Acta Oceanol. Sin., 2014, Vol. 33, No. 10, P. 94–104
DOI: 10.1007/s13131-014-0544-0
http://www.hyxb.org.cn
E-mail: hyxbe@263.net

Microbial community structure and nitrogenase gene diversity of sediment from a deep-sea hydrothermal vent field on the Southwest Indian Ridge

WU Yuehong[1§], CAO Yi[2§], WANG Chunsheng[1], WU Min[3], AHARON Oren[4,5], XU Xuewei[1*]

[1] Laboratory of Marine Ecosystem and Biogeochemistry, Second Institute of Oceanography, State Oceanic Administration, Hangzhou 310012, China
[2] State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China
[3] College of Life Sciences, Zhejiang University, Hangzhou 310058, China
[4] Department of Plant and Environmental Sciences, the Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel
[5] The Hebrew University of Jerusalem, The Edmond J. Safra Campus, Givat Ram, Jerusalem 91904, Israel

Received 25 March 2013; accepted 2 November 2013

Abstract

A sediment sample was collected from a deep-sea hydrothermal vent field located at a depth of 2 951 m on the Southwest Indian Ridge. Phylogenetic analyses were performed on the prokaryotic community using polymerase chain reaction (PCR) amplification of the 16S rRNA and *nifH* genes. Within the Archaea, the dominant clones were from marine benthic group E (MBGE) and marine group I (MGI) belonging to the phyla *Euryarchaeota* and *Thaumarchaeota*, respectively. More than half of the bacterial clones belonged to the *Proteobacteria*, and most fell within the *Gammaproteobacteria*. No epsilonproteobacterial sequence was observed. Additional phyla were detected including the *Actinobacteria*, *Bacteroidetes*, *Planctomycetes*, *Acidobacteria*, *Nitrospirae*, *Chloroflexi*, *Chlorobi*, *Chlamydiae*, *Verrucomicrobia*, and candidate divisions OD1, OP11, WS3 and TM6, confirming their existence in hydrothermal vent environments. The detection of *nifH* gene suggests that biological nitrogen fixation may occur in the hydrothermal vent field of the Southwest Indian Ridge. Phylogenetic analysis indicated that only Clusters I and III NifH were present. This is consistent with the phylogenetic analysis of the microbial 16S rRNA genes, indicating that Bacteria play the main role in nitrogen fixation in this hydrothermal vent environment.

Key words: deep-sea, hydrothermal vent, microbial diversity, 16S rRNA gene, *nifH* gene

Citation: Wu Yuehong, Cao Yi, Wang Chunsheng, Wu Min, Aharon Oren, Xu Xuewei. 2014. Microbial community structure and nitrogenase gene diversity of sediment from a deep-sea hydrothermal vent field on the Southwest Indian Ridge. Acta Oceanologica Sinica, 33(10): 94–104, doi: 10.1007/s13131-014-0544-0

1 Introduction

Since the first discovery of hydrothermal vent ecosystems about thirty-six years ago, vents have been considered oases in benthic environments, accompanied by sharp chemical and physical gradients (pH, temperature, energy and nutrient source). Chemolithoautotrophic microorganisms are the primary producers of hydrothermal microbial communities, and these are able to fix inorganic carbon using chemical energy obtained through the oxidation of reduced compounds such as hydrogen sulfide, hydrogen, and methane. Heterotrophic microorganisms that depend on the energy flow through these chemoautotrophs form another component of the microbial community. They can be free-living or symbiotic, associated with animals (Jørgensen and Boetius, 2007). As a result of successful cultivation efforts, many thermophilic and hyperthermophilic Archaea as well as Bacteria have been isolated from hydrothermal vents (Takai et al., 2002; Nakagawa et al., 2004; Voordeckers et al., 2005; Smith et al., 2008). On the other hand, culture-independent molecular phylogenetic analyses studies of 16S ribosomal RNA (16S rRNA) genes recovered from the Pacific (Schrenk et al., 2003), Atlantic (Nercessian et al., 2005), Indian mid-oceanic ridges (Takai et al., 2004) and other hydrothermal vent environments (Takai and Horikoshi, 1999) have extended our views about the microbial diversity in hydrothermal vents.

High concentrations of organic and inorganic nitrogen compounds exist in the deep-sea environment, which compose the main source of nitrogen in deep-water sediments rather than nitrogen fixation (Burns et al., 2002). However, dissolved dinitrogen gas is abundant in deep-sea hydrothermal vent fluids. The ability to fix nitrogen is widely distributed among phylogenetically diverse Bacteria and Archaea in hydrothermal fields (Mehta et al., 2003; Mehta et al., 2005).

So far, two deep-sea hydrothermal vent systems in the Indian Ocean have been well studied for their microbial community (Takai et al., 2004; Suzuki et al., 2004). The Kairei hydrothermal

Foundation item: The China Ocean Mineral Resources R & D Association (COMRA) Special Foundation under contract No. DY125-15-R-03; the National Natural Science Foundation of China under contract Nos 41276173 and 41206104; the Scientific Research Fund of the Second Institute of Oceanography, State Oceanic Administration under contract No. JT1011.
*Corresponding author, E-mail: xuxw@sio.org.cn
§These authors contributed equally to this work.

field was the first discovered hydrothermal vent system in the Indian Ocean (Hashimoto et al., 2001), followed by the Edmond field (Van Dover et al., 2001). Both are located in the Central Indian Ridge, but no hydrothermal vent system has been reported in other mid-oceanic ridges of the Indian Ocean.

The primary purpose of this study was to characterize the microbial community structure as well as the *nifH* gene diversity of deep-sea sediment from a hydrothermal vent field on the Southwest Indian Ridge. In total, 117 archaeal 16S rRNA gene sequences, 148 bacterial 16S rRNA gene sequences and 104 *nifH* gene sequences were obtained from five clone libraries constructed from this sediment sample. In this study, we performed the first analysis of microbial community structure associated with sediment from a hydrothermal vent field on the Southwest Indian Ridge, and we further examined the presence and origin of *nifH* sequences.

2 Materials and methods

2.1 *Sampling*

A new hydrothermal vent field was discovered during the Chinese cruise on R/V *Dayangyihao* in 2007 at the Southwest Indian Ridge hydrothermal area. A hydrothermal vent sediment sample was obtained from a depth of 2951 m at the TVG3 site (27°50.98′S, 63°56.17′E), southwest of the Kairei and Edmond hydrothermal field (Fig. 1). The sample was collected in a sealed sterile container and frozen in liquid nitrogen until use.

2.2 *Chemical analysis*

Chemical components including nitrite + nitrate (NO_2^-+NO_3^-), nitrite (NO_2^-) and ammonium (NH_4^+) of the hydrothermal vents sediment were measured by a nutrient autoanalyzer (SKALAR San++).

2.3 *DNA extraction, PCR, cloning, and sequencing*

Total DNA was extracted from the sediment sample according to Zhou et al. (1996). Approximately 5 g of sample was used for DNA extraction. Extracted DNA was stored frozen at –20°C. For PCR amplification of prokaryotic 16S rRNA genes we used the primers A2F, A571F, E8F, UA1204R, and U1510R (Baker et al., 2003). Two kinds of primer pairs were introduced to amplify *nifH* gene (Zehr and McReynolds, 1989; Mehta et al., 2003). All PCR reactions followed procedures described in Table 1. PCR products were cleaned with a PCR purification kit (Axygen), and cloned with the pMD18-T vector kit (TakaRa) to construct libraries. Clones from these libraries were randomly selected for sequencing.

2.4 *OTU assignment and phylogenetic analysis*

The 16S rRNA gene sequences were checked for chimeras with the tool of Ribosomal Database Project II (RDP II, http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU). Clones with 97% or higher similarity were assigned as an operational taxonomic unit (OTU) using the Mothur program based on the distance matrix (Schloss et al., 2009). Then, rarefaction curves were constructed by the Dotur program (Schloss Handelsman, 2005). Sequences from active and inactive chimney structures of the Kairei field in the Central Indian Ridge were obtained from GenBank. Venn diagrams were plotted using the Venn Diagram Plotter (http://omics.pnl.gov/software/VennDiagramPlotter.php) and eulerAPE program (http://www.eulerdiagrams.org/eulerAPE/).

