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Microbial Diversity of a Sulfide Black Smoker in Main Endeavour Hydrothermal Vent Field, Juan de Fuca Ridge

Huaiyang Zhou[1,2], Jiangtao Li[1,2]*, Xiaotong Peng[2], Jun Meng[3], Fengping Wang[3], and Yuncan Ai[4]

[1]*Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, P. R. China*
[2]*State Key Laboratory of Marine Geology, Tongji University, Shanghai 200092, P. R. China*
[3]*Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, SOA, Xiamen 361005, P. R. China*
[4]*State Key Laboratory of Biocontrol, Sun Yat-sen University, Guangzhou 510275, P. R. China*

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Submarine hydrothermal vents are among the least-understood habitats on Earth but have been the intense focus of research in the past 30 years. An active hydrothermal sulfide chimney collected from the Dudley site in the Main Endeavour vent Field (MEF) of Juan de Fuca Ridge was investigated using mineralogical and molecular approaches. Mineral analysis indicated that the chimney was composed mainly of Fe-, Zn- and Cu-rich sulfides. According to phylogenetic analysis, within the Crenarchaeota, clones of the order Desulfurococcales predominated, comprising nearly 50% of archaeal clones. *Euryarchaeota* were composed mainly of clones belonging to Thermococcales and deep-sea hydrothermal vent *Euryarchaeota* (DHVE), each of which accounted for about 20% of all clones. Thermophilic or hyperthermophilic physiologies were common to the predominant archaeal groups. More than half of bacterial clones belonged to *ε-Proteobacteria*, which confirmed their prevalence in hydrothermal vent environments. Clones of *Proteobacteria* (*γ*-, *δ*-, *β*-), Cytophaga-Flavobacterium-Bacteroides (CFB) and Deinococcus-Thermus occurred as well. It was remarkable that methanogens and methanotrophs were not detected in our 16S rRNA gene library. Our results indicated that sulfur-related metabolism, which included sulfur-reducing activity carried out by thermophilic archaea and sulfur-oxidizing by mesophilic bacteria, was common and crucial to the vent ecosystem in Dudley hydrothermal site.

***Keywords*:** Dudley, hydrothermal chimney, microbial diversity, sulfur-related metabolism

Since the discovery of deep-sea hydrothermal vents on the Galapagos Rift of the East Pacific Ocean in 1977 (Corlis *et al*., 1979), seafloor hydrothermal system and related geological, chemical and biological phenomena have been the focus of many scientific investigations. Various sulfide structures, or chimneys, are formed by gradual precipitation or crystallization when high-temperature (350∼400°C), metal-rich reduced fluids mix with cold (average temperature about 2°C), oxidative seawater (Tivey and Delaney, 1986; Tivey *et al*., 1999). The unique physical and chemical gradients in the chimney support flourishing microbial communities. These microbial communities have unique physiological characteristics and are mainly chemolithoautotrophs (Prieur, 1997; Corre *et al*., 2001; Kelley *et al*., 2002). So far, culture-independent molecular phylogenetic approaches using the 16S rRNA gene have been successful in investigating the diversity of microbes in different hydrothermal structures (Takai and Horikoshi, 1999; Reysenbach *et al*., 2000; Takai *et al*., 2001; Hoek *et al*., 2003; Schrenk *et al*., 2003; Brazelton *et al*., 2006). It is interesting to reveal that there are abundant microbial groups with various as-yet-uncultivated members. The diversity of microbial communities varies with different geological configures, hydrothermal fluids, mineralogy and age of chimneys (Takai *et al*., 2001; Hoek *et al*., 2003; Schrenk *et al*., 2003).

In 2005, a sulfide black smoker was collected from the Dudley hydrothermal site of the MEF during a China-USA Joint Dive Cruise to Juan de Fuca Ridge by R/V Atlantis/Alvin. Microbial communities of the sample were analyzed by molecular biological techniques. We believe that our results will contribute to the expanding database of hydrothermal microbes.

Materials and Methods

Sampling site, sampling, and subsampling procedure

The Main Endeavour vent Field (MEF, 47°57'N, 129°05'W), with 2200 m water depth, is one of five hydrothermal vent fields in the Endeavour segment of Juan de Fuca Ridge. There are many large, steep-sided active, and inactive hydrothermal sulfide structures located in the active faults and fissures in MEF (Fig. 1) (Delaney *et al*., 1992; Butterfield *et al*., 1994). The sulfide black smoker collected at Dudley site (x=5008, y=6114; depth=2192 m) by DSV Alvin (Dive No.4132) was actively venting fluids of 330°C. The chimney was about 25 cm long and 12 cm wide, with a central conduit varying in diameter from 1 to 3 cm. Numerous tube worms were attached to the exterior of the chimney. The chimney was stored at -20°C immediately after it taken onboard and then maintained in dry ice during transportation to our labo-

* To whom correspondence should be addressed.
(Tel) 86-20-8529-0303; (Fax) 86-20-8529-0303
(E-mail) ljtao98@126.com

