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ORIGINAL PAPER

Vertical distribution of bacterial and archaeal communities along discrete layers of a deep-sea cold sediment sample at the East Pacific Rise (~13°N)

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Abstract The community structure and vertical distribution of prokaryotes in a deep-sea (ca. 3,191 m) cold sediment sample (ca. 43 cm long) collected at the East Pacific Rise (EPR) ~13°N were studied with 16SrDNA-based molecular analyses. Total community DNA was extracted from each of four discrete layers EPRDS-1, -2, -3 and -4 (from top to bottom) and 16S rDNA were amplified by PCR. Cluster analysis of DGGE profiles revealed that the bacterial communities shifted sharply between EPRDS-1 and EPRDS-2 in similarity coefficient at merely 49%. Twenty-three sequences retrieved from DGGE bands fell into 11 groups based on BLAST and bootstrap analysis. The dominant groups in the bacterial communities were *Chloroflexi*, *Gamma proteobacteria*, *Actinobacterium* and unidentified *bacteria*, with their corresponding percentages varying along discrete layers. Pairwise *F*st (*F*-statistics) values between the archaeal clone libraries indicated that the archaeal communities changed distinctly between EPRDS-2 and EPRDS-3. Sequences from the archaeal libraries were divided to eight groups. *Crenarchaea* Marine Group I (MGI) was prevalent in EPRDS-1 at 83%, while Uncultured *Crenarchaea* group II B (UCII B) abounded in EPRDS-4 at 61%. Our results revealed that the vertically stratified distribution of prokaryotic communities might be in response to the geochemical settings and suggested that the sampling area was influenced by hydrothermalism. The copresence of members related to hydrothermalism and cold deep-sea environments in the microbial community indicated that the area might be a transitional region from hydrothermal vents to cold deep-sea sediments.

Keywords East Pacific Rise (EPR) · Cold sediment · Microbial diversity · Vertical distribution · Geochemical features

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Introduction

Analyses on microbial community structures in different environments can be extended to give us an overview of functional and biogeographical relationships of microbes (Staley and Gosink 1999), and also, researches in microbial biodiversity will provide us with a key in understanding biogeochemical cycles and evolution of life on the earth (Martiny et al. 2006). At present, environmental microbiological researches all over the world have revealed the presence of a great amount of diverse bacterial and archaeal communities in different geographically separated sites (Wawrik et al. 2007). Phylogenetic studies have also disclosed that environmental bacterial and archaeal populations are complex, widespread and often consisting of uncultivated or unidentified members (Arakawa et al. 2006; Briee et al. 2007; Hoj et al. 2005; Kanokratana et al. 2004; Munson et al. 1997; Pace 1997; Schwarz et al. 2007; Wilms et al. 2006; Yan et al. 2006b). During the past several decades, molecular techniques that based on

investigations and comparisons of small unit RNA genes or other conserved DNA fragments have provided powerful tools in analyzing various unculturable microbes, their roles and relationships among the whole communities and ecosystems, and therefore greatly enhanced our understanding of physiological functions and ecological roles of environmental microbial assemblages (Fry et al. 2006; Holoman et al. 1998; Inagaki et al. 2006a; Kato et al. 1997; MacGregor et al. 2001; Madrid et al. 2001; Wilms et al. 2006).

The East Pacific Rise (EPR), which consists of different geological units such as hydrothermal vents, is a vast low bulge of the sea floor covered by sediments comparable in size to North and South America (Menard 1960). The EPR 13°N has been of great interest to microbiologists and microbial ecologists, since it has been demonstrated to be a new active hydrothermal field by geographic and microbiological evidences (Hekinian et al. 1983). On the one hand, diverse extreme bacterial and archaeal strains isolated and characterized from the EPR 13°N deep marine indicated that the extreme environment amassed a huge resource of microbes, which at present, has not been understood. For instance, some archaeal strains such as Methanogen M7^T (Jeanthon et al. 1999) and *Pyrococcus glycovorans* sp. AL585^T (Barbier et al. 1999) have enriched our knowledge on both the extreme environmental conditions and the physiology of *archaea*, which is so far the least understood evolutionary domain of life on earth. Besides, bacterial strains including KA3^T (Alain et al. 2002), 525^T (Miroshnichenko et al. 2002) and 6N$^{(T)}$ (Audiffrin et al. 2003) were recognized as sulfur-reducers in hydrothermal environments.

Furthermore, molecular and phylogenetic analyses at community level have demonstrated that environmental microbial populations at the deep-sea hydrothermal vent area of the EPR 13°N are very diversified, complex and often with special functions. Hydrothermal archaeal communities within the basaltic flanks (Ehrhardt et al. 2007) and venting sulphide structures (Nercessian et al. 2003) at the EPR 13°N showed great diversity and spatial variation of different dominant groups. DNA-based analysis of the Fe(III)-reducing enrichments from the deep-sea hydrothermal vents at 13°N demonstrated the existence of thermophilic Fe(III)-reducing microorganisms under the extreme conditions (Slobodkin et al. 2001). 16S rRNA- and *aclB* genes-based in-situ hybridization on the samples from this area provided us the first example of prevalence and ecological significance of free-living *Arcobacter* at deep-sea hydrothermal vents (Moussard et al. 2006a), and as reported recently, genomic approaches such as fosmid environmental libraries were employed for microbiological surveys on environmental archaeal community that inhabited the hydrothermal vents at EPR 13°N, and provided more detailed information on diversity, genetics, physiology and ecology of *archaea* (Moussard et al. 2006b).

All the molecular investigations mentioned above concentrated merely on the environmental samples that were closely related to hydrothermal vents, and the biodiversity and the community structure of the microbes in the vast cold marine sediments at the EPR 13°N were seldom studied. However, detailed information of the community structure and spatial variations of the prokaryotes in this area is essential to assess the biological, geographic and chemical relationships under the extreme conditions. Furthermore, it deserves our attention that to what extent the hydrothermalism could influence the investigation area, with 30 km away from the vent.

In the present study, total environmental genomic DNA was extracted from each of four discrete layers of a cold sediment sample collected at the EPR ~13°N. The composition and variation of bacterial and archaeal communities along these layers were determined and compared using 16S rDNA-based DGGE profiles, clone libraries and sequence analysis. To our current knowledge, this is the first comparison of the prokaryotic communities along discrete layers in this area according to16S rDNA sequence analysis.