All nitrogenase *nifH* gene diversity and phylogenetic analyses were performed with deduced amino acid sequences. 16S rRNA gene and putative NifH protein sequences homology comparisons were carried out using the BLAST tool at the National Center for Biotechnology Information (NCBI, http://

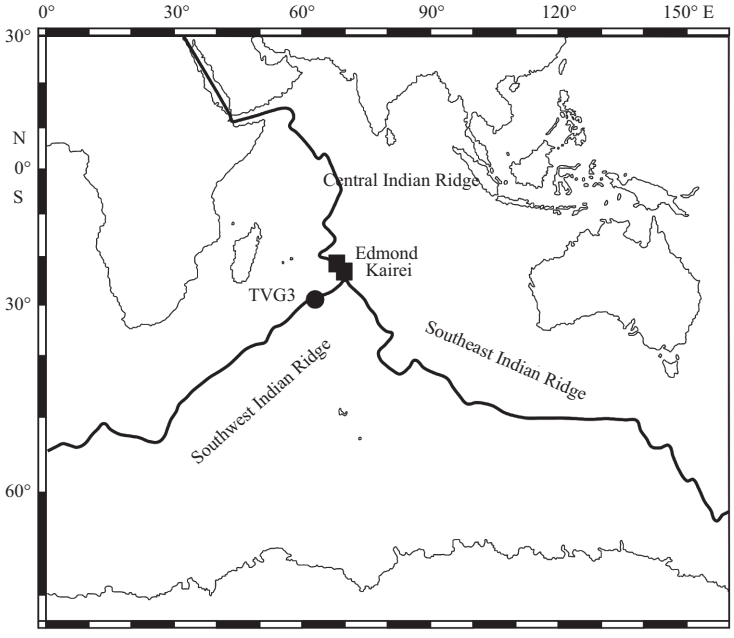

Fig.1. Map of the mid-oceanic ridge of the Indian Ocean showing the location of the TVG3 site on the Southwest Indian Ridge, and the Kairei and Edmond vent fields on the Central Indian Ridge. The map was created using the NOAA satellite global seafloor topography images (www.NOAA.gov).

Table 1. PCR primers and procedures used in this study

Target gene	Primer	Sequences (5′–3′)	PCR cycle conditions
Archaeal 16S rRNA	A2F	TTCCGGTTGATCCTGCCGGA	5 min at 96°C; 30 cycles of 96°C for 20 s, 50°C for 45 s, and 72°C for 2 min; final extension of 72°C for 10 min
	U1510R	GGTTACCTTGTTACGACTT	
Archaeal 16S rRNA	A571F	GCYTAAAGSRICCGTAGC	5 min at 96°C; 30 cycles of 96°C for 20 s, 50°C for 45 s, and 72°C for 2 min; final extension of 72°C for 10 min
	UA1204R	TTMGGGGCATRCIKACCT	
Bacterial 16S rRNA	E8F	AGAGTTTGATCCTGGCTCAG	5 min at 95°C; 30 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 2 min; final extension of 72°C for 10 min
	U1510R	GGTTACCTTGTTACGACTT	
nifH	nifH-1F	GGHAARGGHGGHATHGGNAARTC	2 min at 94°C; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; final extension of 72°C for 10 min
	nifH-1R	GGCATNGCRAANCCVCCRCANAC	
nifH	nifH-2F	TGYGAYCCNAARGCNGA	5 min at 94°C; 35 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min; final extension of 72°C for 10 min
	nifH-2R	ADNGCCATCATYTCNCC	

www.ncbi.nlm.nih.gov/BLAST). Clone sequences and their closest relatives were imported into the MEGA 4.0 program (Tamura et al., 2007). Phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987) with the Jukes and Cantor model (Jukes and Cantor, 1969) for 16S rRNA gene sequences and the Kimura model (Kimura, 1980) for NifH amino acid sequences.

2.5 *Nucleotide sequence accession numbers*

The 16S rRNA sequences for the archaeal 16S rRNA sequences, bacterial 16S rRNA clones and *nifH* sequences from the Southwest Indian Ridge hydrothermal vent chimney were deposited in the GenBank under accession numbers GU195982-GU196019, GU196020-GU196129, and GU232739-GU232766, respectively.

3 Results

3.1 *Chemical compositions of the hydrothermal vent sediment sample*

The chemical compositions of the hydrothermal vent sediment sample were determined. According to the analysis, the concentration of nitrate, nitrite and ammonia were 4.09, 0.23 and 3.97 μg/g, respectively.

3.2 *Characterization of the prokaryotic 16S rRNA and nifH gene libraries*

A cultivation-independent approach was used to characterize the microbial community structure associated with the sediment of this hydrothermal system. High molecular weight DNA was successfully extracted and the 16S rRNA genes of Archaea and Bacteria were amplified. After discarding chimeric sequences, archaeal 16S rRNA gene libraries with different primer pairs designated A1 (primer pair A2F / U1510R) and A2 (primer pair A571F / UA1204R) were constructed, containing 47 and 70 clones, respectively. In addition 148 clones were sequenced from a bacterial library. The archaeal (A1, A2) and bacterial libraries were composed of 22, 16, and 110 phylotypes (sequences less than 97% similar), respectively. *nifH* genes were amplified with two kinds of primer pairs (nifH-1F / nifH-1R and nifH-2F / nifH-2R) and two libraries were generated with 53 and 51 clones, respectively. Eleven and seventeen phylotypes were obtained from these libraries respectively.

3.3 *Rarefaction analysis of bacterial and archaeal libraries*

Significantly lower diversity and fewer OTUs were found in archaeal libraries compared with the bacterial library. It was further supported by the rarefaction analysis. The slope of the rarefaction curves (Fig. 2) for both archaeal libraries became nearly asymptotic and estimated the ultimate richness. In contrast, despite the identification of 148 clones as bacterial, the rarefaction curve indicated that the sampling of bacterial richness was far from complete.

The Venn diagrams showed the relationship of shared and site-specific groups between our study and previous study on active and inactive chimney structures (Takai et al., 2004; Su-

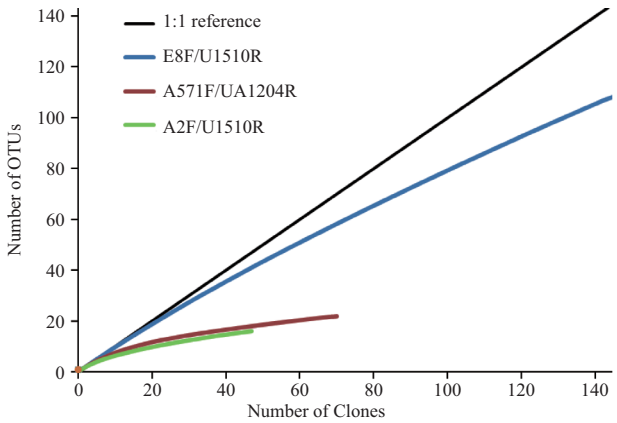

Fig.2. Rarefaction analysis for total bacterial and archaeal clones. OTUs were determined at 3% 16S rRNA gene sequence difference level as assigned by the DOTUR program. 1:1 reference curve indicates that each sequenced clone belonged to a unique OTU.

zuki et al., 2004). The bacterial OTU number for hydrothermal vent sediment (this study), inactive and active chimney structures were 110, 32 and 21, respectively. No overlapped OTU was observed with 97% identity threshold (Fig. 3a); however, with 80% identity threshold, seven OTUs were shared between hydrothermal vent sediment and inactive chimney structures, one OUT was shared between hydrothermal vent sediment and active chimney structures, and four OTUs were shared between inactive and active chimney structures. No OUT was shared by all of them (Fig. 3b). The results suggested that the bacterial abundances differed among the sample areas on the hydrothermal vents field.

3.4 *Archaeal diversity*

The total of 117 archaeal 16S rRNA gene sequences yielded 38 unique phylotypes from the two archaeal libraries. These belonged to five distinct clusters corresponding to the phyla *Euryarchaeota* (76 sequences in 23 phylotypes) and *Thaumarchaeota* (41 sequences in 15 phylotypes), including the marine benthic group E (MBGE), marine group II (MGII), deep-sea hydrothermal vent *Euryarchaeota* group 5 (DHVE5), marine group I (MGI), and undefined *Euryarchaeota* and *Thaumarchaeota* groups (Fig. 4).