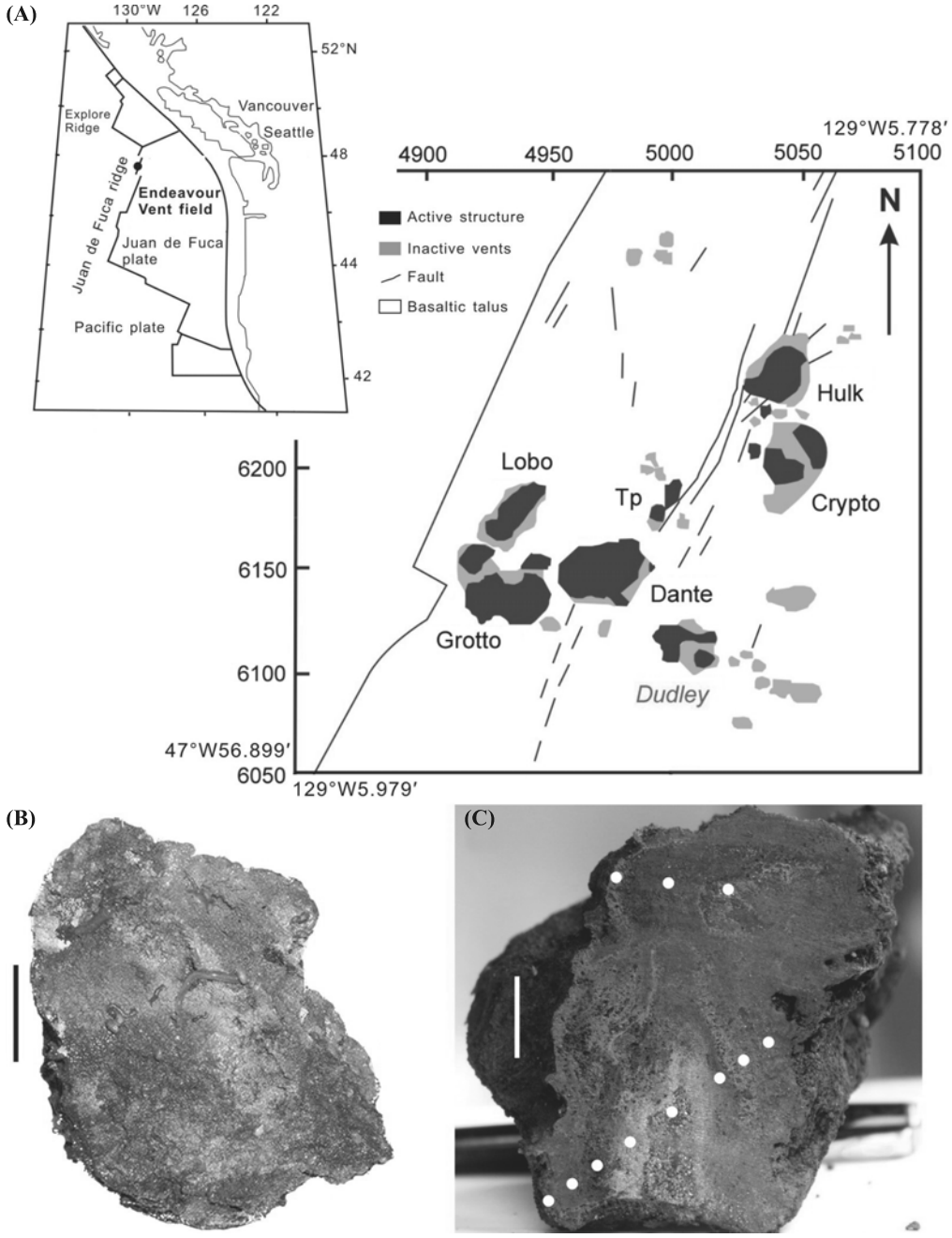

Fig. 1. (A) Location of Dudley hydrothermal vent site in Main Endeavour vent Field (MEF) of Juan de Fuca Ridge where DSV Alvin dived for No.4132 (modified after Delaney *et al.*, 1992; Butterfield *et al.*, 1994). (B) Sulfide structure collected from Dudley hydrothermal site. Some exterior wall formed brown oxides caused by the weathering and inhabited by tube worms. (C) the transect for mineral analysis. The white dots referred the site of sampling from the outer to inner. Scale bar=5 cm.

ratory in China. We subsampled from horizontal transect which extended from the outer side of the chimney wall to the hydrothermal fluid flow channel. Subsamples were pooled, then crushed and mixed well with sterilized tools. A portion of the pooled subsample was milled in liquid nitrogen for the microbial DNA extraction, and another portion was dried for SEM observation. In addition, a small number of mixed subsamples were fixed with 4% paraformaldehyde for Fluorescence *In Situ* Hybridization (FISH).

Mineral analysis

Samples were taken as described above for the identification of representative mineral compositions and phases (Fig. 1). The samples were dried and thoroughly ground using mortar and pestle. Then X-ray diffraction (XRD) pattern analysis was performed using a Rint 2000 X-ray diffrac-

Table 1. Oligonucleotide probes used in this study and optimized experimental conditions

Probe	Specificity	Sequence (5'-3')	Formamide conc.	Reference
Arch915	Archaea	GTGCTCCCCCGCCAATTCCT	30%	Boetius *et al*. (2000)
Eury498	*Euryarchaeota*	CTTGCCCRGCCCTT	10%	Schrenk *et al*. (2003)
Cren499	*Crenarchaeota*	CCAGRCTTGCCCCCCGCT	20%	Schrenk *et al*. (2003)
Eub338	Bacteria	GCTGCCTCCCGTAGGAGT	30%	Amann *et al*. (1995)
EP404	ε-*Proteobacteria*	AAA KGY GTC ATC CTC CA	30%	Zerkle *et al*. (2005)

tometer (Rigaku Co., Japan) with Cu Kα radiation at 40 kV and 30 mA. Diffraction angles (referred to as '2θ') corresponding to the atomic structure unique to each mineral were measured.

Scanning Electron Microscopy (SEM)

Subsamples were observed using FEI Quant 400 environmental scanning electron microscopy (SEM, FEI Company, USA). Gold coating on surfaces was not required for wet samples, which avoided influence of pretreatment on spectral analysis. Micro-area chemical composition of mineral surfaces was analyzed on Genesis energy dispersive spectrometer (EDS) made by EDAX corporation.

Fluorescence *In Situ* Hybridization (FISH)

Following retrieval, sulfide subsamples were fixed for 2∼3 h in 4% paraformaldehyde, washed twice in 1× phosphate-buffered saline (130 mM NaCl, 5 mM $NaHPO_4{\cdot}7H_2O$, 3 mM KCl, 1 mM KH_2PO_4, pH 7.2), and then stored in 1× PBS-ethanol (1:1) at -20°C until analysis. To observe the cellular morphologies of microbes targeted by the domain-specific oligonucleotide probes, FISH was performed using probes Arc915, Eury498, Cren499, Eub338, EP404 (Table 1) labeled with the fluorescent dye FAM (Bond and Banfield, 2001). Hybridizations were carried out in hybridization buffer (0.9 M NaCl, 20 mM Tris-Cl, pH 7.4, 0.01% SDS) containing 30 ng probes and varying amounts of formamide (Table 1). Slides with hybridization buffer were incubated at 46°C for 90 min, rinsed briefly and immediately immersed in wash buffer containing 20 mM Tris/HCl (pH 7.4), 0.01% (w/v) SDS, 5 mM EDTA, and appropriate NaCl for 15 min at 48°C. The NaCl concentration was calculated to achieve the same stringency during washing as during hybridization according to the formula (Lathe, 1985). Slides were rinsed and DAPI stained. Fluorescently stained samples were examined with a Zeiss UEM fluorescence microscope.

DNA extraction and purification

The microbial genomic DNA was extracted directly from the sulfide chimney using Zhou's SDS-based DNA extraction method, with some modification (Zhou *et al*., 1996). Sulfide smoker samples (approximately 10 g) were frozen by immersing in liquid nitrogen and milled 3 times. The powder of sulfide chimney was then mixed with 13.5 ml of DNA extraction buffer (100 mM Tris-HCl; pH 8.0, 100 mM sodium EDTA; pH 8.0, 100 mM sodium phosphate; pH 8.0, 1.5 M NaCl, 1% CTAB) and 100 μl of proteinase K (10 mg/ml, Sigma, USA) in tubes by horizontal shaking at 225 rpm for 30 min at 37°C. After shaking, 1.5 ml of 20% (w/v) SDS were added, and the samples were incubated in a 65°C water bath for 2 h with gentle end-over-end inversions every 15 to 20 min. The supernatants were collected after centrifugation at 6000×g for 10 min at room temperature and transferred into 50 ml centrifuge tubes. Chimney samples were extracted two more times by adding 4.5 ml of the extraction buffer and 0.5 ml of 20% (w/v) SDS, vortexing for 10 sec, incubating at 65°C for 10 min and centrifuging as described above. The supernatants were mixed with an equal volume of chloroformisoamyl alcohol (24:1, v/v). The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at room temperature for at least 1 h. Crude nucleic acids were obtained by centrifugation at 16,000×g for 20 min at room temperature, washed with cold 70% ethanol and resuspended in sterile deionized water, to give a final volume of 100 μl. The crude nucleic acids were purified with cycle-pure kit (Omega, USA) following the manufacturer's suggested protocol, resuspended in elution buffer to a final volume of 50 μl, and stored at -80°C until use.

