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Title: Bacterial and archaeal communities in the surface sediment from the northern slope of the South China Sea

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Abstract: Abstract: Microbial diversity of sediments from the northern slope of the South China Sea was studied by constructing bacterial and archaeal 16*S* rRNA gene clone libraries. Fourteen bacterial phylogenetic groups were detected, including Gammaproteobacteria, Deltaproteobacteria, Planctomycetes, Alphaproteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Chloroflexi, Acidobacteria, Betaproteobacteria, Nitrospirae, candidate divisions OP8 and OP11, and an unknown group. Gammaproteobacteria was the predominant group in bacterial libraries with percentages ranging from 31.8% to 63.2%. However, archaeal libraries had relatively lower diversity, with most clones belonging to marine archaeal group I uncultured Crenarchaeota. In addition, two novel euryarchaeal clones were detected not to match any culture-dependent isolates or culture-independent groups. Compared with other gas hydrate-rich ecosystems and different areas of the South China Sea, a distinct microbial community was revealed in this study.

Response to Reviewers: please see attachment

August 29, 2009

Dear editor,

Attached please find the revised version of the manuscript entitled "Bacterial and archaeal communities in the surface sediment from the northern slope of the South China Sea" – Liao *et al.*, ZUSB-D-09-00181. We want to thank you and the anonymous reviewers for the helpful comments. The changes made, based on the comments raised by the reviewers, are explained below.

Yours sincerely
Xue-Wei Xu
Min Wu

Comments from reviewer 1

1, Strongly suggest having a native English speaker edit the manuscript as there are numerous errors in the choice of words, although the text is usually understandable. Examples:

with percentages arranging	should read	with percentages ranging
considerable low diversity	should read	considerably lower diversity
four unique groups	probably should use	four uncommon groups
Besides, two novel	should read	In addition, two novel

Changed as suggested (page 1, lines 17 to 19). In addition, the English text had been revised by Professor Manyuan Long, the University of Chicago (Email: mlong@midway.uchicago.edu).

2, The small libraries appear to have been sufficient to capture a large fraction of the diversity of archaea, but the authors make no comment regarding how well bacterial diversity is represented. It would have been quite easy to show rarefaction plots for bacteria as well.

The rarefaction analysis for bacterial libraries were performed and showed in Fig. 1b based on the suggestion. The curves showed that bacterial libraries were not saturated. However, we can still get some interesting information from these libraries. What's more, libraries may fail to reach a plateau even if more clones are sequenced. Considering the cost, we have to reduce our sampling effort to get a preliminary analysis of bacterial diversity in South China Sea, which disclosed some useful clues as we hoped.

3, The lengthy recitation of which environmental or cultured organism is mostly closely related is not very useful. The narrative description is very difficult to read and provides little insight into the meaning of the relationships. If you know that a particular clone was most closely related to an organism in uranium mines or rice fields, what have you learned? Most of the results could have been put into a table for anyone who is interested in specific details.

According to the comments, the part of "Phylogenetic analysis of bacterial libraries" was shortened to avoid the lengthy recitation and useless description. Interesting results and specific clones were discussed in the "DISCUSSION" part. Considering most of the results, especially the closest relatives and environments retrieved, were illustrated by phylogenetic trees in Fig. 4a~4c, we did not put them in a table in order to avoid repetition.

4, I would say that the greatest deficiency of the manuscript is the small library size. Because few clones were available, the absence of organisms means very little. However, the presence of some organisms is interesting and the authors have been reasonably careful to avoid overinterpreting their data set.

We know that libraries are small-sized and may miss many organisms. However, just as the reviewer pointed out, the presence of some interesting organisms makes sense in understanding the microbial communities living in hydrate-rich sediments of South China Sea. That's the value of our study. Certainly, your advice is helpful to our future researches.

5, In summary, the authors have a modest amount of information to present, and the information is somewhat interesting. I would advise condensing the manuscript, but otherwise found it suitable for publication after revision.

Based on the comments, the revised manuscript was condensed by reducing 4 pages. We appreciate all your precious comments. Thank you very much!

Comments from reviewer 2

Some minor revisions are indicated below and the english should be improved.

Minor comments:

- Page 1, line 34: "arranging from": bad english

Changed as suggested (page 1, line 17).

- Page 2, line 15: Sentence "The South China Sea harbors...": bad english.

We have changed "The South China Sea harbors great potential gas hydrates, continental slope and basin are in particular." to "The South China Sea exhibits a great potential for gas hydrate presence." (page 2, lines 31 to 32).

- Page 3, line 23, Sampling and sites information. Please give more details on the sediments. Give the date of collection. What type of sediments were collected? (the surface 0-1 cm? 0-5 cm? 0-10 cm?). Were the sediments muddy or sandy? What quantity was sampled?

More details were given on the data of collection and the type of sediments (Sediment samples from the upper 35 cm of the seabed were collected by multicorer), the quality (muddy) and quantity (Muddy surface sediment cores at the depth of 0-5 cm (approximately 50 g wet weight)) of sediments. (page 2, lines 49 to 51)

- Page 3, line 34. Replace "pollution" by "contamination".

Changed as suggested (page 2, line 52).

- Page 3, line 47: replace "were" by "was".

Changed as suggested (page 2, line 55).

- Page 3, line 47: what quantity of sediments was used for DNA extraction?

500 mg sediment for each sample was used for DNA extraction (page 2, line 55).
.

- Page 3, line 59: replace "of" by "by"; replace "were" by "was".

Changed as suggested (page 2, line 58).

- Page 4, line 9. Give the length of the PCR amplicons (primers A571F and UA1204R).

The length was around 650 bp and given based on the suggestion (page 3, line 63).

- Page 4, line 31: Was the same annealing temperature (50°C) used for eubacteria and archaea?

Yes. And we have made it out in the revision (page 3, line 66).

.

- Page 6, line 34: "According to Bonferroni..." : bad english, please rephrase.

We have rephrased this sentence as follows (page 4, lines 98-100).
"A LIBSHUFF comparison of three libraries yielded the following the formula using the Bonferroni correction: $0.05=1-(1-a)^{k(k-1)}$, where a was the critical P-value and k was the number of libraries. The critical P-value was 0.0085 when three libraries were compared."

- Page 8, line 26: replace "were statistically significant different" by "were significantly different".

Changed as suggested (page 5, line 132).

- Page 8, line 58: replace "Total 121 sequences were" by "A total of 121 sequences was".

Changed as suggested (page 5, line 142).

- Page 14, line 50: replace "demonstrated" by "suggested": you have to be careful because the study is entirely based on PCR, which is not a quantitative method.

Changed as suggested (page 7, line 196).

- Page 15, line 20: replace "In the present study, Desulfobulbus is identified in library bS0615" by "In the present study, a Desulfobulbus related sequence was identified in library bS0615". Please give the similarity values between the sequences.

We have replaced "In the present study, Desulfobulbus is identified in library bS0615" by "A Desulfobulbus related sequence (bS0615-24), which shared 95% identity with the relative uncultured Desulfobulbus sp., was identified in library bS0615." (page 7, lines 199-201). And the similarity has been given in the sentence.

- Page 16, line 14: replace "didn't" by "did not".

The sentence has been changed as "Previously, ANME group from methane hydrate

sites was not detected, although sulfate-reducing bacteria were observed (Inagaki et al., 2006)." (page 8, lines 211-213)
We appreciate all your precious comments. Thank you very much!

