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Diversity of bacteria and archaea in the deep-sea low-temperature hydrothermal sulfide chimney of the Northeastern Pacific Ocean

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Our knowledge of the diversity and role of hydrothermal vents microorganisms has considerably expanded over the past decade, while little is known about the diversity of microorganisms in low-temperature hydrothermal sulfide chimney. In this study, denaturing gradient gel electrophoresis (DGGE) and 16S rDNA sequencing were used to examine the abundance and diversity of microorganisms from the exterior to the interior of the deep sea low-temperature hydrothermal sulfide chimney of the Northeastern Pacific Ocean. DGGE profiles revealed that both bacteria and archaea could be examined in all three zones of the chimney wall and the compositions of microbial communities within different zones were vastly different. Overall, for archaea, cell abundance was greatest in the outermost zone of the chimney wall. For bacteria, there was no significant difference in cell abundance among three zones. In addition, phylogenetic analysis revealed that Verrucomicrobia and Deltaproteobacteria were the predominant bacterial members in exterior zone, beta Proteobacteria were the dominant members in middle zone, and *Bacillus* were the abundant microorganisms in interior zone. As to archaea, the middle and exterior were dominated by unclassified archaea, while interior zone was dominated by methanogens. Taken together, these results show that there was a transition in the composition of microbial communities across the sulfide chimney wall. Our findings provide helpful information for understanding the diversity and phylogeny of microorganisms in the deep-sea low temperature hydrothermal sulfide chimney.

Key words: Low-temperature hydrothermal sulfide chimney, bacteria, archaea, DGGE.

INTRODUCTION

Deep-sea hydrothermal vents and their attendant invertebrate communities were discovered in 1977 during exploration of the Galapagos Spreading Center (Van Dover et al., 2002). Vents are now known to occur along all active mid-ocean ridges and back-arc spreading centers and at some seamounts. Hydrothermal sulfide chimneys are found on the deep seafloor, and the mineral structure of chimneys consisted of sulfides minerals such as chalcopyrite ($CuFeS_2$), iron disulfide (FeS_2), [(zinc (Zn), iron (Fe)) sulphide (S)] and others. Thermal and chemical gradients are created by mixing of reduced hot fluids with oxygenated cold seawater (Moyer et al., 1995). Sulfide chimneys present in marine hydrothermal systems are created by hot, metal-enriched fluids (Kato et al., 2010; Schrenk et al., 2003).

Although deep-sea hydrothermal vents are among the most extreme and dynamic environments on earth, biologically diverse communities exist in the immediate vicinity of hydrothermal vent flows (Thornburg et al., 2010). It was discovered that microorganisms are incredibly diverse and believed to be performing critical roles in the

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Table 1. Primers used for PCR of 16S rDNA.

Primer[a]	Target site[b]	Sequence (5' to 3')	Specificity	Reference
BSF8/20	8-27	AGAGTTTGATCCTGGCTCAG	Bacteria	(LaPara et al., 2000)
968F[c]	968–984	AACGCGAAGAACCTTAC	Bacteria	(Felske and Akkermans, 1998)
1401R	1385-1401	CGGTGTGTACAAGACCC	Bacteria	(Felske and Akkermans, 1998)
BSR1541/20	1541-1522	AAGGAGGTGATCCAGCCGCA	Bacteria	(LaPara et al., 2000)
PRA46F	46-60	YTA AGC CAT GCR AGT	Archaea	(O'Connell et al., 2003)
PARCH340F[c]	340-357	CCC TAC GGG GYG CAS CAG	Archaea	(O'Connell et al., 2003)
ARC915R	915-934	GTG CTC CCC CGC CAA TTC CT	Archaea	(O'Connell et al., 2003)
PREA1100R	1100-1117	YGG GTC TCG CTC GTT RCC	Archaea	(O'Connell et al., 2003)

[a]F, forward primer; R, reverse primer. [b]Numbering based on *Escherichia coli* numbering scheme; [c]GC-clamp sequence, 5'-CGC CCG CCG CGC GCG GCG GGG GCG GGG GCA CGG GGG G-3' attached to 5' end of the primer.

