RESEARCH ARTICLE

Microbial diversity in deep-sea sediment from the cobalt-rich crust deposit region in the Pacific Ocean

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Abstract

Cobalt-rich crusts are important metallic mineral resources with great economic potential, usually distributed on seamounts located in the Pacific Ocean. Microorganisms are believed to play a role in the formation of crusts as well as in metal cycling. To explore the microbial diversity related to cobalt-rich crusts, 16S ribosomal RNA gene clone libraries were constructed from three consecutive sediment layers. In total, 417 bacterial clones were obtained from three bacterial clone libraries, representing 17 distinct phylogenetic groups. *Proteobacteria* dominated in the bacterial communities, followed by *Acidobacteria* and *Planctomycetes*. Compared with high bacterial diversity, archaea showed a remarkably low diversity, with all 137 clones belonging to marine archaeal group I except one novel euryarchaeotal clone. The microbial communities were potentially involved in sulfur, nitrogen and metal cycling in the area of cobalt-rich crusts. Sulfur oxidation and metal oxidation were potentially major sources of energy for this ecosystem. This is the first reported investigation of microbial diversity in sediments associated with cobalt-rich crusts, and it casts fresh light on the microbial ecology of these important ecosystems.

Introduction

Seafloor metallic mineral resources occur in many forms including polymetallic nodules, metallic oozes, massive sulfide deposits and cobalt (Co)-rich crusts (Liang & Zhu, 2000). These resources have great economic potentials due to their high content of metals with commercial values, e.g. manganese (Mn), copper (Cu), nickel (Ni) and platinum (Pt). Co-rich crusts, also known as iron-manganese crusts, ferromanganese crusts or Co-rich ferromanganese crusts, can be found globally on the ocean floor. However, the Pacific seamounts are the most concentrated areas. Co-rich crusts usually contain mixed iron and manganese colloids along with other enriched metals, and grow on the hard-rock substrates. Co-rich crusts are unique and differ from polymetallic nodules in many aspects: the crusts occur at shallower depths (< 3000 m) in the deep sea, whereas nodules are formed at much deeper depths (approximately 4000–6000 m) (Wang & Müller, 2009); crusts (up to 80 million years old, Ling *et al.*, 2005) are older than nodules (approximately 12 million years old, Glasby *et al.*, 1982); they have a different appearance and composition, especially Co content (Wang & Müller, 2009). The proportion of Co in these crusts ranges from 0.4% to 2.5%, which is two to three orders of magnitude higher than that of the polymetallic nodules and land-based ores (Hein *et al.*, 2000; Wang *et al.*, 2009b). Because of the strategic role of Co in industrial and military applications (such as making super alloys and electrical appliances), Co-rich crusts have become a focus of deep-sea exploration and marine mining.

Although the mechanism of deep-sea metallic mineral formation is not completely understood, microbes are favorably considered to play an important role. Both endolithic and epilithic microbial communities have been shown to be involved in the formation of nodules and crusts (Wang et al., 2009a, b), suggesting that biomineralization is indispensable in the mineral formation process. Several studies of microbial diversity in the nodule provinces have been performed using cultivation-dependent

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methods and/or molecular approaches (Xu *et al.*, 2005, 2007; Gao *et al.*, 2006). The Pacific nodule province (118–157°W, 9–16°N), spreading over 4.5 million km^2, contains abundant polymetallic nodules with no Co-rich crusts (Xu *et al.*, 2005). Phylogenetic analyses of bacterial and archaeal 16S rRNA gene clone libraries from the deep-sea sediment of this province revealed the predominance of *Gammaproteobacteria* and marine archaeal group I (MGI) (Xu *et al.*, 2005). A metagenomic approach was used to survey the microbial community and genes in the eastern Pacific nodule province (Xu *et al.*, 2007). Results suggested that *Proteobacteria* dominate the province, and a cosmid clone 17H9 was matched to a novel *Alphaproteobacterium* closely related to *Magnetospirillum* species. A bacterial library containing 79 clones from the deep-sea sediment of the northeastern Pacific polymetallic nodule province was also constructed (Xu *et al.*, 2008). Sequence analyses of this library indicated that *Gammaproteobacteria* and *Alphaproteobacteria* are most abundant in 11 phylotypes. The most recent investigation of microbial diversity in polymetallic nodule fields of the Clarion-Clipperton Fracture Zone in the Pacific recovered three dominant groups (*Alpha-*, *Gamma-* and *Deltaproteobacteria*) and 14 other groups (Wang *et al.*, 2010). We compared these studies and concluded that many bacterial phylotypes are common among these metal-rich environments, suggesting similar biological processes in these ecosystems.

Although studies concerning the geological and geographical characters of Co-rich crusts have been published for several decades (Weinberger *et al.*, 1986; Liang & Zhu, 2000), the microbial communities associated with these special areas have not been surveyed yet. For the first time, we investigated the bacterial and archaeal diversity in the deep-sea sediment from a Co-rich crust region by constructing 16S rRNA gene clone libraries. We tried to understand the microbial diversity of sediment from a Co-rich crust region and characterize the microbial community composition in the hope that microorganisms associated with metal (e.g. Mn and Co) cycling would be found. In addition, this study provided us with a primary understanding of microbial diversity in Co-rich crust areas for assessing the environmental impacts of future crusts exploration and marine mining on the indigenous microbial communities.

Materials and methods

Sample collection

Sediment samples were collected from station LX04 (Fig. 1) where many Co-rich crusts occur on seamounts, by the research vessel *DAYANG YIHAO* in July 2007. A TV-multicorer was used to sample the sediments near crusts. The temperature of the sampling station was 1.4 °C. Sediment cores were carefully sectioned at 1-cm intervals for the first 10 cm and at 2-cm intervals for the next 20 cm. All processes were aseptic to avoid contamination. In this study, three layers with depths of 0–3 cm bsf (PC-A), 3–8 cm bsf (PC-B), and 8–16 cm bsf (PC-C) were used for microbial diversity analysis. Samples were stored frozen until use. The biogeochemical properties of three sediment samples were determined by energy dispersive X-ray fluorescence (EDXRF) for major and trace elements and inductively coupled plasma-mass spectrometry (ICP-MS) for rare-earth elements.

DNA extraction and PCR amplification

DNA was extracted directly from each sediment layer using the FastDNA Spin Kit for Soil (Q-BIOgene). Bacterial 16S rRNA genes were amplified by PCR using forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Dojka *et al.*, 1998). Archaeal 16S rRNA genes were amplified using forward primer A571F (5′-GCCTAAAGCGTCCGTAGC-3′) and reverse primer UA1204R (5′-TTCGGGGCATACTGACCT-3′) (Baker *et al.*, 2003). The PCR amplification and purification of PCR products were performed as previously described (Liao *et al.*, 2009).

Construction of 16S rRNA gene clone libraries and sequencing

PCR products from each sediment layer were cloned into pMD19-T vectors (TaKaRa Co., Dalian, China) to construct three bacterial 16S rRNA gene clone libraries (PC-A, PC-B and PC-C) and three archaeal 16S rRNA gene clone libraries (APC-A, APC-B and APC-C), following the method described earlier (Liao *et al.*, 2009). Clones were sequenced using an ABI3730 sequencer in

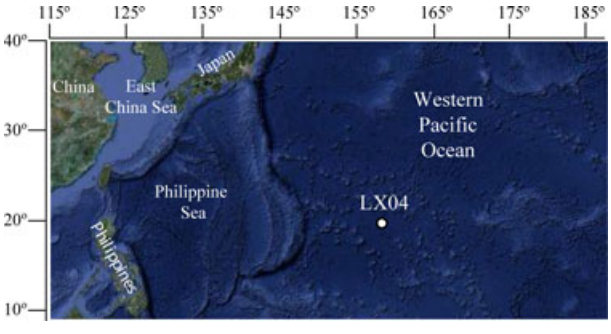

Fig. 1. Map showing the location of station LX04 in the western Pacific Ocean.

the Chinese National Human Genome Center at Shanghai. Chimeras were checked and excluded by the CHECK_CHIMERA program of Ribosomal Database Project II (RDP-II) (Cole *et al.*, 2003). Subsequently, sequences over 600 bp were used for further analysis.

Phylogenetic analysis and statistical analysis of diversity

Sequences in each library were assigned to operational taxonomic units (OTUs) at 3% 16S rRNA gene sequence difference level by the distance-based OTU and richness program (DOTUR) (Schloss & Handelsman, 2005). One representative clone from each OTU was submitted to the BLASTN program (Altschul *et al.*, 1990) and RDP-II to determine the classification and closest matches. Phylogenetic trees of representative clones and their matches were constructed by MEGA software version 4.0 (Tamura *et al.*, 2007) using neighbor-joining method (Saitou & Nei, 1987) with the Kimura two-parameter model. Nodes support was assessed by bootstrapping using 1000 bootstrap replicates.

To estimate species richness and sampling effort, the DOTUR program was used to construct rarefaction curves and calculate diversity indices including abundance-based coverage estimator ACE (Chao & Lee, 1992), species diversity estimator Chao1 (Kemp & Aller, 2004), Simpson index and Shannon index (Zhang *et al.*, 2008). To determine the shared diversity between samples, MOTHUR (Schloss *et al.*, 2009) was used to calculate the shared and unique OTUs (97% sequence similarity) for Venn diagram analysis. Venn diagrams were plotted using the VENN DIAGRAM PLOTTER program (http://omics.pnl.gov/software/VennDiagramPlotter.php), written by Littlefield and Monroe at the Department of Energy, PNNL, Richland, VA (Amaral-Zettler *et al.*, 2009). Sequences from basaltic lavas and seawater of the East Pacific Rise (EPR) were obtained from GenBank, published by Santelli *et al.* (2008).

Nucleotide sequence accession numbers

The sequences of bacterial clones in this study were deposited in the GenBank database under the accession numbers FJ938427–FJ938716. The archaeal sequences were under the accession numbers FJ938360–FJ938426.

Results

Biogeochemical properties of samples

The biogeochemical properties of three sediment samples are summarized in Table 1. In general, the determined properties of three sediment samples were similar, with

Table 1. Biogeochemical properties of the three sediment samples (PC-A, PC-B and PC-C), continental crust and basalts

	PC-A (0–3 cm)	PC-B (3–8 cm)	PC-C (8–16 cm)	Continental crust*	Basalts[†,‡]
SiO_2 (%)	51.30	51.24	51.67	61.50	49.05–51.00
Al_2O_3 (%)	15.86	15.78	15.95	15.10	13.46–16.28
Fe_2O_3 (%)	8.78	8.63	8.75	6.28	NA
CaO (%)	1.33	1.35	1.31	5.50	10.58–12.79
MgO (%)	3.52	3.47	3.43	3.70	6.74–8.80
Na_2O (%)	3.53	3.30	2.88	3.20	2.16–3.54
K_2O (%)	3.01	3.03	3.09	2.40	0.03–0.64
TiO_2 (%)	0.91	0.91	0.92	0.68	1.08–2.38
MnO (%)	0.74	0.72	0.73	0.10	0.14–0.23
P_2O_5 (%)	0.29	0.30	0.30	0.18	0.16–0.29
Ni ($\mu g\ g^{-1}$)	180.00	171.80	166.50	56.00	NA
Co ($\mu g\ g^{-1}$)	98.53	95.24	93.25	24.00	NA
V ($\mu g\ g^{-1}$)	152.67	150.20	151.00	98.00	NA
Cr ($\mu g\ g^{-1}$)	98.57	98.90	97.58	126.00	NA
Cu ($\mu g\ g^{-1}$)	228.67	219.00	220.00	25.00	NA
Zn ($\mu g\ g^{-1}$)	140.67	136.80	136.00	65.00	NA
Cd ($\mu g\ g^{-1}$)	0.25	0.29	0.27	NA	NA
Pb ($\mu g\ g^{-1}$)	37.60	37.00	36.78	14.80	NA
Mo ($\mu g\ g^{-1}$)	10.62	11.18	13.48	NA	NA
Ba ($\mu g\ g^{-1}$)	684.67	777.00	818.00	584.00	3.90–100.00

NA, not available.

