The Journal of Microbiology (2010) Vol. 48, No. 5, pp. 573-585
DOI 10.1007/s12275-010-0151-5

Bacterial Diversity in the Sediment from Polymetallic Nodule Fields of the Clarion-Clipperton Fracture Zone

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(Received April 19, 2010 / Accepted June 21, 2010)

The Clarion-Clipperton Fracture Zone (CCFZ) is located in the northeastern equatorial Pacific and contains abundant polymetallic nodules. To investigate its bacterial diversity, four libraries of 16S rRNA genes were constructed from sediments of four stations in different areas of the CCFZ. In total, 313 clones sequenced from the 4 libraries were assigned into 14 phylogenetic groups and 1 group of 28 unclassified bacteria. High bacterial diversity was predicted by the rarefaction analysis. The most dominant group overall was Proteobacteria, but there was variation in each library: Gammaproteobacteria was the most dominant group in two libraries, E2005-01 and ES0502, while Alphaproteobacteria and Deltaproteobacteria were the most dominant groups in libraries EP2005-03 and WS0505, respectively. Seven groups, including Alphaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Betaproteobacteria, Acidobacteria, Actinobacteria, and Bacteroidetes, were common to all four libraries. The remaining minor groups were distributed in libraries with different patterns. Most clones sequenced in this study were clustered with uncultured bacteria obtained from the environment, such as the ocean crust and marine sediment, but only distantly related to isolates. Bacteria involved in the cycling of metals, sulfur and nitrogen were detected, and their relationship with their habitat was discussed. This study sheds light on the bacterial communities associated with polymetallic nodules in the CCFZ and provides primary data on the bacterial diversity of this area.

***Keywords*:** CCFZ, sediment, bacterial diversity, 16S rRNA gene, polymetallic nodule

The Clarion-Clipperton Fracture Zone (CCFZ) located in the northeastern equatorial Pacific is considered one of the most commercially important nodule areas of the World Ocean (Thiel, 2001). Polymetallic nodules are also known as manganese nodules or ferromanganese nodules because of their high manganese and iron content and the concentration of other heavy metals, such as copper and nickel (Margolis and Burns, 1976). They can be found in many regions in the vast ocean at water depths ranging from 4,000 to 6,000 m, but they mainly aggregate in CCFZ areas as mature and large nodules (Glasby *et al.*, 1982). The mechanism of formation of manganese nodules is as yet unclear, but the function of microbes and the impact of physical and chemical factors in the abyssal environment are critical (Wang and Müller, 2009; Wang *et al.*, 2009a, 2009b). Some iron- and manganese-oxidizing/reducing bacteria have been isolated, such as *Shewanella*, *Hyphomicrobium*, *Leptothrix*, and *Pseudomonas*. In addition, some direct evidence has been provided using the detection of exolithobiontic and endolithobiontic microbial biofilms in manganese nodules to demonstrate their biogenic origin (Wang *et al.*, 2009a, 2009b). Therefore, it is predicted that bacteria that actively participate in metal cycling may be found or even be dominant in the sediments from the CCFZ.

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China Ocean Mineral Resources R & D Association (COMRA) has a contract area for exploration of polymetallic nodules in the CCFZ that covers about 7.5×10^4 km^2, including two regions (east and west regions). The microbial diversity near the west region of the COMRA's contract area has been studied using culture-dependent and -independent methods (Xu *et al.*, 2005). Six phyla have been detected in bacterial communities, with a dominance of Gammaproteobacteria. However, few investigations have been done on the microbial diversity in other Chinese claim areas of the CCFZ. Here we studied the microbial diversity of four stations in the CCFZ by constructing 16S rRNA gene libraries, with the hope of discovering special bacterial communities related to manganese nodules.

Because of the commercial and strategic interests in marine polymetallic nodules, deep-sea mining has great potential in the future. However, mining has some impact on the primary environment by disturbing the original ecosystem and changing the physical and chemical conditions (Alexis *et al.*, 2006). Due to the great economic potential of metal exploitation in the CCFZ, it is very important to understand the natural microbial diversity in this area before mining. A previous study has suggested that the environment requires a very long term to recover from disturbance, making us think before leaping into deep-sea mining (Alexis *et al.*, 2006). Thus, investigation into the natural microbial diversity of the CCFZ is necessary. In

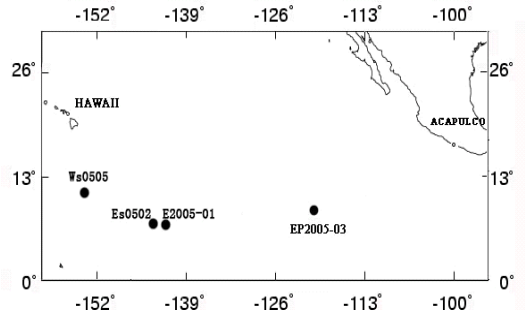

Fig. 1. Map of the four stations sampled in this study

this study, we surveyed the primary composition of bacterial communities of four stations in the CCFZ for the first time. This study serves as a scientific basis for the selection of environmental reference zones for deep-sea mining and as a primary study of bacterial diversity in northeast Pacific polymetallic nodule fields.

Materials and Methods

Sample collection

Deep-sea sediments of the four stations were collected using a TV multicorer from a CCFZ polymetallic nodule province by R/V "DA YANG YI HAO" in August 2005. The sampling stations are characterized in Table 1 and Fig. 1. Core samples of sediment thicker than 10 cm were divided into 10 1-cm sections after collection. Subsamples were put into 10-ml sterile tubes and stored at -20°C until laboratory analysis. To investigate the microbial diversity, sediments from 0-10 cm were mixed for each of three stations, WS0505, ES0502, and E2005-1, while sediments from 0-22 cm were mixed for station EP2005-03. The average biogeochemical properties of the sediment samples were determined using energy dispersive X-ray fluorescence (EDXRF) for major and trace elements.

Table 1. Characteristics of the sampling stations

Parameters	Stations			
	WS0505	ES0502	E2005-01	EP2005-03
Latitude (N)	10°2.3′	8°22.5′	8°00′	9°00′
Longitude (W)	154°0.9′	145°23.8′	145°00′	120°25′
Depth (m)	5120	5307	5197	4299
pH[a]	7.78	7.80	ND	7.82
DO (μM)[a]	360	315	ND	330
DIP (μM)[a]	2.55	2.53	ND	2.50
DIN (μM)[a]	22.31	45.00	ND	45.07
TP (μM)[a]	2.94	3.93	ND	2.92
TN (μM)[a]	39.23	46.92	ND	33.07
SiO_2 (%)[b]	50.83	53.33	54.68	ND
Al_2O_3 (%)[b]	11.13	11.72	11.43	ND
Fe_2O_3 (%)[b]	6.77	5.36	5.57	4.15
CaO (%)[b]	1.61	0.91	1.04	ND
MnO (%)[b]	1.33	0.29	0.47	0.09
Type of sediment	Silicon clay	Silicon clay	Silicon clay	Calcic and Silicon clay

[a] Parameters of near-bottom water, which are the average values of a 50-m water column near the sea floor.
[b] Parameters of sediments. ND, not determined; DO, dissolved oxygen; DIP, dissolved inorganic phosphorus; DIN, dissolved inorganic nitrogen; TP, total phosphorus; TN, total nitrogen.