PCR and cloning of 16S rRNA genes

Using purified genomic DNA as templates, 16S rRNA genes were amplified using universal bacterial and archaeal primers (Lane, 1991; Delong, 1992). Bacterial 16S rDNA was amplified using the primers Eubac27f; 5'-AGA GTT TGA TCC TGG CTC AG-3'/ Eubac1492r; 5'-GGT TAC CTT GTT ACG ACT T-3' and the primers Arch21f; 5'-TTC CGG TTG ATC CYG CCG GA-3'/ Arch958r; 5'-YCC GGC GTT GAM TCC AAT T-3' were used for archaea with products of 1,500 bp and 900 bp, respectively. Each PCR mixture (20 μl) contained 2.5 mM $MgCl_2$, 0.8 mM deoxynucleoside triphosphates, each primer at a concentration of 0.25 μM, 1× PCR buffer and 1 U of *Taq* polymerase (TaKaRa, Japan). Amplification conditions were as follows: an initial denaturalization step of 94°C for 4 min and then denaturation at 94°C for 60 sec, annealing at 55°C for 45 sec, and extension at 72°C for 60 sec for a total of 30 cycles, followed by a final extension step of 72°C for 10 min. The PCR products were purified on a 1% (w/v) agarose gel and extracted by means of a Gel-extraction kit (Omega) following the manufacturer's instructions. Purified PCR products were cloned into the pMD18-T vectors (TaKaRa) and transformed to competent *Escherichia coli* DH5α cells (TaKaRa) according to the instructions. The transformed clones were grown in 1 ml of Luria-Bertani culture medium for 1 h at 37°C with shaking (220 rpm) and then coated on the agar plates con-

taining ampicillin (100 μg/ml) at 37°C for 12~16 h to form individual bacterial colonies. PCR was used to screen for the presence of the correct-size inserts with the vector-specific primers M13f; 5'-GTA AAA CGA CGG CCA G-3' and M13r; 5'-CAG GAA ACA GCT ATG AC-3'. Each PCR mixture (50 μl) contained 1 μl of clone culture, 1.5 mM $MgCl_2$, each primer at a concentration of 0.4 μM, 0.8 mM deoxynucleoside triphosphates, 1× PCR buffer and 1 U of *Taq* polymerase (TaKaRa). The PCR conditions were as follows: an initial denaturalization step of 94°C for 4 min and then denaturation at 94°C for 60 sec, annealing at 55°C for 45 sec, and extension at 72°C for 60 sec for a total of 30 cycles, followed by a final extension step of 72°C for 10 min. PCR products were visualized on a 1% (w/v) agarose gel by staining with Goldview.

Restriction Fragment Length Polymorphism (RFLP) analysis

To screen the clones for grouping into similar clone types, clones containing the target 16S rDNA were subjected to RFLP analysis using restriction enzymes recognizing a 4 bp restriction site. PCR-amplified inserts were digested with the restriction enzymes *Rsa*I and *Msp*I (MBI) according to the manufacturer's instructions (Schrenk *et al*., 2003). The DNA fragments were separated on a 3% (w/v) agarose gel by staining with Goldview. Representative clones of archaea and bacteria with unique RFLP banding patterns were chosen for sequencing.

Sequencing and phylogenetic analysis

The representative clones were directly sequenced by the dideoxynucleotide chain-termination method using an ABI 3730 capillary electrophoresis sequencer (Applied Biosystems, USA) with T-vector universal primers M13f and M13r. The whole insert sequences were spliced by the software DNAMAN (version 6.0). Archaeal and bacterial 16S rDNA sequences of about 900 bp and 1,500 bp, respectively, were obtained. Sequences were checked manually for chimeric sequences using the CHIMERA_CHECK program of Ribosomal Database Project II (Longnecker and Reysenbach, 2001; Maidak *et al*., 2001). Nonchimeric sequences were submitted to the Advanced BLAST search program (available through the National Center for Biotechnology Information) to find closely related sequences in the GenBank and for phylogenetic analysis in EMBL databases. All clone sequences and matched sequences were then aligned with the software

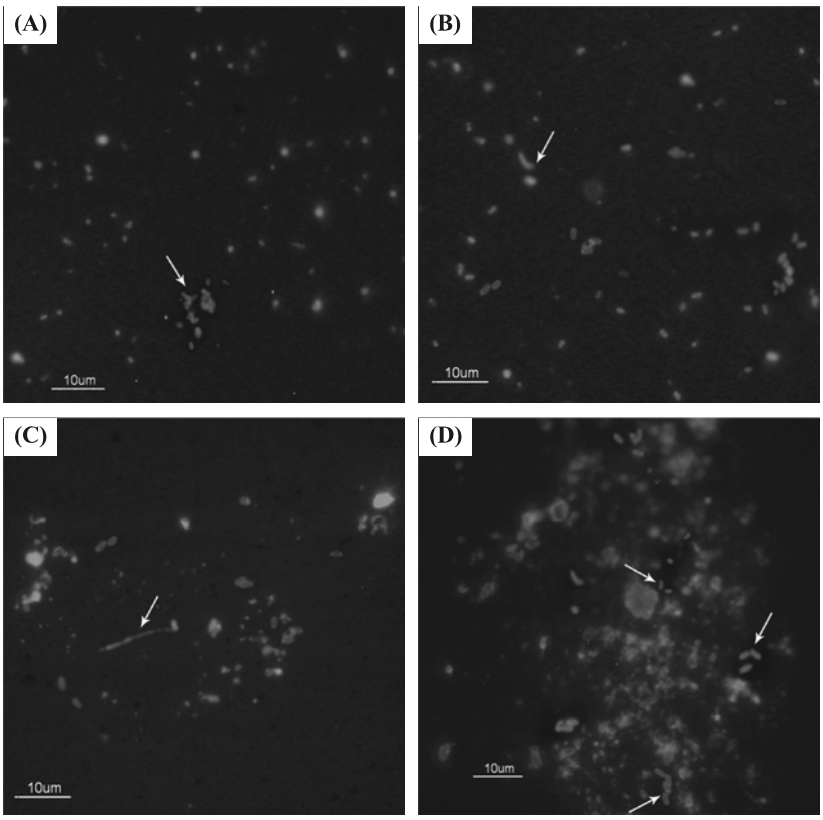

Fig. 2. Epifluorescence photomicrographs of fluorescently stained microbial communities inhabiting the sulfide structures of Dudley site. (A) Corresponding FISH image of *Crenarchaeota* (probe Cren499). They are composed of cells with diverse morphologies, including rods and cocci within the size of 1~5 μm. (B) An image hybridized with specific Arch915 probe (green fluorescence). (C, D) Images obtained by EP404 (C) and Eub338 probe (D), respectively. Bacteria appear more diversity, such as rods, cocci, arc and filaments (indicated by an arrow). Sulfide mineral particles show intensive background fluorescence and some microbes attached to them (indicated by arrows). All images were captured with Zeiss UEM epifluorescence microscope. Scale bar=10 μm.

CLUSTAL X 1.83 and the irregular segments were discarded. Phylogenetic trees were constructed by the neighbor-joining (NJ) method using the software Mega (3.1) (Takai and Horikoshi, 1999; Schrenk *et al*., 2003). Bootstrap analysis was used to provide confidence estimates of tree topologies.