*The values were ranges for several samples of basalts around the EPR.

[†]Data obtained from Wedepohl (1995).

[‡]Data obtained from Reynolds *et al.* (1992).

some slight variations. The concentrations of MgO, Na_2O, Ni, Co, Zn and Pb decreased slightly with depth, whereas the concentrations of Mo, Ba and K_2O increased with depth. The contents of TiO_2, MnO and P_2O_5 were almost the same in the three samples. Compared with continental crust (Wedepohl, 1995), the concentrations of Fe_2O_3, TiO_2, MnO, Ni, Co, V, Cu, Zn, Pb and Ba were higher in the study samples (Table 1). The averaged concentrations of Cu, Ni, Co, Zn and Ba in continental crust were 25, 56, 24, 65 and 584 μg g^{-1} (Wedepohl, 1995), respectively, which were much lower than those in the samples under study (Table 1). Compared with basalts from the EPR (Reynolds *et al.*, 1992), concentrations of CaO, MgO and TiO_2 were lower in the study area, whereas concentrations of MnO, K_2O and Ba were higher (Table 1). The content of Co in deep-sea basalts varied among different areas with an average of 32.71 μg g^{-1} (Nicholls & Islam, 1971). The contents of Co in the three study samples (93.25–98.53 μg g^{-1}) were much higher than in sediments from Maizuru Bay (15.20 μg g^{-1}) and Wakasa Bay (6.70 μg g^{-1}) (Kurata, 1974), and in seawater (1.20×10^{-6} μg g^{-1}) (Glasby *et al.*, 2007). In conclusion, the study samples were composed mainly of SiO_2, Al_2O_3 and Fe_2O_3, and were rich in metals including Co, Cu, Ba, Zn and Ni.

Analysis of bacterial 16S rRNA gene clone libraries

Three bacterial 16S rRNA gene clone libraries, PC-A, PC-B, and PC-C, were constructed from sediment layers 0–3, 3–8 and 8–16 cm, respectively. In total, 417 bacterial 16S rRNA gene partial sequences over 600 bp were analyzed, consisting of 114, 135 and 168 sequences in libraries PC-A, PC-B and PC-C, respectively. All 417 bacterial clones could be assigned to 203 OTUs at 3% 16S rRNA gene sequence difference level. When considering each clone library separately, 85, 97 and 93 OTUs could be found in libraries PC-A, PC-B and PC-C, respectively (Table 2).

Of the 417 clones, 401 clones could be assigned to 17 phylogenetic groups, whereas the remaining 16 clones could not be classified into any known groups (Figs 2a and 8). *Gammaproteobacteria* (23.0%) was the most dominant group, followed by *Alphaproteobacteria* (18.0%) and *Deltaproteobacteria* (17.5%). Non-*Proteobacteria* groups accounted for 41.0% of the 417 bacterial clones and mainly included *Acidobacteria* (7.7%), *Planctomycetes* (7.2%) and *Chloroflexi* (6.2%). The remaining groups were small, representing < 4.0% each in all the 417 bacterial clones (Fig. 2a). The phylogenetic composition of each library is depicted in Fig. 2b. Excluding unclassified bacteria, 13, 14 and 14 groups were identified in libraries PC-A, PC-B and PC-C, respectively. *Gammaproteobacteria* was the predominant group in libraries PC-A and PC-B, and *Alphaproteobacteria* was the predominant group in library PC-C. In total, 11 phylogenetic groups were commonly found in three bacterial libraries, and *Deferribacteres*, *Fibrobacteres*, *Spirochaetes* and candidate division OD1 were specific within an individual library. *Firmicutes* was absent in library PC-A, and increased from two clones in library PC-B to 13 clones in library PC-C.

Most of the sequences recovered in this study tended to cluster with uncultured environmental clones, rather than isolates from established groups. Only a few clones could be assigned to specific genera, such as *Stenotrophomonas*, *Ochrobactrum* and *Enhygromyxa*. The remaining clones could only be identified to phylum or class due to the lack of information required to assign them to a specific genus.

Gammaproteobacteria

Gammaproteobacteria was the most abundant group, with 96 clones detected in the bacterial community, accounting for 23%, 29% and 18% of clones in libraries PC-A, PC-B and PC-C, respectively (Fig. 2b). Clones belonging to *Stenotrophomonas*, *Ectothiorhodospiraceae*, *Alteromonadales* and *Legionellales* were retrieved (Fig. 3). In addition, four unidentified clusters temporarily named in this study were detected, including a Pacific nodule cluster sharing high similarities with clones from the Pacific nodule province, an endosymbiont cluster containing *Olavius ilvae Gamma* 3 endosymbiont, an unknown cluster 1 containing two distant taxa, and an oligotrophic marine

Table 2. Diversity indices including ACE, Chao1, Shannon and Simpson (calculated at 3% 16S rRNA gene sequence difference level) of the bacterial and archaeal libraries

Library	No. of clones	No. of OTUs	ACE	Chao1	Shannon	Simpson
PC-A	114	85	392.740	499.167	4.288	0.008
PC-B	135	97	439.730	548.429	4.395	0.009
PC-C	168	93	213.741	223.714	4.294	0.012
APC-A	62	7	7.000	7.000	1.719	0.197
APC-B	45	10	16.109	13.000	1.912	0.162
APC-C	30	6	6.486	6.000	1.577	0.205

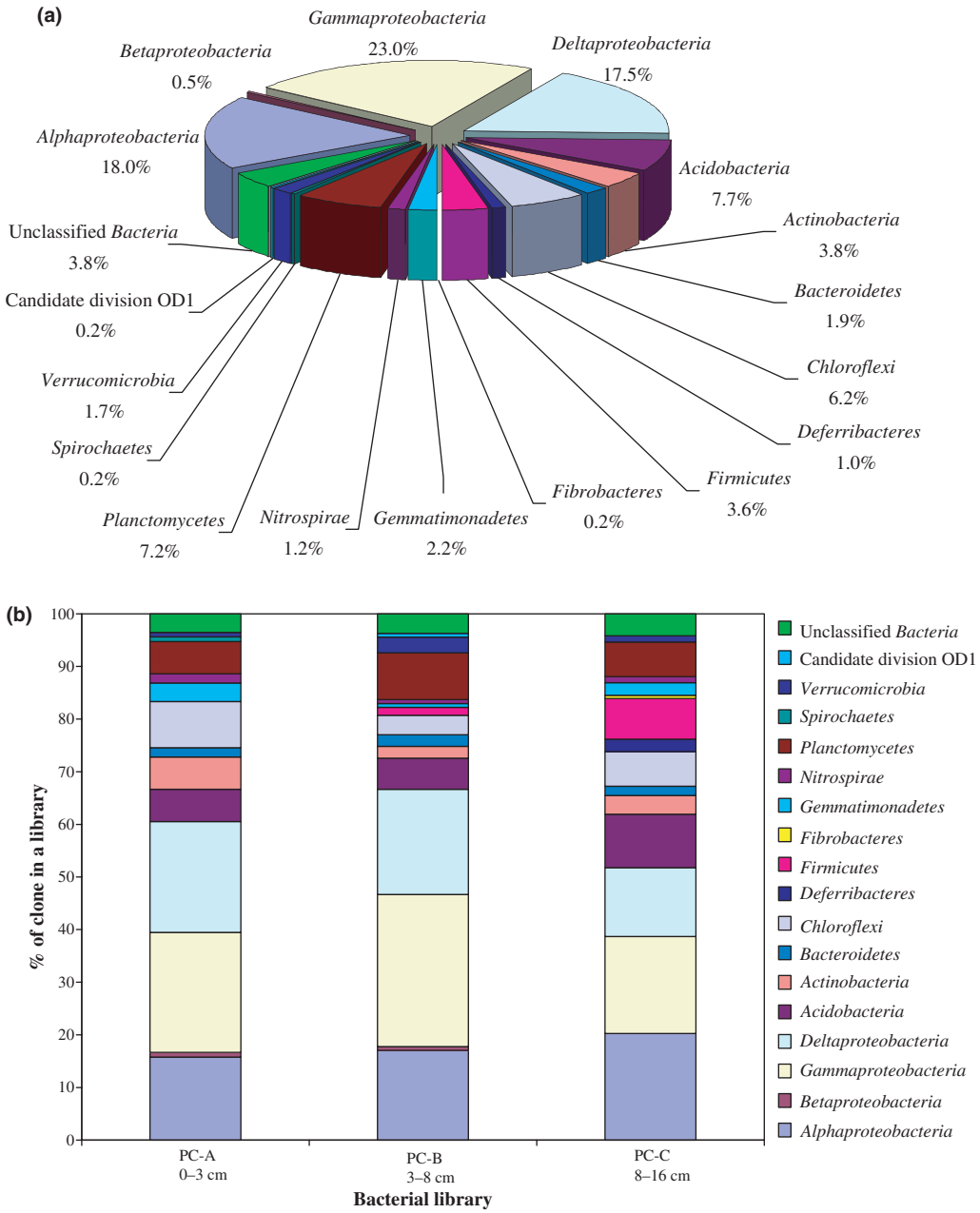

Fig. 2. Bacterial community structure based on 16S rRNA gene analysis: (a) Analysis of the total 417 bacterial clones from three clone libraries. Numbers indicate the percentages in the whole bacterial community; (b) Depth patterns of the bacterial communities in the three clone libraries constructed from the three sediment layers.

Gammaproteobacteria (OMG) cluster containing two clones distantly related to marine *Gammaproteobacterium* strain HTCC2178 (Fig. 3). A novel OTU (PC-B125) sharing very low similarity (< 90%) with the endosymbiont cluster formed a single taxon. The largest family was *Ectothiorhodospiraceae* within the order *Chromatiales*, containing 19, 23 and 21 clones from libraries PC-A, PC-B and PC-C, respectively. One clade within *Ectothiorhodospiraceae* was related to *Thioalkalispira* species. Clusters of *Stenotrophomonas* and *Alteromonadales* consisted exclusively of clones from libraries PC-A and PC-B, whereas clusters of *Legionellales* and Pacific nodule consisted exclusively of clones from libraries PC-B and PC-C. The closest cultured relative was *Stenotrophomonas maltophilia* strain ATCC 19861^T (Anzai *et al.*, 2000), sharing more than 98% identity with OTU PC-B12 (two related clones) and OTU PC-A35 (three related clones) in cluster *Stenotrophomonas*.