Total DNA extraction and purification

Samples were thawed overnight at 4°C before DNA extraction. Two different methods were used to extract DNA from sediment, i.e., using the FastDNA Spin kit for soil (BIO101, USA) and following the established method of (Krsek and Wellington, 1999) with some modifications. In detail, 1 g of sediment sample (wet weight) was mixed with 2 ml of DNA extraction buffer [100 mM Tris-HCl, 100 mM Na_2-EDTA, 100 mM phosphate-buffered solution, 1.5 M NaCl, and 1% cetyltriethylammnonium bromide (CTAB), pH 8.0] and treated three times with liquid nitrogen and boiling water consecutively. Lysozyme was added at a final concentration of 50 mg/ml, the sample was incubated for 1 h at 37°C with shaking at 120 rpm and proteinase K was then added at a final concentration of 100 mg/ml under the same incubation conditions. Next, SDS was added at a final concentration of 2% and incubated for 1 h at 60°C. The supernatant was collected using centrifugation for 1 min at 12,000×g. The precipitate was re-extracted as follows: 1 ml of DNA extraction buffer and 200 μl of 20% SDS were added to the precipitate and incubated for 10 min at 60°C, and the supernatant was collected using centrifugation for 1 min at 12,000×g. Then, 0.6 volume of isopropanol was added for precipitation for 1 h, and the sample was then centrifuged for 30 min at 16,000×g. The precipitate was washed with 70% ethanol and dried at room temperature before being dissolved in 50 μl of TE buffer. The crude DNA extract was purified using a PCR Clean-up kit (QIAGEN, USA).

PCR amplification

The total DNA extracted using the two different methods described above was amplified. PCR was performed using primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTG TTACGACTT-3′) (Webster *et al.*, 2003). The 50 μl PCR reaction system contained 5 μl of 10× buffer, 0.2 mM dNTPs, 20 pM of each primer and 1 U of *Taq* DNA polymerase. The PCR program was performed as follows: denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 75 sec and a final extension at 72°C for 7 min. PCR products were analyzed using 1% agarose gel electrophoresis and recovered using a gel recovery kit (QIAGEN). The recovered DNA products from the two different extraction methods were mixed for each sampling station to construct 16S rRNA gene libraries.

Library construction and sequencing

The recovered PCR products were ligated into pMD18-T vectors (TaKaRa, Japan) at 16°C for 6 h and then transformed into competent *Escherichia coli* TOP10 cells. Four libraries of 16S rRNA genes were constructed from the four sampling stations, respectively: EP2005-03, E2005-01, ES0502, and WS0505. Positive clones were selected using the blue-white selection method and randomly sequenced using M13 primers on an ABI 377 DNA sequencer.

Rarefaction analysis and phylogenetic analysis

Sequences were checked with the CHECK_CHIMERA program in the Ribosomal Database Project (RDP) to exclude possible chimeras (Maidak *et al.*, 2001). The remaining sequences were assigned into

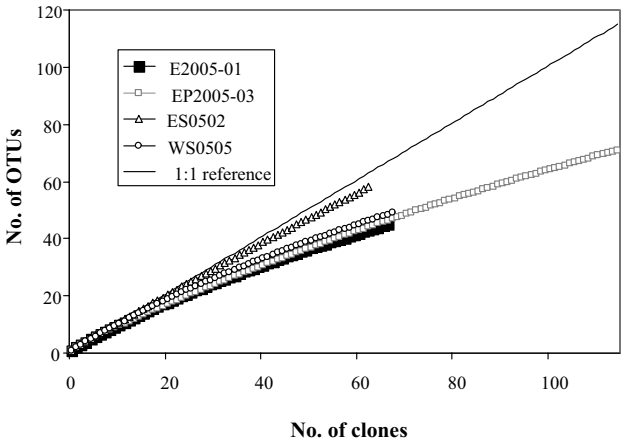

Fig. 2. Rarefaction curves of the four bacterial libraries. The 1:1 reference curve indicates that each sequenced clone belongs to a different OTU.

operational taxonomic units (OTUs) at a level of sequence similarity ≥97%, and rarefaction curves were calculated using the Distance-Based OTU and Richness (DOTUR) program (Schloss and Handelsman, 2005; Liao *et al.*, 2009). All of the OTUs were classified and compared using the RDP. Relatives were downloaded for the construction of phylogenetic trees using MEGA software version 4.0 using the neighbor-joining method with the Kimura 2-parameter model at the bootstrap value of 1000 (Tamura *et al.*, 2007).

Sequence accession numbers

All 16S rRNA gene sequences obtained in this study were deposited in the GenBank nucleotide sequence databank under the accession numbers GU983390 to GU983612.

Results

Rarefaction analysis and phylogenetic analysis

In total, 313 sequences were obtained after excluding chimeras, including 67, 115, 63, and 68 sequences in libraries E2005-01, EP2005-03, ES0502, and WS0505, respectively. The 313 sequences were assigned into 223 OTUs (≥97% similarity), including 45, 71, 58, and 49 in libraries E2005-01, PE2005-03, ES0502, and WS0505, respectively. Rarefaction curves of the four libraries were constructed based on the observed OTUs for each library (Fig. 2). Compared with the 1:1 reference curve, rarefaction curves of the four libraries had smaller slopes but indicated high bacterial diversity. Although rarefaction curves did not reach a plateau, they did reveal the relative diversity of the four libraries; library ES0502 had the highest diversity, while library E2005-01 had the lowest diversity.

The 4 libraries contained 14 phylogenetic groups and 28 unclassified bacteria (Fig. 3). The most dominant group was Alphaproteobacteria, followed by Gammaproteobacteria, accounting for 23.3% and 21.7% of the 313 sequences, respectively. The dominant non-Proteobacteria group was Acidobacteria, which accounted for 7% (22) of the total sequences. Betaproteobacteria, Chloroflexi, Firmicutes, Verrucomicrobia, and candidate division TM6 were minor groups, with ≤9 sequences each. Phylogenetic trees of the 14 phylogenetic groups and unclassified bacteria were constructed to show the phylogenetic relationships with close relatives (Figs. 4-9). Most of the relatives were uncultured bacterial clones from marine environments, such as deep-sea sediments and the ocean crust, whereas only a few clones obtained from the present study could be related to isolates.

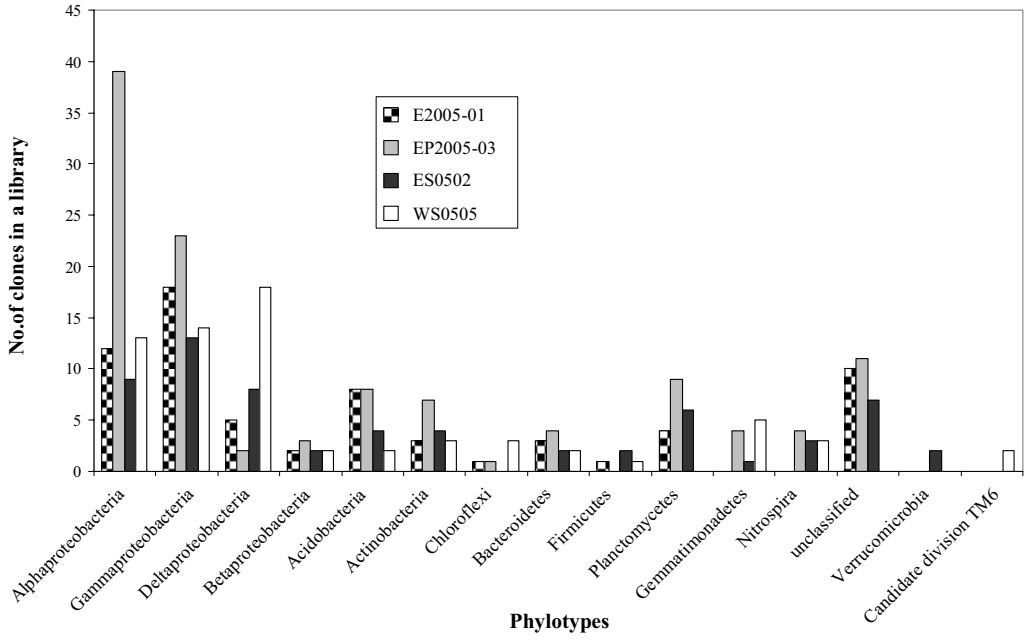

Fig. 3. Phylotype distribution of the four libraries.