Materials and methods

Sample description and DNA extraction

Box core were taken from site E272 at a depth of 3,191 m at the East Pacific Rise (12°36′39"N, 104°19′28"W) on 11 November 2003 during the first scientific cruise of hydrothermal sulfur investigation of China. Subsamples were taken using sterilized corers and then divided into eight layers (0–5, 5–10, 10–15, 15–20, 25–30, 30–35 and 35–40 cm) for further analyses. Subsamples of the discrete layers with depth 5–10 (EPRDS-1), 15–20 (EPRDS-2), 25–30 (EPRDS-3) and 35–40 cm (EPRDS-4) were selected for molecular analysis. The samples were stored at −20°C and kept frozen until used. Total organic carbon, total phosphor and total nitrogen were determined as described by GB18668 (GB18668 2002).

Total genomic DNA of each layer was extracted using the FastDNA Spin Kit for Soil (Q-BIOgene, USA) with the modifications (spin speed: 4.5 and spinning time: 30 s) by Dr. Hongyue Dang (Key Laboratory of Marine Geology and Environment, Institute of Oceanology, Chinese Academy of Sciences), and stored at −80°C until analysis.

PCR amplification, DGGE and clone libraries

The V3 region of bacterial 16S rRNA genes were amplified from the total genomic DNA of each layer using bacterial-

specific DGGE primers 341F-GC and 534R as described previously (Muyzer et al. 1993). Each 25-μl reaction mixture contained 2.5 μl of 10× PCR buffer (Promega, USA), 2 μl of dNTP mix (2.5 nM each), 0.5 μl of 341F-GC primer (50 μM), 0.5 μl of 534R primer (50 μM), 0.2 μl of *Taq* polymerase (TaKaRa, Otsushiga, Japan), 1 μl of template DNA, and RNase/DNase-free water to a final volume of 25 μl. The following PCR program was used: 94°C for 5 min, followed by 30 cycles of 94°C for 45 s, 55°C for 50 s, and 72°C for 50 s, followed by 72°C for 10 min.

The PCR products were examined by agarose gel electrophoresis and then were excised and purified using TIANgel Mini Purification Kit (Tiangen, China). DGGE was performed using a Dcode™ Universal Mutation Detection System (Bio-Rad, USA). Similarly sized and equal amounts of purified PCR products were separated on a vertical gel containing 8% polyacrylamide (acrylamide: bisacrylamide ratio of 37.5:1) and a linear gradient of the denaturants (urea and formamide), which increased from 40% at the top to 70% at the bottom of the gel. Electrophoresis was performed at 60°C in a 1× TAE buffer at 65 V for 10 h. The gel was stained with SYBR Gold and nucleic acid bands were visualized and photographed by cannon 640 digital camera (Cannon Co., Ltd, Japan). Bands of interest were excised from the gel, washed with sterile water and then DNA was eluted from them into 30 μl of sterile water by incubation at 4°C overnight. The eluted DNA was first amplified with the primers 341F and 534R. The products were purified and cloned to TA vector using pBS-T kit (Tiangen, China) with TOP10 chemically competent cells (Invitrogen, Canada) as the host. After cultured overnight, a portion of each culture was used as PCR template directly with primer sets T7 and SP6, and then the product was used as template for PCR amplification with primer sets 341F-GC and 534R. The remainders of the cultures were stored at −20°C with a final glycerol concentration of 10%. The PCR products were purified using TIANquick Mini Purification Kit and analyzed by DGGE as described above. The clones that contained the bands in the correct positions were sequenced, and the sequences were deposited in GenBank database with the accession numbers EU259353–EU259375. To determine the similarities of DGGE profiles among samples, unweighted pair group method with arithmetic mean (UPGMA) tree was generated using NTSYS pc-package (Rohlf 1998), based on the presence or absence of each band. To further estimate the community structure revealed via DGGE, the intensity of each DGGE band was measured using Quantity One Program (Bio-rad, USA) and then analyzed.

The partial archaeal 16S rRNA gene fragments were amplified from the total genomic DNA of each layer with general primers 21F and 958R. Each 25-μl reaction mixture contained 2.5 μl of 10× PCR buffer (Promega, USA), 2 μl of dNTP mix (2.5 nM each), 0.5 μl of 21F primer (50 μM), 0.5 μl of 958R primer (50 μM), 0.2 μl of *Taq* polymerase (TaKaRa, Otsushiga, Japan), 1 μl of template DNA, and RNase/DNase-free water to a final volume of 25 μl. The following PCR program was used: 94°C for 5 min, followed by 30 cycles of 94°C for 60 s, 58°C for 60 s, and 72°C for 60 s, followed by 72°C for 10 min. The products were purified using TIANquick Mini Purification Kit and subsequently cloned into pMD18-T (TaKaRa, Japan) vector using *Escherichia coli* TOP10 (Invitrogen, Canada) as the host. Thirty-six clones of each library containing the fragments of expected length were randomly selected and sequenced. Sequences representing different phylotypes were deposited in GenBank database with the accession numbers EU259323–EU259352.

Sequence and phylogenetic analyses

Sequences were checked with the CHECK_CHIMERA tools from the Ribosomal Database Project II (http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU) and chimeric sequences were excluded from further analyses. The closest relatives of the remaining sequences were obtained from the GenBank database using BLAST program. Sequences representing distinct phylotypes (as the criterion, 97% sequence similarity was used for analysis the archaeal sequences in the same phylogenetic clade) and their closest relatives were aligned with CLUSTAL W (Thompson et al. 1994). Alignments were then checked manually using the secondary structure of 16S rRNA molecule. Evolutionary distances were calculated according to Kimura's two-parameter correction method. Neighbor-joining trees were constructed with the bootstrap value of 1,000 using MEGA version 3.0 (Kumar et al. 2004). The archaeal phylotype diversity was estimated by measuring the relationship between species richness according to Margalef's Species Richness (SR) index (Margalef 1958) and the distribution of individuals among species according to Shannon index (H) of diversity (Shannon and Weaver 1949). Species evenness was calculated after Pielou's evenness index (E) (Pielou 1966). Pairwise Fst (F-statistics) values of the archaeal libraries were calculated with the AMOVA module in Arlequin (Excoffier et al. 2005; Martin 2002) based on Jukes-Cantor corrected distance matrices. The Shannon index (H) of diversity is given as follows: $H = -\sum p_i \ln p_i$, where p_i is the proportion of phylotypes i, that is, $p_i = n_i/N$. Species richness index is given as follows: $\mathrm{SR} = (S-1)/\ln N$, where S is the number of phylotypes and N is the total number of clones. The index of evenness is given as follows: $E = H/\log S$ where H is the Shannon index of diversity, and S is the number of phylotypes.