hydrothermal systems (Zhou et al., 2009). The most widely accepted (or at least hypothesized) mode of metabolism thought to dominate hydrothermal vent microbial communities is chemolithoautotrophy, principally through the oxidation of reduced sulfur and iron compounds as an energy source (Jannasch and Mottl, 1985; Van Dover et al., 2002). Most recent enrichment culture isolation experiments revealed the presence of a physiologically diverse microbial community and led to the characterization of numerous bacterial and archaeal thermophiles (and hyperthermophiles), including both chemolithoautotrophic and chemoorganoheterotrophic strains (Harmsen et al., 1997). The microorganisms isolated at great depths so far were sulfate reducer, chemolithotrophic methanogens (Kelly et al., 2010; Kurr et al., 1991; Takaki et al., 2010), thermophilic aerobic heterotrophic bacteria from the genera *Thermus* and *Bacillus* (Marteinsson et al., 1996), heterotrophic sulfur metabolizers from the order Thermococcales (Gonzalez et al., 1995), a variety of fermenters within the Crenarchaeota (Jannasch et al., 1988) and others.

However, the major limitation of culture-based techniques is that only a relatively small fraction of the microorganisms making up a natural community can generally be cultured, especially for the deep-sea hydrothermal vents ecosystem (Heidelberg et al., 2010; Podosokorskaya et al., 2010). Fortunately, molecular analyses based on 16S rDNA gene fragment such as denaturing gradient gel electrophoresis (DGGE), is more and more frequently used to explore the microbial communities and their dynamics changes recently (Hirasawa et al., 2008; Takai and Horikoshi, 1999a). Furthermore, although successful, the potentials and limitations of these techniques can be sometimes met (Muyzer and Smalla, 1998). New information is rapidly flowing into the field from the cultivation of key organisms, *in situ* hybridization, to metagenomics and ongoing biogeochemical studies (Burgaud et al., 2010; Corre et al., 2001; Ehrhardt et al., 2009; Genilloud et al., 2010; Giovannoni and Stingl, 2005; Xie et al., 2010).

Our knowledge of the diversity and role of hydrothermal vents micro-organisms has considerably expanded over the past decade, while little is known about the diversity of microorganisms in low-temperature hydrothermal sulfide chimney. In this paper, we described the microbial communities within discrete mineralogical zones from clam bed. The zones represent diverse physical-chemical environments due to their spatial location within the low-temperature hydrothermal sulfide chimney wall. The bacterial and archaeal abundance and diversity were analyzed from the exterior to the interior of sulfide chimney using DGGE and 16S rDNA sequencing. In addition, the bacterial and archaeal populations in the deep-sea hydrothermal vent environments were compared with those of other deep-sea hydrothermal vent ecosystems, and the genetic diversity and phylogenetic analyses of the microbes were examined.

MATERIALS AND METHODS

Sampling

China and USA Joint Diving Cruise by R/V Atlantis and Submersible Alvin was made on clam bed (129°5.8' W, 47°48' N) in Northeastern Pacific Ocean in 19th August, 2005. Samples used in the study were obtained from a deep-sea low-temperature hydrothermal sulfide chimney during dive 4136. Three discrete horizontal transects across the chimney wall were taken for analysis. The temperature of the hydrothermal sulfide liquid was 29.2°C. The depth of hydrothermal sulfide chimney was 2181.292 m.

Nucleic acid extraction

Total genomic DNA was extracted from 0.5 g of samples from each of the three vertical zones of the core using an UltraClean™ Soil DNA extraction kit (MO BIO, USA) according to the protocol supplied with the kit. The DNA was resuspended in sterile water and the DNA concentration was measured using a Nano-volume spectrophotometer (Analytik Jena, Germany).