Seventy-two out of seventy-six of these euryarchaeotal sequences belonged to the MBGE group. These sequences were closely related (greater than 90% similarity) to uncultured clones obtained from active deep-sea hydrothermal fields in the Southern Mariana Trough (Kato et al., 2009), the Myojin Knoll, the Izu-Ogasawara area (Takai and Horikoshi, 1999) and the Central Indian Ridge (Takai et al., 2004); deep-sea sediments in the Eastern Mediterranean (Heijs et al., 2008); and shallow methane seep sediments in the Timor Sea (Wasmund et al., 2009). The closest cultured relatives to these euryarchaeotal sequences were the extremely thermophilic methanogens *Methanothermobacter thermophilus*, *Methanothermobacter defluvii* (Kotelnikova et al., 1993), and *Methanothermus fervidus* (Rouvière et al., 1992), with similarities between 83.5% and 86.6%. Euryarchaeotic sequences within phylotype pTVGA17 shared 97% similarity with uncultured clone HF4000_48H06 from a 4 000 m deep-sea station of ALOHA, North Pacific Ocean, belonging to the MGII group (DeLong et al., 2006). Clone pTVGA3 was distantly related (81% similarity) to the cluster DHVE5 clones from sediments associated with a hydrothermal vent site at the Middle Okinawa Trough (Takai and Horikoshi, 1999). The remaining clone pTVGA16 fell into an undefined euryarchaeotal group.

Thirty-eight 16S rRNA gene sequences fell into the MGI group (96% to 99% similarity to the closest matched GenBank sequences), which is now considered part of the new archaeal phylum *Thaumarchaeota* defined as mesophilic Crenarchaeota (Brochier-Armanet et al., 2008). These sequences are affiliated with uncultivated Archaea from deep-sea sediments in the Weddell Sea (Brandt et al., 2007), the East Pacific Rise (Mason et al., 2007), the Nankai Trough (Arakawa et al., 2006), the Pacific nodule province (Xu et al., 2005), the Peru margin (Inagaki et al., 2006), the Eastern Mediterranean (Heijs et al., 2008) and the North Atlantic (Agogue et al., 2008). Phylotypes pTVGA19 and 37 shared 96% similarity with a clone obtained from sediment present in a tropical seawater tank at the Seattle Aquarium, which was closely related to *Nitrosopumilus maritimus* SCM1, the first cultured mesophilic representative of the Crenarchaeota belonging to the MGI group (Könneke et al., 2005). Most of the MGI thaumarchaeotal sequences in our study were affiliated (91% to 97% similarity) with this cultivated archaeon as well. Phylotypes pTVGA11, 15 and 24 fell into an unclassified thaumarchaeotal group. Clones pTVGA11 and 15 were distantly related (90% similarity) with an uncultured clone retrieved from a hydrothermal chimney at the Lost City field, Mid-Atlantic

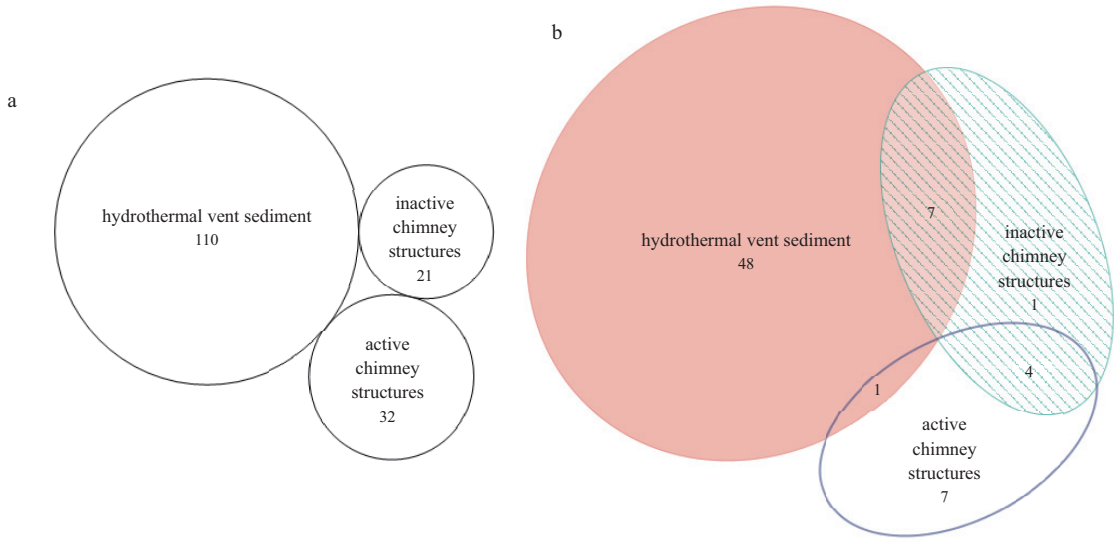

Fig.3. Venn diagrams showing the estimated OTU (97% identity threshold) (a) and OTU (80% identity threshold) (b) richness shared among bacterial communities from hydrothermal vent sediment (this study), inactive chimney structures (Suzuki et al., 2004) and active chimney structures (Takai et al., 2004). Shared OTU richness estimates were calculated using the program MOTHUR. Venn diagrams were plotted using the Venn Diagram Plotter program and eulerAPE program. The object sizes are drawn to show the approximate OTU memberships. Numbers in the Venn diagrams indicate number of OTUs.

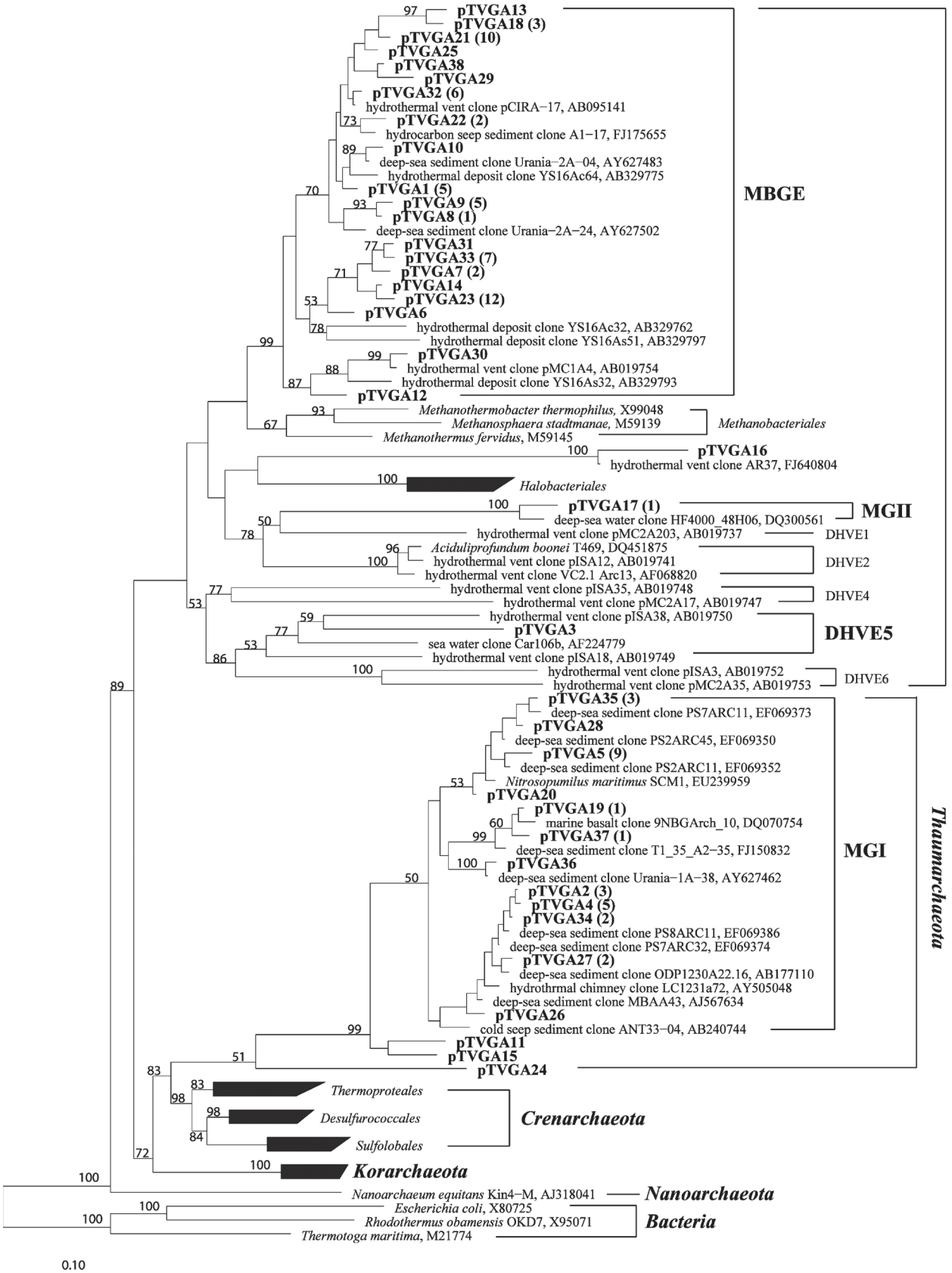

Fig.4. The archaeal 16S rRNA gene phylogenetic tree, constructed using neighbor-joining analysis. Clones detected in this study are shown in bold, the number following the detected clones indicates the number of clones. The numbers at the nodes are the bootstrap values. Bootstrap values (in percent) are based on 1 000 replicates each and are shown for branches with more than 50% bootstrap support. The scale bar represents 0.1 substitutions per nucleotide position.