Alphaproteobacteria

Alphaproteobacteria from library PE2005-03 comprised 14 OTUs with 39 clones, accounting for the largest percentage of Alphaproteobacteria in the four libraries. Three clusters were identified in Alphaproteobacteria, including *Rhodospirillaceae*, *Hyphomicrobiaceae*, and *Marinovum* (Fig. 4). In addition, four unknown clusters, named cluster 1 to cluster 4, were detected that were unrelated to any cultures. The most frequently sequenced OTU was EP2005-03a27, with 20 closely related clones grouped into cluster 4. In *Rhodospirillaceae*, two OTUs were found to be associated with isolates *Pelagibius litoralis* strain CL-UU02^T and *Skermanella* sp. Ph-4-1. In *Hyphomicrobiaceae*, clones were most closely related to the uncultured clone EPR3970-MO1A-Bc67 from the ocean crust, except OTU EP2005-03a33, and formed a sister branch of genera *Pedomicrobium* and *Hyphomicrobium*. In *Marinovum*, OTU EP2005-03b35 shared 96% identity with *Marinovum* sp. YCSA81. The remaining clones were related to uncultured bacteria from marine environments, such as marine basalts, the Pacific nodule province, the South-Atlantic Ocean and hydrothermal regions.

Gammaproteobacteria

Gammaproteobacteria were the second dominant group with 44 OTUs, the maximum found in all the groups. *Pseudomonas*, *Ectothiorhodospiraceae*, and Legionellales were detected with cultured relatives (Fig. 5). Clones grouped into *Pseudomonas* were most similar to uncultured clones from the ocean crust and were closely related to the exclusive isolated relative *Pseudomonas* sp. NB1-h. Clones belonging to *Ectothiorhodospiraceae* were clustered with uncultured bacteria from the ocean crust and the South-Atlantic Ocean and formed a sister branch with isolated strains. Three OTUs in Legionellales shared low similarities with each other and were distantly related to the cultured strains in this group. In the four unidentified clusters (clusters 1-4), most clones shared high similarities (≥98%) with relatives retrieved from the South-Atlantic Ocean. Cluster 3 was characterized by relatives of endosymbionts from marine animals. Cluster 4 was the most diverse, containing isolates from different orders.

Deltaproteobacteria and Betaproteobacteria

Library WS0505 comprised 12 OTUs with 18 clones belonging to Deltaproteobacteria, which was more than the other three libraries. *Nitrospinaceae*, Myxococcales, Syntrophobacterales, and Desulfuromonadales were detected within Deltaproteobacteria as well as two unidentified clusters (Fig. 6). All of the OTUs were closer to uncultured bacterial relatives than cultured strains. However, the only OTU, ES0502c4, in Myxococcales was distantly associated with *Chondromyces pediculatus* strain Cm p17 and had no uncultured relative. The Desulfuromonadales group was the largest in the four identified groups, with the closest isolates *Geoalkalibacter subterraneus* strain Red1 and Desulfuromonadales bacterium Tc37 as the outgroup within Desulfuromonadales.

Two OTUs, WS0505b15 and ES0502a26, were distantly branched from other clones of Betaproteobacteria (Fig. 6), which were almost identical with clones from the South-Atlantic Ocean. OTU ES0502a26 shared high similarity with clone YJQ-2 obtained from a microbial mat in a hot spring, while OTU WS0505b15 only matched the distant clone 004A5 from sediment of the Northern Bering Sea.

Acidobacteria, Actinobacteria, and Nitrospira

As the biggest non-Proteobacteria group, Acidobacteria (Fig. 7) contained six distinct subgroups, including Gp10, Gp21, Gp22, Gp5, Gp9, and Gp6. As of 2007 (Barns *et al.*, 2007), the Acidobacteria phylum contained 26 subgroups or 26 genera recognized in the RDP taxonomic hierarchy. Most of these subgroups were uncultured Acidobacteria obtained from various environments, including metal-contaminated sediment, acidic drainage, and other environments. All of the six subgroups detected in this study were affiliated with uncultured clones from the ocean crust, deep-sea hydrothermal regions, and the South-Atlantic Ocean.

OTU ES0502c21 in Actinobacteria shared 90% similarity with *Solirubrobacter* sp. BXN5-15, forming a deep branch in the phylogenetic tree (Fig. 7). The remaining Actinobacteria clones were associated with uncultured relatives from the ocean crust, the Pacific nodule province, soil and the South-Atlantic Ocean, except OTU EP2005-03b29, which formed a sole branch showing very low identity with others.

Nitrospira formed a sister branch with Actinobacteria in the phylogenetic tree (Fig. 7). Half of the clones belonging to Nitrospira were closely clustered with clone E75 from sediment of the Pacific nodule province and clone P0X3b1A09 from the ocean crust, while the remaining half was associated with clones from the South-Atlantic Ocean.

Planctomycetes and minor groups

Figure 8 shows the phylogenetic relationships of Planctomycetes and other minor groups, including Bacteroidetes, Chloroflexi, Verrucomicrobia, and candidate division TM6. Planctomycetes formed a large branch compared with other minor groups. The OTU EP2005-03c8 (with two related clones) was identical with clone Ucd1519 from the South-Atlantic Ocean. A few clones were related to uncultured relatives from non-marine environments, including mangrove soil and microbial fuel cells. However, clones belonging to Planctomycetes were not found in library WS0505. *Cytophaga* sp. NB1-m was the only isolate detected in Bacteroidetes, sharing 98% similarity with OTU EP2005-03c30. The remaining clones were related to uncultured bacteria from diverse environments, including marine basalts, marine sediments, an iron oxide chimney-like structure and biofilms. Only two OTUs (WS0505b19 and WS0505c24) from library WS0505 were grouped into candidate division TM6, a unique characteristic of library WS0505. Likewise, two OTUs, ES0502c14 and ES0502c20, were found exclusively in library ES0502. Chloroflexi was divided into two separate branches with high bootstrap values and was not found in library ES0502.