Polymerase chain reaction (PCR) - DGGE analysis

The primers used for amplification of 16S rDNA of bacteria and archaea are listed in Table 1. Amplification mixtures with bacteria primers BSF8/20 and BSR1541/20 was performed. The reaction began with an initial 95°C denaturation for 5 min, followed by 35
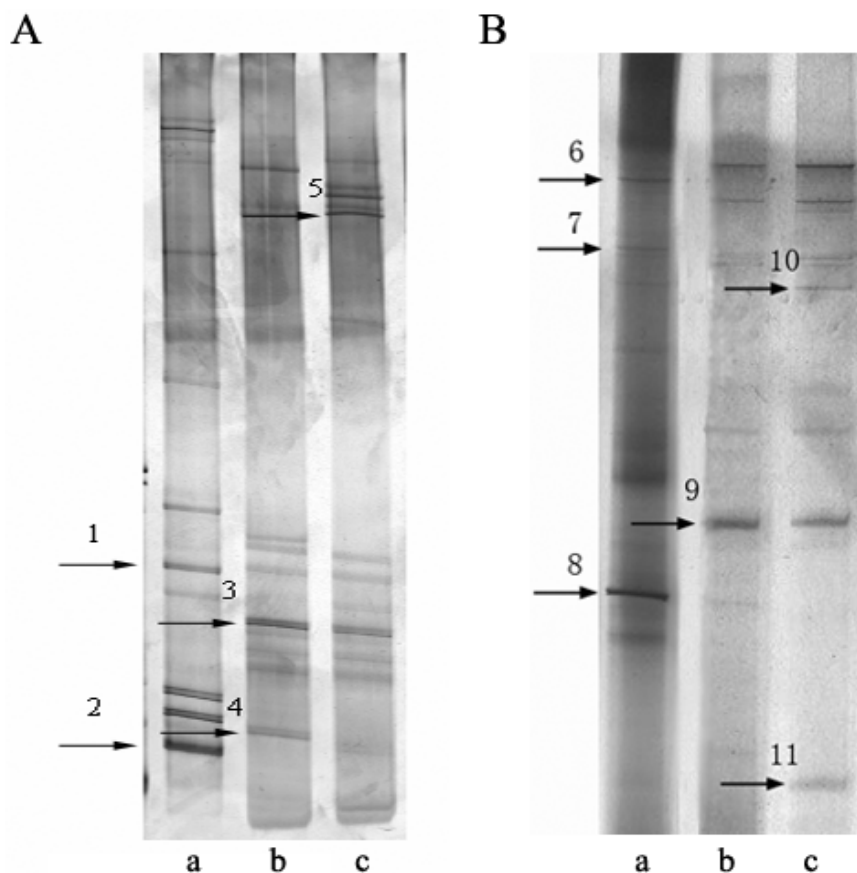
Figure 1. The DGGE profiles of the 16S rDNA fragments of bacteria (A) and archaea (B). 16S rDNA fragments of bacteria and archaea amplified from the exterior to the interior of sulfide chimney were compared by DGGE. Lane a, the exterior of sulfide chimney; lane b, the middle of sulfide chimney; lane c, the interior of sulfide chimney. DGGE, Denaturing gradient gel electrophoresis.

cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1.5 min and a final extension at 72°C for 10 min. The second amplification was performed using the same setting as in the first round of amplification, except with an extension time of 30 s. Moreover, amplification of archaea 16S rDNA sequences was carried out using a nested PCR technique. First, the majority of the archaea 16S rDNA fragment was amplified using the PRA46F and PREA1100R primers (Table 1). The first amplification mixture contained the same concentration of components as in the bacteria mixture. Polymerase chain reaction (PCR) began with a 95°C denaturation for 5 min, followed by 30 cycles of at 95°C for 30 s, 54°C for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min.

For PCR-DGGE analysis, nest-PCR was performed using the same PCR reagents and thermal cycling parameters as aforementioned, except that the primers used were 340F with a stretch of guanine cytosine-rich sequences (GC-clamp) and 915R. DGGE was performed essentially as previously described (Muyzer et al., 1993). Briefly, PCR products were separated on a 1 mm thick, vertical gel containing 6% (w/v) polyacrylamide (37.5:1 acrylamide: bisacrylamide) and a linear gradient of the denaturants urea and formamide, increasing from 30% at the top of the gel to 60% at the bottom. Here, 100% denaturant was defined as 7 mol/L urea plus 40% v/v formamide. The gels were loaded with 50 µL of PCR product. Electrophoresis was performed in a buffer containing 40 mmol/L Tris, 40 m mol/L acetic acid, 1 mmol/L EDTA (pH 7.6) (0.5

Tris–acetate–EDTA buffer) for 5 h at 160 V. Finally, gels were stained by silver staining.

Sequencing of 16S rDNA fragments and comparative sequence analysis

Individual bands were cut from the DGGE gel using new razor blades, placed in 200 µL of sterile distilled recovery buffer (20% (v/v) ethanol, 1 mol/L LiCl, 10 mmol/L Tris-HCl (pH 7.5)), and allowed to incubate overnight at room temperature, then incubated further for 2 h at 65°C. The supernatant containing the eluted DGGE band was recovered and placed in a new sterile microcentrifuge tube. The eluted band was re-amplified with non-GC-clamped primers 968F/1401R and primers PARCH340F/ ARC915R for bacteria and archaea, respectively. Two microliters of PCR product was ligated into pUCm-T vector (Bio Basic Inc.) and transformed to *E. coli* DH5α. The cloning procedure was conducted according to the manufacturer's instructions.

