formula:

$$C = (1 - n_1/N_s) \times 100,$$

where n_1 is the number of phylotypes appearing only once in a library and N_s is the library size. The Shannon's index (H') of diversity and the Simpson's index (D) were calculated by the equation

$$H' = -\sum_{i=1}^s p_i \ln p_i \quad \text{and} \quad D = 1 - \sum_{i=1}^s p_i^2,$$

where p_i is the proportion of clones belonging to the i th phylotype and s is the total number of phylotypes. Evenness was calculated with the formula $E = e^{H'}/N$, where H' is the Shannon index of diversity and N is the total number of phylotypes (Lauritzen and Bottrell, 1994). The phylotype compositions of the clone libraries were compared using the Sorensen index

$$S = 2 \times c/(a + b),$$

where c is the number of phylotypes found in both Sample A and Sample B; a is the number of phylotypes in Sample A; and b is the number of phylotypes in Sample B (Magurran, 1988).

2.9 Nucleotide sequence accession numbers

The 16S rRNA gene sequences obtained in this study have been deposited in the GenBank database under accession numbers GQ848371 to GQ848480.

3 Results

3.1 Physicochemical characteristics of sediments and total cell counts

Physicochemical characteristics of the hydrothermal sediments (Table 1) varied considerably amongst the five samples. All sediments displayed nearly neutral pH, ranging from 6.22 to 7.72. The content of organic carbon and total nitrogen in the sediments was in the range of 0.29%–1.61% and 0.02%–0.07% respectively, with the highest values in the sediment 19-4TVG8 and the lowest values in the sediment 19-4TVG11 (Table 1). The physicochemical characteristics of Samples 19-4TVG8 and 19-4TVG11 represented the greatest difference in the five samples.

Total cell numbers were in the range of 6.89×10^7 to 4.26×10^8 cells/g of wet sediment and showed certain variations among five sediment samples. No significant correlation between total cell numbers and physicochemical characteristics of sediment samples were found. Comparing the physicochemical characteristics of the samples, it was found that Samples 19-4TVG8 and 19-4TVG11 were the most distinct from each other.

3.2 DGGE analysis

DGGE analyses of bacterial and archaeal 16S rRNA genes were performed to compare the microbial compositions of the five sediment samples, and DGGE banding patterns were used to construct dendrograms (Fig. 1). DGGE banding patterns revealed that there were some differentiations in both bacterial and archaeal community structures among hydrothermal sediment samples from different sites. These differences can also be visualized in the inferred genetic relationships of the microbial populations given in the dendrogram for cluster analysis. The bacterial DGGE profiles of the five sediment samples were separated into two clusters, with the lowest similarity coefficient 0.59; the archaeal DGGE profiles were separated into three clusters, with the lowest similarity coefficient 0.47. The bacterial and archaeal patterns of 19-4TVG8 and 19-4TVG11 were clustered to different groups, suggesting that the microbial community structures of both samples greatly differed from each other among the five samples. As mentioned above, the physicochemical characteristics and cell counts of Samples 19-4TVG8

Table 1. Characteristics of the hydrothermal samples and microbial communities analysis

Parameter	Sample				
	19-4TVG2	19-4TVG8	19-4TVG11	19-4TVMC4	19-4TVMC8
South latitude/(°)	19.7418	22.2158	20.9280	19.6927	20.6665
West longitude/(°)	175.9561	176.6072	176.2401	175.9511	176.1253
Depth/m	2 350	1 722	2 255	2 699	2 457
Temperature/°C	ND	84	114	ND	ND
pH	7.66	7.72	6.22	6.25	7.49
Organic carbon (%)	0.51	1.61	0.29	0.77	0.76
Total nitrogen (%)	0.04	0.07	0.02	0.05	0.02
Sulfate concentration/mmol·L ⁻¹	16.9	19.8	13.5	17.4	23.2
Total cell counts/ $\times 10^7$ cells·g ⁻¹	7.48 $\pm$ 0.26	42.6 $\pm$ 1.16	6.89 $\pm$ 0.15	14.7 $\pm$ 0.89	32.1 $\pm$ 1.52

Notes: ND represents not determined.