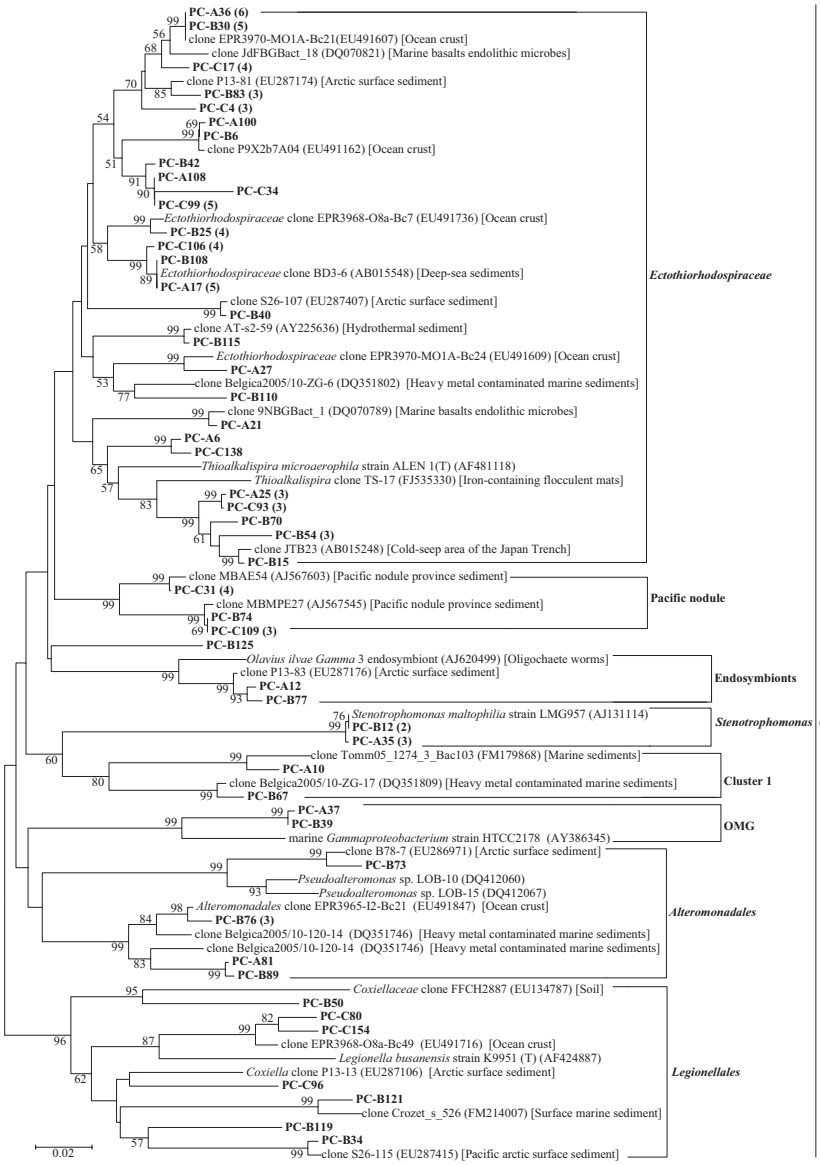

Fig. 3. Neighbor-joining tree of bacterial 16S rRNA gene sequences assigned to *Gammaproteobacteria* from the three clone libraries. Clones in bold were obtained in the present study, and numbers in parentheses represented the total number of clones contained in each OTU. The environments where close relatives were obtained from were given in square brackets. Bootstrap values < 50% are not shown. Scale bar represents a 2% difference in sequence. OMG, oligotrophic marine *Gammaproteobacteria*.

Alphaproteobacteria and *Betaproteobacteria*

The second biggest phylogenetic group was *Alphaproteobacteria*, with 75 clones including 18, 23 and 34 clones from libraries PC-A, PC-B and PC-C, respectively (Fig. 2b). Three genera (*Ochrobactrum*, *Hyphomicrobium* and *Filomicrobium*), one family (*Rhodobacteraceae*) and two orders (*Kordiimonadales* and *Rhodospirillales*) could be identified in *Alphaproteobacteria* (Fig. 4). The remaining two clusters were *Alphaproteobacteria* OCC II (OCC is an abbreviation for ocean crust clades) and cluster 1, containing only uncultured bacterial relatives. The most commonly detected OTU was PC-C18 (eight related clones) in cluster 1, which was identical to OTU PC-B37 (four related clones) and OTU PC-A57 (four related clones), sharing 98% identity with their closest relative clone Therm30-F08 obtained from oxic surface sediment of the eastern Mediterranean Sea (Polymenakou *et al.*, 2005). The three identical OTUs (PC-C18, PC-B37 and PC-A57) could be assigned to one OTU at the bacterial community level. Like *Gammaproteobacteria*, there was a large number of clones (23), including those in cluster *Alphaproteobacteria* OCC II associated with uncultured relatives from oceanic crust. In cluster *Ochrobactrum*, OTU PC-B10 was identical with *Ochrobactrum tritici* strain Y13 and shared high similarity with other two OTUs PC-C148 and PC-A73. In cluster *Kordiimonadales*, clones forming two taxa were distantly related to *Kordiimonas gwangyangensis* strain GW14-5. *Betaproteobacteria* was the smallest group in *Proteobacteria*, with only two

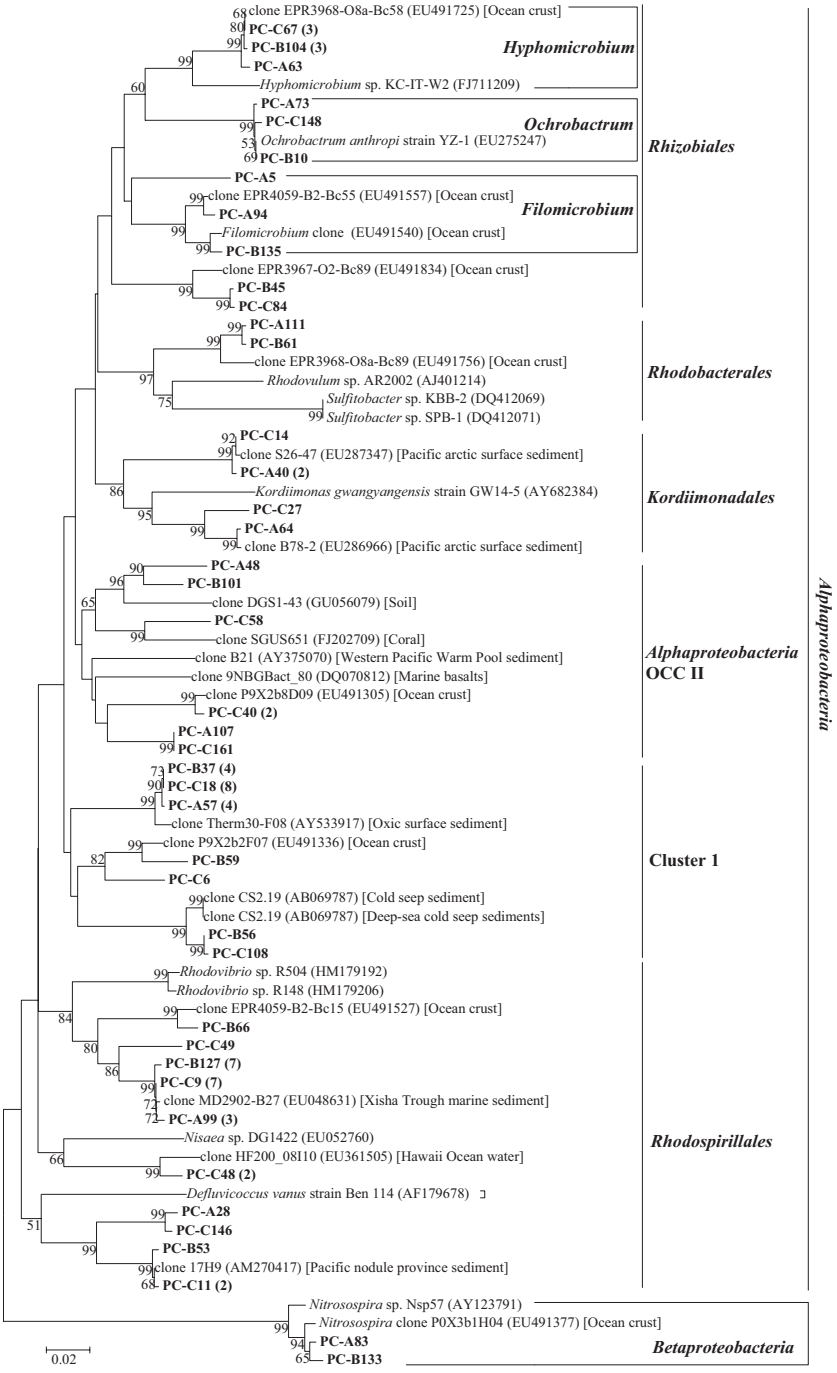

Fig. 4. Neighbor-joining tree of bacterial 16S rRNA gene sequences assigned to *Alphaproteobacteria* and *Betaproteobacteria* from the three clone libraries. Clones in bold were obtained in the present study, and numbers in parentheses represent the total number of clones contained in each OTU. The environments where close relatives were obtained from are given in square brackets. Bootstrap values < 50% are not shown. Scale bar represents a 2% difference in sequence. *Alphaproteobacteria* OCC II, *Alphaproteobacteria* ocean crust clade II.

clones belonging to genus *Nitrosospira*. Both *Betaproteobacteria* clones were closely associated with *Nitrosospira* clone P0X3b1H04 from the ocean crust.

Deltaproteobacteria

The 73 *Deltaproteobacteria* clones were distributed roughly evenly among three bacterial libraries, with 24, 27 and 22 clones in libraries PC-A, PC-B and PC-C, respectively (Fig. 2b). Only two clusters, *Nitrospina* and *Myxococcales*, contained known clones or isolates in *Deltaproteobacteria*. One OTU (PC-A33) was distantly related to clones from the Sargasso Sea and was grouped into SAR324 cluster. The remaining clones were grouped into six distinct clusters designated as clusters 1–6 (Fig. 5). In cluster *Myxococcales*, two OTUs (PC-B41 and PC-B64)

from library PC-B were associated with *Enhygromyxa salina* strain SHK-1^T. Five OTUs (PC-B41, PC-B64, PC-A45, PC-A53 and PC-C149) belonged to *Nannocystineae*, and two OTUs (PC-C12 and PC-B1) belonged to *Sorangineae*. The remaining OTU (PC-C123) was related to clone ESC4 from the Pacific nodule province. Cluster 4 only contained relatives from the ocean crust environment, including marine basalts. The OTU PC-A72 shared very low similarity with others within cluster 3, indicating it is possibly a novel lineage. Another novel OTU (PC-A70) branched distantly outside of *Desulfuromonadales* with a bootstrap value < 50, indicating its uncertain phylogenetic affiliation.