One to two clones from each band as shown in the Figure 1 were sequenced. Sequences were deposited in GenBank (accession numbers EF422853 through EF422863) and aligned with reference sequences, using sequence match software from the Ribosomal Database Project II (RDP II) website (Cole et al., 2005). All sequences   were   aligned   in a two-stage process. Distance matrices

Table 2. Phylogenetic affiliations of clones 16S rDNA genes from the deep-sea hydrothermal sulfide chimney.

	Phylogenetic affiliation	Sequenced clone number	GenBank accession number	Position on site
Bacteria	Verrucomicrobia	1	EF422853	a
	Delta proteobacteria; unclassified Syntrophobacterales	2	EF422854	a
	Beta proteobacteria; Neisseriales	3	EF422855	b
	Beta proteobacteria; Comamonadaceae	4	EF422856	b
	Bacillus	5	EF422857	c
Archaea	Uncultured Archaea	6	EF422858	a
		7	EF422859	a
		8	EF422860	a
		9	EF422861	b
	Methanothermobacter	10	EF422862	c
		11	EF422863	c

a, The exterior of sulfide chimney; b, the middle of sulfide chimney; c, the interior of sulfide chimney.

were constructed from the aligned sequences in the ClustalX v1.8 program. Using the PHYLIP program, a phylogenetic tree was constructed by neighbor-joining method. Bootstrapping was performed using the bootstrap modus of the program.

RESULTS

The diversity of microorganisms from the exterior to the interior of sulfide chimney

The abundance and diversity of microbial communities from the exterior to the interior of sulfide chimney were investigated by PCR-DGGE method. DGGE profiles showed that bacteria and archaea could be examined in all three zones of the chimney wall and samples in the three zones display different migration patterns, which revealed that the compositions of microbial communities within different zones were vastly different. About 20 bacterial and 10 archaeal distinguishable bands were observed in different zones of sulfide chimney, respectively. For bacteria, there was no significant difference in the amount of DNA bands among three zones. While for archaea, the total amount of DNA bands in exterior was higher than both middle and interior, which indicated that cell abundance was greatest in the outermost zone of the chimney wall (Figure 1) as shown by phylogenetic analysis of 16SrDNA of the microorganisms in different zones.

To examine the diversity of the microorganisms communities present at different locations within the sulfide chimney, five most intensive bands from bacterial community and six most thickness bands from archaeal community were excised from DGGE gel respectively and subsequently cloned for sequencing. Results show that

Verrucomicrobia and Deltaproteobacteria were the predominant bacterial members in exterior, beta Proteobacteria were the dominant members in middle zones, and *Bacillus* were the abundant microorganisms in interior. As to these archaea, the six sequences were most similar to two large categories: one consisted of *Methanothermobacter* and the other was unclassified archaea. It was noticed that interior is dominated by methanogens, while the middle and exterior were dominated by unclassified archaea (Table 2). These results suggest that there was a transition in the composition of microbial communities across the sulfide chimney wall.

Furthermore, phylogenetic tree analysis showed that five bacterial DNA fragments detected were related to the several groups (Figure 2). Clone 1 was related to members of the uncultured *Verrucomicrobia* previously detected in association with mucous secretions of the hydrothermal vent polychaete *Paralvinella palmiformis* (*P. palm* C 41, GenBank AJ441225) (Alain et al., 2002) and *Cytophaga* sp. enrichment cultures with *Alvinella pompejana* white tubes collected on East Pacific Rise at 13°N (Dex80-43, GenBank AJ431234; Unpublished results). Hence, we assigned it as *Verrucomicrobia*. In addition, clone 2 was found to be most closely related to unclassified Syntrophobacterales collected in deep sea hydrothermal vent field in Mid Atlantic Ridge (PICO pp37 Rainbow 102, GenBank AJ969442, Unpublished results), unclassified Syntrophobacterales obtained from sediment-hosted carbon(iv)oxide (CO_2) lake of the southern Okinawa Trough hydrothermal system (OT-B08.16, GenBank AB252432) (Inagaki et al., 2006) and unclassified Desulfobulbaceae clone from deep-groundwater microorganism (KNA6-EB15,