Quantitative real-time PCR

Microbial 16S rRNA gene abundance was determined by fluorescence quantitative real-time PCR. Archaeal and bacterial 16S rRNA genes were amplified with the primer sets Arch344f/Arch519r (Bano *et al*., 2004) and Eubac341f/Eubac518r (Dilly *et al*., 2004), respectively. Standard curves were constructed by using a method described previously, with minor modification (He *et al*., 2007). All amplifications were performed on a 7500 Real-time System (Applied Biosystems) in 20 μl reaction mixture containing 1 μl template DNA (1∼10 ng), 0.15 μM of each primer, and 10 μl of Power SYBR® Green PCR Master Mix with ROX and SybrGreen I (Applied Biosystems, UK). Melting curve analysis was performed to confirm PCR product specificity after amplification by measuring fluorescence continuously as the temperature increased from 60°C to 95°C. Cycle thresholds were set automatically by the 7500 system software (Version 1.3). The average of 3 replicates was used.

Nucleotide sequence accession numbers

The sequences reported in this study have been deposited in the GenBank database. Bacterial sequences have GenBank accession no. EU555119 to EU555141 and archaeal sequences EU555142 to EU555153.

Results

Mineralogy and ESEM

According to the result of XRD analysis, the mineral phases were composed mainly of Fe-, Zn-, and Cu-rich sulfides, such as pyrite, marcasite, wurzite, sphalerite, and chalcopyrite. Other minor minerals were also found, including Fe-oxides, element sulfur, barite, and amorphous silica. The mineral assemblages varied from the central passage to the exterior layer according to their forming temperature. The chimney exterior wall contained a few barite and oxides. The amount of wurtzite, sphalerite, and pyrite increased gradually from the outer wall to the inner layers. The central part of the chimney typically contained a high proportion of Cu-rich sulfides. Anhydrites were not found in any part of the sulfide samples.

Some microbes appeared in pellet or rod shapes which were coated with SiO_2 on their surfaces or between the

Table 2. Summary of archaeal 16S rRNA clone sequences from Chimney of Dudley site

Phylogenetic ascription	Clone (n)	Accession numbers	Closest match (accession number)	% Identity	(G+C) %	Environment description	% Clones
Desulfurococceales							47.3
	A8(20)	EU555153	CH8-22 (AY672488)	98	67.62	white smoker of East Pacific Rise 9°N	27.0
	A23(3)	EU555146	pYK04-10A-13 (AB235330)	93	67.59	sulfide chimney of Yonaguni Knoll IV field of Southern Okinawa Trough	4.1
	A14(1)	EU555143	pPACMA (AB052979)	99	66.28	black smoker of PACMANUS site in Manus Basin	1.4
	A4(10)	EU555148	IAN1-22 (AB175580)	98	66.63	deep-sea hydrothermal vent chimney of Iheya North field in Okinawa Trough	13.5
	A3 (1)	EU555147	IAN1-22 (AB175580)	97	66.38	deep-sea hydrothermal vent chimney of Iheya North field in Okinawa Trough	1.4
Thermoproteales							
	A5 (2)	EU555150	CH-A7 (AY280443)	99	62.35	tubeworms attached to exterior of sulfide chimney in Juan de Fuca ridge	2.7
Marine group I							
	A58(1)	EU555151	pIVWA2 (AB019730)	99	51.15	hydrothermal vents environments of Kinawa Iheya Basin	1.4
UC							
	A6 (1)	EU555152	pKB7A-22 (AB247868)	97	64.26	hydrothermal chimney structure in Kermadec Volcanic Arc	1.4
DHVE							20.2
	A12(9)	EU555142	CH-A5 (AY280444)	99	60.10	tubeworms attached to exterior of sulfide chimney in Juan de Fuca ridge	12.2
	A21(6)	EU555145	IAN1-47 (AB175609)	99	63.00	deep-sea hydrothermal vent chimney of Iheya North field in Okinawa Trough	8.1
Archaeogobales							
	A47(7)	EU555149	PICO pp37 25A (AJ969472)	95	64.22	chimney of Rainbow hydrothermal field in the MAR	9.5
Thermococcales							
	A2(13)	EU555144	*Thermococcus celer* (M21529)	99	65.47		17.6

gaps of mineral crystals. This was confirmed by ESEM and EDS analysis.

FISH and Real-time PCR

Under the fluorescence microscope, bacterial and archaeal populations displayed various morphologies, including cocci, arc, rods and filaments (Fig. 2). The size of microbial cells ranged from 1 μm to 15 μm. Some archaeal cocci cells were 0.5 to 2 μm. Cells of filamentary *ε-Proteobacteria* measured up to 15 μm (Fig. 2). Except for the hybridized microbes, some sulfide mineral particles also showed intensive background fluorescence when the samples were inspired (Fig. 2, Image C and D). With the help of DAPI staining, we can partially distinguish the microbes from sulfide mineral particles. SEM-EDS analysis showed similar morphological features to the results of FISH.

Real-time PCR quantification indicated that the archaeal 16S rRNA gene content was 7.30×10^6 copies per gram black smoker samples, and bacteria reached 7.40×10^6 copies/g. The amounts of archaea and bacteria were similar to estimations of some other hydrothermal sulfides (Harmsen *et al*., 1997; Schrenk *et al*., 2003).

Archaeal diversity

The diversity of the archaeal clone library in Dudley chimney was relatively low. Only 12 unique phylotypes were detected from 74 clones (Table 2). About two-thirds of the clones (8 unique phylotypes), including members of Desulfurococcales, Thermoproteales, uncultured *Crenarchaeota* (UC), and marine group I (MGI), fell within the clades of *Crenarchaeota*.