Fig.1. Cluster analysis of the profiles obtained from DGGE banding patterns for bacterial (a) and archaeal (b) communities. The dendrogram was generated with NTSYS version 2.10 using UPGMA.

and 19-4TVG11 were the greatest different in the five samples. The difference in microbial community structure of Samples 19-4TVG8 and 19-4TVG11 may correlate with variations in physicochemical characteristics of the two samples.

3.3 Clone libraries and phylogenetic analysis

DGGE can resolve the diversity of only the most dominant populations (>1%) and separate only relatively small fragments, up to 500 bp. Since the DGGE analysis revealed a certain degree of similarity among banding patterns for the five hydrothermal samples and distinct division of two clusters, we selected two samples (19-4TVG8 and 19-4TVG11) as a representative for a more thorough characterization. Bacterial and archaeal 16S rRNA gene clone libraries were constructed for sample 19-4TVG8 and 19-4TVG11, respectively. A total of 616 clones from the four clone libraries were screened by RFLP and grouped into identical restriction patterns (phylotypes). The phylogenetic trees of the bacteria and archaea from Samples 19-4TVG8 and 19-4TVG11 were constructed on the basis of 16S rRNA sequences as shown in Figs 2 and 3.

In two bacterial clone libraries, 19-4TVG8BA and 19-4TVG11BA were composed of 166 and 160 clones that grouped into 23 and 32 phylotypes, respectively (Fig. 2). In the library 19-4TVG8BA, the phylotypes could be divided into eight lineages, as follows: *Gamma-Proteobacteria* (41.57% of the total clones), *Epsilon-Proteobacteria* (24.1%), *Delta-Proteobacteria* (18.67%), *Alpha-Proteobacteria* (2.41%), *Bacteroidetes* (6.02%), *Firmicutes* (3.01%), *Nitrospirae* (2.41%), *Planctomycetes* (1.81%). In the library 19-4TVG11BA, the phylotypes could be divided into seven lineages: *Gamma-Proteobacteria* (37.5% of the total clones), *Epsilon-Proteobacteria* (8.13%), *Delta-Proteobacteria* (21.86%), *Alpha-Proteobacteria* (20%), *Bacteroidetes* (5.63%), *Nitrospirae* (5%), *Planctomycetes* (1.88%). *Gamma-Proteobacteria* and *Epsilon-Proteobacteria* were the most prominent groups of

microorganisms represented in both bacterial clone libraries. The *Proteobacteria* were highly dominant, accounting for nearly 90% of the total clones in both bacterial libraries and being divided into four subclasses *Alpha*-, *Delta*-, *Epsilon*-, and *Gamma-Proteobacteria*. Within the *Proteobacteria*, the *Gamma-Proteobacteria* was the most abundant phylogenetic group, accounting for 41.57% of the total clones in the library 19-4TVG8BA and 37.5% of the clones in the library 19-4TVG11BA, respectively. The distributions of the *Alpha*- and *Epsilon-Proteobacteria* were different in the two bacterial clone libraries, with a higher richness and greater diversity for *Alpha-Proteobacteria* in the 19-4TVG11BA library and *Epsilon-Proteobacteria* in the 19-4TVG8BA library. The proportions of *Delta-Proteobacteria* in both bacterial libraries were similar, comprising 18.67% of the total clones in library 19-4TVG8BA, and comprising 21.86% in library 19-4TVG11BA. The *Firmicutes* appeared only in library 19-4TVG8BA and did not in the library 19-4TVG11BA. The families of *Bacteroidetes*, *Nitrospirae*, *Planctomycete* were present in both bacterial libraries with similar low frequencies between 1.8% and 6.1%. Some phylotypes classified in the *Proteobacteria* were closely related to cultured species (Fig. 2). However, other bacterial phylotypes had low similarity (<95%) to the sequences of cultured species in public databases. This result suggests that some novel bacterial species are present at the deep-sea hydrothermal vent fields along the ELSC.