A
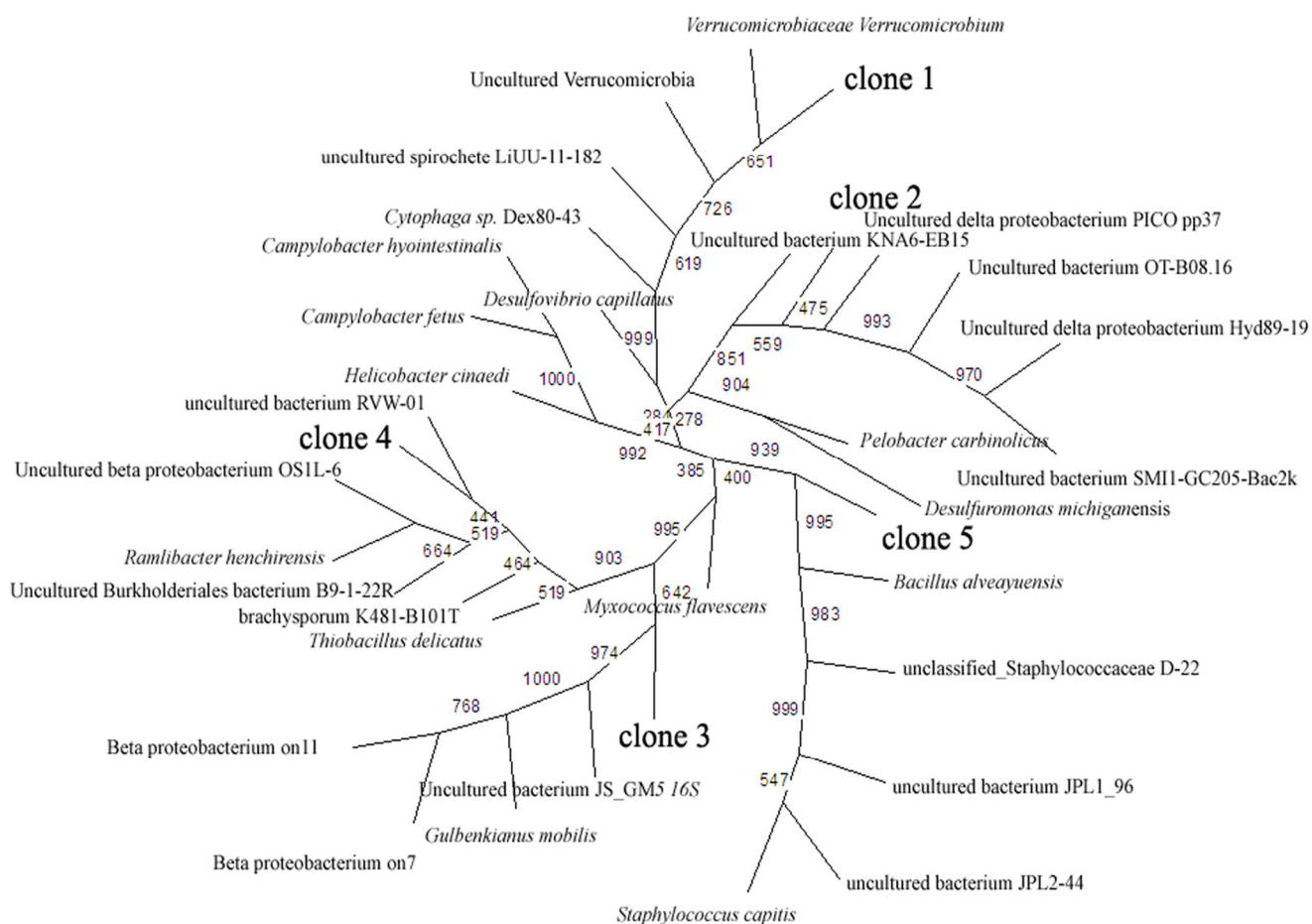
Figure 2. Phylogenetic tree constructed based on 16S rDNA gene sequences of bacteria (A) and archaea (B). 11 clones together with relative clones in the databank were used for phylogenetic tree construction. The phylogenetic tree was constructed by the neighbor-joining method using the phylip program. 1000 trial of bootstrap analysis was used to provide confident estimates for phylogenetic tree topologies. The percentage of 1000 bootstrap re-samplings was indicated. Representative sequences of rDNA clones obtained from the deep-sea hydrothermal vent of the Northeast Pacific in this study are represented by bold letters.