Table 1 Description and geochemical features of the four discrete layers along the deep-sea sediment collected at the EPR ~13°N

	Depth along the sediment column (cm)	Total organic carbon %	Total phosphate %	Total nitrogen %	Sulfur %[b]	*Foraminifera* (yes/no)	Color description
EPRDS-1	5–10	1.10 ± 0.23[a]	0.57 ± 0.13[a]	0.18 ± 0.01[a]	0.38	N	Red brown
EPRDS-2	15–20	0.87 ± 0.19[a]	0.21 ± 0.05[a]	0.14 ± 0.04[a]	0.36	N	Brown
EPRDS-3	25–30	0.98 ± 0.27[a]	0.34 ± 0.04[a]	0.23 ± 0.04[a]	≈0	Y	Yellow green
EPRDS-4	35–40	0.64 ± 0.20[a]	0.18 ± 0.04[a]	0.11 ± 0.02[a]	≈0	Y	Yellow green

[a] Standard errors were based on three observations

[b] Data provided by Shaoxiong YU (unpublished data)

Results

Sample description

The measured geochemical properties of the discrete sediment layers were summarized in Table 1. The sediment sample column collected at a depth of 3,191 m at the EPR ~13°N was clearly stratified in geochemistry. The concentrations of total organic carbon, total phosphor and total nitrogen were comparatively higher in the layers EPRDS-1 and EPRDS-2. Total sulphur content also decreased with depth, where no sulphur was detected in layers EPRDS-3 and EPRDS-4. Planktonic foraminifera shells were found only in the deeper layers EPRDS-3 and EPRDS-4. The color along the layers changed from red-brown to yellow green, reflecting increased reductivity in the overall geochemical settings.

DGGE analysis of bacterial communities inhabited the four discrete sediment layers

DGGE finger-printings of the predominant *bacteria* in the four sediment layers collected at the EPR ~13°N were shown in Fig. 1a. Every layer gave rise to different community profile. More bands were generally observed in the DGGE profiles of upper layers. The number of the predominant bands from EPRDS-1 was the largest among all, whereas the one of EPRDS-4 was the least. Cluster analysis of the DGGE profiles indicated that the bacterial community structure varied with depth (Fig. 1b). The UPGMA tree revealed that the bacterial communities of the four discrete layers were similar to each other in different degrees. The community structure of the top layer EPRDS-1 shared merely 49% similarity to the other three layers, while 81% for EPRDS-4 with EPRDS-2 and EPRDS-3, and 87% between EPRDS-2 and EPRDS-3.

Sequencing analysis of the predominant bacteria that represented the DGGE bands was summarized in Table 2. Blast analysis revealed that the closest relatives of the predominant bacteria in this study were from various environments, particularly in deep-sea sediments and

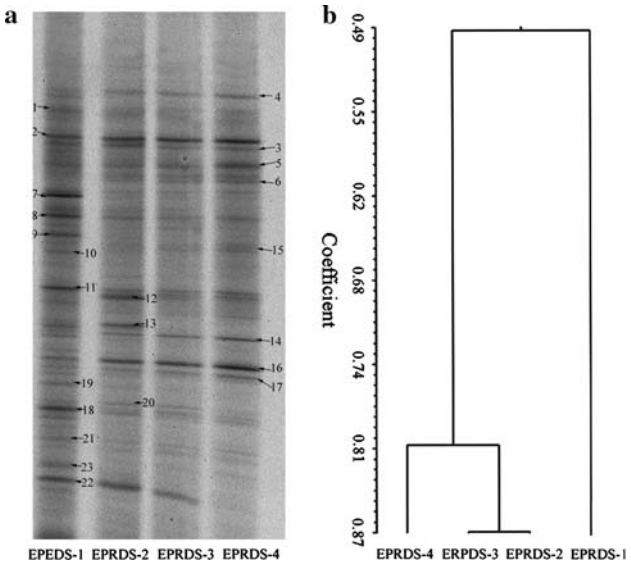

Fig. 1 **a** DGGE profiles of the bacterial communities inhabited the four discrete layers along the sediment sample collected at the EPR ~13°N based on 16S rDNA-V3 fragments. **b** Cluster analysis of the DGGE profiles. The UPMMA tree was generated with NTSYS version 2.02 based on the presence and absence of each band

hydrothermal areas. Statistics showed that most of the sequences were found in this area for the first time. The phylogenetic relationship of all the partial 16S rDNA sequences retrieved from DGGE was shown in Fig. 2. Bootstrap analysis divided all 23 sequences corresponding to the 23 DGGE bands into 11 groups: *Alpha proteobacteria* (three sequences), *Beta proteobacteria* (1), *Gamma proteobacteria* (2), *Delta proteobacteria* (1), *Firmicutes* (1), *Acidobacteria* (1), *Actinobacterium* (5), *Chloroflexi* (4), *Flexibacter* (2), *Planctomycetes* (1) and unidentified *bacteria* (2).

The community structure of bacterial biomass revealed via DGGE was summarized in Fig. 3a, and the structure comparison among the four discrete layers was displayed in Fig. 3b, in percentage of different groups based on the density of their correlated DGGE bands. Considering all sample layers, *Chloroflexi* (19%), *Gamma proteobacteria* (16%) and *Actinobacteria* (16%) dominated the bacterial

Table 2 The overview of bacterial 16S-V3 rDNA sequences detected by PCR-DGGE

Phylogenetic affiliation	Accession number	Closest relatives	% Similarity	Environmental description	Band position
Alpha proteobacteria	EU259369	Clone MD2902-B27	99	Marine sediment	7
	EU259368	Endosymbiont 1b of Inanidrilus leukodermatus	99	Marine sediment	4
	EU259366	Clone LC1-35	100	Marine sediment	23
Beta proteobacteria	EU259370	Clone JH-WH208	99	Soil around iron–manganese nodule	6
Gamma proteobacteria	EU259367	*Acinetobacter* sp. SS-2	99		9
	EU259371	Clone E29	100	Deep-sea sediment	2
Delta proteobacterium	EU259358	Clone ss1_B_02	96	Marine sediment	13
Actinobacteria	EU259373	Clone: NB1-i	96	Deep-sea sediment	1
	EU259355	Clone AT-s2-33	99	Hydrothermal sediment	10
	EU259354	Napoli-1B-02	100	Hydrothermal sediment	11
	EU259353	Napoli-1B-02	99	Hydrothermal sediment	12
	EU259356	Clone A20	100	Deep-sea sediment	21
Flexibacter	EU259365	Clone SC1-26	95	Marine sediment	8
	EU259364	Clone ctg_BRRAA24	99	Deep-sea environment	18
Firmicutes	EU259372	Clone GASP-77KB-862-F12	93	Ancient dune fields	5
Planctomycetes	EU259375	Clone DE6.13	99	Deep-sea sediment	17
Chloroflexi	EU259359	Clone ODP1251B13.5	98	Hydrothermal sediment	14
	EU259360	FS117-47B-02	97	Ridge flank crustal fluids	15
	EU259362	Clone 3G02-01	98	Marine sediment	16
	EU259363	Clone reef138	97	Marine sediment	20
Acidobacteria	EU259374	PCR-derived sequence	97	Hydrothermal sediment	19
Unidentified bacteria	EU259361	Uncultured bacterium	98	Hydrothermal sediment	22
	EU259357	Clone MD2902-B8	100	Deep-sea sediment	3