Acidobacteria and *Chloroflexi*

One phylogenetic tree was inferred for *Acidobacteria* and *Chloroflexi* (Fig. 6), and contained 32 and 26 clones which accounted for 7.7% and 6.2% of the clone libraries, respectively. The distribution of *Acidobacteria* among the three libraries was apparently biased, with 7, 8 and 17 clones in libraries PC-A, PC-B and PC-C, respectively (Fig. 2b). *Acidobacteria* was the biggest non-*Proteobacteria* group detected in the three bacterial libraries, containing six distinct clusters (Gp6, Gp9, Gp10, Gp15, Gp17 and Gp21) classified by the RDP-II classifier program. The largest cluster was Gp21, which contained the most abundant OTU, PC-C52 (six related clones). Besides the six clusters, eight OTUs were unknown and formed sister taxa distant from the identified clusters. A novel OTU (PC-A88) had low similarity (86%) to clone HCM3MC90_1F_RF_RP2 from canyon and slope sediment of southern Cretan margin. Clones belonging to *Chloroflexi* were twice as abundant in libraries PC-A (10 clones) and PC-C (11 clones) compared with library PC-B (five clones). The two named clusters *Anaerolinaceae* and SAR202 were detected in *Chloroflexi* (Fig. 6). The most abundant OTU PC-A7 (three related clones) in *Chloroflexi* shared 98% identity with clone MBMPE25 from the Pacific nodule province. Unlike other bacterial groups, nearly half of the OTUs in *Chloroflexi* were related to uncultured bacterial clones recovered from the ocean waters of Hawaii and the Sargasso Sea.

Planctomycetes, *Firmicutes* and *Verrucomicrobia*

Planctomycetes was the second biggest non-*Proteobacteria* group detected, containing 30 clones which accounted for 7.2% of the bacterial community (Fig. 2a). Three distinct clusters designated as clusters 1–3 contained all of the clones of *Planctomycetes* except OTUs PC-C119 and PC-A18 (Fig. 7). Clones from the three bacterial libraries were detected roughly evenly among the three clusters. In cluster 3, more than half of the closely related clones were obtained from the ocean crust. The most abundant OTU was PC-C116 (four related clones), which showed 100% identity to OTU PC-B3 and clone MD2902-B19 and was associated with clone E50 from the Pacific nodule province. Phylum *Firmicutes* was represented by 13 clones from library PC-C and two clones from library PC-B. No *Firmicutes* were detected in library PC-A. The most abundant OTU PC-C95 (five related clones) was similar to clone ESC10 from the Pacific nodule province. OTU PC-C2 (three related clones) was similar to clone BF0001A093, belonging to genus *Lactobacillus*. Two identical OTUs (PC-B94 and PC-C56) formed a distantly related group with OTU PC-C89. *Verrucomicrobia* was a minor group with only seven clones detected. OTU PC-B55 was identical with *Rubritalea* clone A05_CW03 from cold-water coral. Three clones were associated with relatives from oceanic crust.

Actinobacteria, unclassified bacteria and other minor groups

As shown in Fig. 8, a phylogenetic tree was inferred for *Actinobacteria* (16 clones), unclassified bacteria (16 clones) and other minor groups ($\leq$ 9 clones each). Two genera (*Rhodococcus* and *Conexibacter*) were identified in *Actinobacteria*, and four genera (*Rhodothermus*, *Reichenbachia*, *Chryseobacterium* and *Cytophaga*) were identified in *Bacteroidetes*. Clones belonging to *Gemmatimonadetes* were mainly retrieved in libraries PC-A and PC-C. Groups *Deferribacteres*, *Fibrobacteres*, *Spirochaetes* and candidate division OD1 were found exclusively in a specific library. A significant number of clones in unclassified bacteria groups were scattered among different clades in the phylogenetic tree.

Analysis of archaeal 16S rRNA gene clone libraries and phylogenetic analysis

In total, 137 archaeal 16S rRNA gene clones from three libraries were obtained (62, 45 and 30 clones from libraries APC-A, APC-B and APC-C, respectively). All archaeal clones belonged to *Crenarchaeota* except OTU APC-B37, which was grouped into *Euryarchaeota* (Figs 9 and 10). Interestingly, all crenarchaeotal clones had over 93% similarity to each other and to relatives from environment, including the first isolated ammonia-oxidizing archaeon strain *Nitrosopumilus maritimus* SCM1. All crenarchaeotal clones were assigned to MGI with great confidence (Fig. 9). Three OCC including alpha-MGI OCC VIII, alpha-MGI OCC IX and MGI OCC XI were identified in MGI (Mason *et al.*, 2007). Most of the

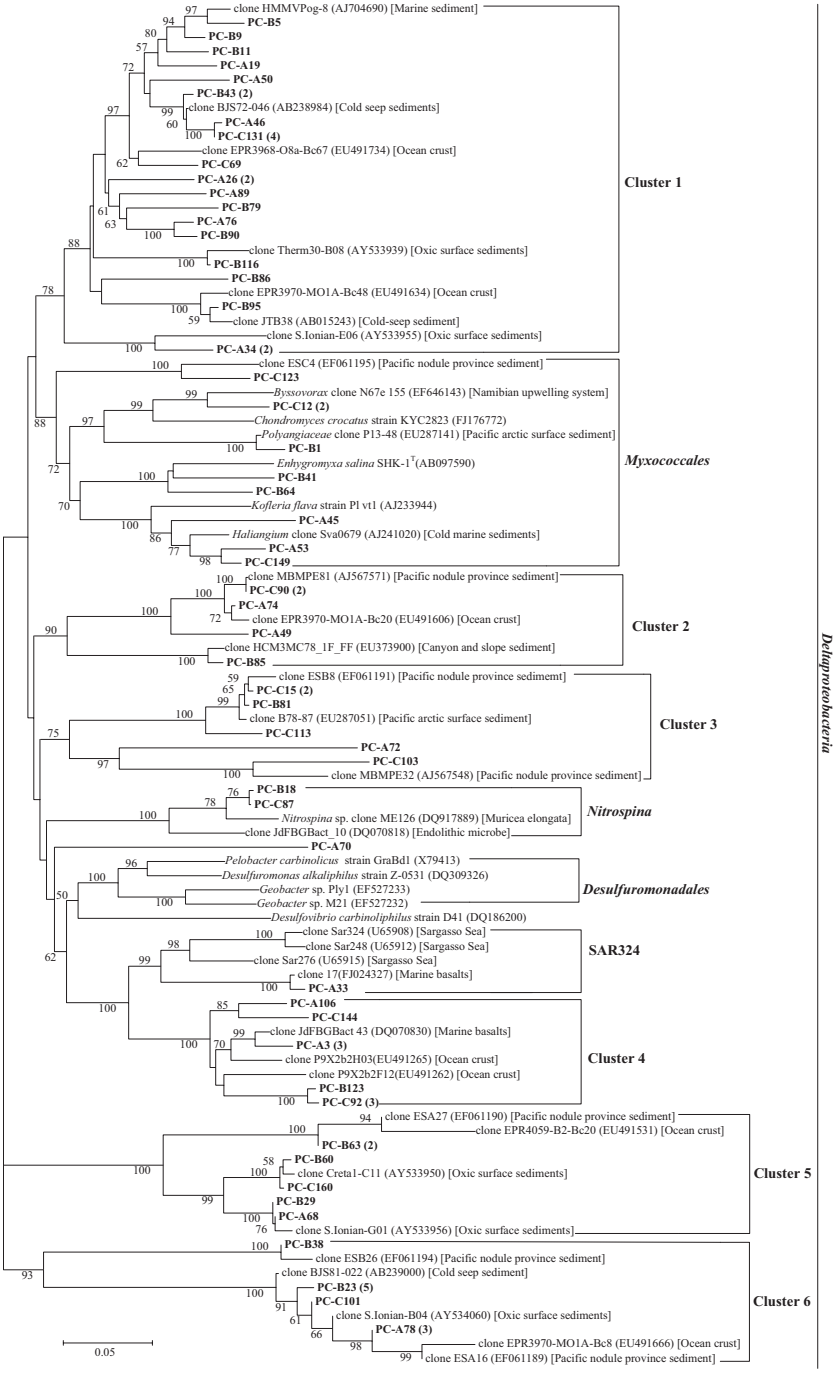

Fig. 5. Neighbor-joining tree of bacterial 16S rRNA gene sequences assigned to *Deltaproteobacteria* from the three clone libraries. Clones in bold were obtained in the present study, and numbers in parentheses represent the total number of clones contained in each OTU. The environments where close relatives were obtained from are given in square brackets. Bootstrap values < 50% are not shown. Scale bar represents a 5% difference in sequence.

closest relatives of *Crenarchaeota* were obtained from marine basalts (Mason *et al.*, 2007), bathypelagic sediment (water depth: 2164–3406 m) of the Weddell Sea in Antarctica (Gillan & Danis, 2007) and sediments collected from a mound near the Urania brine lake in the eastern Mediterranean (Heijs *et al.*, 2008). The euryarchaeotal clone was distantly related to clone 060329_T2S2_S_T_SDP_056 retrieved from lake sediment (Amaral-Zettler *et al.*, 2008), and could not be grouped into any cultured *Euryarchaeota* or identified euryarchaeotal clusters (Fig. 10).

Statistical analysis of bacterial and archaeal libraries

In general, diversity indices showed that the highest bacterial and archaeal diversity was in libraries PC-B and