Firmicutes, Gemmatimonadetes, and unclassified bacteria

Firmicutes and Gemmatimonadetes were not found in libraries PE2005-03 and E2005-01, respectively. OTU ES0502c8 showed low similarity with the other clones of Firmicutes and was distantly related to cultured strains (Fig. 9). The remaining three OTUs of Firmicutes were clustered with uncultured clones and distinctly apart from isolates

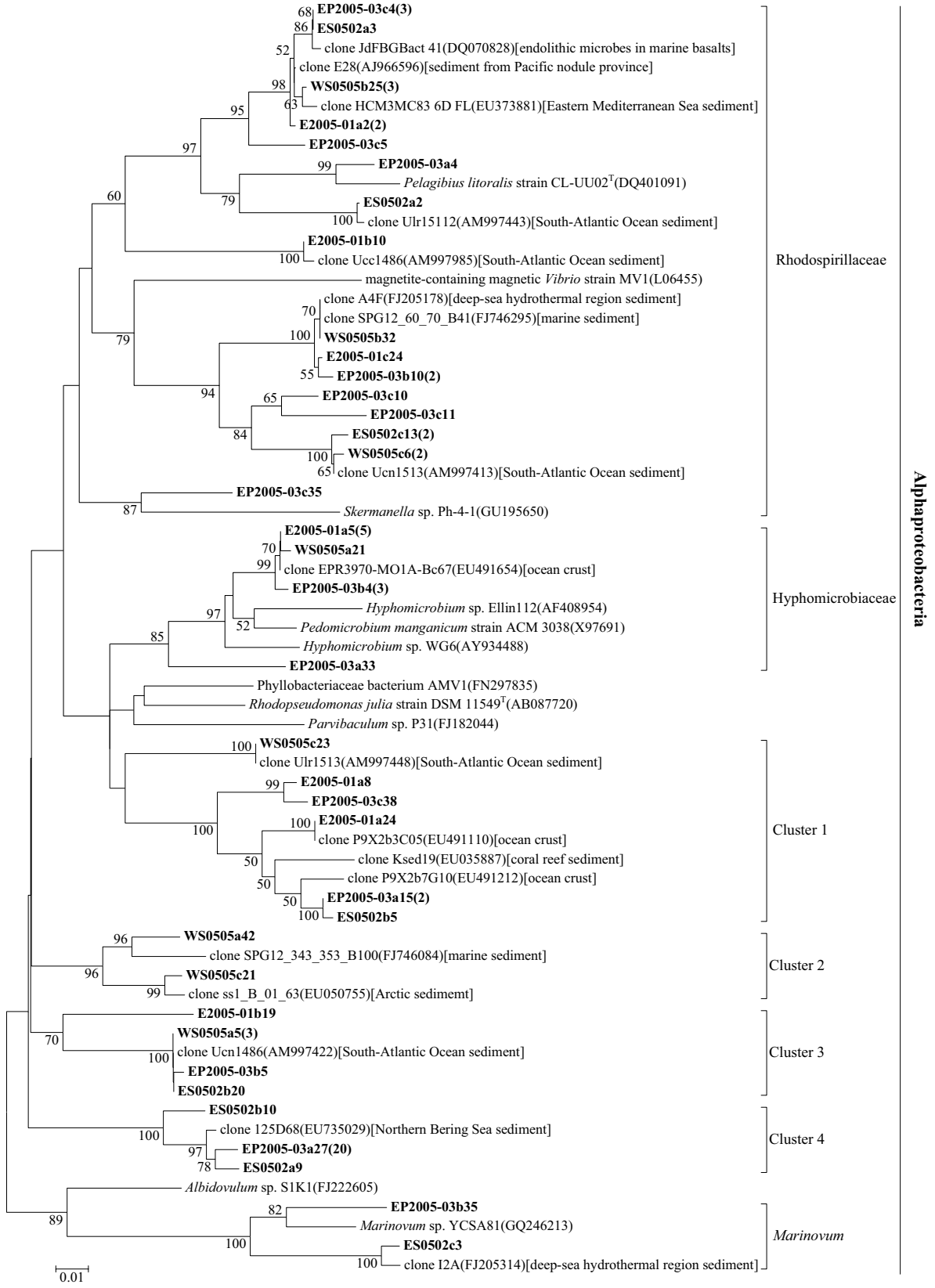

Fig. 4. Phylogenetic tree of Alphaproteobacteria from the four libraries. Clones sequenced in this study are in bold, and numbers in parentheses indicate the total similar sequences (≥2) in OTUs. The environments where relatives were obtained are shown in square brackets. Bootstrap values less than 50% are not shown. Scale represents a 1% difference in sequence.

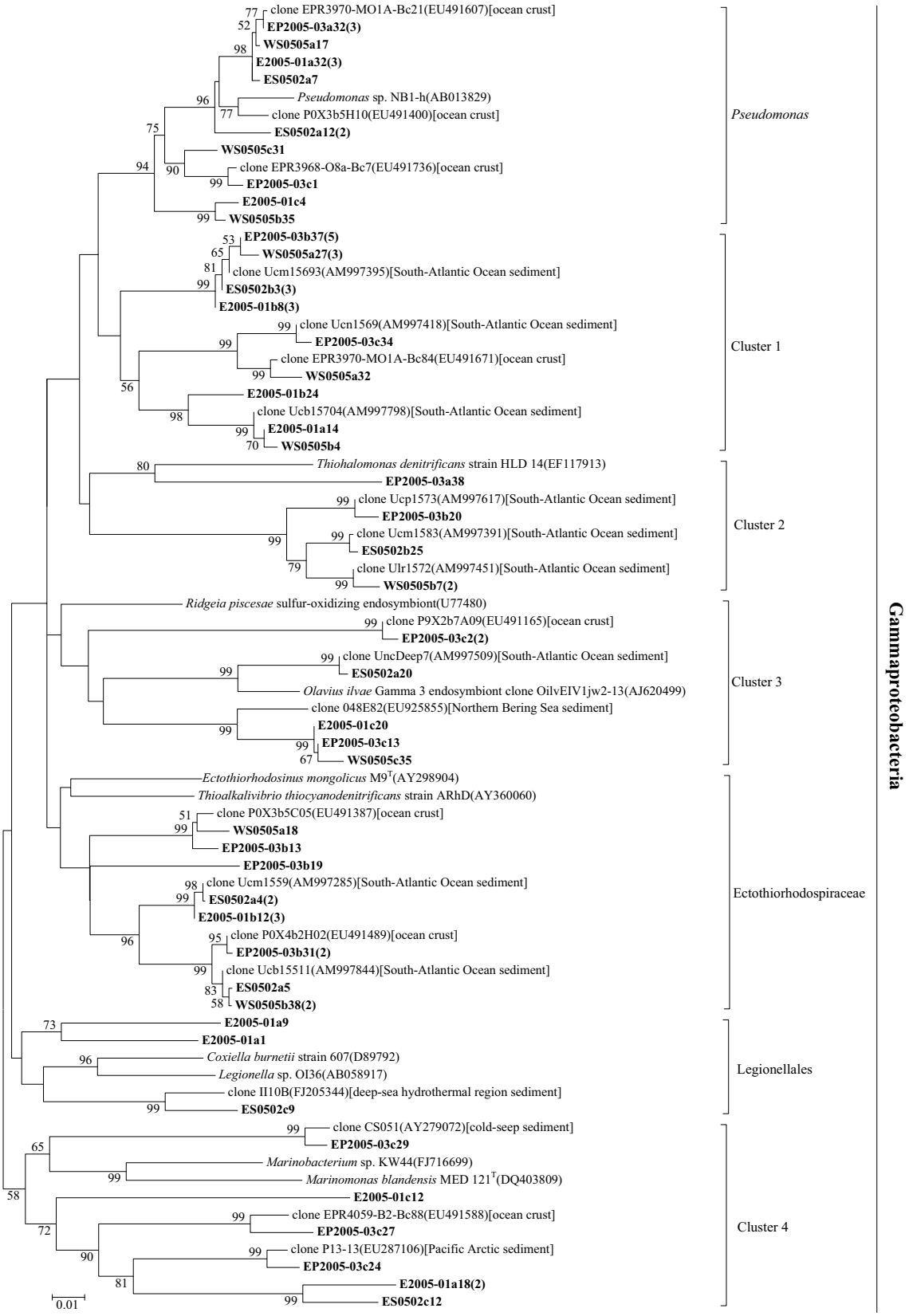

Fig. 5. Phylogenetic tree of Gammaproteobacteria from the four libraries. Clones sequenced in this study are in bold, and numbers in parentheses indicate the total similar sequences (≥2) in OTUs. The environments where relatives were obtained are shown in square brackets. Bootstrap values less than 50% are not shown. Scale represents a 1% difference in sequence.