community. Members belonging to unidentified *bacteria*, *Flexibacter*, *Alpha proteobacteria*, *Firmicutes*, *Delta proteobacteria*, *Beta proteobacteria*, *Planctomycetes* and *Acidobacteria* accounted for 13, 9, 8, 7, 4, 3, 3 and 2% of the community, respectively.

The DGGE profile of the top layer EPRDS-1 produced 17 bands. *Actinobacteria*, *Gamma proteobacteria*, *Flexibacter*, *Alpha proteobacteria* and the unidentified *bacteria* were the predominant groups, which accounted for 20, 16, 16, 16 and 13% of the total DGGE revealed bacterial biomass. In addition, bands that represented *Chloroflexi*, *Firmicutes*, *Acidobacteria* and *Delta proteobacteria* were also detected in the DGGE profile of the top layer. Fourteen bands, representing all bacterial groups in this study, except for the *Acidobacteria*, were detected in the DGGE profile of EPRDS-2. The predominant groups in this layer were *Chloroflexi* (18%), unidentified *bacteria* (17%), *Actinobacteria* (15%) and *Gamma proteobacteria* (13%). There were 14 bands in the DGGE profile of EPRDS-3, covering all the bacterial groups with exception of *Acidobacteria* and *Delta proteobacteria*. The percentage of *Chloroflexi* of the total bacterial biomass dramatically increased to 32% in this layer. *Gamma proteobacteria* and unidentified *bacteria* were another two predominant groups in EPRDS-3 with the percentage of 15 and 12%, respectively. The DGGE profile of the bottom layer EPRDS-4 was made up of 13 main bands. *Chloroflexi* (22%), *Gamma proteobacteria* (15%) and *Actinobacteria* (12%) acted as the dominant groups, while members of *Acidobacteria* and *Delta proteobacteria* were absent from the DGGE detection of this layer.

Diversity, community structure and vertical zonation of *archaea*

A total of 144 clones were randomly sequenced from the four clone libraries. After chimera-check, 132 partial archaeal 16S rDNA sequences were used for further analysis. Considering all sediment layers, archaeal 16S rDNA sequences were grouped into 30 phylotypes according to bootstrap analysis. All of the sequences were found to have their closest relatives of uncultured archaeal clones from various marine environments. The phylogenetic relationship of these phylotypes (Fig. 4) showed that 29

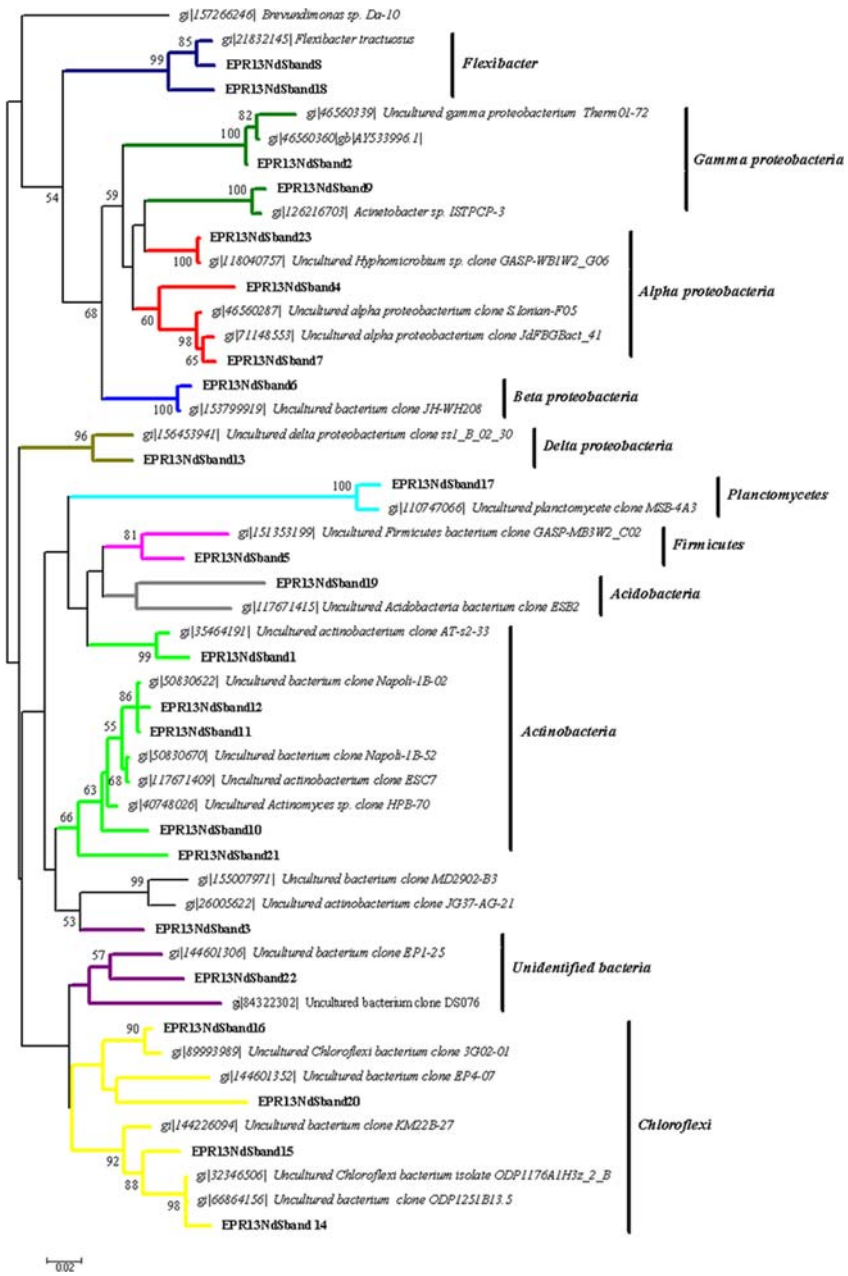

Fig. 2 Phylogenetic analysis on bacterial 16S rDNA-V3 fragments retrieved from DGGE bands. Bootstrap test was based 1,000 replicates and the bootstrap values less than 50% were omitted. Sequences obtained from the cold sediment sample collected at the EPR ~13°N in this study are represented in *bold* letters. The scale bar indicates the inferred frequency of substitutions per nucleotide site