Ridge (Schrenk et al., 2004), and clone pTVGA24 was distantly related to MGI clone ANT33-04 (Arakawa et al., 2006).

3.5 *Bacterial diversity*

A total of 148 bacterial 16S rRNA gene sequences representing 110 phylotypes were retrieved in this study. The library was dominated by sequences affiliated with the *Proteobacteria* (62.2%), *Actinobacteria* (9.5%), *Bacteroidetes* (6.1%), *Planctomycetes* (6.1%), and *Acidobacteria* (6.1%).

The sequences related to *Proteobacteria* were divided into four subgroups (*Alpha-*, *Beta-*, *Gamma-*, and *Deltaproteobacteria*) (Fig. 5a). Fifty-five *Gammaproteobacteria* related sequences formed the largest division. Phylotypes pTVGB18 and 93 were closely related to cultured species (99% similarity). However, most phylotypes were related to uncultured clones obtained from other hydrothermal vent sediments (López-Garcia et al., 2003; Nercessian et al., 2005), seafloor lava environments (Santelli et al., 2008), deep-sea water samples (Pham et al., 2008), and marine sediments (Gillan and Pernet, 2007; Sørensen et al., 2007). We also detected 16 and 17 sequences related to *Alphaproteobacteria* and *Deltaproteobacteria*, respectively. These sequences were closely related to uncultured clones from deep-sea sediment (Santelli et al., 2008; Hunter et al., 2006) and other habitats. Only four *Betaproteobacteria*-related sequences were recovered, and these were related to sequences earlier retrieved from seafloor lava sediments (Santelli et al., 2008). *Epsilonproteobacteria*-related sequences which are typical for hot vents (Jørgensen and Boetius, 2007) were not encountered in this study.

Fourteen *Actinobacteria*-related sequences belonged to the family of high G+C Gram positive bacteria. They showed relationship with uncultured organisms retrieved from Mid-Atlantic Ridge hydrothermal fields (López-Garcia et al., 2003; Nercessian et al., 2005); deep-sea sediment in the Mediterranean (Heijs et al., 2008); methane-seep sediment in the Eel River Basin in California (Beal et al., 2009); and Arctic surface sediment (Li et al., 2009). Nine sequences were identified to be members of the *Bacteroidetes* group, within the subdivision *Cytophaga-Flavobacterium*, which consists of heterotrophic bacteria (Kirchman, 2002). They are related to clones identified from various marine environments (López-Garcia et al., 2003; Musat et al., 2006; Crump et al., 2007; Heijs et al., 2008; Polymenakou et al., 2009; Li et al., 2009). Nine clones belonged to the *Planctomycetes*-related cluster. These sequences showed high similarity with uncultured relatives encountered in a hydrothermal vent (Nercessian et al., 2005), in marine sediments (Polymenakou et al., 2005; Hunter et al., 2006; Santelli et al., 2008), and in sea water from the Barents Sea (Schmid et al., 2007). Phylotype pTVGB6 shared 86% similarity with cultured anaerobic ammonium-oxidizing planctomycete KOLL2a (Egli et al., 2001), obtained from a deep-sea hydrothermal system. Nine phylotypes were identified as *Acidobacteria* related sequences, closely affiliated with uncultured organisms obtained from various marine habitats including deep-sea hydrothermal vents (López-Garcia et al., 2003). Other clones of the bacterial library were affiliated with the *Nitrospirae*, *Chloroflexi*, *Chlorobi*, *Chlamydiae*, *Verrucomicrobia*, and candidate divisions OD1, OP11, WS3, TM6, found previously in marine environments (Nercessian et al., 2005; Heijs et al., 2008) (Fig. 5b).

3.6 *nifH diversity*

A total of 104 *nifH* clones were sequenced from two libraries obtained by using two different primer pairs (Table 1). Twenty-eight diverse *nifH* gene phylotypes were obtained which shared less than 97% similarity. The deduced protein sequences shared 80% to 98% similarity with the GenBank published sequences. All the sequences were imported and aligned with the NifH database (http://pmc.ucsc.edu/~wwwzehr/research/database/). Phylogenetic analysis showed that all NifH sequences retrieved in this study fell into Cluster I or III and within seven groups as defined by Chien and Zinder (1994) and Zehr et al. (2003) (Fig. 6). Eleven hydrothermal vent NifH phylotypes of twenty-six sequences fell into Cluster I of NifH within the 1C, 1H, 1P groups including alpha-, beta-, and gammaproteobacterial nitrogenases, based on sequences retrieved from cultivated organisms. One unclassified clone N-2-13 was obtained, which was closely related to the 1C group. Sixteen phylotypes with seventy-seven hydrothermal vent NifH sequences fell within the 3I, 3P, 3K and 3J groups of NifH Cluster III. Based on the phylogenetic distribution of the *nifH* gene among cultivated representatives, the 3P and 3K group belong to the *Deltaproteobacteria* and the *Spirochaetes*, respectively. All the sequences within the 3I group are derived from environmental clones. Clones N-1-11, N-2-4, N-2-17, N-1-3, N-1-4, N-2-12, N-2-2 and clones N-2-5, N-2-8, N-2-6, N-2-10, N-1-10, N-2-7 formed two large diverse clades within the 3I and 3P groups, respectively, which indicates the existence of distinct deep-sea hydrothermal vent nitrogenase groups. Genome studies show that some archaeal *nifH* genes fall into the 3J group. Clones N-2-14 and N-2-15 are closely related (84% similarity) to uncultured methanogenic archaeon RC-I *nifH* (Erkel et al., 2006).

4 Discussion

Phylogenetic analysis of the 16S rRNA gene clones from the Southwest Indian Ridge hydrothermal vent sediment provided a view of its microbial diversity; however, because of the biases associated with the polymerase chain reaction (von Wintzingerode et al., 1997), the abundance of the phylotypes in the libraries obtained cannot be assumed to reflect their abundance in the environment.

The Archaea in this vent sample were found to possess relatively little diversity. Most archaeal sequences fell into the groups of MBGE and MGI (61.5% and 32.5% of the sequences, respectively). Microorganisms belonging to the MBGE group could take part in methane cycling, possibly representing methanogens (Vetriani et al., 1999). Previous studies showed that only a small proportion of methanogens in hydrate-bearing sediments could be detected using methanogen-specific primers (Inagaki et al., 2006). Based on the chemical analysis of sediments from the sampling stations, the concentration of ammonia is 3.97 μg/g. It indicated that ammonia oxidizers may play an important role in the sediment. Most of the MGI thaumarchaeotal sequences in this study were affiliated with this cultivated archaeon *Nitrosopumilus maritimus* SCM1, which is nonthermophilic and able to grow by aerobically oxidizing ammonia to nitrite. These features are consistent with functional gene investigations (Francis et al., 2005; Wang et al., 2009), indicating that some members of the MGI Archaea are autotrophic ammonia oxidizers. *Betaproteobacteria*-related sequences were related to sequences earlier retrieved from seafloor lava sediments (Santelli et al., 2008), probably belonging to ammonia oxidizers (Teske et al., 1994).