In the two archaeal clone libraries, 31 and 25 archaeal phylotypes were defined from 160 and 130 clones in the 19-4TVG8AR and 19-4TVG11AR libraries, respectively (Fig. 3). The 19-4TVG8AR clone library contained 58% of *Crenarchaeota* and 42% of *Euryarchaeote*, whereas there was a higher richness and greater diversity for *Crenarchaeota* (85%) in the 19-4TVG11AR library. These phylotypes had a low similarity (less than 90%) to those isolated species in the database and were difficult to confidently determine their taxonomic status. Thus, the physiolog-

Fig.2. Phylogenetic trees of the bacterial 16S rRNA gene sequences obtained from the clone library 19-4TVG8BA and 19-4TVG11BA. Clones detected in this study are indicated in bold. The tree was conducted using sequences of comparable region of the 16S rRNA gene sequences available in public databases. Neighbor-joining analysis using 1 000 bootstrap replicates was used to infer tree topology.

Fig.3. Phylogenetic trees of the archaeal 16S rRNA gene sequences obtained from the clone library 19-4TVG8AR and 19-4TVG11AR. Clones detected in this study are indicated in bold. The tree was conducted using sequences of comparable region of the 16S rRNA gene sequences available in public databases. Neighbor-joining analysis using 1 000 bootstrap replicates was used to infer tree topology.

ical characteristics of these uncultured phylotypes are unclear. According to the previous studies, the archaeal phylotypes recovered were associated with the following groups: the Miscellaneous Crenarchaeotic Group (MCG) (Inagaki et al., 2003a), Marine Group I and III (MGI and MGIII) (Fuhrman et al., 1992; Fuhrman and Davis, 1997), Marine Benthic Group E (MBGE) (Vetriani et al., 1999), Terrestrial Hot Spring Crenarchaeota (THSC) and Deep-sea Hydrothermal Vent Euryarchaeota (DHVE) (Takai and Horikoshi, 1999) (Fig. 3). In addition, the phylotypes TVG11AR07 and TVG11AR08 were not clearly classified in these groups. The TVG11AR07 clone had 99% similarity to uncultured clone FnvA87 from crustal fluids in back-arc hydrothermal fields of the Southern Mariana Trough. The TVG11AR08 clone exhibited 92% similarity to the clone LP30MA94 obtained from phreatic limestone sinkholes in northeastern Mexico. Until now, no cultured member of all these archaeal groups, with the exception of MGI, has been discovered.

3.4 Comparison and statistical analysis of clone libraries

The number of clones, phylotypes, and biodiversity indices calculated for the four clone libraries were summarized in Table 2. The coverage of the clone libraries was high, ranging from 80% to 86%. The high coverage values combined with the saturated rarefaction curves (data not shown) suggest that most

phylotypes present in the microbial communities were identified in this study. The results of the Shannon index, Simpson index and Coverage, indicate that the 19-4TVG8 archaeal population was the most diverse, while the 19-4TVG8 bacterial population was the least diverse. In addition, the 19-4TVG11 bacterial diversity was higher in both species richness and evenness than that of 19-4TVG8, while species richness and evenness were similar between two archaeal populations.

The microbial compositions of the clone libraries were compared by calculating the Sorensen similarity index. Phylotype comparisons between the 19-4TVG8 and 19-4TVG11 archaeal libraries reveal that four phylotypes gave the same best-BLAST matches, for a lower similarity value of 0.14. The similarity value of the two bacterial libraries (0.36) was substantially higher than that determined for the archaeal libraries (0.14). About 43% of the total 19-4TVG8 phylotypes had the same best-BLAST matches as the 19-4TVG11 phylotypes. The uniqueness of the microbial communities in this study was estimated by comparing the degree of relatedness of the microbial sequences we obtained with their closest matches in GenBank. Overall, 36% of the bacterial phylotypes and 39% of the archaeal phylotypes were related to the existing sequences at being less than 95% identity, which suggests that these sequences represent novel microbial genera not yet recovered from any other environment.