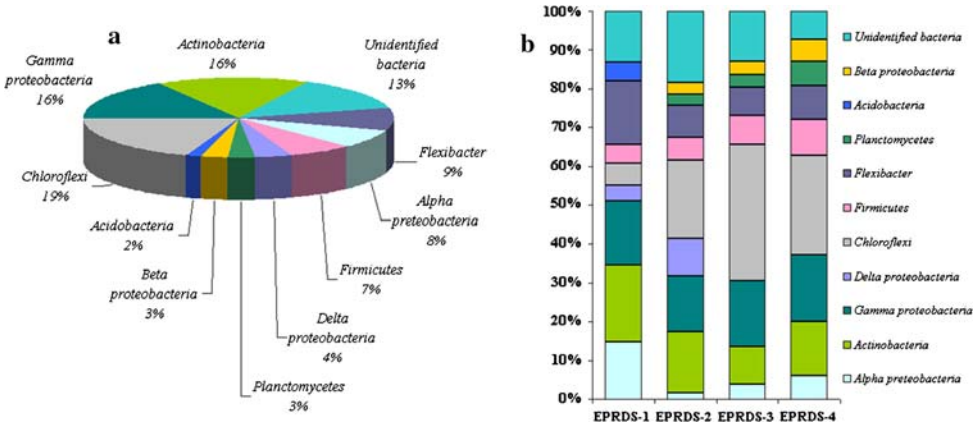

Fig. 3 Bacterial community structures based on the quantitative analysis of the DGGE bands: **a** Analysis of the total DGGE detected bacterial biomass; **b** Depth patterns of the DGGE detected bacterial biomass along the four discrete layers

phylotypes belonged to the Crenarchaeota and only one phylotype belonged to the Euryarchaeota. Bootstrap analysis divided the phylotypes in the Crenarchaeota into seven groups: Marine Benthic Group A *Crenarchaea* (MBGA) (two phylotypes), Marine Benthic Group B *Crenarchaea* (MBGB) (2), Uncultured *Crenarchaea* Group II B (UCII B) (1), *Thermoprotei* (TH) (2), Novel *Crenarchaea* (NC) (4), Soil *Crenarchaea* Group (SCG) (1) and Marine Group I *Crenarchaea* (MGI) (17). Statistics of all the archaeal sequences (Fig. 5a) showed that members of MGI (36%) and UCII B (26%) dominated the whole archaeal community. Sequences related to *Thermoprotei* accounted for 12% of all clones, and for the rest, 8% belonged to MBGA, 8% to MBGB, 6% to NC, 2% to SSG and 2% to the Euryarchaeota (EU).

The distribution of archaeal clones along the four discrete layers was summarized in Table 3 and compared in Fig. 5b. Members of MGI decreased with depth, whereas members of MGBA, MBGB and UCII B increased. Clones related to NC were detected only in EPRDS-2 and EPRDS-3. The presence of *Thermoprotei* ranged from 12 to 19% in the upper three layers and members of SSG and EU were mainly detected in EPRDS-4.

Eleven phylotypes of partial archaeal 16S rDNA sequences were identified from 31 sequenced archaeal clones from EPRDS-1. The majority of the clones from this layer belonged to MGI at the percentage of 83%. *Thermoprotei* and MBGA accounted for 12 and 5% of all clones, respectively. It was found that the sequences from this layer had their closest relatives in deep marine sediments of the Weddell Sea near Antarctica (Gillan and Danis, unpublished data), Madovi Estuary sediment (Singh et al., unpublished data), sediment of the Pearl River Estuary of China (Jiang et al., unpublished data) and Xisha Trough surface sediment (Li et al., unpublished data). The Shannon index (*H*) of diversity measure was 2.26, the Margalef's richness measure was 2.05, the species evenness value was 2.17 and the *F*st value of AMOVA within group was 0.41 (Table 4).

Thirty-four partial archaeal 16S rDNA sequences from EPRDS-2 fell into 17 phylotypes. Members of MGI accounted for 58% of all sequences. Clones related to *Thermoprotei* accounted for 19% of the clone library and clones associated with the MBGA accounted for 10% of all sequences. Sequences belonging to NC and UCII B represented 8 and 2% of all clones in this layer, respectively. The closest relatives of the sequences from this layer were from the uncultured clones in deep marine sediments of the Weddell Sea (Gillan and Danis, unpublished data), hydrothermal sediments from the Yonaguni Knoll IV of Southern Okinawa Trough (Nunoura et al., unpublished data), methane hydrate bearing sub-seafloor sediment at the Cascadia margin (Inagaki et al. 2006b), sediment of the Pearl River Estuary in China (Jiang et al., unpublished data), Xisha Trough surface sediment (Li et al., unpublished data), metal-rich and low-activity deep subsurface sediment of the Peru Basin (Sorensen et al. 2004), sediment of Napoli mud volcano at Eastern Mediterranean (Heijs et al., unpublished data) and mangrove soil (Yan et al. 2006a). The values of Shannon index (*H*) of diversity, Margalef's richness, species evenness and *F*st of AMOVA within group were 2.73, 2.87, 2.26 and 0.39, respectively, in Table 4.

The archaeal clone library of EPRDS-3 was composed of 35 sequences that could be divided into eight phylotypes. UCBII, MBGA and *Thermoprotei* accounted for 31, 17 and 16% of the library, respectively. MGI, NC and MBGA each accounted for 15, 14 and 8% of all clones. The archaeal 16S rDNA sequences from this layer were most closely related to the uncultured archaeal clones from deep marine sediments of the Weddell Sea near Antarctica (Gillan and Danis, unpublished data), tropical estuary (Singh et al., unpublished data), organic poor sediments of the central Pacific Ocean (Lauer et al., unpublished data), metal-rich and low-activity deep subsurface sediment of the Peru Basin (Sorensen et al. 2004), marine sediment from Skan Bay (Kendall et al. 2007) and mangrove soil (Yan et al. 2006a). The value of the Shannon index (*H*) of diversity, the value of the Margalef's richness, the value of species evenness and the value of *F*st of AMOVA within group were 1.88, 1.43, 2.08 and 0.39, respectively (Table 4).