Bacterial and archaeal communities in the surface sediment from the northern slope of the South China Sea

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Abstract: Microbial diversity of sediments from the northern slope of the South China Sea was studied by constructing bacterial and archaeal 16S rRNA gene clone libraries. Fourteen bacterial phylogenetic groups were detected, including *Gammaproteobacteria*, *Deltaproteobacteria*, *Planctomycetes*, *Alphaproteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Chloroflexi*, *Acidobacteria*, *Betaproteobacteria*, *Nitrospirae*, candidate divisions OP8 and OP11, and an unknown group. *Gammaproteobacteria* was the predominant group in bacterial libraries with percentages ranging from 31.8% to 63.2%. However, archaeal libraries had relatively lower diversity, with most clones belonging to marine archaeal group I uncultured *Crenarchaeota*. In addition, two novel euryarchaeal clones were detected not to match any culture-dependent isolates or culture-independent groups. Compared with other gas hydrate-rich ecosystems and different areas of the South China Sea, a distinct microbial community was revealed in this study.

Key words: 16S rRNA; Library; Diversity; South China Sea

INTRODUCTION

The South China Sea (SCS) close to the West Pacific "warm pool" is the biggest and deepest sea of China, as well as one of the largest marginal seas in the world (Lai *et al.*, 2007, Li *et al.*, 2008b). Gas hydrates in deep marine environments are solid compounds which contain mainly

methane and water, formed due to high pressure, low temperature, abundant gas, and other unknown factors under deep-sea conditions (Mills *et al.*, 2005). The South China Sea exhibits a great potential for gas hydrates presence. The north slope covers an area of 21×10^4 km^2, which takes up approximately 6% of total area of the South China Sea (Yu *et al.*, 2004). Like many other continental slope margins, it is an important component of gas hydrate-bearing area in the South China Sea (Lin *et al.*, 2005; Wu *et al.*, 2008).

Microorganisms play a significant role in the formation of gas hydrates (Kvenvolden, 1995). Microbial communities can be apparently influenced by the presence of gas hydrates (Inagaki *et al.*, 2006). However, only a few studies have been carried out to survey the microbial diversity of the South China Sea, including Qiongdongnan Basin (Jiang *et al.*, 2007), Xisha Trough (Li *et al.*, 2008b), north slope (17º57.70' N, 114º57.33' E) (Wang and Li, 2008) and south slope (Li *et al.*, 2008a). The diverse microbial communities living in vast area of the South China Sea are still poorly known. In this study, we investigated the diversity of bacteria and archaea in marine sediments from three sites on the northern slope of the South China Sea.

MATERIALS AND METHODS

Sampling and sites information

Characteristics of three sampling sites were described in Table 1. Sediment samples from the upper 35 cm of the seabed were collected by multicorer on the northern slope of the South China Sea in the summer of 2006, and stored at −80°C until transported to laboratory for storage at −20°C. Muddy sediment cores with depth 0−5 cm (approximately 50 g wet weight) from three sampling sites were used for diversity analysis. All processes were aseptic to avoid contamination.

DNA extraction and PCR amplification

Total genomic DNA in 500 mg sediment from each sample was extracted directly using FastDNA-Spin Kit for soil (Q-BIOgene, Carlsbad, CA, USA), as described previously (Polymenakou *et al.*, 2005). The DNA extracts were diluted 10-fold prior to PCR amplification to reduce inhibition by contaminants. Bacterial 16S rRNA gene was amplified by PCR with universal primer 1492r (5'-GGTTACCTTGTTACGACTT-3') (Eden, 1991; Dojka *et al.*, 1998b) and

bacterial specific primer 27f (5'-AGAGTTTGATCCTGGCTCAG-3') (Dojka *et al.*, 1998b; Tanner *et al.*, 1998). Forward primer A571F (5'-GCCTAAAGCGTCCGTAGC-3') and reverse primer UA1204R (5'-TTCGGGGCATACTGACCT-3') were used to amplify archaeal partial 16S rRNA gene (around 650 bp) (Baker *et al.*, 2003). PCR reaction mixtures contained 1 to 4 ng diluted DNA extracts, 1× PCR buffer (1.5 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl), 0.4 μmol/L of each primer, 200 μmol/L dNTPs and 2 U *Taq* DNA polymerase (TaKaRa, Japan), adding MillQ water to a final volume of 50 μl. Thermal cycling for both bacteria and archaea was modified to reduce PCR bias as follows, 94°C for 5 min, followed by 29 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min, and a final extension at 72°C for 10 min. Each site was amplified in two replicate PCR reactions of 50 μl. Negative (no-template) control was used to exclude contamination. PCR products were electrophoresed in 1.5% (w/v) low-melting agarose gels and extracted with the AxyPrep DNA Gel Extraction kit (Axygen, USA).

Cloning and sequencing

Bacterial and archaeal libraries were constructed for each site with the corresponding name bS0610, bS0615 and bS0604 for bacterial libraries, and aS0610, aS0615 and aS0604 for archaeal libraries, respectively. The equivalent amount of PCR products of each sample were cloned into pMD19-T vectors (TaKaRa, Japan), and transformed into *Escherichia coli* DH5α competent cells. Transformants were screened out by the blue-white screening system and picked out randomly for plasmids extraction. Recombinant plasmids were identified by agarose gel electrophoresis after digestion with two restriction enzymes *Bam*HI and *Hind*III (TaKaRa, Japan), and sequenced using primer 27f for bacterial clones and A571F for archaeal clones on an ABI 3730 sequencer at Chinese National Human Genome Center at Shanghai. Only sequences over 600 bases with sound quality were used for further analysis. Sequences were checked for chimeras with CHECK_CHIMERA software of Ribosomal Database Project II (http://rdp8.cme.msu.edu/html/index.html) (Maidak *et al.*, 2001), and cross-checked with Pintail program (http://www.bioinformatics-toolkit.org) (Ashelford *et al.*, 2005).

Statistical analysis of diversity and differences between libraries

Chimera-free sequences in each library were aligned separately by CLUSTALW online

(http://align.genome.jp/), and the phylip output format files were used to calculate distance matrices by DNADIST program contained in Phylip 3.67 program package (http://evolution.genetics.washington.edu/phylip.html). Distance-based OTU and Richness program (DOTUR, http://www.plantpath.wisc.edu/fac/joh/dotur.html) (Schloss and Handelsman, 2005) was used to assign OTUs and calculate diversity indices including ACE (Chao, 1987), Chao1 (Kemp and Aller, 2004), simpson and shannon (Zhang *et al.*, 2008).

To determine the significance of differences between bacterial libraries, web LIBSHUFF version 0.96 (http://libshuff.mib.uga.edu/) was used following Singleton method (Singleton *et al.*, 2001). A LIBSHUFF comparison of libraries yielded the following the formula using the Bonferroni correction: $0.05=1-(1-a)^{k(k-1)}$, where a was the critical P-value and k was the number of libraries. The critical P-value was 0.0085 when three libraries were compared. If any comparison of two libraries had a lower P-value below or at 0.0085, then there was a 95% confidence to believe those two libraries were significantly different in community composition.

Phylogenetic analysis

One representative clone was chosen for each OTU, and then submitted to BLAST program and Ribosomal Database Project II program online to obtain the closest published relatives. Phylogenetic trees were constructed by MEGA software version 4.0 using Neighbor-Joining method (Saitou and Nei, 1987) with Kimura 2-parameter model.

GenBank accession numbers

All partial 16S rRNA gene sequences determined in the present study were deposited in GenBank under the accession numbers EU886378 to EU886464 for bacterial clones, and GQ180871 to GQ180903 for archaeal clones.