a

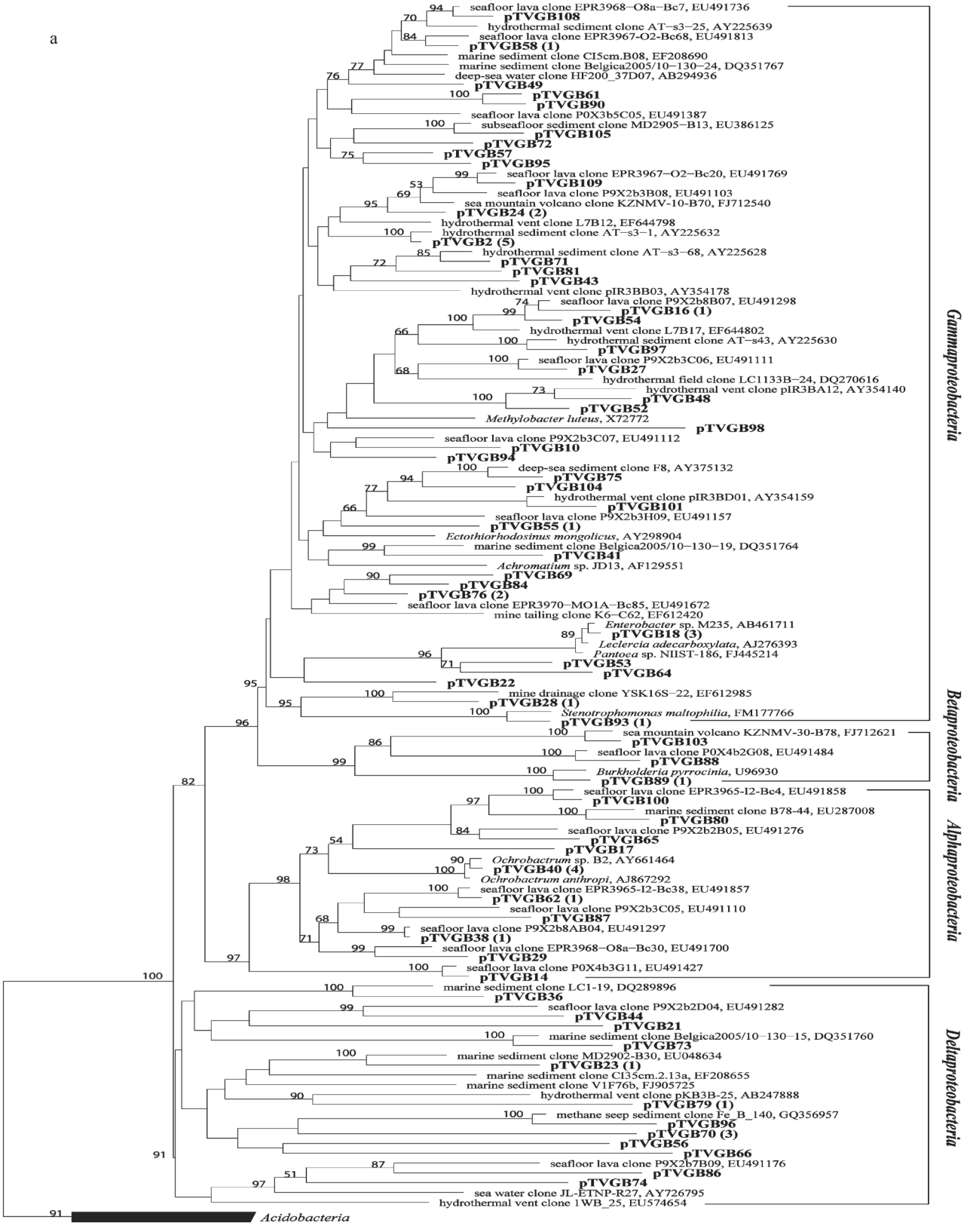
seafloor lava clone EPR3968−O8a−Bc7, EU491736
pTVGB108
hydrothermal sediment clone AT−s3−25, AY225639
seafloor lava clone EPR3967-O2-Bc68, EU491813
pTVGB58 (1)
marine sediment clone CI5cm.B08, EF208690
marine sediment clone Belgica2005/10−130−24, DQ351767
deep-sea water clone HF200_37D07, AB294936
pTVGB49
pTVGB61
pTVGB90
seafloor lava clone P0X3b5C05, EU491387
subseafloor sediment clone MD2905−B13, EU386125
pTVGB105
pTVGB72
pTVGB57
pTVGB95
seafloor lava clone EPR3967−O2−Bc20, EU491769
pTVGB109
seafloor lava clone P9X2b3B08, EU491103
sea mountain volcano clone KZNMV-10-B70, FJ712540
pTVGB24 (2)
hydrothermal vent clone L7B12, EF644798
hydrothermal sediment clone AT−s3−1, AY225632
pTVGB2 (5)
hydrothermal sediment clone AT−s3−68, AY225628
pTVGB71
pTVGB81
pTVGB43
hydrothermal vent clone pIR3BB03, AY354178
seafloor lava clone P9X2b8B07, EU491298
pTVGB16 (1)
pTVGB54
hydrothermal vent clone L7B17, EF644802
hydrothermal sediment clone AT−s43, AY225630
pTVGB97
seafloor lava clone P9X2b3C06, EU491111
pTVGB27
hydrothermal field clone LC1133B−24, DQ270616
hydrothermal vent clone pIR3BA12, AY354140
pTVGB48
pTVGB52
Methylobacter luteus, X72772
pTVGB98
seafloor lava clone P9X2b3C07, EU491112
pTVGB10
pTVGB94
deep-sea sediment clone F8, AY375132
pTVGB75
pTVGB104
hydrothermal vent clone pIR3BD01, AY354159
pTVGB101
seafloor lava clone P9X2b3H09, EU491157
pTVGB55 (1)
Ectothiorhodosinus mongolicus, AY298904
marine sediment clone Belgica2005/10−130−19, DQ351764
pTVGB41
Achromatium sp. JD13, AF129551
pTVGB69
pTVGB84
pTVGB76 (2)
seafloor lava clone EPR3970−MO1A−Bc85, EU491672
mine tailing clone K6−C62, EF612420
Enterobacter sp. M235, AB461711
pTVGB18 (3)
Leclercia adecarboxylata, AJ276393
Pantoea sp. NIIST-186, FJ445214
pTVGB53
pTVGB64
pTVGB22
mine drainage clone YSK16S−22, EF612985
pTVGB28 (1)
Stenotrophomonas maltophilia, FM177766
pTVGB93 (1)
sea mountain volcano KZNMV-30-B78, FJ712621
pTVGB103
seafloor lava clone P0X4b2G08, EU491484
pTVGB88
Burkholderia pyrrocinia, U96930
pTVGB89 (1)
seafloor lava clone EPR3965-I2-Bc4, EU491858
pTVGB100
marine sediment clone B78-44, EU287008
pTVGB80
seafloor lava clone P9X2b2B05, EU491276
pTVGB65
pTVGB17
Ochrobactrum sp. B2, AY661464
pTVGB40 (4)
Ochrobactrum anthropi, AJ867292
seafloor lava clone EPR3965-I2-Bc38, EU491857
pTVGB62 (1)
seafloor lava clone P9X2b3C05, EU491110
pTVGB87
seafloor lava clone P9X2b8AB04, EU491297
pTVGB38 (1)
seafloor lava clone EPR3968−O8a−Bc30, EU491700
pTVGB29
seafloor lava clone P0X4b3G11, EU491427
pTVGB14
marine sediment clone LC1-19, DQ289896
pTVGB36
seafloor lava clone P9X2b2D04, EU491282
pTVGB44
pTVGB21
marine sediment clone Belgica2005/10−130−15, DQ351760
pTVGB73
marine sediment clone MD2902-B30, EU048634
pTVGB23 (1)
marine sediment clone CI35cm.2.13a, EF208655
marine sediment clone V1F76b, FJ905725
hydrothermal vent clone pKB3B-25, AB247888
pTVGB79 (1)
methane seep sediment clone Fe_B_140, GQ356957
pTVGB96
pTVGB70 (3)
pTVGB56
pTVGB66
seafloor lava clone P9X2b7B09, EU491176
pTVGB86
pTVGB74
sea water clone JL-ETNP-R27, AY726795
hydrothermal vent clone 1WB_25, EU574654
Acidobacteria
0.10
Gammaproteobacteria
Betaproteobacteria
Alphaproteobacteria
Deltaproteobacteria

Fig.5.