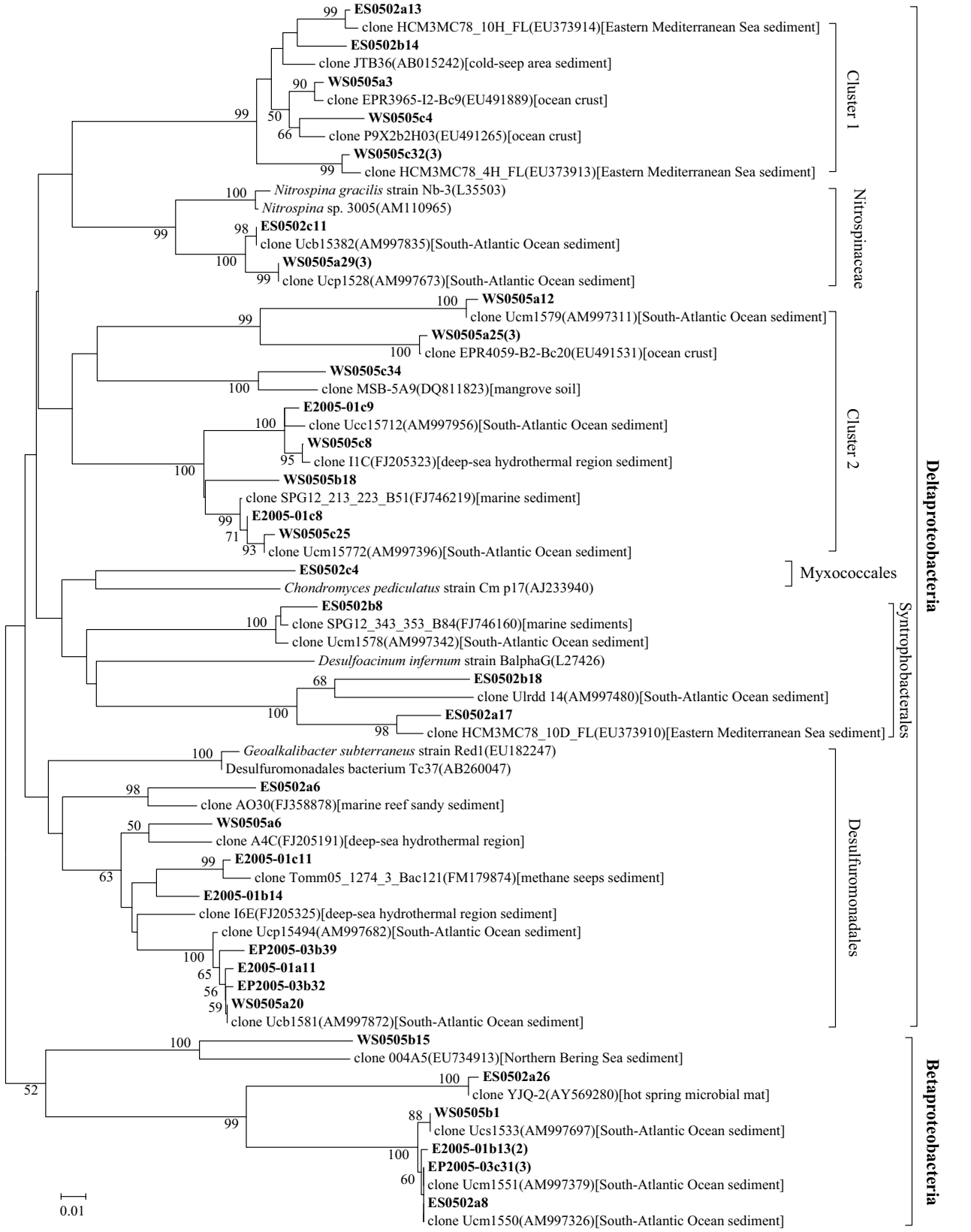

Fig. 6. Phylogenetic tree of Betaproteobacteria and Deltaproteobacteria from the four libraries. Clones sequenced in this study are in bold, and numbers in parentheses indicate the total similar sequences (≥2) in OTUs. The environments where relatives were obtained are shown in square brackets. Bootstrap values less than 50% are not shown. Scale represents a 1% difference in sequence.