GenBank AB179691) (Miyoshi et al., 2005). Clones 3 and 4 were classified as members of the beta Proteobacteria. Clone 4 was related to sequences retrieved from thermophiles and hyperthermophiles in deep-subsurface geothermal environments (RVW-01, GenBank AB199568) (Kimura et al., 2006) and a *Ramlibacter henchirensis* isolated from subdesert soil in Tunisia (GenBank AF439400) (Heulin et al., 2003). Clone 5 was placed in the phylum of *Bacillus*.

Phylogenetic tree analysis indicated that the 6 archaeal

rDNA fragments obtained from three zones were found to fall into three large phylogenetic assemblages: one consisted of the very deep lineages of rDNA sequences within the methanogens and the other two were placed in an intermediate position between the uncultured Crenarchaeota and Euryarchaeota kingdoms and assigned as unclassified Archaea. Additionally, clone 6 was closely related to three other sequences, one from an active deep-sea vent chimney sample (PS-A8, GenBank AY280451) (Page et al., 2004), one from nascent

B
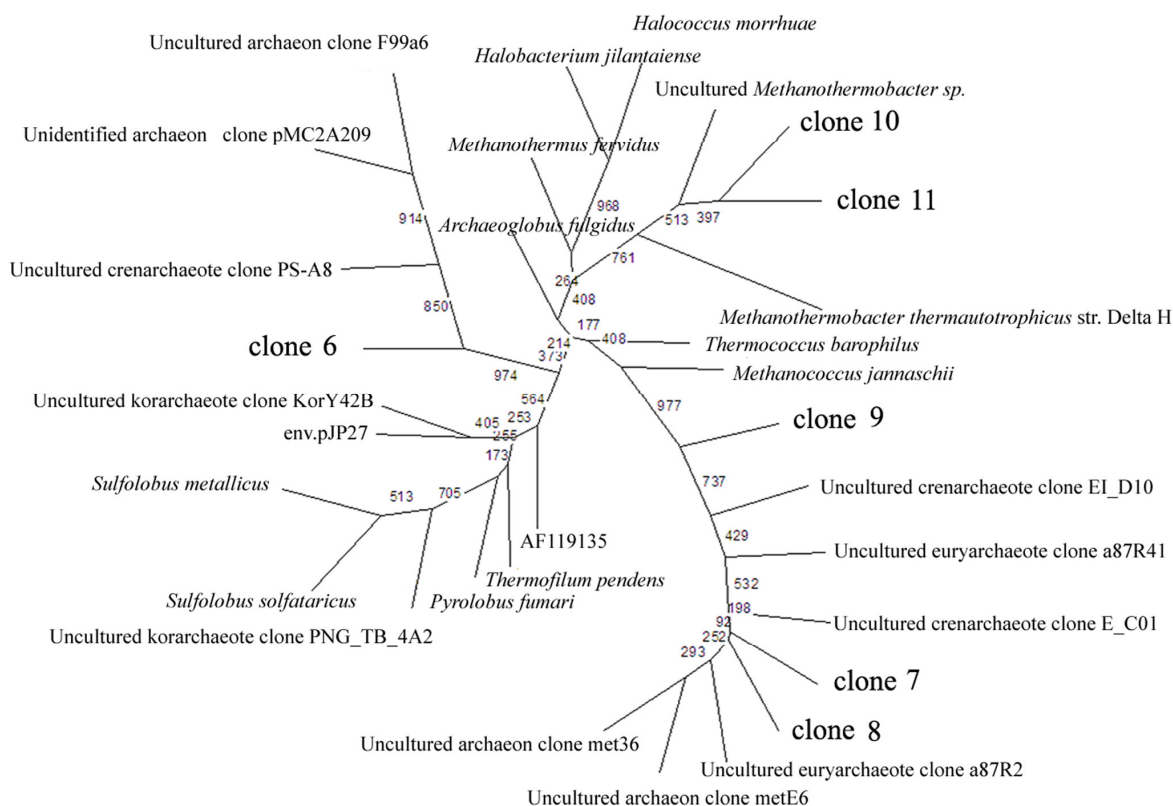
Figure 2. Contd.

hydrothermal chimney (F99a6. GenBank DQ228515. Schrenk et al., unpublished results), and one from a deep-sea hydrothermal vent environments (pMC2A209, GenBank AB019719) (Takai and Horikoshi, 1999b). The clones 7, 8 and 9 were in the same cluster of sequences with 96 to 99% sequence identity to other uncultured archaen clones (Figure 2b). The sequence corresponding to clones 10 and 11 was a deep lineage within the archaea, and most closely associated with the *Methano-thermobacter*.