Only four phylotypes were identified to represent all the 33 sequences from the clone library of the bottom layer EPRDS-4. Members of UCII B accounted for 61% of all clones. Sequences belonging to MBGB, SCG and EU represented 27, 6 and 6% of all sequences, respectively. The closest relatives of the sequences in this library were from sediment of Norway Haakon Mosby Mud Volcano (Barents Sea) (Losekann et al. 2007), deep marine sediment of Weddell Sea near Antarctica (Gillan and Danis, unpublished data), metal-rich and low-activity deep subsurface sediment of the Peru Basin (Sorensen et al. 2004) and Lonar Soda Lake in India (Wani et al. 2006). The Shannon index (*H*) of diversity measure was 0.96, the Margalef's richness measure was 0.61, the species evenness value was 1.60, whereas the *F*st value of AMOVA within group was 0.40 (Table 4).

Discussion

This study reported the vertical distribution of the prokaryotic communities inhabiting the cold sediment at EPR ~13°N, using 16S rDNA-based techniques. We refer to this paper for an extensive description on the gradient and dynamics of some detailed composition of the prokaryotic

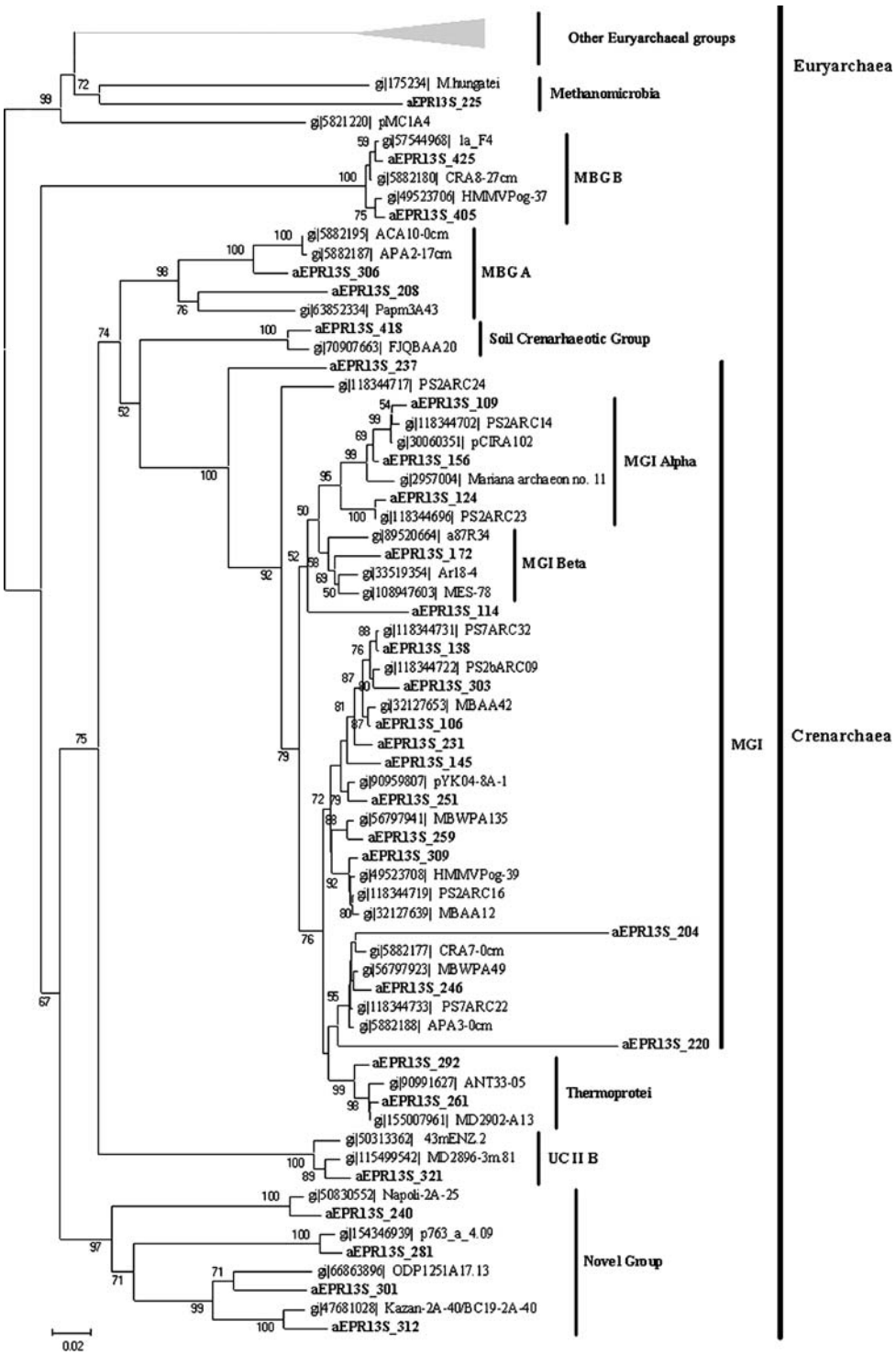

Fig. 4 Phylogenetic analysis of the phylotypes of partial archaeal 16S rDNA sequences obtained in this study. Bootstrap test was based 1,000 replicates and the bootstrap values less than 50% were omitted. Sequences obtained from the cold sediment sample collected at the EPR ~13°N in this study are represented in *bold* letters. The scale bar indicates the inferred frequency of substitutions per nucleotide site

communities in this area. It must be acknowledged that, limited by present techniques, many research procedures such as sample collection, DNA extraction, PCR amplification (Kanagawa 2003; von Wintzingerode et al. 1997; Zeidner and Beja 2004) and the other following operations might introduce bias to our results. We also have to admit that restricted by the mechanism of DGGE techniques (Muyzer et al. 1993), the retrieved bacterial 16S rDNA V3 fragments were only about 190 bp in length and merely representing dominant groups. The former length restriction was a major limitation to phylogenetic analysis and the latter might lead to underestimation on the diversity of the

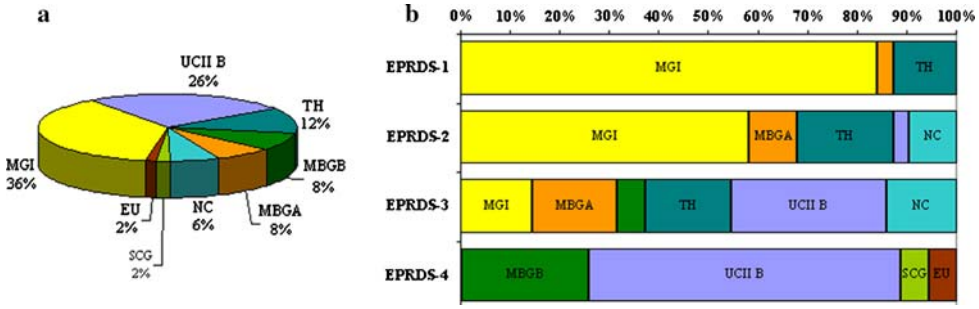

Fig. 5 Archaeal community structures based on the 16S rDNA libraries: **a** Analysis of the total clones sequenced in this study; **b** Depth patterns of the archaeal communities along four discrete layers