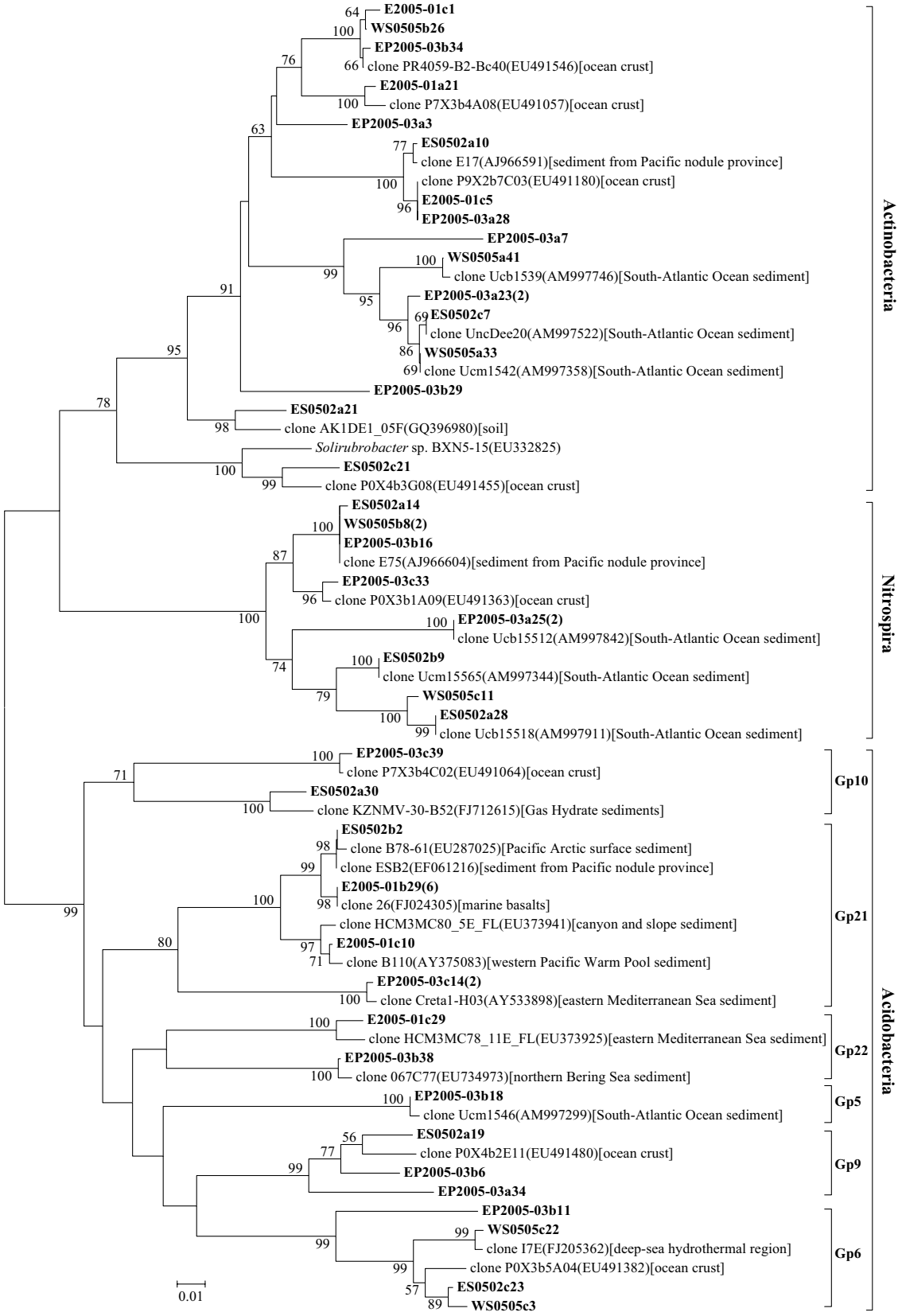

Fig. 7. Phylogenetic tree of Acidobacteria, Actinobacteria, and Nitrospira from the four libraries. Clones sequenced in this study are in bold, and numbers in parentheses indicate the total similar sequences (≥2) in OTUs. The environments where relatives were obtained are shown in square brackets. Bootstrap values less than 50% are not shown. Scale represents a 1% difference in sequence.

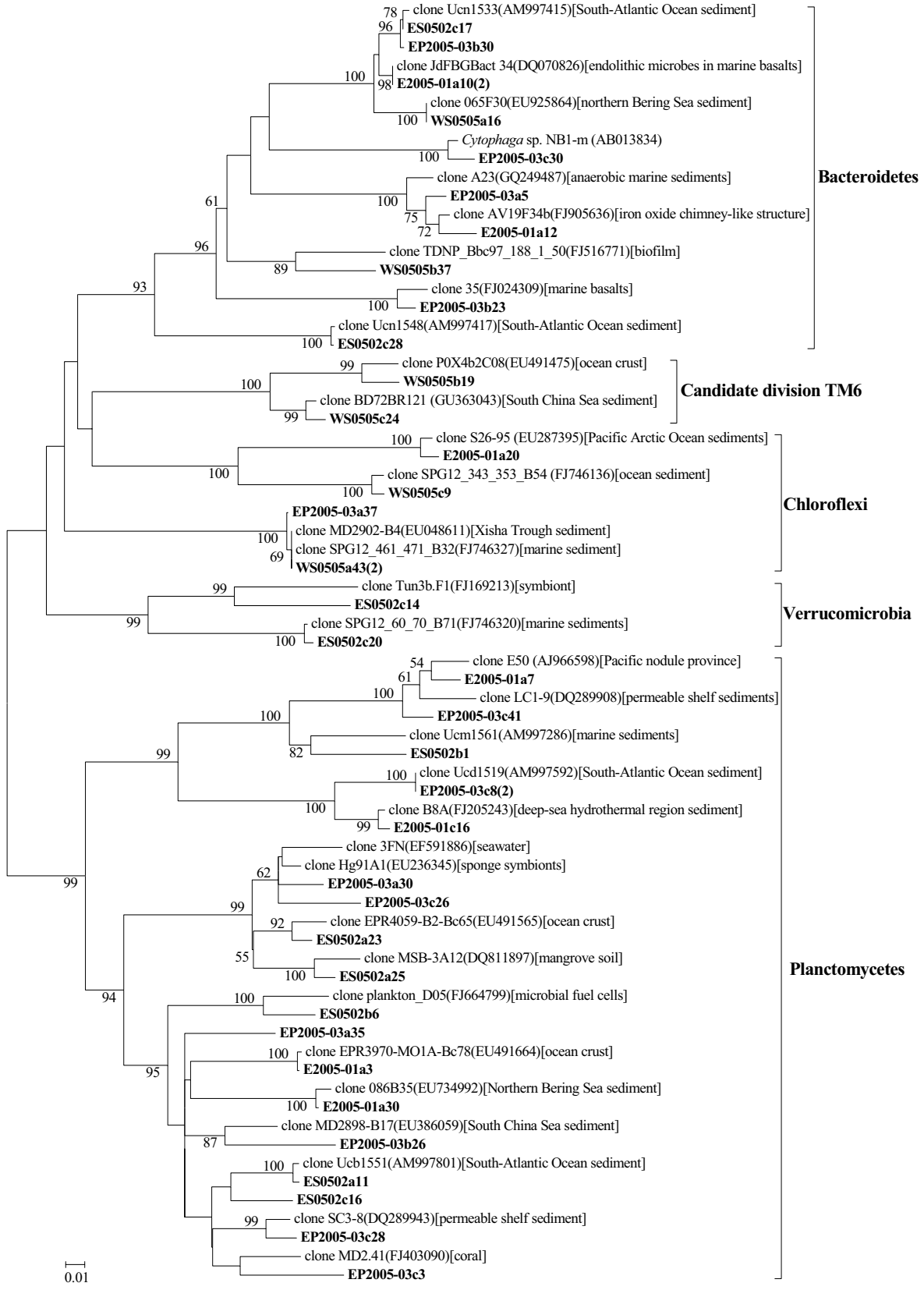

Fig. 8. Phylogenetic tree of Bacteroidetes, Chloroflexi, Candidate division TM6, Planctomycetes, and Verrucomicrobia from the four libraries. Clones sequenced in this study are in bold, and numbers in parentheses indicate the total similar sequences (≥2) in OTUs. The environments where relatives were obtained are shown in square brackets. Bootstrap values less than 50% are not shown. Scale represents a 1% difference in sequence.