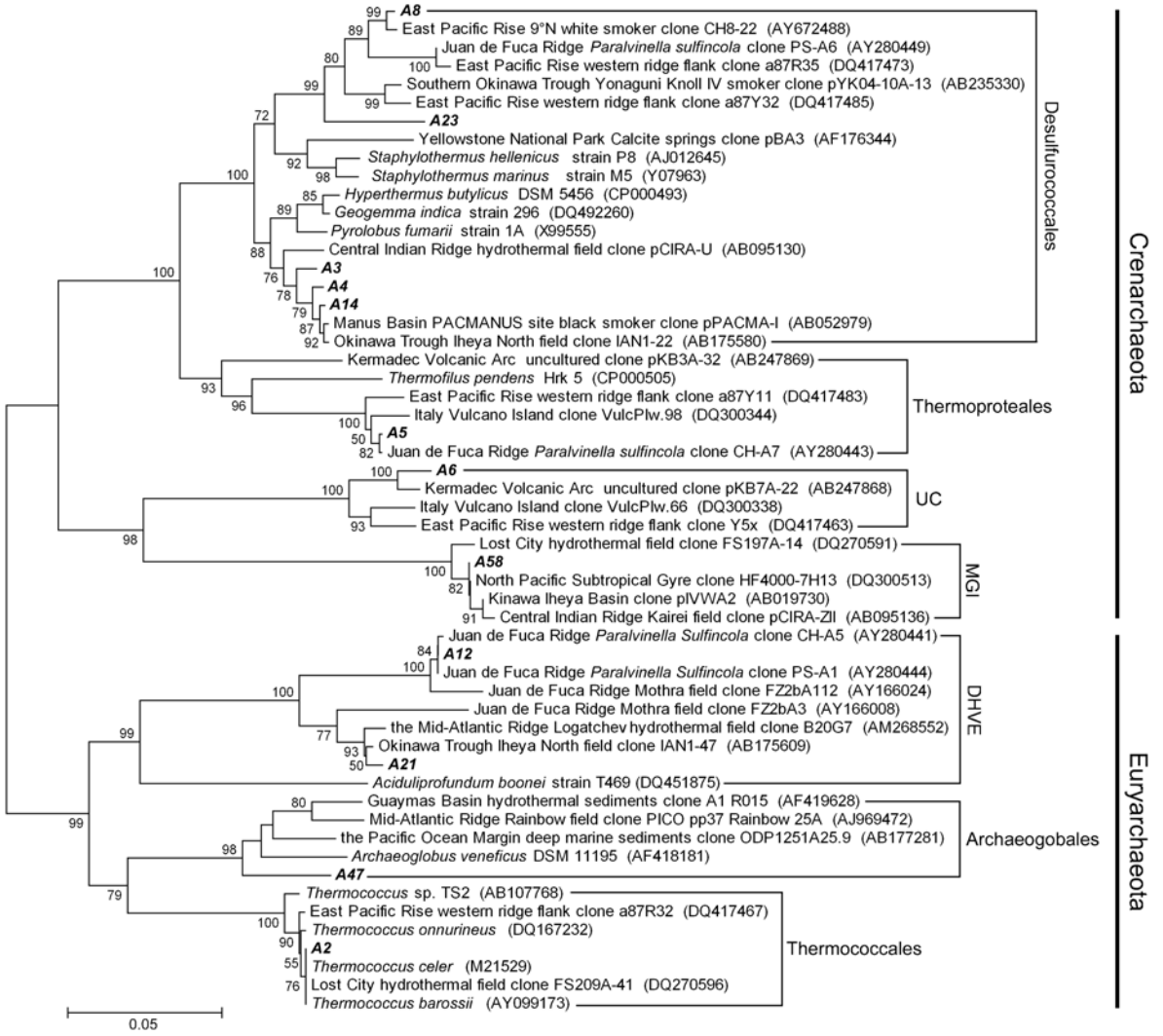

Fig. 3. Phylogenetic relationships of representative archaea clones obtained from the black smoker chimney at the Dudley hydrothermal site in Main Endeavour hydrothermal field. The tree was inferred by neighbor-joining analysis of 16S rRNA gene sequences with software Mega 3.1. Clones from this study are denoted in bold and italic type face. The numbers in parentheses are the GenBank accession no. for sequences obtained from NCBI except for our clones. Bootstrap percentages are obtained by using 1,000 replicates, and values greater than 50% are indicated at the nodes. The scale bar represents the expected number of changes per nucleotide position.

Table 3. Summary of bacterial 16S rRNA clone sequences from Chimney of Dudley site

Phylogenetic ascription	Clone (n)	Accession numbers	Closest match (accession number)	% Identity	Environment description	% Clones
ε-Proteobacteria						52.44
	B3 (4)	EU555129	*Sulfurovum* sp. (AP009179)	96	vent of Iheya North deep-sea hydrothermal field	4.88
	B33(3)	EU555132	R76-B25(AF449244)	99	tubeworms of EPR hydrothermal vent	3.66
	B15(4)	EU555122	ScaleC23C6(AY531602)	96	gastropod inhabited in vent of Indian Ocean	4.88
	B14(1)	EU555121	CH1-25(AY672507)	97	hydrothermal vent smoker of East Pacific Rise 9°N	1.22
	B49(2)	EU555135	ScaleC23C6(AY531602)	93	gastropod inhabited in vent of Indian Ocean	2.44
	B37(4)	EU555133	ScaleC23D9(AY531589)	91	gastropod inhabited in vent of Indian Ocean	4.88
	B1 (2)	EU555119	FT17B18(AY251062)	91	sulfide spire of Edmond field at Central Indian Ridge	2.44
	B2 (3)	EU555126	ScaleC23D7(AY531590)	92	gastropod inhabited in vent of Indian Ocean	3.66
	B63(5)	EU555139	CH1-28(AY672509)	95	hydrothermal vent smoker of East Pacific Rise 9°N	6.10
	B27(14)	EU555128	CH2b105(DQ228652)	98	mineral surface within the sulfide-microbial incubator	17.07
	B5 (1)	EU555136	CH2b105(DQ228652)	95	mineral surface within the sulfide-microbial incubator	1.22
γ-Proteobacteria						17.07
	B40(1)	EU555134	NDII 1.1(AF170424)	88	shallow water hydrothermal vent	1.22
	B32(6)	EU555131	B185(AY375060)	93	western Pacific warm pool sediments	7.32
	B13(4)	EU555120	ScaleC7E4(AY327876)	94	surface of vent snail foot of Indian Ocean	4.88
	B17(3)	EU555124	ScaleC7E4(AY327876)	96	surface of vent snail foot of Indian Ocean	3.66
δ-Proteobacteria						9.76
	B18(3)	EU555125	R103-B27(AF449228)	95	tubeworm's tube of EPR hydrothermal vent	3.66
	B79(2)	EU555141	MS12-1-E09(AM712338)	87	hydrothermal vent of Brothers Seamount in New Zealand	2.44
	B60(3)	EU555138	A2 B027(AF42034)	97	Guaymas Basin hydrothermal ediments	3.66
β-Proteobacteria						
	B24(3)	EU555127	ODP-62B-02(DQ490034)	99	Hawaiian volcanic deposits	3.66
Deinococcus-Thermus						6.10
	B16(4)	EU555123	*O. profundus*(AJ30586)	99	deep-sea hydrothermal vent at East Pacific Rise 13°N	4.88
	B65(1)	EU555140	*O. profundus*(AJ30586)	98	deep-sea hydrothermal vent at East Pacific Rise 13°N	1.22
CFB						3.66
	B31(2)	EU555130	P. palm C/A 221(AJ441218)	98	mucous secretions of hydrothermal vent polychaete	2.44
	B51(1)	EU555137	ScaleC4E6(AY327875)	89	gastropod inhabited in vent of Indian Ocean	1.22
Others (chimeric)						
	6 clones					7.32

Among the *Crenarchaeota*, clones of the order Desulfurococcales, including cultured hyperthermophilic species, predominated. Phylogenetically, these sequences had higher similarity to those clones or cultures obtained from deep-sea hydrothermal chimneys and from invertebrates that inhabited near the vents (Fig. 3). *Staphylothermus marinus* and *Hyperthermus butylicus*, which showed high similarity to our sequences, were hyperthermophiles with optimum growth temperature over 90°C (Blöchl *et al*., 1997). Only one phylotype (A5, 2 clones), which had 97% similarity to the sequence obtained from hydrothermal tube worm, was affiliated with the Thermoproteales. The sequence of the phylotype A58 (1 clone) came from the subgroup of *Crenarchaeota*, marine group I (MGI). MGI is very common in the deep-sea environments, including deep-sea hydrothermal fields, sedimentary basins, and water columns. In addition, there was one clone (A6) belonging to uncultured *Crenarchaeota* (UC).

As shown in the phylogenetic tree, clones within *Euryarchaeota* fell into three separate groups: deep-sea hydrothermal vent *Euryarchaeota* (DHVE), Thermococcales, and Archaeoglobales (Fig. 3). Several phylotypes were members of DHVE (Takai and Horikoshi, 1999; Page *et al*., 2004). Generally, habitats of DHVE are restricted to high temperature niches of vents (Takai and Horikoshi, 1999; Hoek *et al*., 2003; Nercessian *et al*., 2003; Schrenk *et al*., 2003). Two phylotypes were closely related to organisms belonging to the orders Thermococcales and Archaeoglobales. Within the Thermococcales, A2 (13 clones) showed high sequence similarity (99%) to the cultured isolations of *Thermococcus*, anaerobic hyperthermophiles (optimum growth temperature 85∼90°C) which can be isolated from diverse geothermal environments, such as terrestrial hot springs, deep-sea hydrothermal vents and abyssal sediments (Lepage *et al*., 2004).