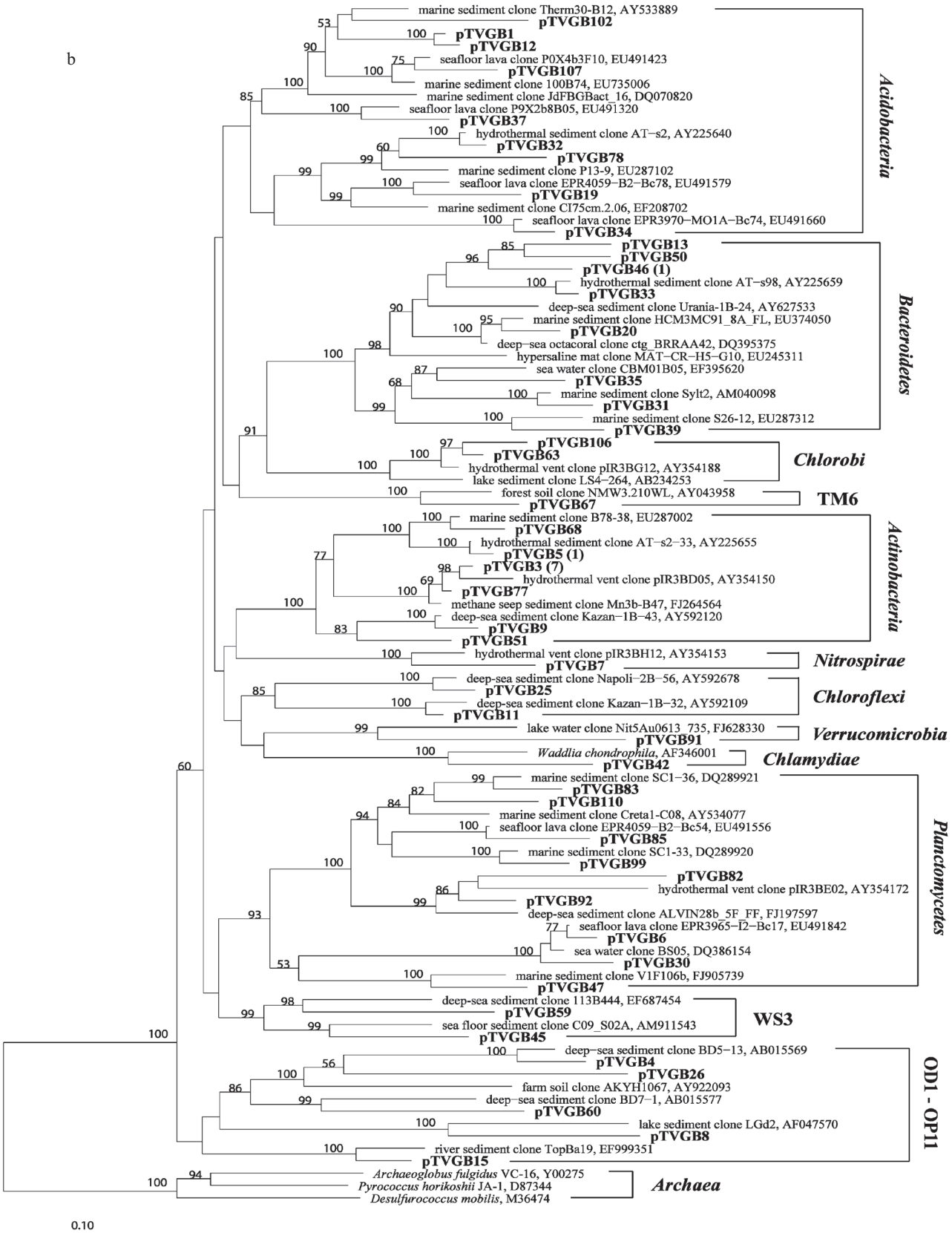

Fig.5. The bacterial 16S rRNA gene phylogenetic tree as determined by neighbor-joining analysis. a. *Proteobacteria*-related sequences and b. *Actinobacteria, Bacteroidetes, Planctomycetes, Acidobacteria, Nitrospirae, Chloroflexi, Chlorobi, Chlamydiae, Verrucomicrobia,* and candidate divisions OD1, OP11, WS3, and TM6 related sequences. Clones detected in this study are shown in bold, the number following the detected clones indicates the number of clones. The numbers at the nodes are the bootstrap values. Bootstrap values (in percent) are based on 1 000 replicates each and are shown for branches with more than 50% bootstrap support. The scale bar represents 0.1 substitutions per nucleotide position.

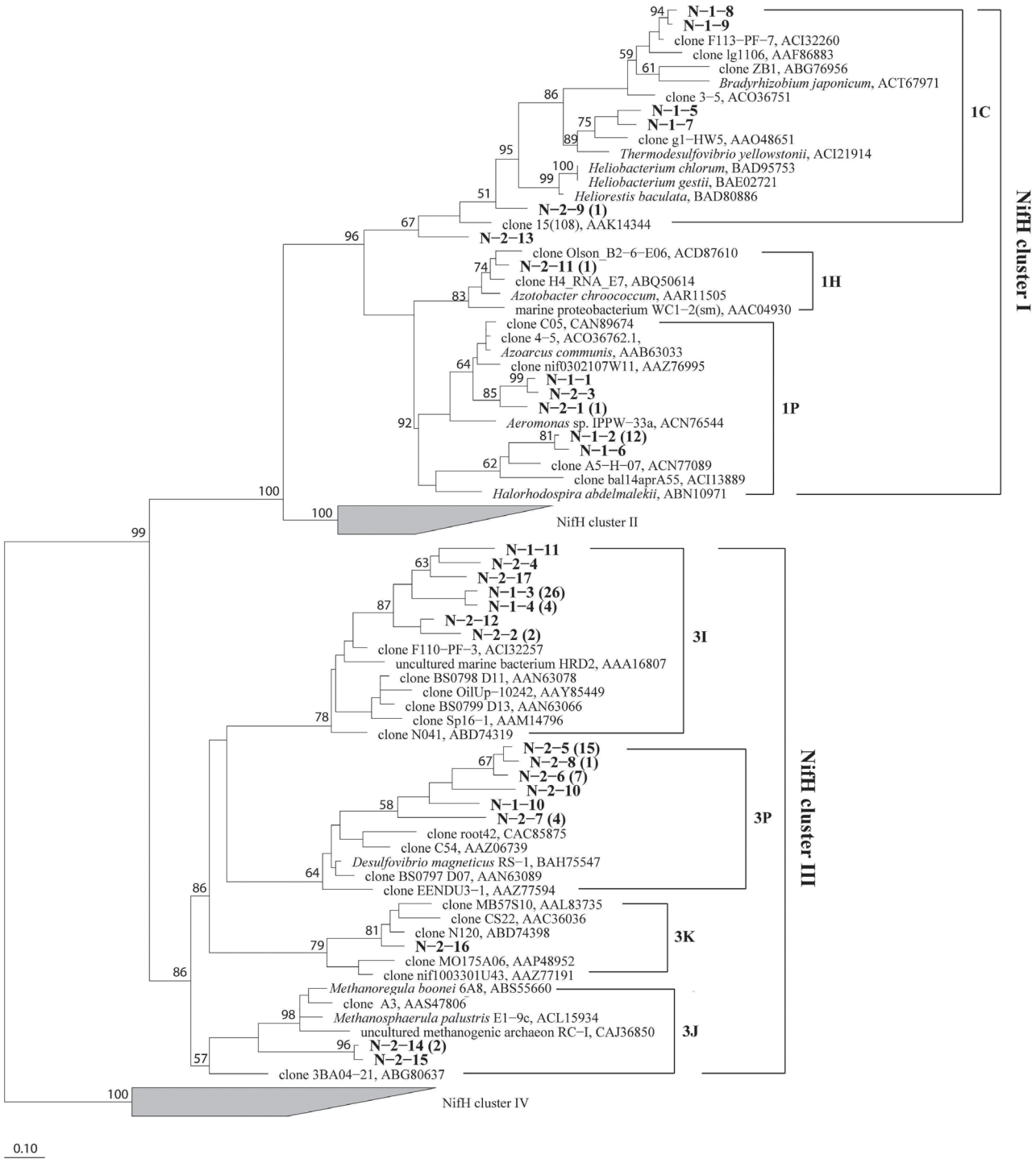

Fig.6. Phylogenetic tree of NifH (Clusters I to IV), constructed using neighbor-joining analysis. Clones detected in this study are shown in bold, the number following the detected clones indicates the number of clones. The numbers at the nodes are the bootstrap values. Bootstrap values (in per cent) are based on 1 000 replicates each and are shown for branches with more than 50% bootstrap support. The scale bar represents 0.1 substitutions per amino acid position.