Table 2. Number of clones and phylotypes analyzed for the four 16S rRNA gene clone libraries and their diversity indices

Clone library	Total No. of clones	No. of phylotypes	Coverage (%)	Shannon index (H')	Simpson's index (D)	Evenness (E)	Sorensen similarity index
19-4TVG8BA	166	23	86	2.12	0.89	0.82	0.36
19-4TVG11BA	160	32	80	2.39	0.95	0.93	
19-4TVG8AR	160	31	81	2.42	0.96	0.95	0.14
19-4TVG11AR	130	25	82	2.33	0.95	0.95	

4 Discussion

The present study provides the first characterization of the microbial diversity present in the deep-sea hydrothermal vent fields found at the ELSC of Lau Basin and the results show the existence of a variety of bacterial and archaeal phylotypes in hydrothermal samples along the ELSC, as represented in the DGGE gels and 16S rRNA gene clone libraries. Our results suggest that deep-sea hydrothermal vents along the ELSC are likely to provide habitats for phylogenetically and metabolically diverse microorganisms.

4.1 Diversity of bacteria and archaea

All bacterial groups identified in this study are widespread in deep-sea hydrothermal vent environments and may participate in a variety of biogeochemical processes. Generally, *Gamma-Proteobacteria* are very diverse in marine plankton, and also are universal and abundant in deep-sea hydrothermal fields (López-García et al., 2001). *Gamma-Proteobacteria* not only accounted for the largest proportion in our bacterial clone libraries, but also they were very diverse. Similar results were also found at the Juan de Fuca Ridge hydrothermal vent based on geochip analysis, which demonstrate the prevalence and predominance of bacteria belonging to the gamma subdivision of *Proteobacteria* (Wang et al., 2009).

Most members of *Delta-Proteobacteria* are strictly anaerobic sulfate reducing bacteria, reducing sulfate or sulfite using H_2 or an organic molecule as electron donors, and may par-

ticipate in the reduction processes of sulfate, sulfur or Fe^{3+} (Aubildgaard et al., 2006; Vandieken et al., 2006). In this study, sequences related to *Delta-Proteobacteria* belonged to the genera *Desulfobulbus* and *Desulfuromonas*, and clustered with those hydrothermal sediment clones obtained from the Mid-Atlantic Ridge (MAR) and the EPR (Nercessian et al., 2005). This suggests that potentially active sulfate-reducing metabolism is present in deep-sea hydrothermal fields along the ELSC.

Interestingly, the distributions of *Epsilon-Proteobacteria*, *Alpha-Proteobacteria* and *Firmicutes* in the clone libraries 19-4TVG8BA and 19-4TVG11BA were significantly different (Fig. 2). Several detected phylotypes belonging to *Epsilon-Proteobacteria* were related to either *Sulfurimonas* sp. or environmental sequences. The previous studies indicate that *Epsilon-Proteobacteria* inhabited in deep-sea hydrothermal fields are comprised of mesophilic to moderately thermophilic chemolithoautotrophs capable of oxidizing hydrogen and sulfur compounds with nitrate, oxygen, and sulfur compounds as terminal electron acceptors (Nercessian et al., 2005; Campbell et al., 2006; Moussard et al., 2006). In the present study, the proportion of *Epsilon-Proteobacteria* in the library 19-4TVG8BA was significantly higher than that in the library 19-4TVG11BA. The *Alpha-Proteobacteria*, represented in our study by *Rhodobacterales* and *Rhizobiales*, were found in both bacterial libraries, with a higher proportion in the library 19-4TVG11BA. Members of the *Alpha-Proteobacteria*, especially *Roseobacter* clade, are metabolically versatile and widespread