DISCUSSION

Thermal and chemical gradients within the wall of hydro-thermal sulfide chimney can select for and sustain organisms adapted to specific environmental conditions. On this basis, the compositions of microbial communities within different microenvironments, such as the exterior and interior of a sulfide chimney, are expected to be vastly different (Schrenk et al., 2003). However, large numbers

of research are focus on the high-temperature hydro-thermal chimney, hence little is known about the diversity of microorganisms in low-temperature hydrothermal sulfide chimney.

The low-temperature hydrothermal sulfide chimney from clam bed is characterized by an abundance of oxidized sulfide minerals and presence of macrofauna, which may support unique microbial communities adapted to this favorable metabolic couple. Previous microbial diversity studies in high-temperature hydrothermal sulfide vent areas have shown that anaerobic archaea (e.g. *Thermo-coccales, Archaeoglobales* and *Methanococcales*) and sulfate reducer bacteria (e.g. delta and epsilon Proteobacteria) can be dominant members and may be important mediators of both the sulfur and nitrogen cycling in hydrothermal vent ecosystems (Longnecker and Reysenbach, 2001; Reed et al., 2006; Voordeckers et al., 2005). Our results indicate that the microbial community structure shifted, significantly alters across the sulfide chimney wall in the low-temperature hydrothermal sulfide chimney, ranging from delta Proteobacteria and
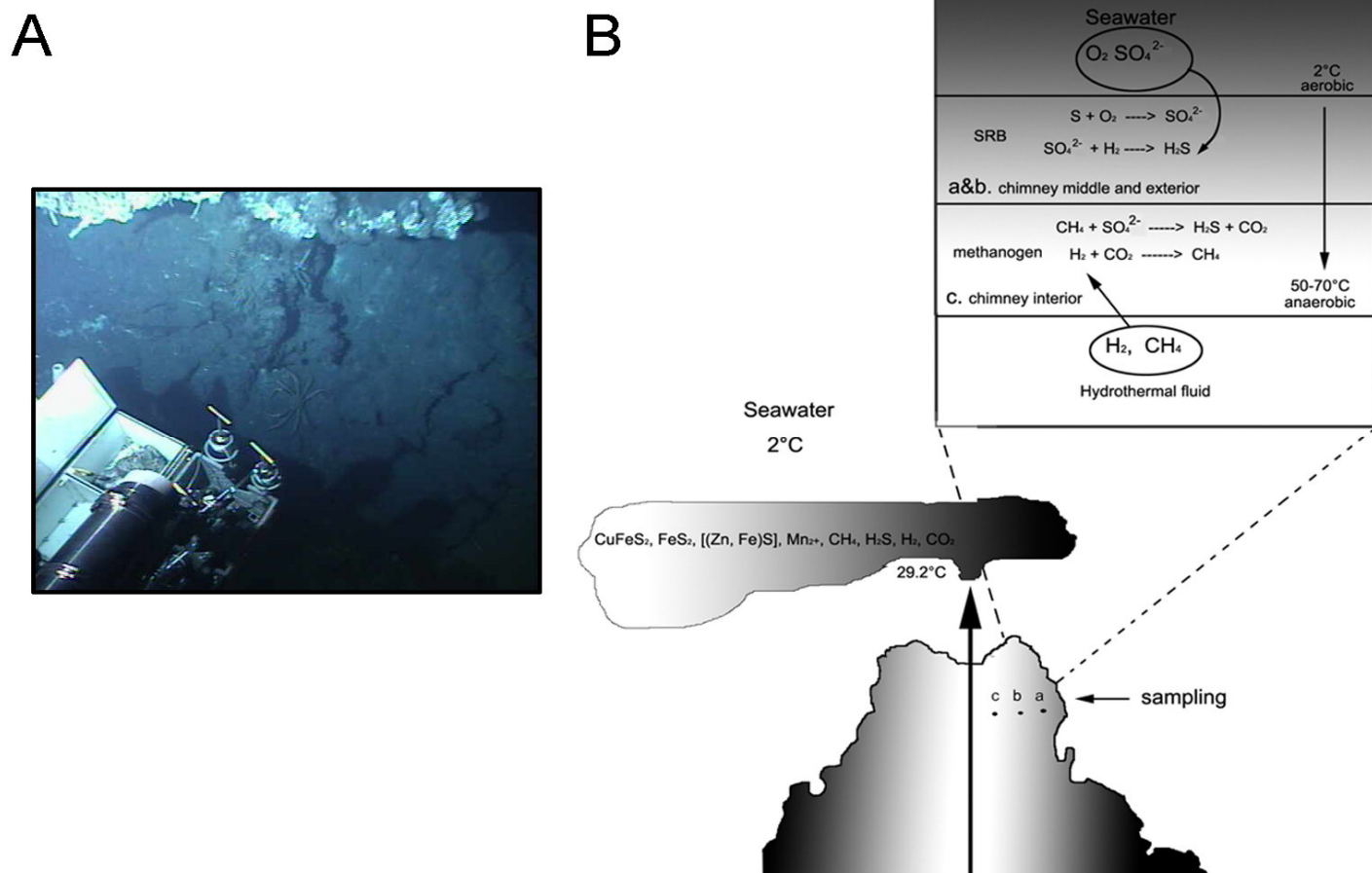
Figure 3. (A) Photograph of the site of chimney taken just before sampling (http://4dgeo.whoi.edu/alvin). (B) Model of biogeochemical zonation at the clam bed hydrothermal field.