In addition, phylotype A47 fell into the order Archaeoglobales. The relative sequences of A47 included those obtained from Guaymas Basin hydrothermal sediments, black chimney of Rainbow and Pacific Ocean deep-sea sediment.

Bacterial diversity

ε-Proteobacteria

Members of *ε-Proteobacteria* contained 11 phylotypes (43

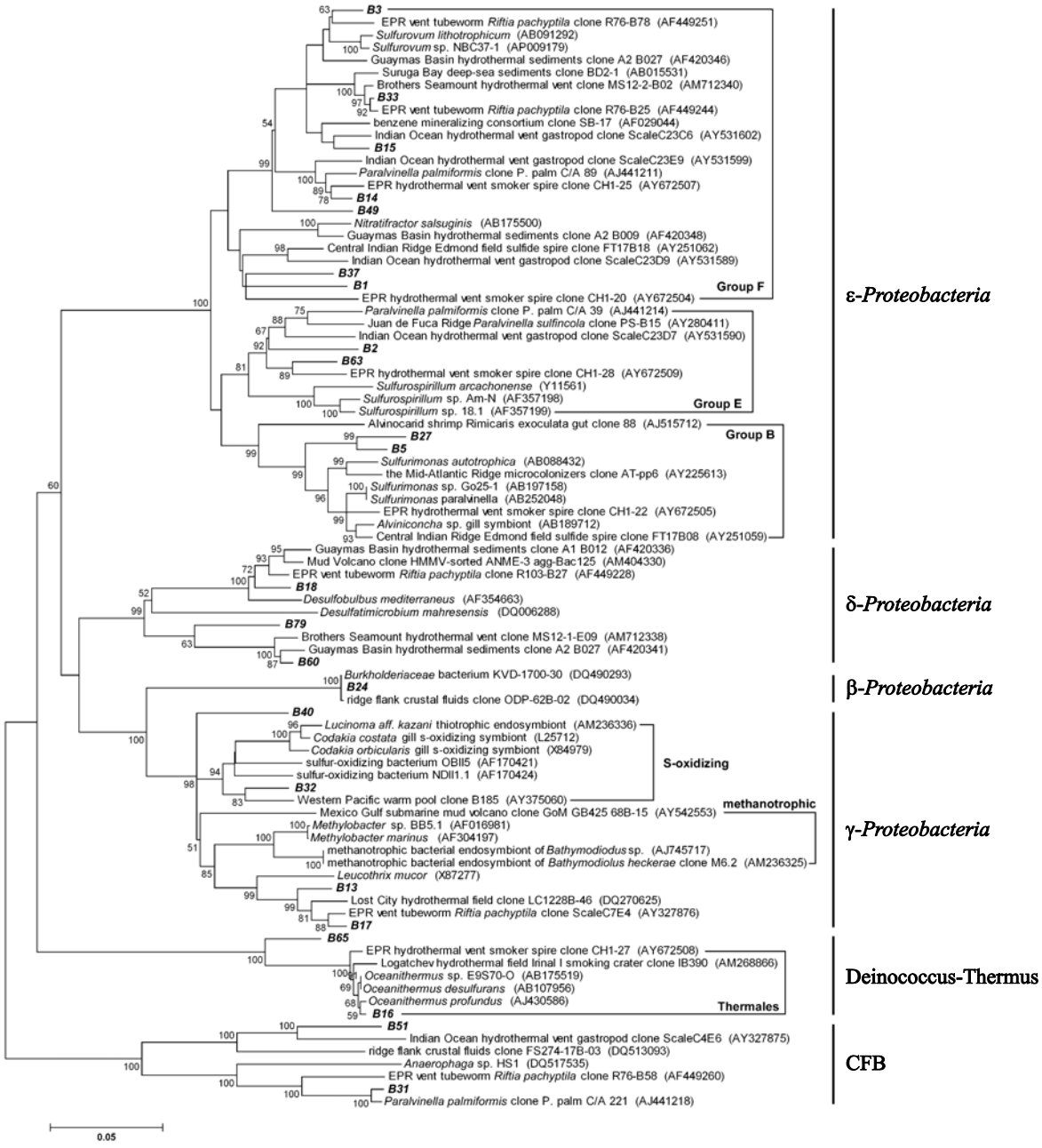

Fig. 4. Phylogenetic relationships of representative bacterial clones obtained from the black smoker chimney at the Dudley hydrothermal site in Main Endeavour hydrothermal field. The tree was inferred by neighbor-joining analysis of 16S rRNA gene sequences with software Mega 3.1. Clones from this study are denoted in bold and italic type face. Definition of Group B, E, and F is according to Corre *et al.* (2001). The numbers in parentheses are the GenBank accession no. for sequences obtained from NCBI except for our clones. Bootstrap percentages are obtained by using 1,000 replicates, and values greater than 50% are indicated at the nodes. The scale bar represents the expected number of changes per nucleotide position.

clones) and fell into three clusters, B, E, and F (Corre *et al*., 2001). Our sequences of group F [7 phylotypes, 20 clones) mainly clustered with those obtained from the bacterial symbionts of vent invertebrates (including *Riftia pachyptila* in the East Pacific Rise (EPR), gastropods in the Indian Ocean and *Paralvinella palmiformis* in Northeastern Pacific] and from the bacterial clones inhabiting other hydrothermal chimneys (Fig. 4). Some of our sequences show high similarity to mesophilic and chemoautotrophic pure cultures, *Sulfurovum lithotrophicum* and *Sulfurovum* sp., which were recently isolated from hydrothermal sediments (Inagaki *et al*., 2004). Group E included two phylotypes (7 clones). They were closely related to cultured members of heterotrophic sulfur-reducing bacteria *Sulfurospirillum* was isolated

Table 4. Occurrence of different microbial groups detected at different hydrothermal vents

Sample	Location	Types of samples	T(°C)	Crenarchaeota (%)			Euryenarchaeota (%)			Proteobacteria (%)			References
				Des	Thp	MGI	DHVE	Arg	Thm	ε-	γ-	δ-	
Dudley	MEF/JDF	active sulfide chimney	330	47.3	2.7	1.4	20.2	9.5	17.6	52.4	17.1	9.8	the present study
Mothra	JDF	active black smoker	302	nd	0.5	23.2	30.3	0.5	1.3				Schrenk *et al*. (2003)
ASHES	Axial Volcano/JDF	inferno chimney	>300	nd	nd	nd	90.9	nd	2.2	20.2	nd	3.9	Page *et al*. (2004)
Bio9	9°N EPR	white smoker spire	350	7.8	6.3	nd	1.6	25	45.3	67.4	2.2	4.3	Kormas *et al*. (2006)
Edmond	CIR	sulphide spire		nd	nd	nd	93	nd	7	99	nd	nd	Hoek *et al*. (2003)
Pacmanus	Manus Basin	black smoker chimney	>250	21.7	nd	nd	6.4	nd	28.1				Takai *et al*. (2001)
Logatchev	MAR	high-temperature chimney	300-350	nd	nd	nd	little	nd	ND	19-49	8	8-15	Perner *et al*. (2007)
Lost City	MAR	white carbonate chimney	70-90	nd	nd	+	nd	nd	+	+	+	nd	Brazelton *et al*. (2006)
MM	EPR	materias within ridge flanks	>110	31.9	24.4	nd	18.6	nd	3.7				Ehrhardt *et al*. (2007)
BBS	JDF	ridge flank crustal fluids	20	nd	nd	+	nd	nd	+	+	nd	+	Huber *et al*. (2006)