in marine habitats. Strikingly, several phylotypes detected in this study were related to the genera *Nitrobacter* and *Sulfitobacter*, which are autotrophic nitrite-oxidizing bacteria and heterotrophic sulfite-reducing bacteria, respectively. *Firmicutes* were detected only in the libraries 19-4TVG8BA, and absent in the libraries 19-4TVG11BA. Representatives of *Firmicutes* from the libraries 19-4TVG8BA are *Bacillus* sp., moderately thermophilic bacteria oxidizing a wide range of organic substrates (Miroshnichenko and Bonch-Osmolovskaya, 2006). The differences in physicochemical conditions of the two sites, such as temperature, oxygen, nutrient conditions and substrate availability, may explain the observed variability in bacterial community compositions, e.g., the lack of *Firmicutes* in the library 19-4TVG11BA may be attributed to the high sampling temperature (114°C) and the low organic carbon content of the corresponding sample. It is generally believed that bacterial community structures in deep-sea hydrothermal vent fields are mainly defined by complex interactions of biogeochemical parameters.

Bacteroidetes, *Nitrospirae* and *Planctomycete* were also found in both two bacterial clone libraries although with a small portion (<10%). The *Nitrospirae* bacteria may also be nitrifiers, important in the nitrite oxidation process and carbon fixation in the deep-sea environments (Ehrich et al., 1995). *Bacteroidetes* and *Planctomycete* are ubiquitous in marine environments, which mainly reside in microaerobic environments and are related to degradation of carbohydrate polymer (Ravenschlag et al., 2001).

Our study show the presence of MCG, MBGE, MGI, MGII-I, THSC and DHVE in deep-sea hydrothermal vent fields along the ELSC, which is the first report of the detection of various unique archaeal phylotypes in these fields. MBGE and MCG clones were the most abundant groups in both archaeal clone libraries. MBGE consists of a cluster of closely related sequences within the *Euryarchaeota* and the detected phylotypes affiliated in this group were related to environmental sequences detected in deep-sea environments (Huber et al., 2005; Heijs et al., 2008; Takai et al., 2008). In this study, no MBGE clones appeared to be specifically related to known archaeal isolates, but their phylogenetic position between members of the *Methanobacteriales* and the *Methanosarcinales* suggests a possible methanogenic phenotype. MCG was previously designated the terrestrial miscellaneous crenarchaeotic group, but was given the recent reports of marine, hot and cold, surface and subsurface environments (Vetriani et al., 1999; Inagaki et al., 2003a; Heijs et al., 2008; Parkes et al., 2005; Rogers and Amend, 2005; Teske, 2006). Most of the phylotypes in the MBGE and MCG in the present study were related to the sequences retrieved from hydrothermal fields of the MAR, Southern Mariana Trough, or Taktetomi Island (Fig. 3).

Phylotypes in the MGI and MGIII were also retrieved from the 19-4TVG8 and 19-4TVG11 samples. The MGI and MGIII archaea are abundant and ubiquitous in ocean environments (Fuhrman et al., 1992; Fuhrman and Davis, 1997). The clone TVG11AR23 was classified within the DHVE group which is considered to have a significant impact in hydrothermal habitats. It has been observed that members of DHVE group represented a widespread euryarchaeotal lineage in deep-sea hydrothermal fields of the EPR and Central Indian Ocean Ridge (Hoek et al., 2003; Kormas et al., 2006). But cultured member of this group has been poorly reported. Reysenbach et al. (2006) reported the isolation and cultivation of an archaeon belonging to the D-

HVE2 group, which is an obligate thermoacidophilic sulphur or iron reducing heterotrophic archaeon.

The physiological characteristics of all these groups, with the exception of MGI, are unknown because no cultivated species exist. In this study, most archaeal sequences may be regarded as novel (not previously cultured) phylotypes and have a high probability of representing new taxa. Nevertheless, our results suggest the presence of unique and diverse archaeal communities at the deep-sea hydrothermal vent fields along the ELSC.

The recent studies based on analysis of 16S rRNA gene sequences have demonstrated that archaea are much more diverse and widespread than previously suspected (Massana et al., 2000; Kormas et al., 2006). Also, several reports have indicated a distinct distribution of the marine environment between *Crenarchaeota* and *Euryarchaeota*, with *Euryarchaeota* dominant in the surface zone and *Crenarchaeota* dominant in deep-sea area (Massana et al., 2000; Pernthaler et al., 2002). In the present study, it was found that *Crenarchaeota* were dominant in deep-sea hydrothermal sediments along the ELSC, which is consistent with other studies on deep-sea hydrothermal environments (Page et al., 2008; Brazelton et al., 2006).