Table 3 Phylotype distribution and their phylogenetic grouping of archaeal 16S rDNA sequences in the four discrete layers along the deep-sea sediment sample collected at the EPR ~13°N

Phylogenetic group	Phylotype sequences			
	EPRDS-1	EPRDS-2	EPRDS-3	EPRDS-4
Marine group I *Crenarchaea*	aEPR13S-109 (7), aEPR13S-156 (2), aEPR13S-124 (4), aEPR13S-172 (2), aEPR13S-114 (2), aEPR13S-138 (2)[a], aEPR13S-106 (2), aEPR13S-145 (3), aEPR13S-133 (2)[b]	aEPR13S-237(2), aEPR13S-231 (2), aEPR13S-251 (2), aEPR13S-259 (1), aEPR13S-233 (1)[a], aEPR13S-274 (3)[b], aEPR13S-204 (2), aEPR13S-220 (1), aEPR13S-246 (4)	aEPR13S-303 (3), aEPR13S-309 (2)[b]	Not found
Marine benthic group A *Crenarchaea*	aEPR13S-154(1)[c]	aEPR13S-208 (3)[c]	aEPR13S-306 (6)	Not found
Marine benthic group B *Crenarchaea*	Not found	Not found	aEPR13S-315 (2)[d]	aEPR13S-405 (3), aEPR13S-425(6)[d]
Thermoprotei	aEPR13S-150 (4)[e]	aEPR13S-292 (3), aEPR13S-261 (3)[e]	aEPR13S-317 (6)[e]	
Uncultured *Crenarchaea* II B	Not found	aEPR13S-216 (1) [f]	aEPR13S-321 (11)[f]	aEPR13S-412 (22)[f]
Soil *Crenarchaea* group	Not found	Not found	Not found	aEPR13S-418(2)
Novel *Crenarhaea*	Not found	aEPR13S-281 (1), aEPR13S-240 (2)	aEPR13S-301 (3), aEPR13S-312 (2)	Not found
Euryarchaea	Not found	aEPR13S-225 (2)	Not found	Not found

Numbers between within *brackets* represent the total of sequences detected belonging to each phylotype

[a–f] Sequences belonging the same phylotype but inhabited different layers

bacterial communities. The quantification of intensity of DGGE bands added bias to the analysis, whereas it provided more detailed information of the bacterial community structure and the dominant groups. In addition, according to the rarefaction curves of clone libraries (data not shown), archaeal phylotypes were not saturated in the clone library of layer EPRDS-2, which indicated that the full diversity of the archaeal communities were far to be covered. Nevertheless, the results of this study provided new insights through an overall picture of clear heterogeneity between the prokaryotic communities that inhabited discrete sediment layers at the EPR ~13°N deep marine.

The phylogenetic analysis of 16S rDNA sequences obtained from the sediment samples revealed a significant diversity and a clearly vertical zonation of the bacterial community. All the environments related to the closest relatives of sequences obtained in this study shared two common backgrounds: deep-sea sediment or influence by hydrothermal vents or hot springs. Some of the sequences were recently reported, while the others have never appeared in publications yet. Therefore, detailed description on these organisms was very limited or unavailable (Table 2). The *Chloroflexi* was frequently found in deep-sea environments since it was firstly recognized as a division-level bacterial group about two decades ago (Woese 1987), whereas the functions of this group remained unidentified so far. Much like the recent report on the sediment samples of Florida Escarpment (Reed et al.

Table 4 Margalef's species richness, Shannon index (*H*) of diversity indices, and species evenness of phylotypes in archaeal 16S rDNA libraries and DNA sequence based *F*st indices of AMOVA within libraries

	Margalef's species richness	Shannon index (*H*) of diversity	Species evenness	*F*st
EPRDS-1	2.05	2.26	2.17	0.41
EPRDS-2	2.87	2.73	2.26	0.39
EPRDS-3	1.43	1.88	2.08	0.39
EPRDS-4	0.61	0.96	1.60	0.40

Table 5 Pairwise *F*st indices of the archaeal communities that inhabited discrete layers at the EPR ~13°N based on Jukes-Cantor method

	EPRDS-1	EPRDS-2	EPRDS-3	EPRDS-4
EPRDS-1	0.00	–	–	–
EPRDS-2	0.06	0.00	–	–
EPRDS-3	0.46	0.27	0.00	–
EPRDS-4	0.67	0.49	0.05	0.00

2006), the increasing percentage of *Chloroflexi* with depth in this study might indicate its unique role in more anoxic environments. *Gamma Proteobacteria* was wildly found in deep-sea sediments, working as the sulfide-oxidizer in both hydrothermal vents (Nercessian et al. 2005) and cold seeps (Larkin and Henk 1996). The prevalence of chemoautotrophic sulfide-oxidizing organisms such as *Gamma Proteobacteria* in all layers in this study resembled the previous report on Nankai Trough (Newberry et al. 2004) and Rain Bow Vent field (Nercessian et al. 2005), and might indicate a reducing environment of the cold sediment around the hydrothermal vent at the EPR 13°N. *Actinobacteria* were small but very significant and ubiquitous members in marine communities (Venter et al. 2004). The dominance of *Actinobacteria* in the bacterial communities in this study coincided with the results of a previous research on the deep-sea sediment samples from Nankai Trough (Colwell et al. 2004); however, the roles or functions of *Actinobacteria* in these extreme environments remained untouched to a great extent. The unidentified *bacteria* in this study, together with its closest relatives from the Xisha Trough surface sediment (Li et al. unpublished data) and the Japan Trench (Yanagibayashi et al. 1999), provided a novel bacterial subdivision and might indicate special functions.