[a] MEF-Main Endeavour Field; JDF, Juan de Fuca ridge; EPR, East Pacific Rise; CIR, the Central Indian Ridge; MAR, the Mid-Atlantic Ridge; BBS, the Baby Bare Seamount
[b] %, referring the percentage in clone libraries
[c] Des, Desulfurococcales; Thp, Thermoproteales; MGI, Marine group I Crenarchaeota; Arg, Archaeogobales; Thm, Thermococcales
[d] nd corresponds to not detected; blank denotes no data available; + refers the occurrence of groups but without quantitative data

from marine environments. Nearly 50% of *ε-Proteobacteria* clones (2 phylotypes) were affiliated with group B or group II, as defined by Teske *et al*. (2002). The sequences related with group B mainly included those clones retrieved from deep-sea vents or symbionts of invertebrates (Urakawa *et al*., 2005).

γ-Proteobacteria

Four phylotypes, including 14 clones making up a proportion of 17.1%, were most closely related to *γ-Proteobacteria*. Among them, several phylotypes branched with symbionts (sulfur-oxidizer) obtained from hydrothermal vent invertebrates (Fig. 4). Generally, *γ-Proteobacteria* are universal and abundant in various marine environments, including hydrothermal vents, cold-seeps, sediments and water column.

δ-Proteobacteria

Three phylotypes (8 clones), which accounted for about 10% of the whole bacterial library, fell into the *δ-Proteobacteria*. The majority of these sequences clustered with representatives obtained from vents, hydrothermal sediments and symbionts of tube worms (Fig. 4). Almost all of the cultured members of this phylum belonged to sulfate reducing bacteria (SRB). Phylotype B18 branched with the pure culture, *Desulfobulbus mediterraneus*, which could reduce sulfate, sulfite and sulfur.

Other groups

There were two phylotypes (3 clones) belonging to Cytophaga-Flavobacterium-Bacteroides (CFB) which are ubiquitous in marine environments. As one kind of degraders of carbohydrate polymer, they mainly reside in microaerobic environments, such as plumes and sediments, and play an important role in deep-sea carbon cycle. Five clones in our analysis fell into the order Thermales. They were closely related to the facultatively chemolithotrophic, thermophilic culture, *Oceanithermus profundus* (>99% sequence similarity). In addition, there were 3 clones within *β-Proteobacteria*.

Discussion

Diversity of microbes of the chimney

The comparison of microbial diversity of our results and those of other hydrothermal fields is listed in Table 4. In our library, the members within the order Desulfurococcales predominated, and comprised as much as 50% of the total (5 unique phylotypes). Sequences related to Desulfurococcales were also found in the 9°N EPR and Manus Basin hydrothermal chimneys, although their abundance was not quite high (Takai *et al*., 2001; Kormas *et al*., 2006). In contrast, they were absent from the spires collected from the Mothra and the Axial Volcano field and from the Edmond field (Hoek *et al*., 2003; Shrenk *et al*., 2003; Page *et al*., 2004). Some isolates within this order have been cultured successfully, including many hyperthermophilic, autotrophic and heterotrophic, sulfur-reducing or fermentative genera (Huber and Stetter, 1998; Kashefi and Lovely, 2003; Kormas *et al*., 2006). A recent study by Ehrhardt and his colleagues (2007) found that Desulfurococcales predominated in basaltic ridge flanks, which suggests the existence of a subsurface biosphere. Although the factors determining their occurrence are unclear, it seems that relative higher temper-

atures (>85°C) may be necessary (Kormas *et al*., 2006). However, members of the Desulfurococcales were absent in the structures of Mothra and ASHES, even though the temperature regimes were similar.

Sequences related to the order Thermoproteales were detected in our library, despite their very low proportion, with only one phylotype representing 2 clones. Except for the result detected by Ehrhardt *et al*. (2007), the Thermoproteales are hardly detected in hydrothermal vents (Cowen, 2004; Nercessian *et al*., 2004; Page *et al*., 2004). Although it appears in libraries of the chimneys of the Bio 9 and Mothra hydrothermal field, its proportion was also very low (Schrenk *et al*., 2003; Kormas *et al*., 2006). Kormas *et al*. (2006) suggested that Thermoproteales might play significant role in hydrothermal habitats. To date, the pure cultures within Thermoproteales come only from high-temperature terrestrial or freshwater environments (Huber and Stetter, 2001; Kormas *et al*., 2006).

In the *Euryarchaeota*, the branches of DHVE and Thermococcales predominated, with a proportion of about 20% each (Table 2). DHVE, considered to be endemic to hydrothermal vents, has been detected in hydrothermal chimneys distributed in MAR, EPR, JDF, Guaymas Basin, etc. (Reysenbach *et al*., 2000; Teske *et al*., 2002; Shrenk *et al*., 2003; Kormas *et al*., 2006). In the Edmond and ASHES hydrothermal vents, their proportions were more than 90% (Hoek *et al*., 2003; Page *et al*., 2004). In contrast to their wide distribution, almost nothing is known about their physiological characterizations because of the lack of pure isolations. So far, only one pure culture, *Aciduliprofundum boonei* from the venting sulfides of the Eastern Lau Spreading Centre (ELSC), has been successfully isolated (Reysenbach *et al*., 2006). It is an obligate thermoacidophilic sulphur or iron reducing heterotroph capable of growing with pH from 3.3 to 5.8 and temperature between 55°C to 75°C.

The emergence of Thermococcales was not unexpected in the Dudley sulfide chimney. The members of Thermococcales (clones or pure cultures) are common and they appear in almost all surveys of hydrothermal environments (Takai *et al*., 2001; Hoek *et al*., 2003; Perner *et al*., 2007; Page *et al*., 2008). They sometimes predominate in vent communities or in specific parts of a chimney (Takai *et al*., 2001; Kormas *et al*., 2006). It is interesting to note that the members of hyperthermophilic *Thermococcus* were also found in mesophilic (about 20°C) crustal fluid (Huber *et al*., 2006). It was inferred that these groups probably came from the thermophilic habitats beneath the ridge flanks.