uncultured archaea near the exterior of the chimney to predominantly beta Proteobacteria, *Methanothermobacter* and uncultured archaea near the interior of the chimney. A broad diversity of phylotypes belonging to other bacterial divisions was detected, including Verrucomicrobia and *Bacillus*. Clone 1 was assigned to phylum *Verrucomicrobia*. It was related to members of the uncultured *Verrucomicrobia* previously detected in association with mucous secretions of the hydrothermal vent polychaete *P. palmiformis*, which were two sulfide chimneys of CASM vent field (on T and S chimney, 130° 01' W 45° 59' N; depth, 1546 m) and ASHES vent field (on Hell chimney, 130° 01'W 45° 56' N; depth, 1580 m) close to our sampling site (Alain et al., 2002). *Verrucomicrobia* was also detected with the chitin tubes of the giant vent tubeworm *Riftia pachyptila* (collected at the East Pacific Rise, 9° N and 13° N) (Lopez-Garcia et al., 2002). Moreover, it was not strange to find some ectosymbionts or symbionts in the clam bed.

The presence of delta Proteobacteria (Syntrophobacteraceae) in the venting chimney suggested that sulfur-related metabolism was common and crucial to the vent ecosystem. The chimney's abundant uncultured archaea and *Methanothermobacter*, likely reflected sulfate, elevated hydrogen and CO_2 providing important energy sources for microbial life at the hydrothermal vents, and the chimney structures are likely to favor anaerobic niches and the temperature ranges from 50 to 70° in the interior zone of the chimney wall. Diverse uncultivated archaea species are known to be associated with chimney. Cultivation work was done in the laboratory, but there was no archaea isolated. Figure 3 depicts a biogeochemical model of carbon and sulfur cycling in the low-temperature hydrothermal sulfide chimney. The system is characterized by the interaction of H_2- and CH_4- rich hydrothermal fluid with oxygenated seawater. Sulfur-related metabolism is common and crucial to the ecosystem. Biofilms of methanogen are restricted to high-temperature anoxic zones; hence clam and shrimp are common there.

Conclusion

Ongoing analyses of several sulfide chimneys are providing multifaceted maps of the vent chimneys and are

allowing integration of multiple data sets into a coherent biogeochemical model. Our findings provide helpful information for understanding the diversity and phylogeny of microorganisms in the deep-sea low-temperature hydrothermal sulfide chimney. In the future, we hope to perform fluorescent *in situ* hybridization (FISH) and PCR with primers on functional genes etc., to uncover more information in the deep-sea low-temperature hydro-thermal sulfide chimney.

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