Proteobacteria dominated the main group of bacterial sequences. *Gammaproteobacteria* were the most abundant in the bacterial library (37.2% of the sequences), followed by *Alphaproteobacteria* and *Deltaproteobacteria* (10.8 and 11.5%, respectively). They are likely to be involved in sulfur oxidization and sulfate reduction. Sulfur is a critical element in nature. Sulfur oxidization and sulfate reduction are two important metabolic pathways in sulfur cycling. Study of cultivated

organisms indicated that the *Alphaproteobacteria* are potential sulfur oxidizers (Blazejak et al., 2005) and *Deltaproteobacteria* function as sulfate reducers in the deep-sea (Nakagawa et al., 2006). Sulfate-reducing bacteria (SRB) are a group of anaerobic bacteria that reduce sulfate or other oxidized sulfur compounds to sulfide. In the present study, clones belonging to *Desulfobacterales*, *Desulfovibrionales* and *Syntrophobacterales* were retrieved within *Deltaproteobacteria* that were distantly related to identified bacteria *Desulforegula conservatrix* Mb1Pa, *Desulfothermus okinawensis* TFISO9, *Desulfovibrionales* bacterium Spi55 and *Desulfobacca acetoxidans* ASRBZ. *Desulforegula conservatrix* Mb1Pa is a sulfate-reducing bacterium isolated from sediments of a freshwater lake (Rees and Patel, 2001). *Desulfothermus okinawensis* TFISO9 is a thermophilic and heterotrophic sulfate-reducing bacterium isolated from a deep-sea hydrothermal field at the Yonaguni Knoll IV in the Southern Okinawa Trough (Nunoura et al., 2007). *Desulfovibrionales* bacterium Spi55 was isolated from black rust exposed to hot ridge flank crustal fluids (Nakagawa et al., 2006). *Desulfobacca acetoxidans* ASRBZ is a mesophilic sulfate reducer, isolated with acetate as sole carbon and energy source from granular sludge of a laboratory-scale upflow anaerobic sludge bed reactor fed with acetate and sulfate (Oude Elferink et al., 1999). Interestingly, phylotype pTVGB94 showed close relationship (93% 16S rRNA gene similarity) with *Thioprofundum lithotrophica*, a novel thermophilic piezophile recently isolated from a deep-sea hydrothermal vent chimney in the Mid Atlantic Ridge, which performed as a chemolithoautotroph with reduced sulfur compounds (Takai et al., 2009). These sulfate-reducing bacteria may cooperate with sulfur-oxidizing bacteria in the ecosystem and play an indispensable role in sulfur cycling.

The mechanism of fix nitrogen is complex. According to the previous studies, the *nifH* gene encodes the iron protein and led to the recognition of four clusters: Cluster I, standard molybdenum nitrogenases from *Cyanobacteria* and *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*, as well as *Gammaproteobacteria vnfH*; Cluster II, methanogen nitrogenases and bacterial *anfH*; Cluster III, nitrogenases from diverse anaerobic bacteria including Gram-positive, low G+C microorganisms (*Clostridia*) and sulfate reducers (*Deltaproteobacteria*); Cluster IV, divergent nitrogenases from Archaea (Mehta et al., 2003). In this study, the detection of *nifH* suggested that nitrogen fixation may occur in the hydrothermal vent chimneys of the Southwestern Indian Ridge. Phylogenetic analysis of the NifH library suggested that only Cluster I NifH from *Proteobacteria* (Alpha, Beta, and Gamma group) and Cluster III NifH from *Deltaproteobacteria* and uncultured bacteria are present. This is consistent with the results of the phylogenetic analysis of the microbial 16S rRNA genes, which indicates that bacteria can be expected to play a principal role in nitrogen fixation in this sample.

References

Agogue H, Brink M, Dinasquet J, et al. 2008. Major gradients in putatively nitrifying and non-nitrifying Archaea in the deep North Atlantic. Nature, 456(7223): 788–791

Arakawa S, Sato T, Yoshida Y, et al. 2006. Comparison of the microbial diversity in cold-seep sediments from different depths in the Nankai Trough. J Gen Appl Microbiol, 52(1): 47–54

Baker G C, Smith J J, Cowan D A. 2003. Review and re-analysis of domain-specific 16S primers. J Microbiol Methods, 55(3): 541–555

Beal E J, House C H, Orphan V J. 2009. Manganese- and iron-dependent marine methane oxidation. Science, 325(5937): 184–187

Blazejak A, Erseus C, Amann R, et al. 2005. Coexistence of bacterial sulfide oxidizers, sulfate reducers, and spirochetes in a gutless worm (Oligochaeta) from the Peru margin. Appl Environ Microbiol, 71(3): 1553–1561

Brandt A, Gooday A J, Brandao S N, et al. 2007. First insights into the biodiversity and biogeography of the Southern Ocean deep sea. Nature, 447(7142): 307–311

Brochier-Armanet C, Boussau B, Gribaldo S, et al. 2008. Mesophilic Crenarchaeota: proposal for a third archaeal phylum, the *Thaumarchaeota*. Nat Rev Microbiol, 6(3): 245–252

Burns J A, Zehr J P, Capone D G. 2002. Nitrogen-fixing phylotypes of Chesapeake Bay and Neuse River estuary sediments. Microb Ecol, 44(4): 336–343

Chien Y T, Zinder S H. 1994. Cloning, DNA sequencing, and characterization of a *nifD*-homologous gene from the archaeon *Methanosarcina barkeri* 227 which resembles *nifD* 1 from the eubacterium *Clostridium pasteurianum*. J Bacteriol, 176: 6590–6598

Crump B C, Peranteau C, Beckingham B, et al. 2007. Respiratory succession and community succession of bacterioplankton in seasonally anoxic estuarine waters. Appl Environ Microbiol, 73(21): 6802–6810

DeLong E F, Preston C M, Mincer T, et al. 2006. Community genomics among stratified microbial assemblages in the ocean's interior. Science, 311(5760): 496–503

Egli K, Fanger U, Alvarez P J, et al. 2001. Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate. Arch Microbiol, 175(3): 198–207

Erkel C, Kube M, Reinhardt R, et al. 2006. Genome of Rice Cluster I archaea - the key methane producers in the rice rhizosphere. Science, 313(5785): 370–372

Francis C A, Roberts K J, Beman J M, et al. 2005. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. Proc Natl Acad Sci USA, 102(41): 14683–14688

Gillan D C, Pernet P. 2007. Adherent bacteria in heavy metal contaminated marine sediments. Biofouling, 23(1): 1–13

Hashimoto J, Ohta S, Gamo T, et al. 2001. First hydrothermal vent communities from the Indian Ocean discovered. Zool Sci, 18(5): 717–721

Heijs S K, Laverman A M, Forney L J, et al. 2008. Comparison of deep-sea sediment microbial communities in the Eastern Mediterranean. FEMS Microbiol Ecol, 64(3): 362–377

Hunter E M, Mills H J, Kostka J E. 2006. Microbial community diversity associated with carbon and nitrogen cycling in permeable shelf sediments. Appl Environ Microbiol, 72(9): 5689–5701

Inagaki F, Nunoura T, Nakagawa S, et al. 2006. Biogeographical distribution and diversity of microbes in methane hydrate-bearing deep marine sediments on the Pacific Ocean Margin. Proc Natl Acad Sci USA, 103(8): 2815–2820

Jørgensen B B, Boetius A. 2007. Feast and famine—microbial life in the deep-sea bed. Nat Rev Microbiol, 5(10): 770–781

Jukes T H, Cantor C R. 1969. Evolution of protein molecules. In: Munro H N, ed. Mammalian Protein Metabolism. New York: Academic Press, 21–132

Kato S, Kobayashi C, Kakegawa T, et al. 2009. Microbial communities in iron-silica-rich microbial mats at deep-sea hydrothermal fields of the Southern Mariana Trough. Environ Microbiol, 11(8): 2094–2111

Kimura M. 1980. A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. J Mol Evol, 16(2): 111–120

Kirchman D L. 2002. The ecology of *Cytophaga–Flavobacteria* in aquatic environments. FEMS Microbiol Ecol, 39(2): 91–100

Kotelnikova S V, Obraztsova A Y, Blotevogel K H, et al. 1993. Taxonomic analysis of thermophilic strains of the genus *Methanobacterium*: Reclassification of *Methanobacterium thermoalcaliphilum* as a synonym of *Methanobacterium thermoautotrophicum*. Int J Syst Bacteriol, 43(3): 591–596

Könneke M, Bernhard A E, de la Torre J R, et al. 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature,