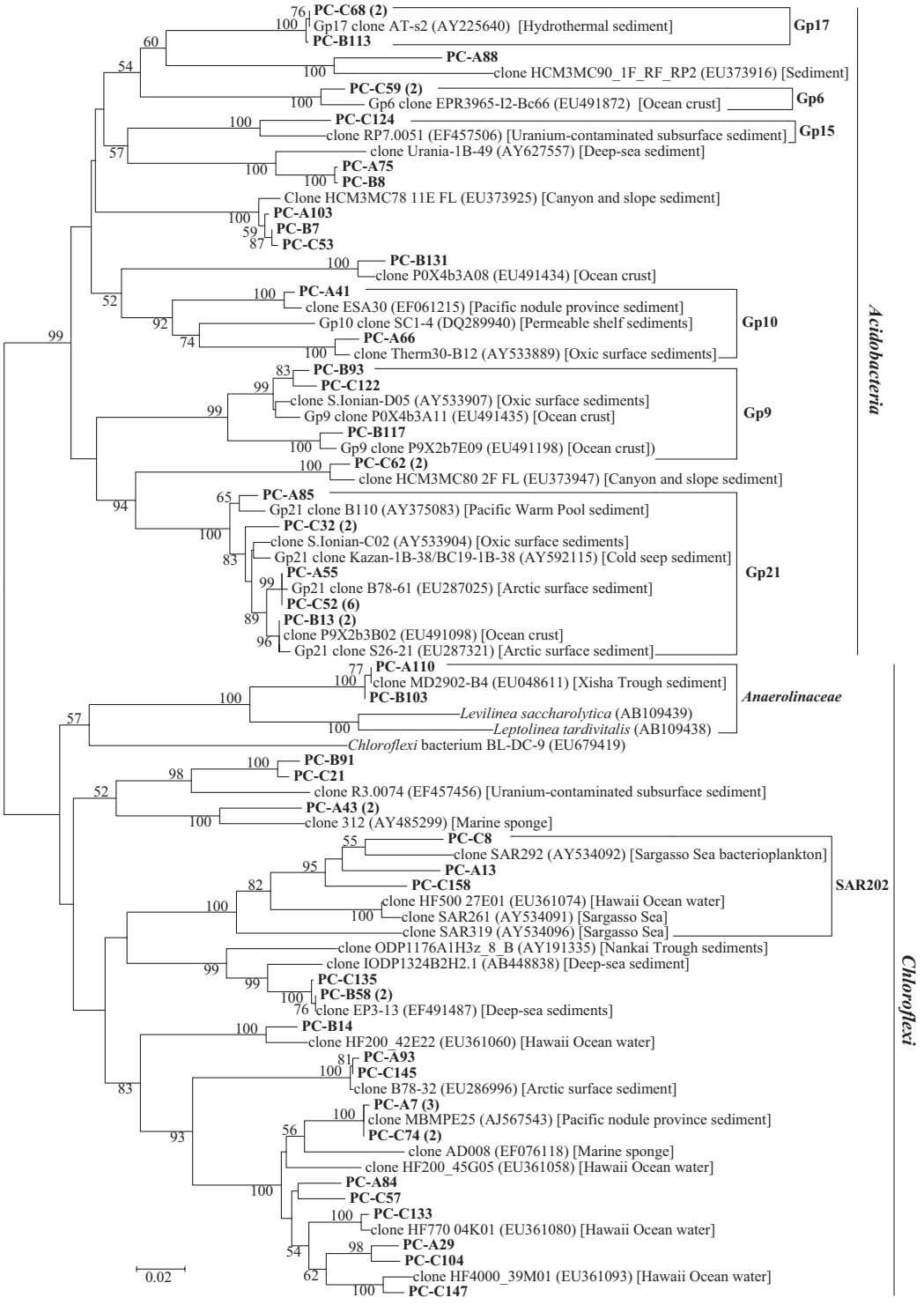

Fig. 6. Neighbor-joining tree of bacterial 16S rRNA gene sequences assigned to *Acidobacteria* and *Chloroflexi* from the three clone libraries. Clones in bold were obtained in the present study, and numbers in parentheses represent the total number of clones contained in each OTU. The environments where close relatives were obtained from are given in square brackets. Bootstrap values < 50% are not shown. Scale bar represents a 2% difference in sequence.

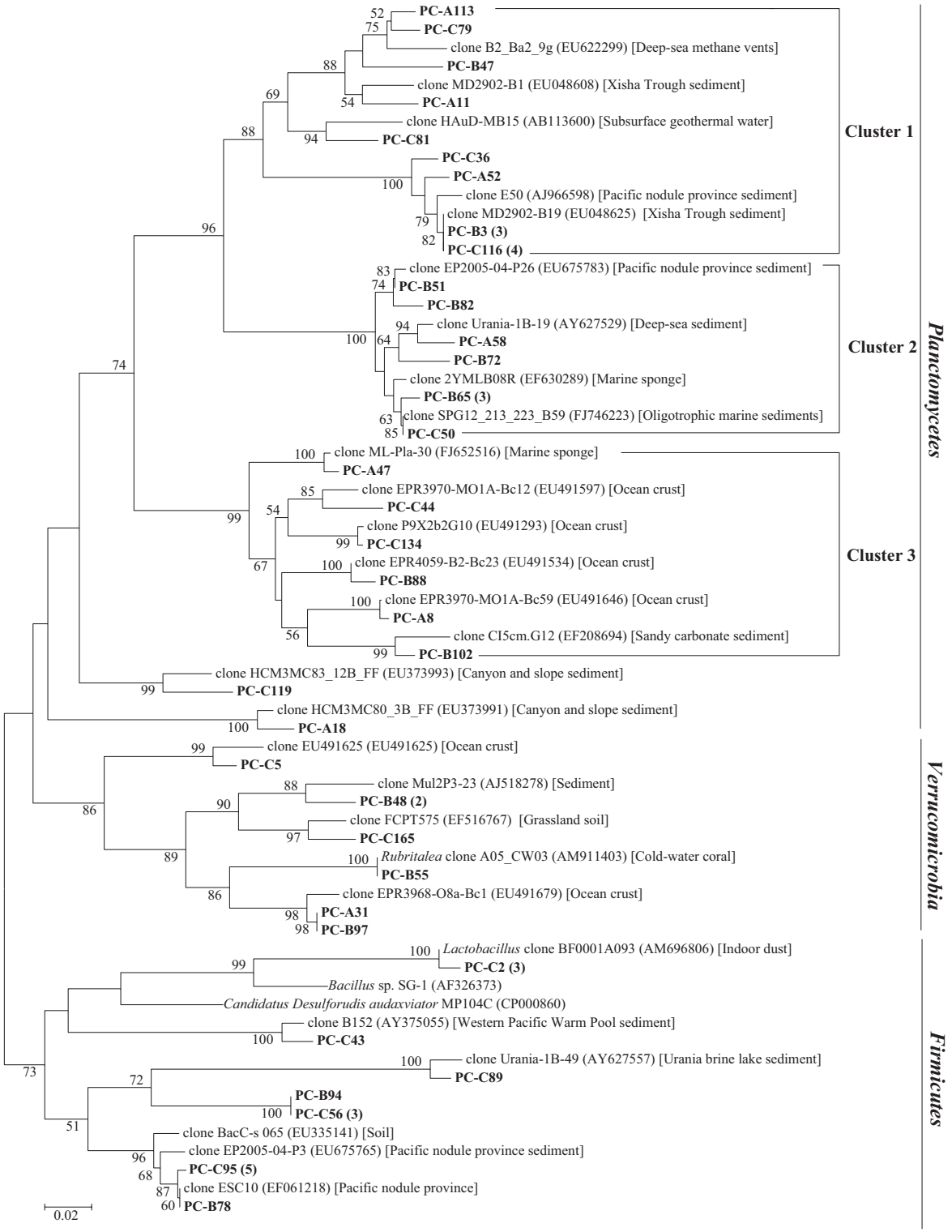

Fig. 7. Neighbor-joining tree of bacterial 16S rRNA gene sequences assigned to *Planctomycetes*, *Firmicutes* and *Verrucomicrobia* from the three clone libraries. Clones in bold were obtained in the present study, and numbers in parentheses represent the total number of clones contained i-n each OTU. The environments where close relatives were obtained from are given in square brackets. Bootstrap values < 50% are not shown. Scale bar represents a 2% difference in sequence.

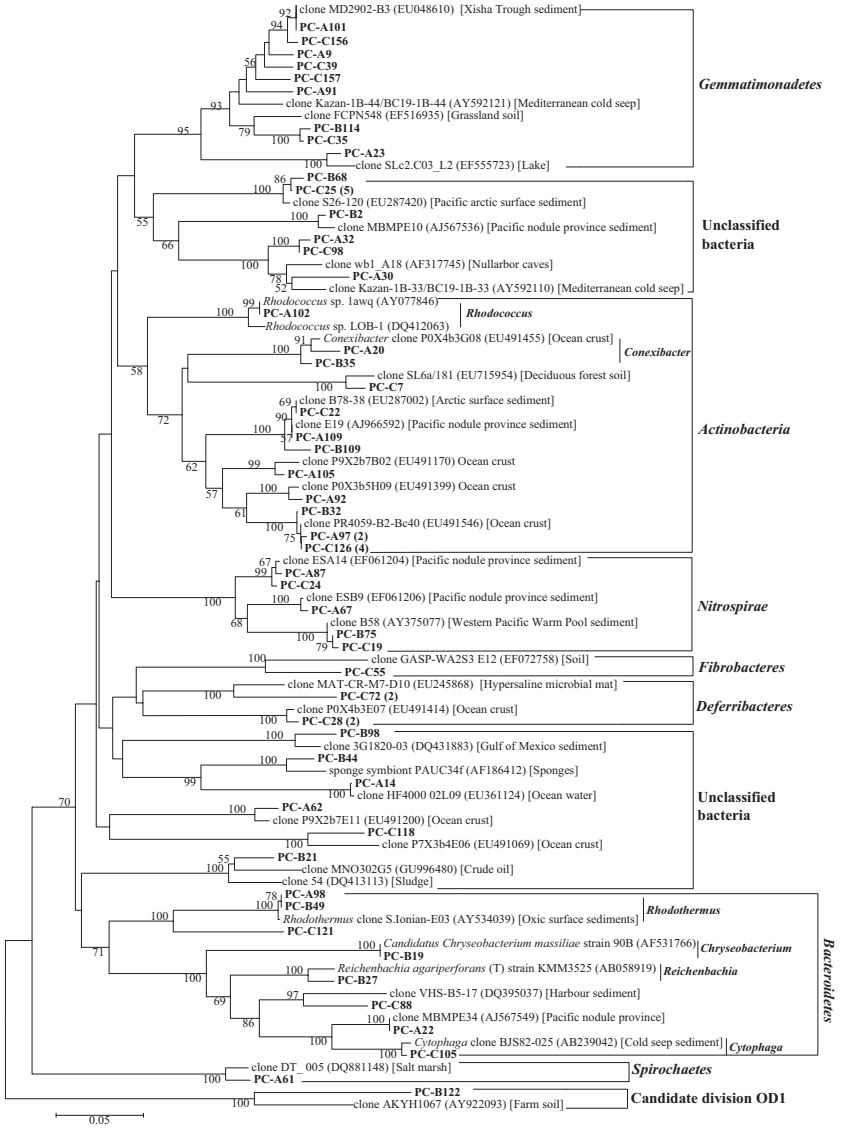

Fig. 8. Neighbor-joining tree of bacterial 16S rRNA gene sequences assigned to *Actinobacteria* and other minor groups from the three clone libraries. Clones in bold were obtained in the present study, and numbers in parentheses represent the total number of clones contained in each OTU. The environments where close relatives were obtained from are given in square brackets. Bootstrap values < 50% are not shown. Scale bar represents a 2% difference in sequence.

APC-B, respectively, and both were constructed from the sediment layer of 3–8 cm (Table 2). Compared with bacterial libraries, archaeal libraries contained apparently lower diversity and fewer OTUs. This was further supported by the rarefaction analysis, which showed a large difference in estimated diversity between bacteria and archaea (Fig. 11a). The rarefaction curve for total archaeal clones in three libraries reached a plateau at a very low sampling effort, whereas the rarefaction curve for total bacterial clones tended to approach asymptote only after sampling more than 400 clones. Rarefaction curves for three dominant subgroups in *Proteobacteria* suggested that they were well sampled (Fig. 11b). The highest diversity was found in *Deltaproteobacteria* and the lowest in *Alphaproteobacteria*.