RESULTS

Diversity analysis and differences between bacterial libraries

OTUs and diversity estimators were all determined at 3% 16S rDNA sequence difference level by DOTUR program (Table 2). In all, 22, 26 and 32 OTUs were obtained in three bacterial

libraries bS0610, bS0604 and bS0615, respectively. However, archaeal libraries had much fewer OTUs, with only 5, 5 and 6 OTUs assigned in libraries aS0610, aS0604 and aS0615, respectively. As depicted in Fig. 1a, rarefaction curves for archaeal libraries had almost reached asymptote, indicating that the archaeal community was well sampled with low diversity. In contrast, rarefaction curves for bacterial libraries failed to approach a plateau (Fig. 1b), indicating a high bacterial diversity. Previous studies suggested that the rarefaction curves were not saturated even if hundreds of bacterial 16S rRNA gene clones were retrieved. But they did reveal important information about the relative diversity. The curves for bacterial libraries (Fig. 1b) revealed the same tendency with the curves for archaeal libraries (Fig. 1a) in which diversity appeared to increase with depth, supported by the diversity estimators (Table 2).

The web LIBSHUFF program (for a review see Singleton *et al.*, 2001) was used to determine the significance of differences between libraries. *P*-value of pairwise comparisons was 0.001 (< the critical *P*-value 0.0085) in our comparisons, indicating libraries were significantly different in community composition, with a 95% confidence. Besides, the difference between homologous coverage curve and heterologous coverage curve was determined by the distribution of delta-*C* (ΔC) as a function of evolutionary distance (*D*) (solid red curves, Fig. 2). If the two libraries were identical, the value of delta-*C* (ΔC) would have been very small. Our results of all the comparisons showed significant differences between libraries, with considerable delta-*C* (ΔC) values at evolutionary Distance *(D*) below 0.2. All these results support a conclusion that bacterial libraries had significantly different community composition.

Analysis of bacterial and archaeal libraries

A total of 121 sequences were generated from three bacterial libraries, with fourteen different phylogenetic groups being identified (Table 3). *Proteobacteria* (with 76 clones) dominated in the bacterial community, *Gammaproteobacteria* in particular, which took up 63.2%, 41.0% and 31.8% in libraries bS0610, bS0604 and bS0615, respectively (Fig. 3). *Gammaproteobacteria*, *Deltaproteobacteria*, *Firmicutes*, *Bacteroidetes* and *Chloroflexi* were commonly detected in three libraries, while *Betaproteobacteria*, *Nitrospirae*, Candidate divisions OP8 and OP11 were seldom detected, with only one clone in each group. Taking three libraries as a whole represent of the South China Sea, bacteria affiliated with *Alphaproteobacteria*, *Gammaproteobacteria*,

Deltaproteobacteria and *Planctomycetes* took up 9.1%, 44.6%, 8.3% and 9.9% of the total 121 clones, respectively. The percentages of remaining phylogenetic groups ranged from 0.8% to 6.6%.

Archaeal libraries were much simpler with a lower diversity (Fig. 5). Marine archaeal group I was the dominant group, which took up 100%, 83% and 78.6% of aS0604, aS0610 and aS0615, respectively. Furthermore, candidatus *Nitrososphaera*, Deep-Sea Archaeal Group (DSAG) and an unknown group were found within *Crenarchaea*. *Crenarchaea* took up 96% of the total archaeal clones. Merely two clones from library aS0610 (aS0610-10 and aS0610-16) belonged to *Euryarchaea*.

Phylogenetic analysis of bacterial libraries

In total, seven, nine and thirteen phylogenetic groups were identified in bacterial libraries bS0610, bS0604 and bS0615, respectively (Fig. 4). The most abundant OTU was affiliated to *Gammaproteobacteria* with the closest relative *Pantoea agglomerans* sp. RI22 (DQ530141). A few OTUs were affiliated with established groups which contained isolated representatives, including *Pantoea*, *Pseudomonas* and *Stenotrophomonas* within *Gammaproteobacteria*, *Ochrobactrum* and *Sphingomonas* within *Alphaproteobacteria*, *Desulfobulbus* and *Enhygromyxa* within *Deltaproteobacteria*, and *Clostridium* within *Firmicutes*. However, the majority of bacterial clones were closely related to uncultured clones from marine environments, except for a few ones from non-marine environments such as Chinese rice field and uranium mining waste piles. Two novel OTUs (bS0604-37 and bS0615-35), assigned into unknown group, were found in libraries bS0604 and bS0615. OTU bS0604-37 (two related clones) was closest to clone Creta1-C11 (AY533950) obtained from oxic surface sediments, and formed an independent branch which was far away from the remaining groups (Fig. 4b). OTU bS0615-35, distantly related to clone VHS-B3-74 (DQ394961) from harbor sediment with 86% identity, formed a sister branch with candidate division OP8 (Fig. 4c).

Phylogenetic analysis of archaeal libraries

A distinct diversity of archaeal community was revealed (Fig. 5). In total, 100%, 83% and 78.6% of archaeal clones in libraries aS0604, aS0610 and aS0615 belonged to marine archaeal

group I, respectively. Relatives of this group included uncultured clones retrieved from surface sediments at 2164 m and 3406 m depth of the Weddel Sea, Antarctica (Gillan and Danis, 2007) and the isolate *Nitrosopumilus maritimus* SCM1 (DQ085097). All clones of marine archaeal group I shared high similarity (> 96%) with each other, and formed a sister branch with candidatus *Nitrososphaera* containing clone aS0615-4. In addition, Deep-Sea Archaeal Group (DSAG) and an unknown group were detected within *Crenarchaeaota*. Only two clones aS0610-16 and aS0610-10 were grouped into *Euryarchaeota*, and they shared low similarity with other isolates or established groups within *Euryarchaeota*.

DISCUSSION

Proteobacteria are the dominant bacteria in all three libraries, which is consistent with previous diversity investigation of marine sediments from the South China Sea (Xu *et al.*, 2004; Lai *et al.*, 2007; Li *et al.*, 2008a; 2008b). Because they are the most metabolically diverse bacteria by far, *Proteobacteria* appear in various environments and play crucial roles in cycling of chemical elements. Even in deep-sea environments, they can still dominate in the bacterial community, which is also suggested by our results.

The northern slope of the South China Sea is considered to contain large amount of oil/gas/gas-hydrate resources (Jiang *et al.*, 2007). The microbial community may be special in such an environment. *Deltaproteobacteria* are found in all three libraries. A *Desulfobulbus* related sequence (bS0615-24), which shared 95% identity with the relative uncultured *Desulfobulbus* sp., was identified in library bS0615. Some species isolated from oilfields or water-oil separation system belong to *Desulfobulbus* (Lien *et al.*, 1998). Furthermore, it is an interesting discovery that sulfate-reducing bacteria (SRB) are syntrophically associated with uncultured anaerobic methane-oxidizing archaea (ANME) to form a complex consortia in methane-rich deep marine sediments, and exhaust a large portion of methane from marine ecosystem (Pernthaler *et al.*, 2008). *Desulfobulbus* is a commonly found syntrophic partner in the consortia (Niemann *et al.*, 2006). Thus, the detection of *Desulfobulbus* may give some clues to understand the particular role of bacterial community inhabiting in our sampling environment. These related clones may participate in sulfur cycle through sulfate reduction, and cooperate with ANME group to consume methane.

However, no ANME group is detected in our archaeal libraries, except two *Euryarchaeota* clones which are distantly related to methanogenic bacteria such as *Methanosphaera*. Previously, ANME group from methane hydrate sites was not detected, although sulfate-reducing bacteria were observed (Inagaki et al., 2006). A possible explanation is that bacterial biomass is much greater than archaeal biomass in these ecosystems, which leads to the miss of ANME groups in culture-independent analysis.