437(7058): 543–546
Li Huirong, Yu Yong, Luo Wei, et al. 2009. Bacterial diversity in surface sediments from the Pacific Arctic Ocean. Extremophiles, 13(2): 233–246
López-Garcia P, Duperron S, Philippot P, et al. 2003. Bacterial diversity in hydrothermal sediment and epsilonproteobacterial dominance in experimental microcolonizers at the Mid-Atlantic Ridge. Environ Microbiol, 5(10): 961–976
Mason O U, Stingl U, Wilhelm L J, et al. 2007. The phylogeny of endolithic microbes associated with marine basalts. Environ Microbiol, 9(10): 2539–2550
Mehta M P, Butterfield D A, Baross J A. 2003. Phylogenetic diversity of nitrogenase (*nifH*) genes in deep-sea and hydrothermal vent environments of the Juan de Fuca Ridge. Appl Environ Microbiol, 69(2): 960–970
Mehta M P, Huber J A, Baross J A. 2005. Incidence of novel and potentially archaeal nitrogenase genes in the deep Northeast Pacific Ocean. Environ Microbiol, 7(10): 1525–1534
Musat N, Werner U, Knittel K, et al. 2006. Microbial community structure of sandy intertidal sediments in the North Sea, Sylt-Romo Basin, Wadden Sea. Syst Appl Microbiol, 29(4): 333–348
Nakagawa S, Inagaki F, Suzuki Y, et al. 2006. Microbial community in black rust exposed to hot ridge flank crustal fluids. Appl Environ Microbiol, 72(10): 6789–6799
Nakagawa S, Takai K, Horikoshi K, et al. 2004. *Aeropyrum camini* sp. nov., a strictly aerobic, hyperthermophilic archaeon from a deep-sea hydrothermal vent chimney. Int J Syst Evol Microbiol, 54(Pt2): 329–335
Nercessian O, Fouquet Y, Pierre C, et al. 2005. Diversity of Bacteria and Archaea associated with a carbonate-rich metalliferous sediment sample from the Rainbow vent field on the Mid-Atlantic Ridge. Environ Microbiol, 7(5): 698–714
Nunoura T, Oida H, Miyazaki M, et al. 2007. *Desulfothermus okinawensis* sp. nov., a thermophilic and heterotrophic sulfate-reducing bacterium isolated from a deep-sea hydrothermal field. Int J Syst Evol Microbiol, 57(10): 2360–2364
Oude Elferink S J, Akkermans-van Vliet W M, Bogte J J, et al. 1999. *Desulfobacca acetoxidans* gen. nov., sp. nov., a novel acetate-degrading sulfate reducer isolated from sulfidogenic granular sludge. Int J Syst Bacteriol, 49(2): 345–350
Pham V D, Konstantinidis K T, Palden T, et al. 2008. Phylogenetic analyses of ribosomal DNA-containing bacterioplankton genome fragments from a 4000 m vertical profile in the North Pacific Subtropical Gyre. Environ Microbiol, 10(9): 2313–2330
Polymenakou P N, Bertilsson S, Tselepides A, et al. 2005. Bacterial community composition in different sediments from the Eastern Mediterranean Sea: a comparison of four 16S ribosomal DNA clone libraries. Microb Ecol, 50(3): 447–462
Rees G N, Patel B K. 2001. *Desulforegula conservatrix* gen. nov., sp. nov., a long-chain fatty acid-oxidizing, sulfate-reducing bacterium isolated from sediments of a freshwater lake. Int J Syst Evol Microbiol, 51(5): 1911–1916
Polymenakou P N, Lampadariou N, Mandalakis M. 2009. Phylogenetic diversity of sediment bacteria from the southern Cretan margin, Eastern Mediterranean Sea. Syst Appl Microbiol, 32(1): 17–26
Rouvière P, Mandelco L, Winker S, et al. 1992. A detailed phylogeny for the Methanomicrobiales. Syst Appl Microbiol, 15(3): 363–371
Saitou N, Nei M. 1987. The neighbour joining method: a new tool for reconstructing phylogenetic trees. Mol Biol Evol, 4(4): 406–425
Santelli C M, Orcutt B N, Banning E, et al. 2008. Abundance and diversity of microbial life in ocean crust. Nature, 453(7195): 653–656
Schloss P D, Handelsman J. 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl Environ Microbiol, 71(3): 1501–1506
Schloss P D, Westcott S L, Ryabin T, et al. 2009. Introducing Mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol, 75(23): 7537–7541
Schmid M C, Risgaard-Petersen N, van de Vossenberg J, et al. 2007. Anaerobic ammonium-oxidizing bacteria in marine environments: widespread occurrence but low diversity. Environ Microbiol, 9(6): 1476–1484
Schrenk M O, Kelley D S, Bolton S A, et al. 2004. Low archaeal diversity linked to subseafloor geochemical processes at the Lost City Hydrothermal Field, Mid-Atlantic Ridge. Environ Microbiol, 6(10): 1086–1095
Schrenk M O, Kelley D S, Delaney J R, et al. 2003. Incidence and diversity of microorganisms within the walls of an active deep-sea sulfide chimney. Appl Environ Microbiol, 69(6): 3580–3592
Smith J L, Campbell B J, Hanson T E, et al. 2008. Nautilia profundicola sp. nov., a thermophilic, sulfur-reducing epsilonproteobacterium from deep-sea hydrothermal vents. Int J Syst Evol Microbiol, 58(7): 1598–1602
Sørensen K B, Glazer B, Hannides A, et al. 2007. Spatial structure of the microbial community in sandy carbonate sediment. Mar Ecol Prog Ser, 346: 61–74
Suzuki Y, Inagaki F, Takai K, et al. 2004. Microbial diversity in inactive chimney structures from deep-sea hydrothermal systems. Microb Ecol, 47(2): 186–196
Takai K, Gamo T, Tsunogai U, et al. 2004. Geochemical and microbiological evidence for a hydrogen-based, hyperthermophilic subsurface lithoautotrophic microbial ecosystem (HyperSLiME) beneath an active deep-sea hydrothermal field. Extremophiles, 8(4): 269–282
Takai K, Horikoshi K. 1999. Genetic diversity of archaea in deep-sea hydrothermal vent environments. Genetics, 152(4): 1285–1297
Takai K, Inoue A, Horikoshi K. 2002. *Methanothermococcus okinawensis* sp. nov., a thermophilic, methane-producing archaeon isolated from a Western Pacific deep-sea hydrothermal vent system. Int J Syst Evol Microbiol, 52(Pt4): 1089–1095
Takai K, Miyazaki M, Hirayama H, et al. 2009. Isolation and physiological characterization of two novel, piezophilic, thermophilic chemolithoautotrophs from a deep-sea hydrothermal vent chimney. Environ Microbiol, 11(8): 1983–1997
Tamura K, Dudley J, Nei M, et al. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol, 24(8): 1596–1599
Teske A, Alm E, Regan J M, et al. 1994. Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. J Bacteriol, 176(21): 6623–6630
Van Dover C L, Humphris S E, Fornari D, et al. 2001. Biogeography and ecological setting of Indian Ocean hydrothermal vents. Science, 294(5543): 818–823
Vetriani C, Jannasch H W, MacGregor B J, et al. 1999. Population structure and phylogenetic characterization of marine benthic Archaea in deep-sea sediments. Appl Environ Microbiol, 65(10): 4375–4384
von Wintzingerode F, Göbel U B, Stackebrandt E. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev, 21(3): 213–229
Voordeckers J W, Starovoytov V, Vetriani C. 2005. *Caminibacter mediatlanticus* sp. nov., a thermophilic, chemolithoautotrophic, nitrate-ammonifying bacterium isolated from a deep-sea hydrothermal vent on the Mid-Atlantic Ridge. Int J Syst Evol Microbiol, 55(2): 773–779
Wang Shufang, Xiao Xiang, Jiang Lijing, et al. 2009. Diversity and abundance of ammonia-oxidizing archaea in hydrothermal vent chimneys of the Juan de Fuca Ridge. Appl Environ Microbiol, 75(12): 4216–4220
Wasmund K, Kurtböke D I, Burns K A, et al. 2009. Microbial diversity in sediments associated with a shallow methane seep in the tropical Timor Sea of Australia reveals a novel aerobic methanotroph diversity. FEMS Microbiol Ecol, 68(2): 142–151
Xu Meixiang, Wang Peng, Wang Fengping, et al. 2005. Microbial diversity at a deep-sea station of the Pacific nodule province. Biodivers Conserv, 14(14): 3363–3380
Zehr J P, Jenkins B D, Short S M, et al. 2003. Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environ Microbiol, 5(7): 539–554
Zehr J P, McReynolds L A. 1989. Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. Appl Environ Microbiol, 55(10): 2522–2526
Zhou J, Bruns M A, Tiedje J M. 1996. DNA recovery from soils of diverse composition. Appl Environ Microbiol, 62(2): 316–322