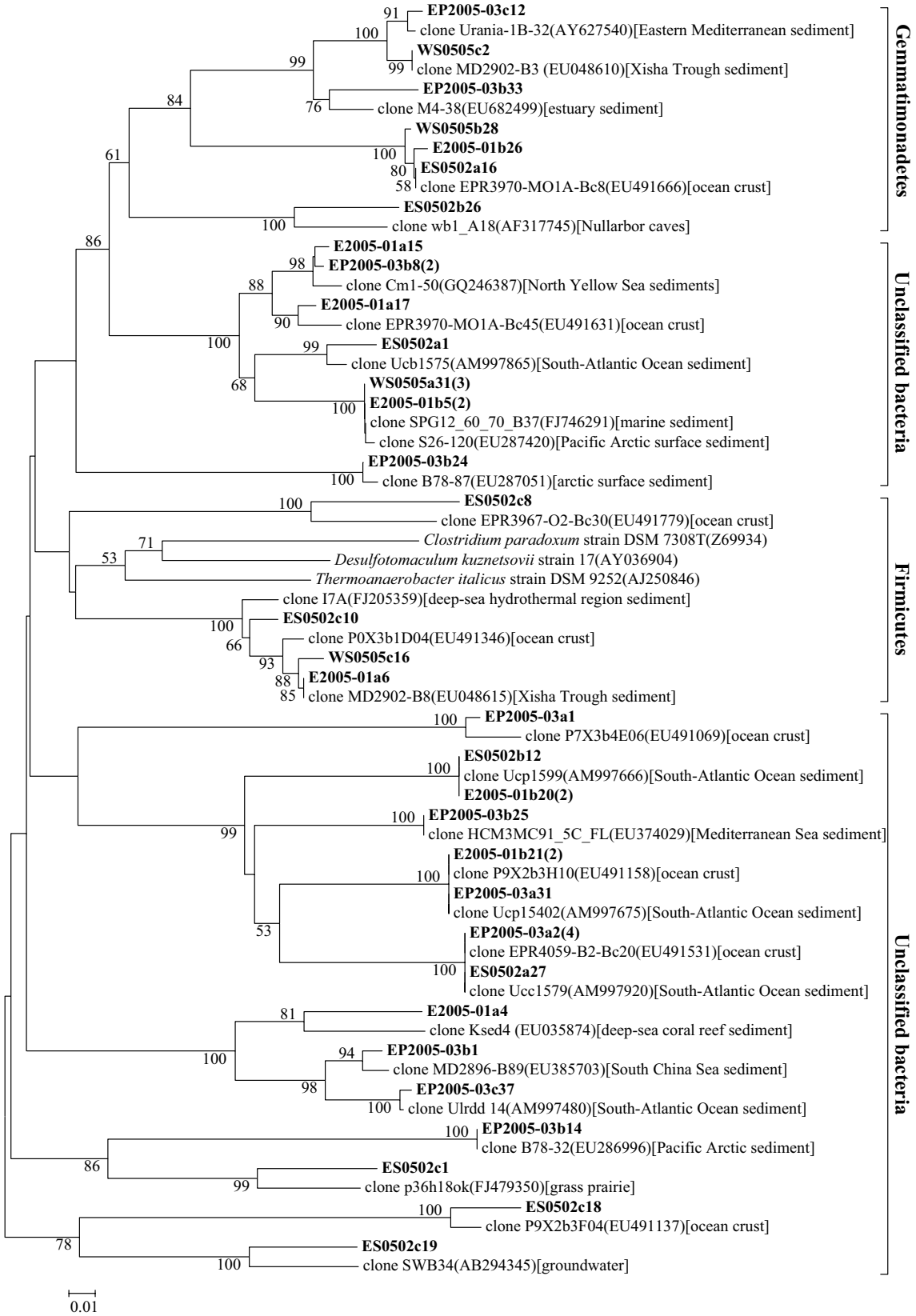

Fig. 9. Phylogenetic tree of Gemmatimonadetes, Firmicutes, and unclassified bacteria from the four libraries. Clones sequenced in this study are in bold, and numbers in parentheses indicate the total similar sequences (≥2) in OTUs. The environments where relatives were obtained are shown in square brackets. Bootstrap values less than 50% are not shown. Scale represents a 1% difference in sequence.

Thermoanaerobacter italicus strain DSM 9252, *Desulfotomaculum kuznetsovii* strain 17, and *Clostridium paradoxum* strain DSM 7308^T. Unlike the other clones in Gemmatimonadetes, OTU ES0502b26 was associated with a clone from Nullarbor caves, a nonaqueous environment, and formed a distant branch from other clones within Gemmatimonadetes. Unclassified bacteria, comprising 28 clones that were unable to be assigned into a taxonomic group, distributed in three libraries, except library WS0505. A total of 11 unclassified bacteria were branched between Firmicutes and Gemmatimonadetes, including a sister branch of Gemmatimonadetes (cluster 1) and a distantly branched OTU EP2005-03b24 (cluster 2). The remaining 17 unclassified clones formed 4 distinct branches, including cluster 3 to cluster 6, and belonged to different phylogenetic groups that were unable to be identified. These unclassified clones may belong to new groups waiting to be identified and may be unique in the studied environment.

Discussion

Comparison of the four sampling stations

The four sampling stations in this study were located in different areas of the CCFZ (Fig. 1 and Table 1). Station WS0505 belonged to the west part of COMRA's contract area, while stations ES0502 and E2005-01 belonged to the east part. Station EP2005-03 was close to Mexico and outside COMRA's contract area. The abundance of polymetallic nodules was highest in station WS0505 and decreased in stations ES0502 and E2005-01. However, much fewer nodules could be found around station EP2005-03. The Fe_2O_3 and MnO content in sediment was highest in station WS0505 and lowest in station EP2005-03, while it was similar in stations ES0502 and E2005-01 (Table 1). In addition, other characteristics varied between stations (Table 1). Different bacterial communities were assumed to be associated with the four different stations. Actually, bacterial diversity and community structure were dissimilar in the four libraries constructed from the four stations. In libraries E2005-01 and ES0502, the most dominant group was Gammaproteobacteria, followed by Alphaproteobacteria, while the predominant groups were Alphaproteobacteria and Deltaproteobacteria in libraries EP2005-03 and WS0505, respectively. It was considered that the dominant bacterial groups were largely related to their corresponding living environment, which is consistent with the geographic relationship and characteristics of the four sampling stations. In addition, the subordinate groups distributed in the four libraries in different patterns (Fig. 3). The bacterial diversity also varied between libraries. Rarefaction analysis revealed that the most diverse library was ES0502, while the three other libraries had lower diversity, according to the rarefaction curves (Fig. 2). Previous studies have shown that almost all environments are dominated by only a few preponderant phylotypes but with a long tail of rare taxa (Fuhrman, 2009). Thus, a small library containing approximately 100 clones reveals the most abundant taxa but misses most of the rare taxa, except in samples with extremely low diversity (Fuhrman, 2009). Because of the high diversity revealed by the rarefaction analysis, many rare taxa may not have been detected in our libraries.

Metal cycling and nodule formation

The CCFZ is a polymetallic nodule province where nodules are abundant. Because of the high concentration of heavy metals, such as iron (Fe), manganese (Mn), nickel (Ni), and cobalt (Co), the CCFZ is a distinctive and important environment. Bacterial communities living in this area are predicted to be unique, with some bacteria involved in metal cycling and nodule formation. In fact, nearly half of the sequences recovered from the sampling stations were related to uncultured representatives from the ocean crust, the Pacific nodule province and marine basalts. The ocean crust and marine basalts belong to the big oceanic lithosphere in which the rock is very reactive and contains large amount of Fe and Mn compared with seawater (Santelli *et al.*, 2008). Thus, the corresponding phylotypes represented by these sequences may potentially be involved in metal cycling or in the formation of nodules or crust. However, an interesting result was that many clones obtained in this study shared high similarities with uncultured relatives recovered from the sediment of three basins (Angola, Cape, and Guinea basins) of the South-Atlantic Ocean (Schauer *et al.*, 2009). The geographic distance between the sampling stations in the tropical northeast Pacific and the South-Atlantic Ocean is large, but clones obtained from both areas were highly overlapped, suggesting that some similar environment factors (possibly high metal levels) may exist.

Mn(II)-oxidizing bacteria have been found in diverse phylotypes, including low G+C Gram-positive bacteria (e.g., *Bacillus* spp.), Alphaproteobacteria (e.g., *Hyphomicrobium* sp. and *Pedomicrobium* sp.), Betaproteobacteria (e.g., *Leptothrix* spp.), Gammaproteobacteria (e.g., *Pseudomonas* spp.), and Bacteroidetes (e.g., *Cytophaga* sp.) (Cahyani *et al.*, 2007; Wang *et al.*, 2009a). The Mn-reducing bacteria have been identified in the genera *Shewanella*, *Geobacter*, *Bacillus*, *Desulfovibrio*, and *Desulfotomaculum*, among others (Cahyani *et al*., 2007). Usually, Mn-cycling bacteria can also catalyze the redox reaction of iron or some other metals. Identified phylotypes related to metal metabolism were detected in the present study. Clones related to *Hyphomicrobium* sp. Ellin112 and *Pedomicrobium manganicum* strain ACM 3038 were found within *Hyphomicrobiaceae* of Alphaproteobacteria. *Pedomicrobium* is a genus of budding hyphal bacteria that have the ability to oxidize Mn through either enzymatic mechanisms or iron oxidation (Cox and Sly, 1997; Larsen *et al.*, 1999). *P. manganicum* is able to oxidize Mn by first adsorbing Mn to the surface and subsequently catalyzing the oxidation with the help of a multicopper oxidase (Sly and Arunpairojana, 1987; Larsen *et al.*, 1999; Ridge *et al.*, 2007). These bacteria are predicted to be involved in Mn cycling based on the physiology of their relatives.