Bacterial JS1 candidate group is a major methane-associated group recovered from Pacific Ocean margins including Peru margin and Cascadia margin, where methane hydrates are in great concentration, and acts as an indicator of methane presence (Inagaki *et al.*, 2006). *Planctomycetes* are also found abundant in these methane hydrate-rich sediments. However, no bacterial clone is affiliated with JS1 group in our libraries. Considering previous studies of the South China Sea (Xu *et al.*, 2004; Jiang *et al.*, 2007; Lai *et al.*, 2007; Li *et al.*, 2008a; 2008b, Wang and Li, 2008), no JS1 group has ever been discovered in the South China Sea margins, thus, a hypothesis is proposed that JS1 group is location-specific and can not be used as an universal indicator for methane presence in different marine ecosystems. In our study, *Planctomycetes* is detected as the most abundant group besides *Proteobacteria* in two libraries (bS0604 and bS0615) constructed from deeper sediment (> 1200 m water depth), but absent in library bS0610 constructed from shallow sediment (546 m water depth). This result suggests *Planctomycetes* prefer deeper depth in our sampling environment.

Chloroflexi (or Green non-sulfur bacteria) can be detected in all three libraries with only one clone in each library. Previous study of late Pleistocene organic-rich sediments (sapropels) from the eastern Mediterranean Sea has showed as high as 70% of total bacteria belonged to uncultured green non-sulfur bacteria, and this high percentage of green non-sulfur bacteria was associated with organic-rich sediments (Coolen *et al.*, 2002). *Chloroflexi* is also found to be abundant in organic-rich, hydrate-free sites of the Pacific Ocean margins, while consists a very small portion in hydrate-rich sediment (Inagaki *et al.*, 2006). The detection of few *Chloroflexi* clones in our libraries is consistent with the character of methane hydrate-bearing ecosystem.

Candidate divisions OP8 and OP11 are unique in library bS0615 constructed from the deepest sediment sample. The candidate division OP series were firstly discovered in a hot spring Obsidian Pool in Yellowstone National Park of America (Hugenholtz *et al.*, 1998). These bacteria

are distantly related to known isolates and clones, and have not been cultured till now. Our discovery of the candidate divisions OP8 and OP11 is consistent with previous studies in which these OP series were also identified in hydrocarbon-containing soil samples under methanogenic conditions (Dojka *et al.*, 1998a; Hugenholtz *et al.*, 1998) and sediments from the South China Sea (Li *et al.*, 2008a). This consistency may indicate a non-negligible role of these candidate divisions in hydrocarbon or methane-bearing environments.

Besides, some OTUs are affiliated with clones obtained from carbonate sediments (bS0610-31, bS0604-25 and bS0604-38), and methane hydrate-bearing sediments (bS0615-50 and bS0615-29). These environments are characterized by high content of carbon, indicating a possible role in carbon cycling of these related clones.

Archaeal community inhabiting the northern slope of the South China Sea has a low diversity. Only two clones (aS0610-10 and aS0610-16) in library aS0610 belong to *Euryarchaeota*, while the remaining clones are all grouped within *Crenarchaeota*. Our results are consistent with previous studies which have discovered that *Euryarchaeota* are more abundant in upper marine water column, while *Crenarchaeota* are predominant in deeper sediments (Massana *et al.*, 1997; Karner *et al.*, 2001; Church *et al.*, 2003). Marine archaeal group I is the most dominant part within *Crenarchaeota*, which accounts for 83%, 78.6% and 100% in libraries aS0610, aS0615 and aS0604, respectively. Marine archaeal group I, also named as archaeobacterium group 1, archaeal group I and marine group I *Crenarchaeota*, was first found in oxygenated coastal surface waters of North America and abundant in marine environments (Delong, 1992). Previous studies have revealed that marine archaeal group I *Crenarchaeota*, including the first isolated ammonia-oxidizing archaeon *Nitrosopumilus maritimus* (Konneke *et al.*, 2005), play important roles in nitrogen cycling (Leininger *et al.*, 2006; Gillan and Danis, 2007). Due to the ubiquity of the marine archaeal group I in various environments, the roles of these *Crenarchaeota* are supposed to be more versatile and key. Marine archaeal group I is possibly the most abundant archaeal group on Earth (Wang *et al.*, 2005). Unexpectedly, marine archaeal group I was also found in a large proportion in methane hydrate-rich sediments from Peru margin and Cascadia margin. Thus, it is believed that marine archaeal group I might be an unusual group which plays an unknown role in methane metabolism.

Besides marine archaeal group I, candidatus *Nitrososphaera* and Deep-Sea Archaeal Group

(DSAG, also named as Marine Benthic Group B) are also detected within *Crenarchaeota*. Candidatus *Nitrososphaera* is the first described thermophilic ammonia-oxidizing *Crenarchaea* obtained from Garga hot spring enrichments (Hatzenpichler *et al.*, 2008), and plays an important role in nitrogen cycling. DSAG is a dominant group detected in methane hydrates sites of Peru margin and Cascadia margin (Inagaki *et al.*, 2006). DSAG also presents in our library, although there is only one related clone.

No methanogens was found in our archaeal libraries. 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). Thus, it is possible that methanogens might be missed using universal archaeal primers.

Therefore, a hypothesis can be proposed that microbial community inhabiting in sampling sediments from northern slope of the South China Sea may participate in nitrogen, carbon and sulfur cycling, with the dominance of nitrogen cycling in archaeal community. Further study is needed to capture more organisms. Since our clones are mostly close to uncultured relatives from environments, culture-dependent experiments should be taken to obtain isolates. Novel bacteria and archaea detected in the present study are also worth analyzing further. This study provides us a primary knowledge of microbial diversity in sediments from the northern slope of the South China Sea, and indicates a distinct microbial community that contains potential novel species and possibly even more that can be expected.

CONCLUSION

To conclude, both bacterial and archaeal diversity were studied by 16S rRNA gene clone libraries constructed from sediments on the northern slope of the South China Sea at different depth. Fourteen phylogenetic groups including an unknown group were detected among three bacterial libraries. *Proteobacteria* dominated in the bacterial community, followed by *Planctomycetes* and *Firmicutes*. Most clones obtained in the present study were affiliated with uncultured bacteria from marine ecosystems, including methane hydrate-bearing environment, deep-sea sediments and hydrothermal vents and so on. Archaeal community was much simpler with a lower diversity, and marine archaeal group I dominated significantly in archaeal libraries.

Besides, two novel clones were found within *Euryarchaeota*. Microbial communities in the sampling sediments from northern slope of the South China Sea played an important role in nitrogen, carbon and sulfur cycling. The present study disclosed a distinct microbial community, and provided a primary analysis of microbial diversity of this special marine environment.

ACKNOWLEDGMENTS

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Table 1. Information of sampling sites.

Sites	Longitude	Latitude	Depth of water (m)	Seafloor
S0610	118°53′	22°08′	546	Mud
S0604	118°40′	21°57′	1211	Mud
S0615	119°06′	22°05′	1285	Mud

Table 2. Diversity indices (Calculated at 0.03 difference level) of bacterial and archaeal libraries.

Library	No. of Clones	OTUs	ACE	Chao1	Shannon	Simpson
bS0610	38	22	232.000	117	2.434	0.172
bS0604	39	26	156.114	79	2.965	0.057
bS0615	44	32	422.395	235	3.154	0.052
aS0610	18	5	11.000	7	0.961	0.517
aS0604	18	5	5.628	5	1.382	0.249
aS0615	14	6	13.656	12	1.475	0.231

Table 3. 16S rDNA phylotype distribution in three bacterial libraries.