The Venn diagrams showed the shared and site-specific groups among study samples from Co-rich crusts area, EPR basaltic lavas and seawater (Fig. 12a) (Santelli *et al.*, 2008). There were 21 OTUs shared between EPR basaltic lavas and study samples and four OTUs shared between EPR basaltic lavas and seawater, but none shared between EPR seawater and study samples. As shown in Fig. 12b,

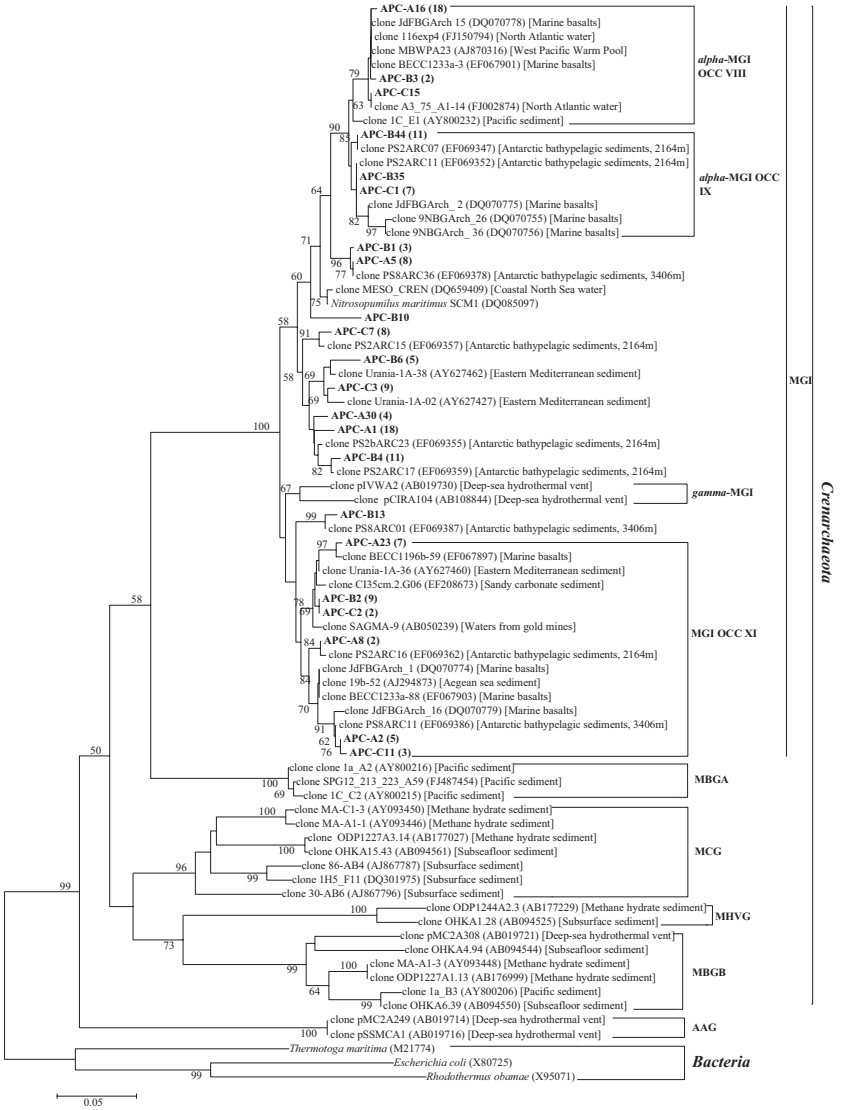

Fig. 9. Neighbor-joining tree of crenarchaeal 16S rRNA gene sequences from the three archaeal clone libraries. Clones in bold were obtained in the present study, and numbers in parentheses represent the total number of clones contained in each OTU. The environments where close relatives were obtained from are given in square brackets. Bootstrap values < 50% are not shown. Scale represents a 5% difference in sequence. MGI, marine archaeal group I; OCC, ocean crust clade; MBGA, marine benthic group A; MCG, miscellaneous Crenarchaeotic group; MHVG, marine hydrothermal vent group; MBGB, marine benthic group B; AAG, ancient archaeal group.

203 OTUs were found in the three bacterial clone libraries constructed in this study; 13 OTUs were shared by all three of them, and 26–32 OTUs overlapped two of the three bacterial clone libraries. The unique OTU counts in libraries PC-A, PC-B and PC-C were 45, 51 and 48, respectively. Ten OTUs were found in the three archaeal clone libraries, and four OTUs were shared (Fig. 12c). All five OTUs from library APC-A were included in library APC-C. No OTUs were detected only in libraries APC-A or APC-C, whereas four OTUs were unique to library APC-B.

Discussion

The primary purpose of the present study was to characterize the bacterial and archaeal diversity of deep-sea sediment from Co-rich crust areas. In total, 417 bacterial clones and 137 archaeal clones were sequenced from six clone libraries constructed from three consecutive layers of sediments. To our knowledge, no reference to microbial diversity from Co-rich crusts is available and this is the first report of microbial diversity analysis concerning Co-rich crusts in a deep-sea environment.

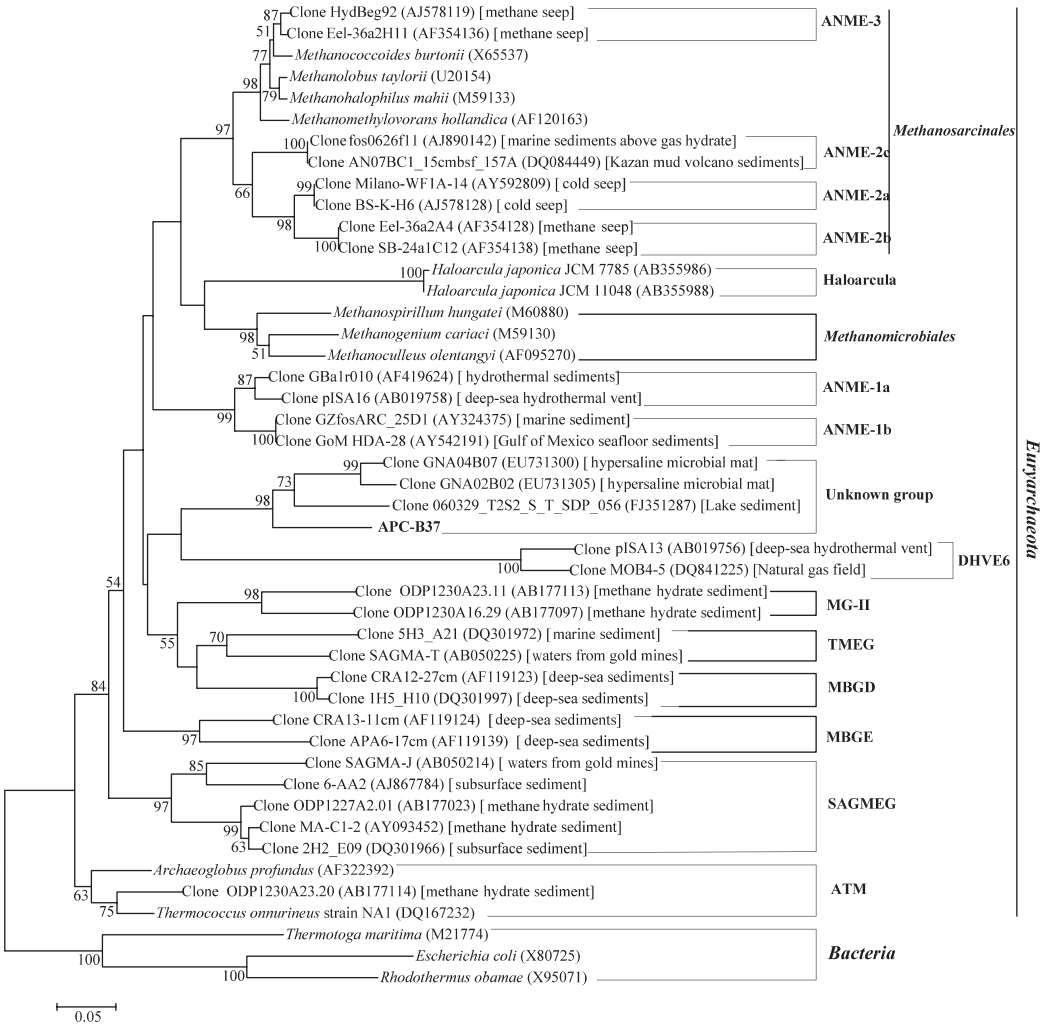

Fig. 10. Neighbor-joining tree of the only euryarchaeal 16S rRNA gene sequence from this study. Clone in bold was obtained in the present study. The environments where close relatives were obtained from are given in square brackets. Bootstrap values < 50% are not shown. Scale represents a 5% difference in sequence. ANME, anaerobic methane-oxidizing *Archaea*; DHVE6, deep-sea hydrothermal vent euryarchaeotal group 6; MG-II, marine group II; TMEG, terrestrial miscellaneous euryarchaeotal group; MBGD, marine benthic group D; MBGE, marine benthic group E; SAGMEG, South African gold mine euryarchaeotic group; ATM, *Archaeoglobales*, *Thermococcales* and *Methanococcales*-cluster.

The mechanism of forming Co-rich crusts is complex and still unclear. According to early reports, crusts are usually formed at the mixing zone where the upper oxygen-minimum zone (OMZ) meets the lower oxygen-rich bottom zone (ORZ) (Wang & Müller, 2009; Wang *et al.*, 2009b). In addition to the chemical mineralization, biomineralization also plays an important role in the crust formation (Wang & Müller, 2009). Bacteria and other unicellular organisms could act as bio-seeds and promote the formation of crusts (Wang & Müller, 2009). Although coccoliths/coccolithophores, rod-shaped and spherical microorganisms were found in nodules and crusts (Wang et al., 2009a, b), we do not know exactly what they are and how they work in the crust formation. Microbial species potentially involved in the formation of crusts are expected in the examined ocean environments. We cannot predict the mechanism of crust formation only based on 16S rRNA gene sequences, but this study discovered a diverse microbial community and gained important information about the microorganisms living in a Co-rich crust area (as discussed below).

The study samples were collected from a Co-rich crust area which was characterized by a high level of metals, including Co. Co has two oxidation states in nature, soluble Co(II) and insoluble Co(III). Co(III) is enriched in ferromanganese nodules and crusts (Lee & Tebo, 1994). It is debatable whether Co(II) oxidation is direct or indirect. Two hypotheses were proposed based on the model of *Bacillus* sp. strain SG-1: Co is oxidized either directly by a multicopper oxidase (MCO)-like enzyme

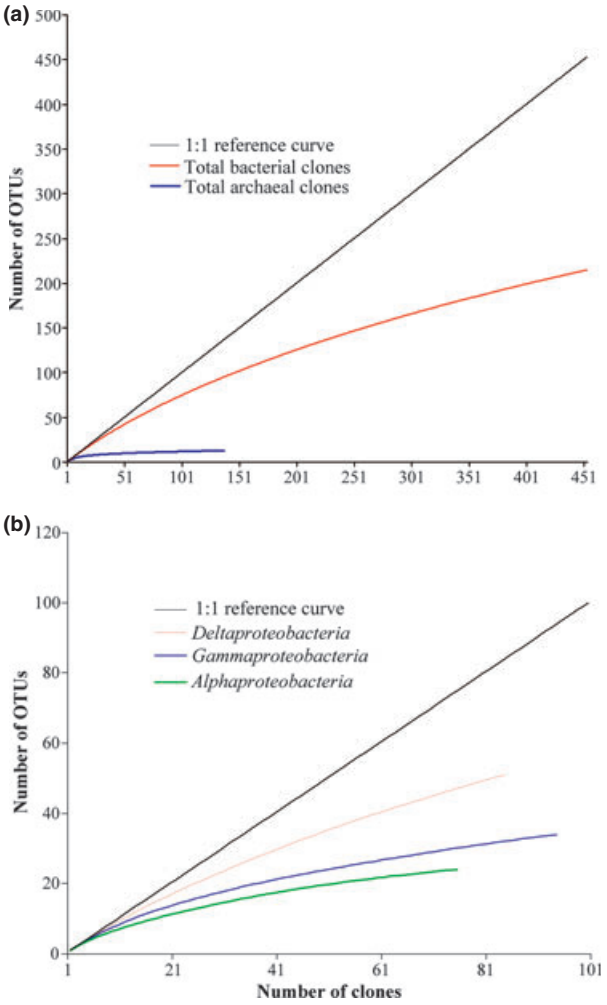

Fig. 11. Rarefaction analysis for total bacterial and archaeal clones (a), and the dominant *Proteobacteria* including *Alpha-*, *Delta-* and *Gamma*-subgroups (b). OTUs were determined at 3% 16S rRNA gene sequence difference level as assigned by the DOTUR program. 1 : 1 reference curve indicates that each sequenced clone belonged to a unique OTU.