4.2 Sulfur-related metabolism in hydrothermal vents

The ability to metabolize sulfur compounds appears to be widespread among microorganisms of widely different phylogenetic and physiological types in deep-sea hydrothermal environments (Sievert et al., 2007). The detection of sequences involved in sulfur cycles, such as those belonging to *Sulfuricurvum*, *Sulfurimonas*, *sulfitobacter*, *Desulfuromonas*, *Desulfobulbus* and *Desulfurococcaceae* genera, suggests the presence of sulfur-oxidizers and sulfate-reducers in deep-sea hydrothermal vent sediments along the ELSC. Although the metabolic capabilities of phylotypes can not be directly determined from the phylogenetic relationship to cultured species, the 16S rRNA gene analysis supports the presence of putative sulfur-oxidizers and sulfate-reducers in our samples.

Sulfur is a key substrate at hydrothermal vents, particularly at higher temperatures, as a number of thermophilic and hyperthermophilic bacteria and archaea can use sulfur as an electron donor in either autotrophic or heterotrophic metabolism. In the present study, numerous sequences show high similarities with those related to sulfur metabolism. *Epsilon-Proteobacteria* belonging to *Sulfuricurvum* and *Sulfurimonas* genera identified in this study are typically sulfur oxidizers, which obtain energy through oxidation of some sulfur types, such as sulfide, thiosulfate and elemental sulfur (Takai et al., 2003; Inagaki et al., 2003b; López-García et al., 2003). Some sequences which are similar to gammaproteobacterial endosymbionts, are also putative sulfur-oxidizers (Goffredi et al., 2004). Phylotypes related to *Marinobacter* and *Natronocella* sp. were also detected in the two hydrothermal samples, and representatives from both genera are capable of heterotrophic sulfur oxidation (Sorokin, 2003; Podgorsek et al., 2004). Reduction of oxidized sulfur compounds, as a part of deep-sea hydrothermal vent microbial metabolism, is mainly performed by *Delta-Proteobacteria* that are comprised of phylotypes closely related to diverse genera (*Desulfuromonas* and *Desulfobulbus*) of sulfur- and sulfate-reducing bacteria. Additionally, the phylotype TVG11BA10 belonging to *Alpha-Proteobacteria* shared 99% similarity with sequences of the *sulfitobacter* genera, which is involved in the oxidation of sulfite and would be important in

the active sulfuretum. It is presumed that archaea belonging to the orders of *Desulfurococcales*, *Thermoproteales*, *Archaeoglobales*, and *Thermococcales*, are the important participaters in the sulfur metabolism, which were found to utilize SO_4^{2-} , $\text{S}_2\text{O}_3^{2-}$, or S^0 with H_2S as their ultimate metabolite (Sievert et al., 2007). Sulfur oxidation had been expected to be the primary energy metabolism driving deep-sea vent ecosystems (Nakagawa et al., 2005). It is generally thought that the mechanisms of sulfur oxidation in deep-sea vent ecosystems is different from shallow water environments, with the more various sulfur-metabolizing prokaryotes and more important ecological significance. Usually, sulfate is depleted in surface water environments but becomes available in deep-sea vent ecosystems, which may be used as an electron acceptor by a microbial consortium oxidizing methane.

In conclusion, this work demonstrates a diversity of bacteria and archaea, and also provided insights into microbial diversity in deep-sea hydrothermal fields along the ESLC in the Lau Back Arc Basin. Some sequences especially archaeal sequences were distantly related to these known so far and therefore we expanded the known diversity of microbes in deep-sea hydrothermal environments. Moreover, we provided a basis for further analysis of microbial sulfur metabolism mechanisms and ecological significance in deep-sea hydrothermal systems. In addition, we provided a guide to develop appropriate culturing methods for isolating novel indigenous bacteria and archaea.

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