Phylogenetic groups	Libraries [a]		
	bS0610	bS0604	bS0615
Alphaproteobacteria	0	4	7
Beltaproteobacteria	0	0	1
Gammaproteobacteria	24	16	14
Deltaproteobacteria	4	2	4
Planctomycetes	0	7	5
Firmicutes	2	3	3
Actinobacteria	0	3	2
Acidobacteria	3	0	2
Bacteroidetes	3	1	2
Nitrospirae	1	0	0
Chloroflexi	1	1	1
Candidate division OP8	0	0	1
Candidate division OP11	0	0	1
Unknown group	0	2	1

a. Number of clones affiliated with each phylogenetic group.

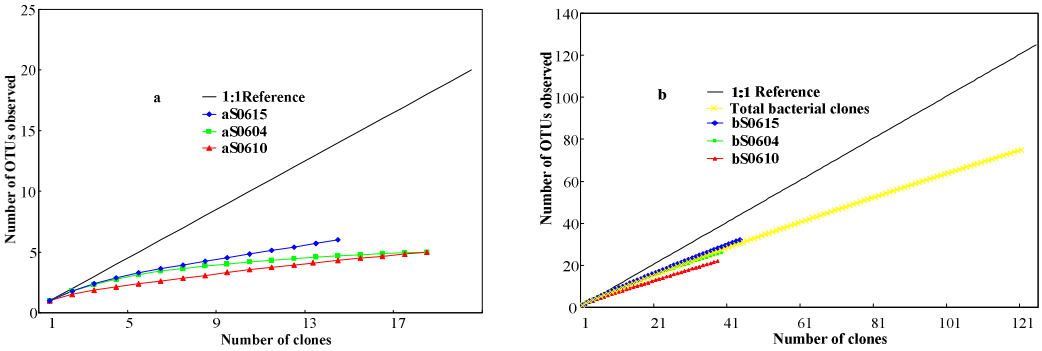


Fig. 1. Rarefaction curves for archaeal (a) and bacterial (b) clone libraries. The observed numbers of OTUs identified by DOTUR program at 3% difference level are plotted against number of clones in library. The curve of 1:1 reference means that each sequenced clone belongs to a unique OTU. Yellow curve (forks) represents the rarefaction curve for total bacterial clones from three libraries (b).

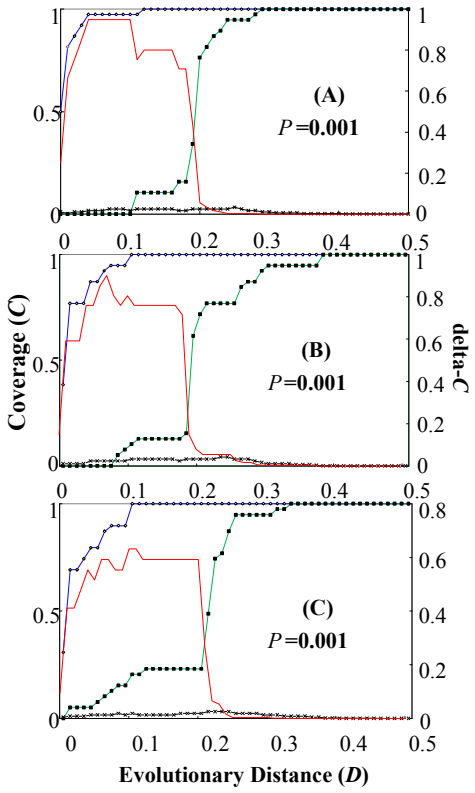


Fig. 2. Results of selected LIBSHUFF comparisons of (A) libraries bS0610 (*X*) to bS0615 (*Y*), (B) libraries bS0604 (*X*) to bS0610 (*Y*) and (C) libraries bS0604 (*X*) to bS0615 (*Y*). Solid blue line with hollow diamonds and solid green line with solid rectangles represent homologous and heterologous coverage curves, respectively. Solid red lines indicate delta-*C* (Δ*C*) for original samples at different value of evolutionary distance. Solid lines with forks indicate the 95% delta-*C* (Δ*C*) for the randomized samples. The *P*-value of comparisons is 0.001.