Our results reconfirmed the predominance of ε-*Proteobacteria* in vent environments (Table 2). *ε-Proteobacteria* was previously shown to be prevalent and predominant in microbial communities of various hydrothermal environments (Table 4). By comparative analysis of 16S rRNA genes, *ε-Proteobacteria* were clustered into seven subgroups (A to G) (Corre *et al*., 2001). Our sequences fell into three clusters (group B, E, and F), including free-living species and symbionts of invertebrates. Because of their ability to utilize a wide spectrum of electron donors and acceptors, including arsenate, selenate, manganese, Fe^{3+}, H_2, sulfur compounds, nitrate and oxygen (Hoek *et al*., 2003; Nakagawa *et al*., 2005), *ε-Proteobacteria* can flourish in multifarious hydrothermal niches. Hoek *et al*. (2003) found that the majority (>99%) of the bacterial phylotypes were *ε-Proteobacteria* in a sulfide spire collected from the Edmond field. It was also discovered by several *in situ* incubation experiments that *ε-Proteobacteria* were generally the first colonizers in hydrothermal environments and that they could rapidly colonize all available exposed surfaces, including animal teguments, mineral precipitates or artificial substrates, regardless of the nature of the surface (Reysenbach *et al*., 2000; Hoek *et al*., 2003; López-Garcia *et al*., 2003). It was suggested that *ε-Proteobacteria* plays a significant role in the sulfur, nitrogen, and hydrogen cycles in vent environments (Nakagawa *et al*., 2005).

A portion of our clones were grouped into CFB and *β-Proteobacteria*. It was generally difficult to distinguish their source because they are distributed widely in both ocean water and hydrothermal environments. Similar to the source of MGI in archaea, the contamination of ambient seawater drawn into the sample with the contraction of hot fluid caused by cooling might be an important source of these groups (Kormas *et al*., 2006).

Another characteristic of the archaeal groups dwelling in the Dudley structure was their unique thermo-adaptation. Thermophilic or hyperthermophilic physiologies were common to those inhabitants, including Desulfurococcales, Thermoproteales, DHVE, Thermococcales, and Archaeogobales. According to our results (Table 2), the thermophilics-related clones accounted for approximately 95% of archaeal clone library. The habitats in the Dudley sulfide chimney were caused or determined by venting of high temperature fluids, which had a maximum value of 330°C. The mixing processes between hot fluids and seawater provide optimal zones (oxygen-depleted, high-temperature) for these hyperthermophilic members. Culturable relatives of these groups generally have an optimal growing temperature of 80°C or higher (Zillig and Reysenbach, 2001; Kormas *et al*., 2006). To date, the growing temperature of 121°C of an isolated member of the order Desulfurococcales (Kashefi and Lovely, 2003) is the upper limit of life. Although very few members of these clades (DHVE, UE and UC) have been cultured, higher G+C contents (average more than 60% shown in Table 2) are evidence of their unique thermo-adaptation (Ehrhardt *et al*., 2007).

By contrast, bacterial communities in our chimney were generally mesophilic or moderately mesophilic (with optimal temperature near 30°C) except for the order Thermales. *Oceanithermus profundus*, related most closely to our bacterial B16, grows at 40~70°C, with an optimum at 60°C (Miroshnichenko *et al*., 2003).

Sulfur-related metabolism in the chimney

Based on our data of microbial diversity and phylogenetic relationship, it was obvious that the metabolism of most clusters were closely related to sulfur cycles. Those thermophilic or hyperthermophilic (the optimal growing temperature over 80°C) in our library, whether autotrophic or heterotrophic, belonged to the orders Desulfurococcales, Thermoproteales, Archaeoglobales, and Thermococcales. They were found to utilize a wide spectrum of substrates, including SO_4^{2-}, $S_2O_3^{2-}$, or S^0 with H_2S, as their ultimate metabolite.

Autotrophs get their primary metabolic energy from reduction reactions in anaerobic, hot environments because of thermodynamic constraints (McCollom and Shock, 1997). For most heterotrophs, sulfur and related compounds were necessarily required for their growth (Huber and Stetter, 1998; Hao and Ma, 2003; Bae *et al*., 2006). Recently, Kuwabara and colleagues (2007) isolated a hyperthermophilic culture, *Thermococcus celericrescens*, from a hydrothermal vent. Growth of *Thermococcus celericrescens* was enhanced remarkably by element sulfur, although it was not essential.

There were a proportion of sulfur-reducing bacteria grouped into *ε-Proteobacteria* and *δ-Proteobacteria* in our chimney sample. In group E of *ε-Proteobacteria*, culturable heterotrophic *Sulfurospirillum* were sulfur-reducers. They were able to grow at low oxygen levels and to oxidize organic compounds with element sulfur as electron acceptor (Finster *et al*., 1997). Most members of *δ-Proteobacteria* are strictly anaerobic sulfate reducers, reducing sulfate using H_2 or an organic molecule as an electron donor.

The sulfur oxidizing clusters were mainly bacteria with aerobic and mesophilic physiologies. In our library, *ε-Proteobacteria* contributed substantially to the sulfur oxidizers. Members of groups B and F within *ε-Proteobacteria* included the genera *Sulfurimonas* and *Sulfurovum,* respectively, which were typically mesophilic or moderate thermophilic chemoautotrophs (Inagaki *et al*., 2003; Takai *et al*., 2003; Nakagawa *et al*., 2005). They obtain energy through oxidation of some sulfur species, such as sulfide, thiosulfate and elemental sulfur (López-García *et al*., 2002). Members of sulfur-oxidizers also appeared in *γ-Proteobacteria* and CFB groups.

Other branches of sulfur-oxidizers were endosymbionts that fell into *γ-Proteobacteria* and *ε-Proteobacteria* groups (Fig. 4). They oxidize H_2S directly with the participation of oxygen, which is the most direct approach to obtain energy to support the chemosynthesis-based faunal communities such as tube worms, shrimps, and gastropods (Poltz and Cavanaugh, 1995; Goffredi *et al*., 2004; Urakawa *et al*., 2005). The source of those endosymbionts in our sample was not clear. They might come from the minor mixture of some symbiotic fauna on the surface of the chimney, although they were removed to the best of our ability.

It was interesting that methanogens or methanotrophs did not occur in our library. Similar results were also reported in the East Pacific Rise (9°N), the Central Indian Ridge, and the Manus Basin (Takai *et al*., 2001; Hoek *et al*., 2003; Kormas *et al*., 2006). Chemical characteristics of hydrothermal fluids, as well as of supported communities, were determined by the type of host rock. In three famous hydrothermal fields (the Lost City, Rainbow and Logatchev) hosted by ultramafic rocks in Mid-Atlantic Ridge, microbial populations were dominated by methane-related groups (such as ANME-1, Methanococcales, Methanosarcinales) and H_2-oxidizers, which corresponds to the high concentrations of dissolved H_2 and CH_4 in hydrothermal fluids (Brazelton *et al*., 2006; Perner *et al*., 2007). In contrast, concentrations of H_2 and CH_4 in basalt-hosted hydrothermal fluids are generally low (Donval *et al*., 1997; Kelley *et al*., 2001; Van Dover *et al*., 2001; Von Damm and Lilley, 2004). However, the methane concentration of end member hot fluids emitting from Endeavour Segment (basalt-hosted) had CH_4 concentrations as high as 1.8~3.4 mmol/kg, which was suspected to be influenced by sediments (Lilley *et al*., 1993, 2003). Similarly, Schrenk *et al*. (2003) surveyed the archaeal communities in the Mothra field and found only a very low proportion of methane-related populations (about 2%).

According to our data, sulfur-related metabolism is common and pivotal to vent microbial communities in Dudley, which also probably acts as the basis to maintain the hydrothermal vent ecosystem. Molecular phylogenetic techniques that we used expanded our view about microbial communities, especially those of the uncultured groups that inhabit extreme seafloor environments. It is obvious that there is a uniqueness in the diversity of hydrothermal communities and, therefore, its key factors need to be further investigated by multiple approaches.

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