In addition, *Pseudomonas* was detected in Gammaproteobacteria, which contained 14 clones related to *Pseudomonas* sp. NB1-h. Many strains of *Pseudomonas* isolated from diverse environments have the ability to oxidize Mn(II), especially the well-studied strains *Pseudomonas putida* MnB1 and GB-1 (Francis and Tebo, 2001). However, *Pseudomonas* sp. NB1-h, isolated from Japan Trench sediment at a depth of 6292 m (Miki Yanagibayashi, 1999), has not been reported to oxidize Mn(II). Because *Pseudomonas* stains have diverse metabolic abilities, it is only hypothesized that these bacteria grouped

into *Pseudomonas* may play a role in Mn cycling. Nevertheless, *Pseudomonas* strains can form biofilms easily, which is helpful in the formation and growth of nodules.

Bacteria grouped into Desulfuromonadales within Deltaproteobacteria were associated with the isolates *G. subterraneus* strain Red1 and Desulfuromonadales bacterium Tc37. *G. subterraneus* strain Red1 is an anaerobic Fe(III)- and Mn(IV)-reducing bacterium and can receive energy from the reduction of Fe(III) and Mn(IV) (Greene *et al.*, 2009). Bacteria belonging to Desulfuromonadales have also been detected in the bacterial communities inhabiting manganese nodules in rice field subsoils (Cahyani *et al*., 2007), indicating that Desulfuromonadales are possibly involved in manganese cycling. *Cytophaga* sp. has been reported to be involved in Mn(II) oxidation (Gregory and Staley, 1982; Cahyani *et al*., 2007). Bacteria closely associated with *Cytophaga* sp. NB1-m may play a role in Mn(II) oxidation.

Sulfur cycling-related bacteria

Sulfur is a critical element in nature. Sulfuration and sulfate reduction are two indispensable metabolic pathways in the cycling of sulfur. In this study, a cluster of clones belonging to *Ectothiorhodospiraceae* was detected. *Ectothiorhodospiraceae* is a family of purple sulfur bacteria, including phototrophic and chemoautotrophic bacteria, that can produce sulfur globules outside of their cells (Imhoff, 2006; Tourova *et al.*, 2007). Twelve genera have been established in the *Ectothiorhodospiraceae* family according to List of Prokaryotic names with Standing in Nomenclature (LPSN). *Ectothiorhodospira*, *Ectothiorhodosinus*, *Halorhodospira*, and *Thiorhodospira* are phototrophic genera, while *Thioalkalispira* and *Thioalkalivibrio* are chemolithotrophic sulfur-oxidizing genera (Tourova *et al.*, 2007). However, it is hard to conclude whether these bacteria are phototrophic or chemotrophic based merely on the phylogenetic analysis of 16S rRNA. We hypothesize that these bacteria may be related to sulfur oxidation through non-phototrophic metabolism because the deep ocean is considered to be dark.

Interestingly, a group of clones belonging to *Rhodospirillaceae* within Alphaproteobacteria were obtained. The *Rhodospirillaceae* family contains mainly purple non-sulfur bacteria, which produce energy by photosynthesis. However, some bacteria of *Rhodospirillaceae*, e.g., *Rhodospirillum rubrum*, can grow either chemotrophically without light or phototrophically in light (Golecki and Oelze, 1980). The phototrophs are intermingled with non-phototrophs in the phylogenetic tree (Kawasaki *et al.*, 1993). Clones were associated with *P. litoralis* and *Skermanella* as well as a distantly related magnetite-containing magnetic *Vibrio* strain MV1. These bacteria are predicted to play a role in the oxidation of hydrogen sulfide at low concentrations. Likewise, because it is dark in the deep sea, these bacteria may oxidize sulfur compounds chemotrophically.

Sulfate-reducing bacteria (SRB) are a group of anaerobic bacteria that reduce sulfate or other oxidized sulfur compounds to sulfide, including Desulfobacterales, Desulfovibrionales, and Syntrophobacterales. In the present study, clones belonging to Syntrophobacterales and Desulfuromonadales were retrieved within Deltaproteobacteria that were distantly related to identified bacteria *Desulfoacinum infernum* strain BalphaG, *G. subterraneus* strain Red1 and Desulfuromonadales bacterium Tc37, respectively (Fig. 6). *D. infernum* strain BalphaG is a thermophilic sulfate-reducing bacterium isolated from a North Sea petroleum reservoir (Rees *et al.*, 1995). *G. subterraneus* strain Red1 can reduce elemental sulfur as well as Fe(III) and Mn(IV) (Greene *et al.*, 2009). These sulfate-reducing bacteria may cooperate with sulfur-oxidizing bacteria in the ecosystem and play an indispensable role in sulfur cycling.

Nitrogen cycling-related bacteria

Nitrification and denitrification, carried out by nitrifiers and denitrifiers respectively, are two important parts in nitrogen cycling, especially in marine environments. Nitrifiers are usually aerobic chemoautotrophs, such as *Nitrosospira* and *Nitrosomonas*, while denitrifiers are generally anaerobic heterotrophs (Ward, 1996). In this study, four clones associated with *Nitrospina* sp. 3005 and *Nitrospina gracilis* strain Nb-3 belonging to *Nitrospinaceae* were detected. *N. gracilis* is a nitrite-oxidizing bacteria isolated from the marine environment (Watson and Waterbury, 1971). Thus, it is predicted that these bacteria are nitrite-oxidizing bacteria. Based on the chemical analysis of sediments from the sampling stations, the average concentration of nitrate is approximately 40-45 μM and increases slightly with depth (data not shown), indicating that nitrification is dominant in these samples. However, other nitrogen cycling-related bacteria are waiting to be discovered by expanding the libraries.

Conclusion

Phylogenetic and diversity analyses of bacterial communities were performed for four stations in the CCFZ. The 313 total clones sequenced from the 4 libraries, except for 28 unclassified bacteria, were divided into 14 phylogenetic groups. The most dominant phylum was Proteobacteria, but the predominant class within Proteobacteria was different in each library. High bacterial diversity was revealed by the rarefaction analysis. Because the CCFZ is abundant in polymetallic nodules, we attempted to relate the bacterial communities with their habitats with the hope that special bacterial patterns would be discovered. The 16S rRNA gene libraries have revealed some bacteria potentially related to metals (e.g., Mn and Fe) cycling, which is a very important process in the formation and growth of polymetallic nodules. In addition, bacteria possibly involved in sulfur and nitrogen cycling have been detected. This study gives a primary view of bacterial communities living in the fields of deep-sea polymetallic nodules and is the first report of a molecular ecological study of four stations in the CCFZ. Some interesting discoveries of special bacteria, such as metal cycling-related bacteria and unclassified novel bacteria, are worth further studies. More information about the bacterial communities living in the studied area can be obtained through culture experiments as a complement.

Acknowledgements

This work was supported by grants from China Ocean Mineral Resources R & D Association (COMRA) Special Foundation

(DYXM-115-01-3-01, DYXM-115-01-3-02), the Ministry of Science and Technology of China (863 Program, 2007AA021305) and the National Natural Science Foundation of China (40806066).

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