The archaeal communities observed in clone libraries obtained from the four discrete layers were also very diverse and showed obviously stratified features. The unpublished sequences, which were most closely related to the archaeal communities in this study, accounted for a much higher percentage than the bacterial ones, and this phenomenon indicated that the archaeal community was a mysterious world, which was far to be understood for human. *Crenarchaea* is a predominant phylum in various environments such as soil, seawater, sediment and so forth. The high percentage of *Crenarchaea* in this study was much like the phylogenetic analysis of the archaeal community concentrated on the deep-sea sediment samples from West Pacific Warm Pool (Wang et al. 2005). It was reported that MGI were largest population of *archaea* in our world and found ubiquitous in marine environments such as the deep-sea and coastal hydrothermal areas (Huber et al. 2002; Takai et al. 2004). The decrease of MGI with depth in this study indicated that MGI might not be suitable for extreme anaerobic conditions in the deeper layer of the sediment. UCII B were found in the walls of an active deep-sea sulfide chimney (Schrenk et al. 2003), hot spring of Yellow Stone National Park (Barns et al. 1994), Iceland (Marteinsson et al. 2001) and metal-rich and low-activity deep subsurface sediment of the Peru Basin (Sorensen et al. 2004). Members of UCII B were rarely cultured so that we could only infer that they might play a special role in anoxic metabolism. In our data, the distribution of UCII B might reflect different physical and chemical factors in different sediment layer such as anaerobic conditions and PH status. *Thermoprotei* was a big group of *Crenarchaea* including *Desulfurococcales*, *Sulfolobales*, *Thermoproteales*, etc., and these *Crenarchaea* were considered to play crucial roles in sulfur cycling. The dominance of *Thermoprotei* in the top three layers provided clues of the influence of hydrothermal activities. MGBA were firstly defined in deep-sea sediment from northwest Atlantic Ocean (Chandler et al. 1998) and MBGB were mainly related to methane-rich benthic sediment, whereas their functions were not determined yet.

Comparison of the prokaryotic community structures of the sediment sample revealed a clearly stratified distribution along discrete layers; however, the dividing lines of bacterial and archaeal communities were not identical. The bacterial communities shifted sharply from EPRDS-1 toEPDS-2 according to DGGE profiles and cluster analysis (Fig. 1a, b), while the changes along the bottom three layers were rather moderate. The pairwise *F*st values (Table 5) between groups revealed that the dividing line of the archaeal communities along four discrete layers was between the middle two layers EPRDS-2 and EPRDS-3. The different dividing lines of bacterial and archaeal communities along the layers indicated that different prokaryotic groups held distinct sensitivities to the environmental conditions such as physical and chemical factors. According to the values of Margalef's species richness, species evenness and Shannon index (*H*) of diversity indices of 16S rDNA phylotypes, the structure

and diversity of archaeal community in bottom layer differed much from the other layers, although the DNA-based *F*st indices of AMOVA within groups that revealed their divergence on nucleic level are quite similar (Table 4). This phenomenon suggested that archaeal 16S rRNA genes shared a similar mutation rate despite the fact that they differed on phylotypes. A recent study on the inorganic elements in this area also demonstrated a vertical geochemical distribution (Yuan et al., unpublished data) as the total stratified geochemistry settings in this study. The accordance between geochemical features and vertical shifts of prokaryotic communities indicated the crucial impact of the environmental characteristics such as the physical and chemical factors on the microbial communities.

Analysis on the prokaryotic communities proved that the sampling area in this study was influenced by hydrothermal activity. Although physiology cannot be assumed from phylogeny, comparative phylogenetic analysis on environmental sequences may reveal some features of the prokaryotic communities, and also may provide the indications of geochemical conditions. On the one hand, both bacterial and archaeal communities in this study contained a great portion of members (34.7% of bacterial sequences retrieved from DGGE bands and 31.0% of the archaeal phylotypes obtained from clone libraries) whose closest relatives were from various hydrothermal-related environments, and therefore we could infer the presence of influence by hydrothermalism in this area, and our colleagues Chun-wei Yuan and Zhigang Zeng also proved the influence by hydrothermal plume in this area through element analyses (Yuan et al., unpublished data). The influence of hydrothermalism to the microbial communities of surrounding environments has been demonstrated by previous studies such as at Rainbow vent field on the Mid-Atlantic Ridge (Nercessian et al. 2005) and in the basaltic flanks of the EPR (Ehrhardt et al. 2007). Compared with previous studies, the sampling area of this study was more distant from the vent, for approximately 30 km. The influence of hydrothermal vent to the biosphere in this area was owing to several oceanic and geographic factors, i.e., the deep marine currents and the relative altitude between the vent (ca. 2,600 m depth) and our sampling region (ca. 3,191 m depth at a deep-sea trench). On the other hand, the prevalence of other members in the bacterial and archaeal communities indicated spatial distinction between our sampling area and the hydrothermal vent. The dominance of *Crenarchaea* in archaeal communities in all layers shared more similarity to the archaeal communities at West Pacific Warm Pool (Wang et al. 2005) than to the hydrothermal vent at EPR 13°N (Nercessian et al. 2003). Therefore, the results presented here presented a transitional prokaryotic community from hydrothermal vents to cold sediments, coupling with the transitional geochemical features in this area under the influence of hydrothermalism.

Studying endemic features of bacterial and archaeal communities provided not only an integrated view of the natural ecological system, but also some clues to historical events. According to the C to N ratios of biomass, organic matter in surface sediment in this area was mainly influenced by marine depositions, whereas in sediment deeper than 30 cm, it was mainly affected by terrigenous depositions (Yu et al., unpublished data). This viewpoint was proved by some of the results in this study. For instance, the hydrothermal-related organisms thriving in the top layers indicated the influence of the hydrothermal fluids. However, the bacteria corresponding to DGGE band five, whose closest relative is an uncultured organism living at subtropical Altamaha and Ohopee River Valley inland dune fields that formed thousands of years before A.D (Tarlera and unpublished data), in the bottom layer of the sediment might record the original information of the biosphere in this area. Therefore, the depth profiles of the prokaryotic communities might reflect some occurrence and vicissitude of different niches in the sampling area. Combining with our common knowledge on the geographic sediment depositing-speed, we could roughly infer that the influence of hydrothermalism in this area increased during the past several thousand years, and this opinion also coincided with the results of element analysis by our colleagues (Yuan et al., unpublished). A further study on the prokaryotic community structure that inhabits deeper sediment layers beyond our sample could provide prospect to test this hypothesis.

In conclusion, this study revealed a quite complex prokaryotic community in a cold sediment sample collected at the EPR ~13°N, and the dominant groups of each community were determined. Both bacterial and archaeal communities exhibited a clearly vertical and stratified distribution along four layers due to changes in geochemical settings. The high percentage of hydrothermal-related members in prokaryotic communities indicated a considerable impact of the hydrothermalism on the surrounding environments, whereas total community analysis revealed that this area was a transitional region from the vents to common cold floor in great biological and ecological significance. Further studies on functional genes or large genomic fragments of the environmental communities will be necessary for better understanding of the special ecosystem in this area.

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