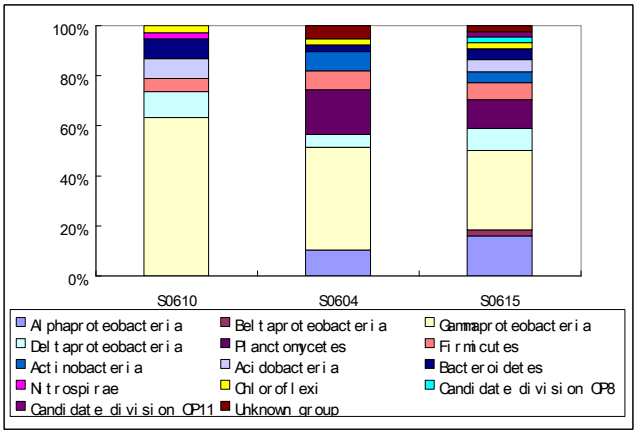
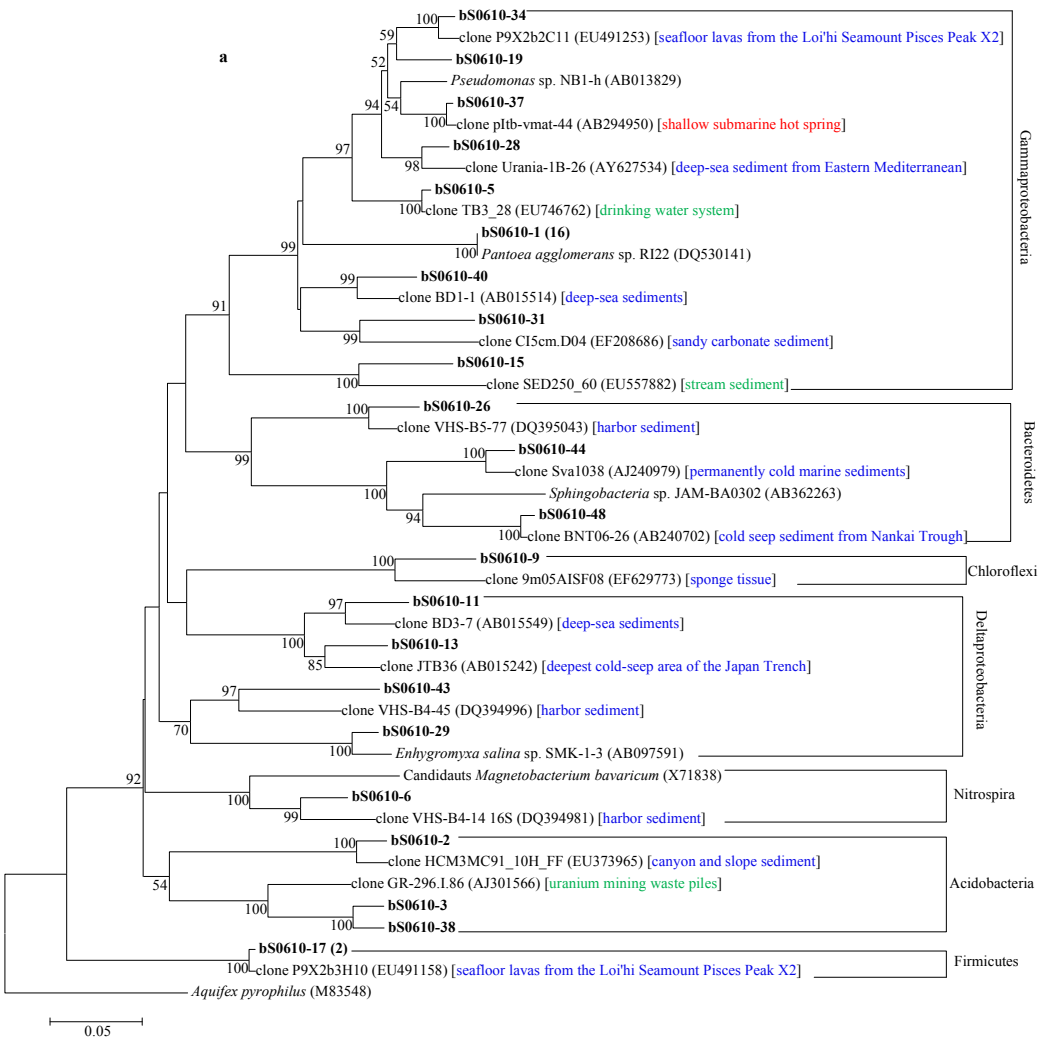
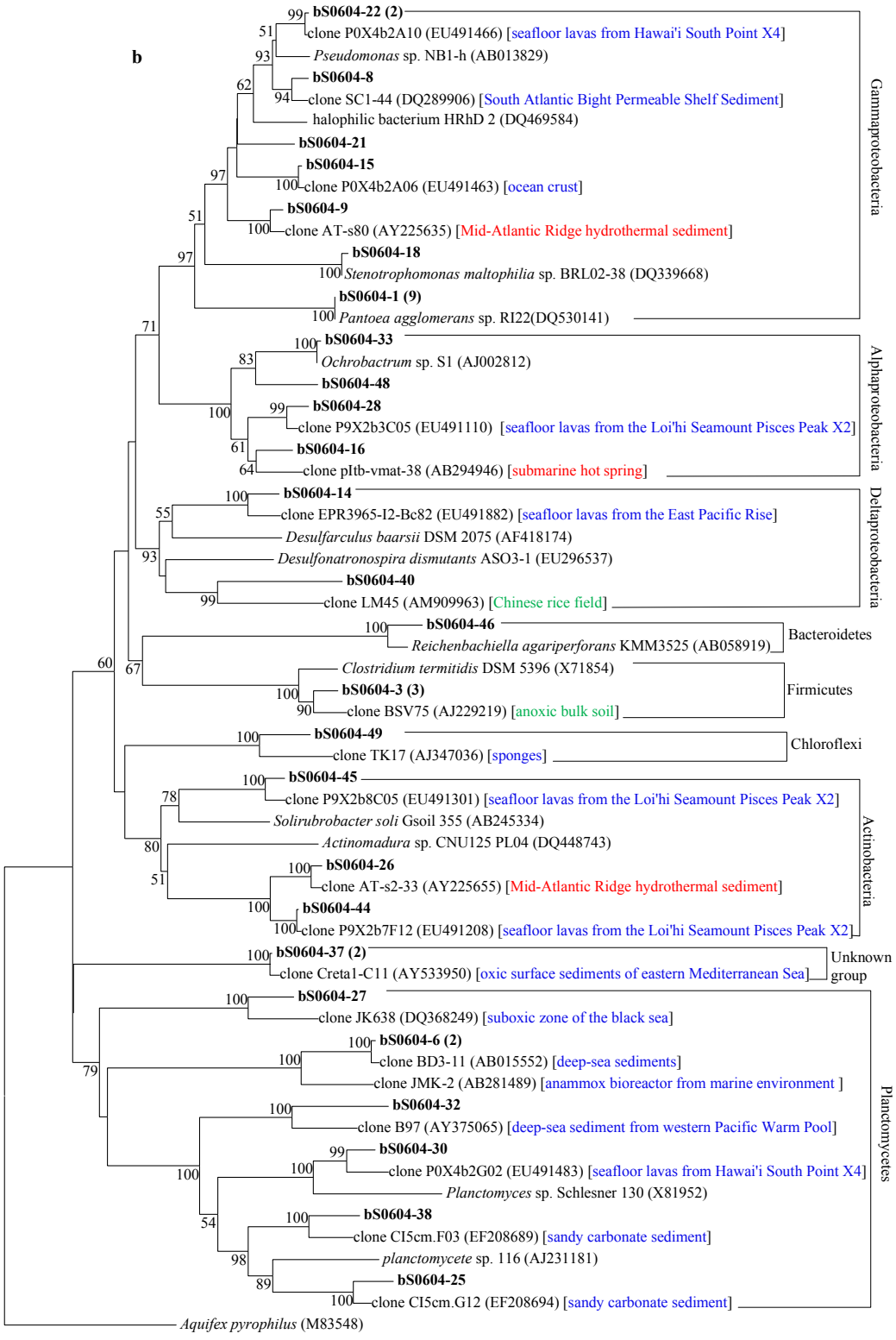
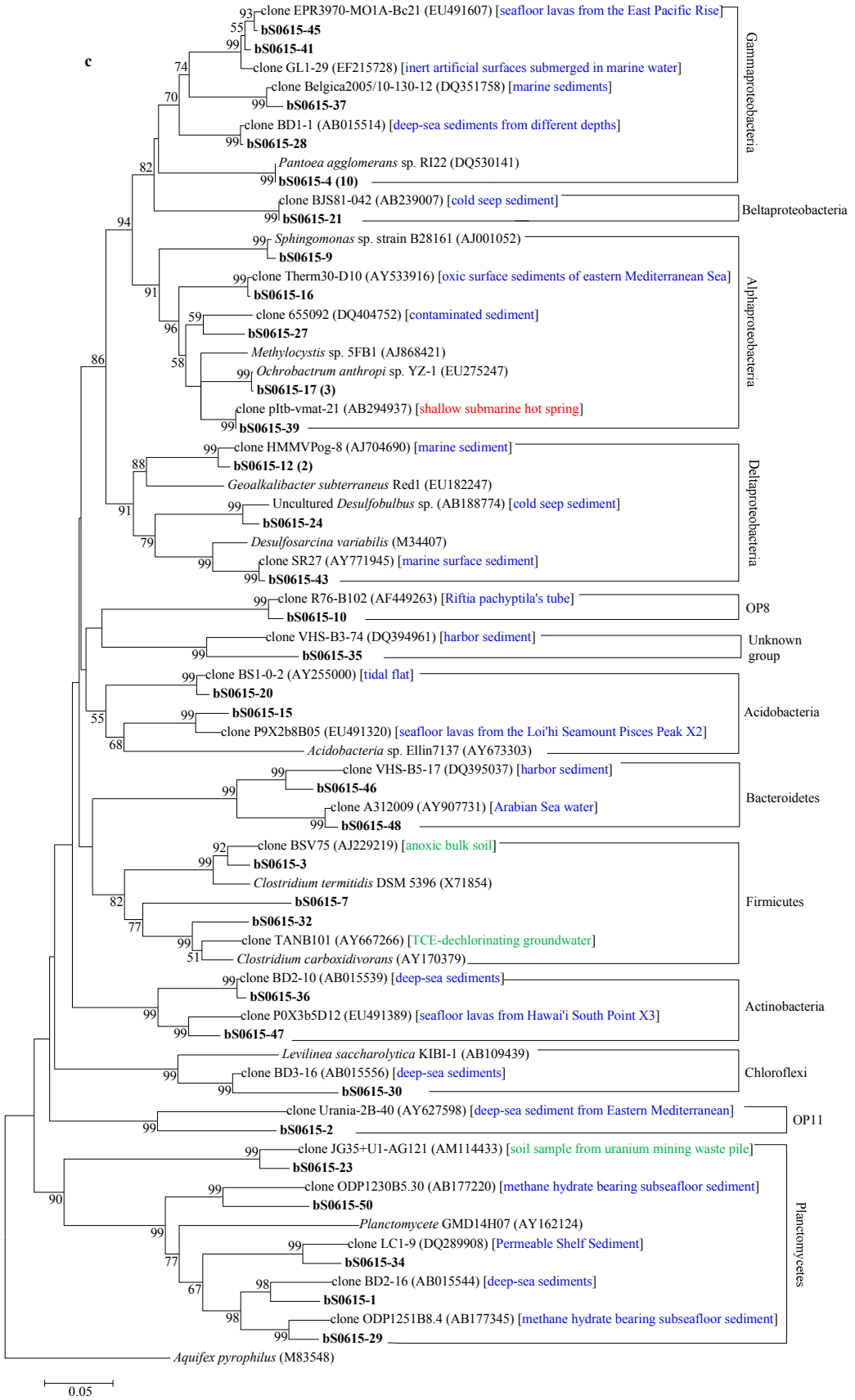