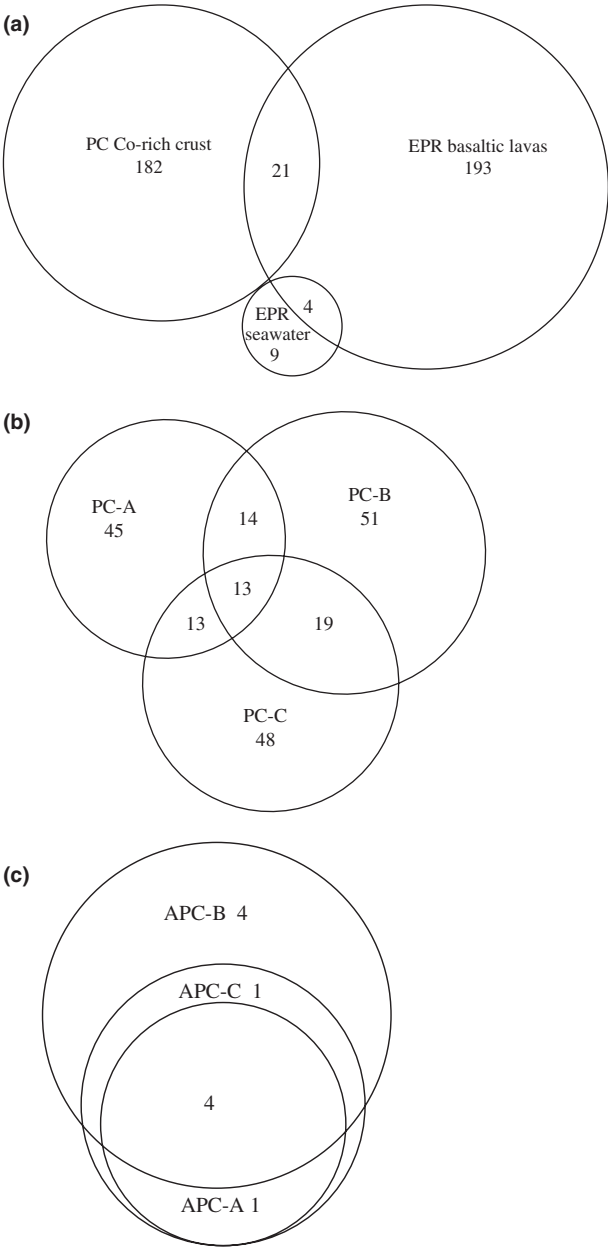

Fig. 12. Venn diagrams showing the estimated OTU (97% sequence similarity) richness shared among bacterial communities from PC (the Pacific Ocean) Cobalt-rich Crust (this study), EPR basaltic lavas (Santelli *et al.*, 2008) and EPR deep seawater (Santelli *et al.*, 2008) (a); the three clone bacterial libraries PC-A, PC-B and PC-C in this study (b); and the three archaeal libraries APC-A, APC-B and APC-C in this study (c). Shared OTU richness estimates were calculated using the program MOTHUR. Venn diagrams were plotted using the Venn Diagram Plotter program. The object sizes are drawn to show the approximate OTU memberships. Numbers in the Venn diagrams indicate number of OTUs.

that can also catalyze the oxidation of Mn(II) to Mn(IV) (Lee & Tebo, 1994), or indirectly by oxidized Mn, such as Mn(IV) or Mn(III) produced by Mn(II)-oxidizing bacteria (Murray *et al.*, 2007). Although there is still no direct evidence that can show the actual mechanism of Co oxidation, there is reason to suspect that there may be some relationship between Co and Mn oxidation. Co cycling is considered to be coupled to Mn cycling, and Mn-reducing or -oxidizing bacteria could also participate in Co metabolism. For example, Co(II) oxidation in aquatic environments has been shown to be linked to Mn (II) oxidation, a process primarily mediated by bacteria (Murray *et al.*, 2007). Mn-oxidizing bacteria are a group of phylogenetically diverse bacteria which can oxidize soluble Mn(II) to insoluble Mn(III) and Mn(IV), an important process in the formation of manganese deposits (Nealson, 2006). Many more Mn(II)-oxidizing bacteria

were found in environments containing high content of Mn such as ferromanganese crusts where Mn cycling is supposed to be an active process (Nealson, 2006). The most well-characterized Mn(II)-oxidizing bacteria are *Bacillus* sp. strain SG-1 isolated from sandy marine sediments in La Jolla, CA (Rosson & Nealson, 1982), *Leptothrix discophora* strains SS-1 and SP-6, and *Pseudomonas putida* strains MnB1 and GB-1 isolated from freshwater (Tebo *et al.*, 1997; Francis *et al.*, 2001). No bacterium from this study was found to be closely related to the above Mn(II)-oxidizing bacteria. However, two Mn(II)-oxidizing bacteria, *Pseudoalteromonas* sp. LOB-10 and LOB-15, isolated from submarine basalts at the Loihi seamount (Templeton *et al.*, 2005), are related to OTU PC-B73 (92% similarity) within *Alteromonadales* (Fig. 3). In addition, *Rhodococcus* sp. LOB-1, a Mn(II)-oxidizing bacterium, also isolated from submarine basalts at the Loihi seamount (Templeton *et al.*, 2005), had 97% similarity to OTU PC-A102 within *Actinobacteria* (Fig. 8). Considering the high frequency of Mn(II)-oxidizing bacteria in environments containing high levels of Mn, and their phylogenetic diversity, Mn(II)-oxidizing bacteria were expected to be recovered in this study. However, we were not able to identify them only based on 16S rRNA gene sequences.

The Mn oxides produced by Mn(II)-oxidizing bacteria can adsorb trace metals such as Co, Cu, Ni and Mn, which is helpful in the enrichment of such metals (Nealson, 2006). In addition, many other phylogenetically distinct bacteria have the abilities of biosorption and metabolism of metals such as Mn, Co, Fe and Ur, such as *Shewanella* species (Roh *et al.*, 2006), *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* within *Alphaproteobacteria* (Konishi *et al.*, 1997). However, we failed to detect such bacteria in our libraries as most of the clones retrieved were related to uncultured bacteria in which physiological function is unknown. Thus, cultivation experiments should be conducted to isolate any potential bacteria responsible for Co metabolism. Although Co-resistant microorganisms were isolated by Krishnan *et al.* (2006), none of them participated directly in Co cycling. However, some bacteria have been found that are responsible for the immobilization of metals such as Co in the environment, including isolates belonging to the genera *Flavobacterium*, *Pseudomonas* and *Acinetobacter* (Krishnan *et al.*, 2006). Isolation of Co-metabolizing bacteria will aid immensely in understanding the formation of Co-rich crusts and establishing the mechanism of Co oxidation. Cultivation experiments are currently being performed in our laboratory, with the aim of obtaining isolates that can oxidize Co directly. However, no such strain has been cultivated yet. Whether Co-metabolizing microorganisms exist or not requires further research, such as investigation of functional genes potentially involved in metal cycling.

In comparing research of microbial diversity in this study to another high metal content environment, the Pacific nodule province (Xu *et al.*, 2005; Li et al., 2008a, b), similarities and differences in the microbial community composition were found. The phylum *Proteobacteria* was quantitatively predominant in all the taxonomic groups recovered in the present study, especially the subdivisions *Gammaproteobacteria*, *Alphaproteobacteria* and *Deltaproteobacteria*, similar to previous studies of the Pacific nodule province (Xu *et al.*, 2005, 2007, 2008). In addition, quite a few clones in our study are closely related (generally over 97% identity) to those from the Pacific nodules, such as clones from the Pacific nodule cluster in *Gammaproteobacteria* (Fig. 3), clusters 2 and 3 in *Deltaproteobacteria* (Fig. 5) and Gp10 in *Acidobacteria* (Fig. 6), etc. However, microbial communities are different in nodules and crusts. Although *Proteobacteria* was predominant in both nodules and crusts, the dominant subdivisions of *Proteobacteria* are different. *Gammaproteobacteria* (92.9% of the *Proteobacteria* and 69.7% in the clone library) absolutely dominated the 16S rRNA gene clone library constructed from the Pacific nodule province A station, whereas *Alphaproteobacteria* and *Deltaproteobacteria* only accounted for 4% and 1.4% in the clone library, respectively (Xu *et al.*, 2005). *Deltaproteobacteria* (48.6% in the clone library) dominated the metagenomic library from the ES0303 station of the east Pacific Nodule Province, whereas *Alphaproteobacteria* and *Deltaproteobacteria* only accounted for 5.4% and 8.1%, respectively (Xu *et al.*, 2007). *Gammaproteobacteria* (23% in the three clone libraries) was the most abundant group in our study, but *Alphaproteobacteria* (18% in the three clone libraries) and *Deltaproteobacteria* (17.5% in the three clone libraries) also accounted for a large proportion of the bacterial communities. In addition, the dominant orders in *Proteobacteria* subdivisions were also different in these studies. *Gammaproteobacteria* consisted of 73.6% *Alteromonadaceae* in the Pacific nodule province A station (Xu *et al.*, 2005), whereas *Ectothiorhodospiraceae* accounted for 70% of total *Gammaproteobacteria* in this study.

The *Ectothiorhodospiraceae* is a family of purple sulfur bacteria containing chemolithotrophic and photoautotrophic genera retrieved from marine, hypersaline and haloalkaline environments, and are distinguished by the depositing of elemental sulfur outside of their cells (Tourova *et al.*, 2007). The closest cultured relative was *Thioalkalispira microaerophila* strain ALEN 1^T isolated from the soda lake Fazda in Egypt (Sorokin *et al.*, 2002). Genus *Thioalkalispira* contains obligately chemolithoautotrophic sulfur-oxidizing bacteria, which play an important role in sulfur cycling under micro-oxic conditions. It is

speculated that *Ectothiorhodospiraceae* recovered in this study may be involved in sulfur oxidation through chemotrophic metabolism and support the lithosphere microbial ecosystem. Chemoautotrophs are very important in deep-sea environments with poor nutrition and light, as they constitute the primary producers.

The genus *Stenotrophomonas* was detected within *Gammaproteobacteria*, containing five clones with the closest ($\geq$ 98% identity) relative *S. maltophilia* strain LMG 957 (Hauben *et al.*, 1999). *Stenotrophomonas* was reclassified from the genus *Xanthomonas* (Palleroni & Bradbury, 1993) and is a genus of gram-negative bacteria containing species distributed in soil and various aquatic environments. Interestingly, *S. maltophilia*, an opportunistic pathogen widespread in the environment, is able to tolerate high levels (0.1–50 mM) of several heavy metals such as Cd, Pb, Co, Zn, Hg and Ag (Pages *et al.*, 2008), and the heavy metal resistance genes were reported to be acquired from gram-positive bacteria (Alonso *et al.*, 2000). Previously, *S. maltophilia* was found to be the closest relative of novel circumneutral Fe-oxidizing bacterial clones (Emerson & Moyer, 1997), and *S. maltophilia*-like clones were retrieved from ferromanganese deposits in Lechuguilla and Spider Caves (Northup *et al.*, 2003). Here, the detection of *Stenotrophomonas* clones may also indicate these bacteria are Co- and Mn-tolerant.

Genera *Ochrobactrum*, *Hyphomicrobium* and *Filomicrobium* were detected in the phylum of *Alphaproteobacteria* (Fig. 4). Three clones had 99–100% identity with *Ochrobactrum anthropi* strain YZ-1, an opportunistic pathogen and also an exoelectrogenic bacterium which can transfer extracellular electrons to insoluble electron acceptors, such as metal oxides or the anodes of microbial fuel cells (Zuo *et al.*, 2008). Many exoelectrogens are dissimilatory metal-reducing bacteria, such as *Shewanella* and *Pseudomonas* of *Gammaproteobacteria*, *Desulfuromonas* and *Geobacter* of *Deltaproteobacteria*, which could reduce insoluble metals (Fe or Mn oxides etc.).

Three OTUs (PC-C18, PC-B37 and PC-A57), containing 16 clones in cluster 1 in the phylum of *Alphaproteobacteria*, had high similarity (98%) to clone Therm30-F08 recovered from the Thermaikos Gulf of eastern Mediterranean Sea at a depth of 86 m (Polymenakou *et al.*, 2005). Clone Therm30-F08 was retrieved from oxic surface sediment near the coast, in contrast to the closely related clones from this study, which were obtained from deep-sea sediment. In addition, seven clones from *Deltaproteobacteria* were also associated with relatives from oxic surface sediments. It is possible that these bacteria are facultative anaerobes that originated in coastal sediments but have since adapted to life in deep-sea environments.

Bacteria possibly involved in nitrogen-cycling were detected. The only two clones in *Betaproteobacteria* were affiliated with genus *Nitrosospira*, a group of ammonia-oxidizing bacteria (AOB), indicating that *Betaproteobacteria* play an important role in nitrogen cycling through nitrification. The genus *Nitrospina* belongs to the family *Nitrospinaceae* in *Deltaproteobacteria*. *Nitrospina* species are mainly found in marine environments and play an important role in nitrogen cycling by converting nitrites to nitrates (Northup *et al.*, 2003; Weidler *et al.*, 2007). These nitrogen-cycling bacteria were also retrieved from metal-rich sediments from Green Bay (Stein *et al.*, 2001), and ferromanganese deposits in Lechuguilla and Spider caves as well (Northup *et al.*, 2003).

Interestingly, 89 bacterial clones (Figs 3–8, indicated as ocean crust in square brackets) were associated with uncultured clones from oceanic crust (Santelli *et al.*, 2008), including seafloor lavas from the EPR and Hawaiian basalts. These 89 bacterial clones were from *Gammaproteobacteria* (24 clones), *Alphaproteobacteria* (20 clones), *Betaproteobacteria* (two clones), *Deltaproteobacteria* (15 clones), *Acidobacteria* (six clones), *Planctomycetes* (four clones), *Verrucomicrobia* (three clones), *Actinobacteria* (11 clones), *Deferribacteres* (two clones), and unclassified bacteria (two clones). The resemblance of such a large number of bacterial clones (89 clones) between these two studies indicates some relationship or similarity of the sampled environments, and these bacteria may perform similar processes in their respective environments. Like Co-rich crusts, lava crust also contains many reduced elemental species such as Fe, S and Mn (Santelli *et al.*, 2008). The similarity in microbial communities leads us to speculate about a possible relationship of these bacteria to metal metabolism and rock weathering. In addition, the oxidation of metals such as Fe and Mn may fuel the chemoautotrophs in this oceanic lithosphere. In fact, chemoautotrophic Fe-oxidizing bacteria (FeOB) belonging to *Alphaproteobacteria* and *Gammaproteobacteria* have been isolated from the surfaces of weathered rock and metalliferous sediments on the seafloor (Edwards *et al.*, 2003). Thus, we hypothesized that the bacterial communities in Co-rich crusts play an important role in metal metabolic pathways, and the shared 89 clones may be involved in metal cycling.

To make community-wide comparisons between different sites, Venn diagrams were plotted to show the shared and unique OTUs (Fig. 12). The shared OTUs between clone libraries indicate these bacteria had $\geq$ 97% similarity in 16S rRNA gene sequence to each other, and can be found in more than one sample. The unique OTUs indicate these bacteria can be only found in an individual sample. There are 21 OTUs shared between the EPR basaltic lavas from oceanic crust and the study samples, distributed in six groups including *Gammaproteobacteria* (eight OTUs), *Alphaproteobacteria*

(five OTUs), *Deltaproteobacteria* (four OTUs), *Acidobacteria* (two OTUs), *Actinobacteria* (one OTU), and *Betaproteobacteria* (one OTU) (Fig. 12a). The Venn diagram analyses are consistent with the above results of phylogenetic tree analyses, which showed that many bacterial clones were associated with clones from oceanic crust. However, there are no OTUs shared between the EPR seawater and the study samples, indicating that bacterial communities between these two sites had no similarity at species level (97% identity) (Fig. 12a). In the 203 OTUs comprising the 417 bacterial clones, 13 OTUs were shared by the three libraries and 26–32 OTUs overlapped two of the three libraries (Fig. 12b). The common 13 OTUs consisted of *Gammaproteobacteria* (38 clones), *Alphaproteobacteria* (43 clones), *Deltaproteobacteria* (16 clones), *Acidobacteria* (12 clones) and *Actinobacteria* (seven clones), indicating these bacteria were distributed throughout the three sediment layers. Almost half the OTUs were shared between libraries and the remaining half were unique to a particular library. A higher percentage of archaeal OTUs overlapped (Fig. 12c). Four OTUs were shared by all three archaeal clone libraries, ranging from 44% of OTUs in library APC-B to 80% of OTUs in library APC-A. Library APC-C contained all five OTUs in library APC-A, and library APC-B contained all but one of the OTUs in libraries APC-A and APC-C (Fig. 12c). Only library APC-B had four unique OTUs that were not shared by the other two libraries. Both diversity indices and Venn diagram analyses suggest that archaeal communities tend to be homogeneous and less diverse than bacterial communities revealed in this study.

Several OCC were identified, including *Alphaproteobacteria* OCC II and *alpha*-MGI OCC VIII, *alpha*-MGI OCC IX and MGI OCC XI from MGI. These OCCs were recovered from oceanic crust environments such as sediment, basalt and gabbro. Eleven OCCs were named in the study of Mason *et al.* (2007), including seven bacterial OCCs and four OCCs from MGI. Only the *alpha*-MGI OCC IX clade consisted entirely of sequences from basalts and was predicted to be a basalt-adapted ecotype (Mason *et al.*, 2007). The remaining OCC clades were composed of sequences from both sediments and basalts (Mason *et al.*, 2007). However, clones belonging to *alpha*-MGI OCC IX clade were found in the sediments from the Co-rich crust area in this study and Antarctic bathypelagic sediments as indicated by phylogenetic tree (Fig. 9), providing evidence that the *alpha*-MGI OCC IX clade may not be exclusively endemic to basalts. However, these OCC bacteria are most likely indigenous to oceanic crust including basalts and surrounding sediments. The 16S rRNA gene sequence of *N. maritimus* SCM1 is 96–97% similar to those of *Alpha*-MGI OCC VIII and *alpha*-MGI OCC IX (Fig. 9), which is consistent with the results of Mason *et al.* (2007). The recovery of a large number of these archaea in our libraries also supports the previous prediction that ammonium-oxidizing archaea may exist actively in basalt environments (Mason *et al.*, 2007). All the *Crenarchaeota* were classified as MGI (Fig. 9). MGI was first identified in seawater (Delong, 1992; McCallum & Davis, 1992) and later discovered in marine sediments as well (Sørensen *et al.*, 2004). In 2005, the first isolated ammonia-oxidizing archaeon *N. maritimus* SCM1 within MGI confirmed aerobic nitrification in the *Archaea* domain (Konneke *et al.*, 2005). All the *Crenarchaeota* obtained in this study shared $\geq$ 96% similarity with *N. maritimus* SCM1, indicating a potential role in nitrogen cycling. However, such a potential role should be tested by an investigation of functional genes such as *amo*A encoding ammonia monooxygenase. Our results were consistent with previous studies in which only MGI was detected by hybridization with specific probe in the Pacific nodule province A core (Xu *et al.*, 2005). In addition, abundant mesophilic *Crenarchaeota* were also found from ferromanganese deposits in Lechuguilla and Spider caves (Northup *et al.*, 2003). However, the ecological roles and importance of these archaeal phylotypes were poorly understood due to little cultivation success, which excluded analysis of the interaction with crust formation and potential metabolic pathways. Thus, isolation experiments and functional gene analysis would be beneficial to our understanding of this abundant group.

Only one clone was found to be affiliated with *Euryarchaeota*. This euryarchaeotal clone in OTU APC-B37 may present a novel lineage, because it formed a distant cluster from known lineages and had only 90% identity to the most similar clone 060329_T2S2_S_T_SDP_056, retrieved from sediment of Lake Pontchartrain (Amaral-Zettler *et al.*, 2008) (Fig. 10). One possible explanation for the huge bias between *Crenarchaeota* and *Euryarchaeota* detected in our libraries is that *Crenarchaeota* increased significantly with depth in the water column, whereas *Euryarchaeota* were more abundant in surface water (Karner *et al.*, 2001; Church *et al.*, 2003). Therefore, it is not surprising that more *Crenarchaeota* were retrieved in sediments close to the bottom water.

During 1970s, deep-sea mining was promoted by the skyrocketing price of metals, especially critical and strategic metals. Afterwards, the deep-sea metal rush was tempered due to falling metal prices and the lack of mining technology (more information in Glasby, 2000). However, the huge metal resources in the ocean are promising for future exploitation. An important aspect of deep-sea mining is the impact on environment, especially on microbial ecosystems. As mining will inevitably change the microbial communities by removing some species and

bringing others from the surface or other environments, and by changing the physical and chemical conditions (Halfar & Fujita, 2002), it is critical to assess the microbial diversity before and after mining. Thus, a better understanding of the immense microbial diversity inhabiting Co-rich crusts ecosystems is helpful in evaluating the possible impacts of deep-sea mining on microbial life.

In conclusion, this is the first study of microbial diversity in deep-sea sediment from the Co-rich crust region in the Pacific Ocean. Bacterial diversity of the studied samples is much higher than archaeal diversity. Bacterial communities were potentially involved in metal cycling and sulfur oxidizing in Co-rich crusts, whereas archael communities were possibly involved in nitrogen cycling. The overlap of bacterial communities between oceanic crust and Co-rich crusts in the present study indicates that these bacteria may be involved in some similar processes performed in the deep-sea floor. This study gives us the first up-close view of microbial communities indigenous to Co-rich crust environments, which will be helpful in guiding crust exploration and deep-sea mining. As there are hundreds of thousands of seamounts in the vast ocean on which Co-rich crusts may form, researchers are expected to explore the ecosystems and the microbial communities in these poorly known crust regions soon.

Acknowledgements

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