Fig. 3. 16S rDNA phylotype comparison of three bacterial libraries. Each color represents the corresponding phylogenetic group as given by cutline.


a
bS0610-34
clone P9X2b2C11 (EU491253) [seafloor lavas from the Loi'hi Seamount Pisces Peak X2]
bS0610-19
Pseudomonas sp. NB1-h (AB013829)
bS0610-37
clone pItb-vmat-44 (AB294950) [shallow submarine hot spring]
bS0610-28
clone Urania-1B-26 (AY627534) [deep-sea sediment from Eastern Mediterranean]
bS0610-5
clone TB3_28 (EU746762) [drinking water system]
bS0610-1 (16)
Pantoea agglomerans sp. RI22 (DQ530141)
bS0610-40
clone BD1-1 (AB015514) [deep-sea sediments]
bS0610-31
clone CI5cm.D04 (EF208686) [sandy carbonate sediment]
bS0610-15
clone SED250_60 (EU557882) [stream sediment]
Gammaproteobacteria
bS0610-26
clone VHS-B5-77 (DQ395043) [harbor sediment]
bS0610-44
clone Sva1038 (AJ240979) [permanently cold marine sediments]
Sphingobacteria sp. JAM-BA0302 (AB362263)
bS0610-48
clone BNT06-26 (AB240702) [cold seep sediment from Nankai Trough]
Bacteroidetes
bS0610-9
clone 9m05AISF08 (EF629773) [sponge tissue]
Chloroflexi
bS0610-11
clone BD3-7 (AB015549) [deep-sea sediments]
bS0610-13
clone JTB36 (AB015242) [deepest cold-seep area of the Japan Trench]
bS0610-43
clone VHS-B4-45 (DQ394996) [harbor sediment]
bS0610-29
Enhygromyxa salina sp. SMK-1-3 (AB097591)
Deltaproteobacteria
Candidauts Magnetobacterium bavaricum (X71838)
bS0610-6
clone VHS-B4-14 16S (DQ394981) [harbor sediment]
Nitrospira
bS0610-2
clone HCM3MC91_10H_FF (EU373965) [canyon and slope sediment]
clone GR-296.I.86 (AJ301566) [uranium mining waste piles]
bS0610-3
bS0610-38
Acidobacteria
bS0610-17 (2)
clone P9X2b3H10 (EU491158) [seafloor lavas from the Loi'hi Seamount Pisces Peak X2]
Firmicutes
Aquifex pyrophilus (M83548)
0.05

510


b
bS0604-22 (2)
clone P0X4b2A10 (EU491466) [seafloor lavas from Hawai'i South Point X4]
Pseudomonas sp. NB1-h (AB013829)
bS0604-8
clone SC1-44 (DQ289906) [South Atlantic Bight Permeable Shelf Sediment]
halophilic bacterium HRhD 2 (DQ469584)
bS0604-21
bS0604-15
clone P0X4b2A06 (EU491463) [ocean crust]
bS0604-9
clone AT-s80 (AY225635) [Mid-Atlantic Ridge hydrothermal sediment]
bS0604-18
Stenotrophomonas maltophilia sp. BRL02-38 (DQ339668)
bS0604-1 (9)
Pantoea agglomerans sp. RI22(DQ530141)
Gammaproteobacteria
bS0604-33
Ochrobactrum sp. S1 (AJ002812)
bS0604-48
bS0604-28
clone P9X2b3C05 (EU491110) [seafloor lavas from the Loi'hi Seamount Pisces Peak X2]
bS0604-16
clone pItb-vmat-38 (AB294946) [submarine hot spring]
Alphaproteobacteria
bS0604-14
clone EPR3965-I2-Bc82 (EU491882) [seafloor lavas from the East Pacific Rise]
Desulfarculus baarsii DSM 2075 (AF418174)
Desulfonatronospira dismutants ASO3-1 (EU296537)
bS0604-40
clone LM45 (AM909963) [Chinese rice field]
Deltaproteobacteria
bS0604-46
Reichenbachiella agariperforans KMM3525 (AB058919)
Bacteroidetes
Clostridium termitidis DSM 5396 (X71854)
bS0604-3 (3)
clone BSV75 (AJ229219) [anoxic bulk soil]
Firmicutes
bS0604-49
clone TK17 (AJ347036) [sponges]
Chloroflexi
bS0604-45
clone P9X2b8C05 (EU491301) [seafloor lavas from the Loi'hi Seamount Pisces Peak X2]
Solirubrobacter soli Gsoil 355 (AB245334)
Actinomadura sp. CNU125 PL04 (DQ448743)
bS0604-26
clone AT-s2-33 (AY225655) [Mid-Atlantic Ridge hydrothermal sediment]
bS0604-44
clone P9X2b7F12 (EU491208) [seafloor lavas from the Loi'hi Seamount Pisces Peak X2]
Actinobacteria
bS0604-37 (2)
clone Creta1-C11 (AY533950) [oxic surface sediments of eastern Mediterranean Sea]
Unknown group
bS0604-27
clone JK638 (DQ368249) [suboxic zone of the black sea]
bS0604-6 (2)
clone BD3-11 (AB015552) [deep-sea sediments]
clone JMK-2 (AB281489) [anammox bioreactor from marine environment]
bS0604-32
clone B97 (AY375065) [deep-sea sediment from western Pacific Warm Pool]
bS0604-30
clone P0X4b2G02 (EU491483) [seafloor lavas from Hawai'i South Point X4]
Planctomyces sp. Schlesner 130 (X81952)
bS0604-38
clone CI5cm.F03 (EF208689) [sandy carbonate sediment]
planctomycete sp. 116 (AJ231181)
bS0604-25
clone CI5cm.G12 (EF208694) [sandy carbonate sediment]
Planctomycetes
Aquifex pyrophilus (M83548)
0.05


c
clone EPR3970-MO1A-Bc21 (EU491607) [seafloor lavas from the East Pacific Rise]
bS0615-45
bS0615-41
clone GL1-29 (EF215728) [inert artificial surfaces submerged in marine water]
clone Belgica2005/10-130-12 (DQ351758) [marine sediments]
bS0615-37
clone BD1-1 (AB015514) [deep-sea sediments from different depths]
bS0615-28
Pantoea agglomerans sp. RI22 (DQ530141)
bS0615-4 (10)
Gammaproteobacteria
clone BJS81-042 (AB239007) [cold seep sediment]
bS0615-21
Beltaproteobacteria
Sphingomonas sp. strain B28161 (AJ001052)
bS0615-9
clone Therm30-D10 (AY533916) [oxic surface sediments of eastern Mediterranean Sea]
bS0615-16
clone 655092 (DQ404752) [contaminated sediment]
bS0615-27
Methylocystis sp. 5FB1 (AJ868421)
Ochrobactrum anthropi sp. YZ-1 (EU275247)
bS0615-17 (3)
clone pItb-vmat-21 (AB294937) [shallow submarine hot spring]
bS0615-39
Alphaproteobacteria
clone HMMVPog-8 (AJ704690) [marine sediment]
bS0615-12 (2)
Geoalkalibacter subterraneus Red1 (EU182247)
Uncultured Desulfobulbus sp. (AB188774) [cold seep sediment]
bS0615-24
Desulfosarcina variabilis (M34407)
clone SR27 (AY771945) [marine surface sediment]
bS0615-43
Deltaproteobacteria
clone R76-B102 (AF449263) [Riftia pachyptila's tube]
bS0615-10
OP8
clone VHS-B3-74 (DQ394961) [harbor sediment]
bS0615-35
Unknown group
clone BS1-0-2 (AY255000) [tidal flat]
bS0615-20
bS0615-15
clone P9X2b8B05 (EU491320) [seafloor lavas from the Loi'hi Seamount Pisces Peak X2]
Acidobacteria sp. Ellin7137 (AY673303)
Acidobacteria
clone VHS-B5-17 (DQ395037) [harbor sediment]
bS0615-46
clone A312009 (AY907731) [Arabian Sea water]
bS0615-48
Bacteroidetes
clone BSV75 (AJ229219) [anoxic bulk soil]
bS0615-3
Clostridium termitidis DSM 5396 (X71854)
bS0615-7
bS0615-32
clone TANB101 (AY667266) [TCE-dechlorinating groundwater]
Clostridium carboxidivorans (AY170379)
Firmicutes
clone BD2-10 (AB015539) [deep-sea sediments]
bS0615-36
clone P0X3b5D12 (EU491389) [seafloor lavas from Hawai'i South Point X3]
bS0615-47
Actinobacteria
Levilinea saccharolytica KIBI-1 (AB109439)
clone BD3-16 (AB015556) [deep-sea sediments]
bS0615-30
Chloroflexi
clone Urania-2B-40 (AY627598) [deep-sea sediment from Eastern Mediterranean]
bS0615-2
OP11
clone JG35+U1-AG121 (AM114433) [soil sample from uranium mining waste pile]
bS0615-23
clone ODP1230B5.30 (AB177220) [methane hydrate bearing subseafloor sediment]
bS0615-50
Planctomycete GMD14H07 (AY162124)
clone LC1-9 (DQ289908) [Permeable Shelf Sediment]
bS0615-34
clone BD2-16 (AB015544) [deep-sea sediments]
bS0615-1
clone ODP1251B8.4 (AB177345) [methane hydrate bearing subseafloor sediment]
bS0615-29
Planctomycetes
Aquifex pyrophilus (M83548)
0.05

513

Fig. 4. Phylogenetic trees showing the relationship of bacterial 16S rDNA sequences in libraries bS0610 (a), bS0604 (b) and bS0615 (c) to relatives in GenBank. The trees were constructed by bootstrap neighbor-joining method in MEGA 4.0. Clones in bold were obtained in the present study, and numbers in parentheses showed the number of related clones. The environments where relative clones were obtained from were given in square brackets using different font color, i.e., non-hydrothermal marine environments were specified in blue font, hydrothermal environments in red font, and non-marine environments in green font. Bootstrap values under 50% were not shown. *Aquifex pyrophilus* was used as the outgroup. Bar, 0.05 substitutions per nucleotide position.

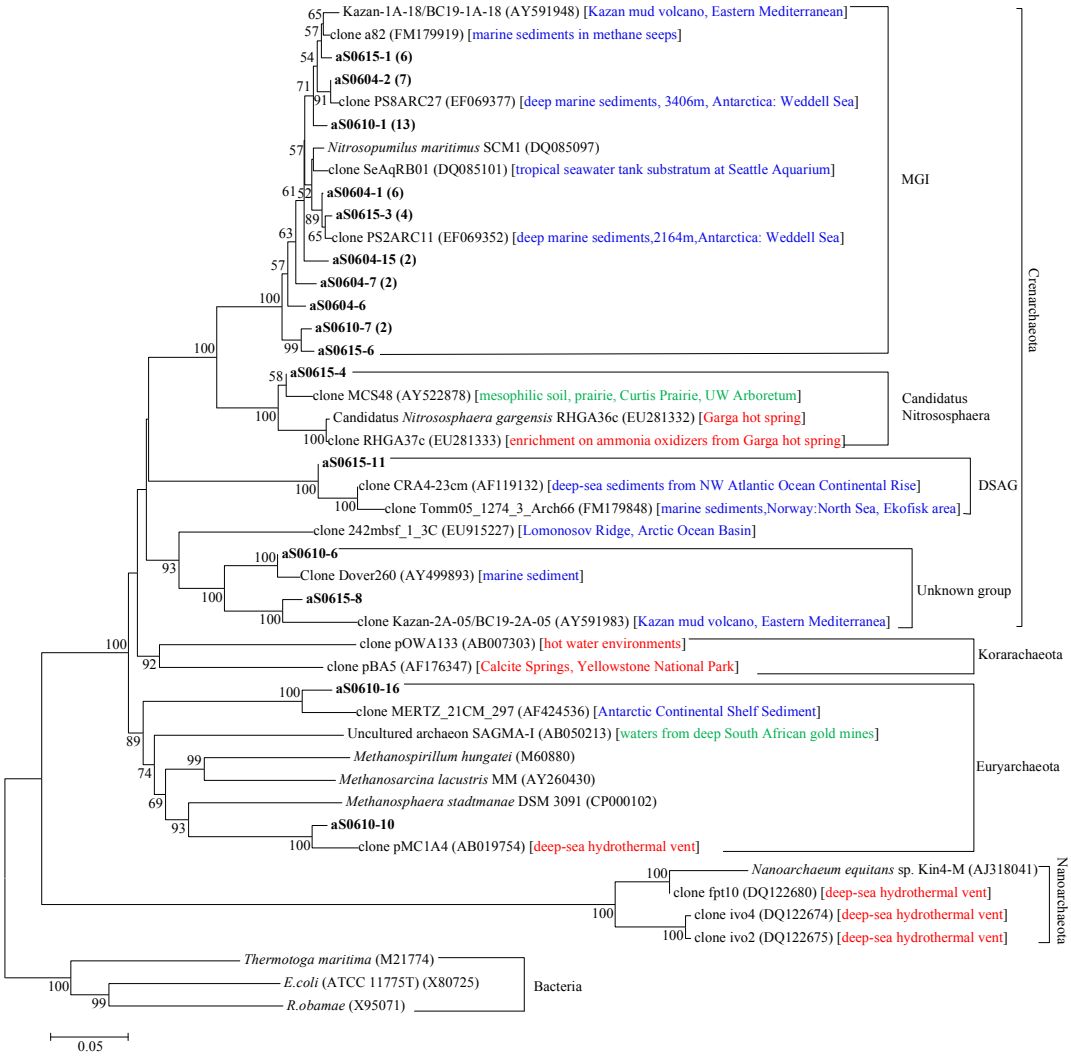


Fig. 5. Phylogenetic trees showing the relationship of archaeal 16S rDNA sequences in libraries aS0610, aS0604 and aS0615 to relatives in GenBank. The trees were constructed by bootstrap neighbor-joining method in MEGA 4.0. Clones in bold were obtained in the present study, and numbers in parentheses showed the number of related clones. The environments where relative clones were obtained from were given in square brackets by using different font color, i.e., non-hydrothermal marine environments were specified in blue font, hydrothermal environments in red font and non-marine environments in green font. Bootstrap values under 50% were not shown. Bar, 0.05 substitutions per nucleotide position.