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Cold-Seep Sediment Harbors Phylogenetically Diverse Uncultured Bacteria

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Abstract A culture-independent molecular phylogenetic survey was carried out on the bacterial community in cold-seep sediment at Edison Seamount, south of Lihir Island, Papua New Guinea. Small-subunit rRNA genes were amplified directly from the sediment DNA by PCR and cloned. The majority of the cloned 16S rRNA gene sequences were most closely related to as-yet-uncultivated microorganisms found in deep-sea sediments, and were primarily affiliated with one of four groups: the γ-, δ-, and ε-subdivisions of *Proteobacteria*, and *Cytophaga-Flavobacterium-Bacteroides*. We did not recover any sequences related to cyanobacteria, prochlorophytes, and α-*Proteobacteria*, which are known to occur in great abundance within the surface mixed layer of the Atlantic and Pacific Oceans. The majority of the cloned γ- and ε-*Proteobacterial* sequences were closely related to chemoautotrophic sulfur-oxidizing symbionts of marine benthic fauna, and the δ-*Proteobacterial* sequences to sulfate- and sulfur-reducing bacteria, indicating that they might play an important role in chemoautotrophic primary production and the sulfur cycle in the cold-seep area. These results demonstrate the high diversity of the bacterial community in the cold-seep sediment, and substantially expand knowledge of the extent of bacterial diversity in this formidable and unique habitat.

Key words: Cold-seep, bacteria, diversity

Cold-seeps are sites of fluid release from the sea floor that are derived from various sources, including artesian flow and processes producing sediment compaction. Deep-sea cold-seep areas have been found in subduction zones off the coasts of Oregon and Peru, in the Japan Trench, and in the Nankai Trough [28, 43, 53]. Deep-sea sediments are potentially unique habitats for microbial communities, and the discovery of dense organisms associated with deep-sea hydrothermal vents and cold-seeps profoundly altered traditional views that life on this planet depends solely on photosynthetic primary production [2, 34]. An estimated 95% of the organic matter produced photosynthetically in surface waters recycles in the upper 100–300 m, and about 1% of this photosynthetic primary production settles to the deep-sea floor [25]. The source of energy for these ecosystems appears to be oxidation of reduced inorganic substrates such as sulfide and thiosulfate, and chemoautotrophic microorganisms are considered to be the base of the deep-sea sediment food chain [25].

Although deep-sea sediments play a significant role in global biogeochemical cycling, little is known about the diversity and ecology of the microbial communities that occupy these unique niches. The majority of microbiological studies of deep-sea sediment have relied on enrichment culture techniques for growing thermophiles [1, 17, 21, 26] and mesophiles [15, 41], and some studies on biogeochemistry have elucidated the role of sulfur-oxidizing bacteria [50, 57], methane-oxidizing bacteria [10], and endosymbionts [5, 8] in this ecosystem. A few cultivation-independent studies of microbial diversity in marine sediments have been conducted [12, 47, 49, 55]. The sequences obtained in these studies revealed the presence of mostly unknown organisms only distantly related to known isolates.

To further uncover microbial diversity in cold-seep sediment and to identify potentially dominant groups in this habitat, we surveyed the diversity of cold-seep microbial communities using a molecular phylogenetic approach. Here, we describe the phylogenetic affiliation and diversity of the prokaryotic community in this unique ecosystem.

Materials and Methods

Study Site

Cold-seep sediment samples (depth, 1,450 m) were collected with a camera-guided grab sampler at Edison Seamount

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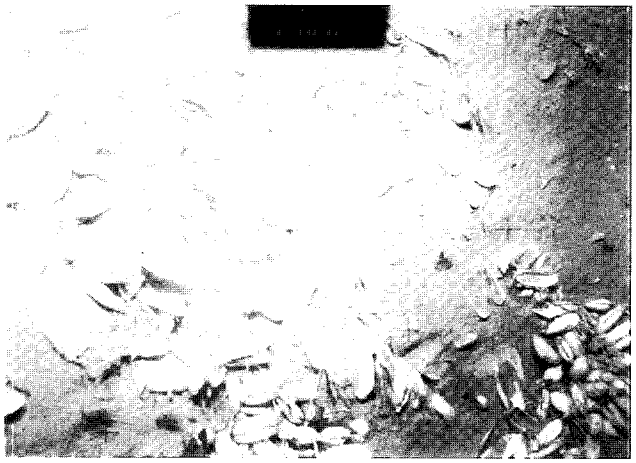

Fig. 1. Photograph of sampling site at Edison Seamount taken by a camera-guided grab sampler.

[03°19' S, 152°34' E], south of Lihir Island in the New Ireland Fore-arc, near Papua New Guinea, from the R/V Sonne during the 2002 research cruise SO-166 (September 2002). The Edison Seamount pyroclastic cone rises 150 m above the surrounding seafloor at 1,600 m depth. The striking feature of the site was the extensive clam beds associated with low-temperature venting at the crater rim (Fig. 1). Sulfidic sediment, which was notably darker than the local pelagic ooze, locally surrounded the clam beds. The clam beds (mainly *Calyptogena* species) were extremely dense, with populations at the center of the fields exceeding 50 individuals per square meter and covering 100% of the seafloor. Samples of the sediment consisted of foraminiferal carbonate ooze, minor smectite, and disseminated Fe-monosulfide. Other details about the sampling site are described by Herzig *et al.* [22]. Onboard, the intact samples devoid of contamination were taken from inside the sediment, aliquoted into sterile tubes, and preserved at 4°C during transportation for 2 weeks, before being stored at 80°C.

Molecular Phylogenetic Analysis

Community DNA was extracted from the sediment samples by the method of Cho *et al.* [9] with minor modifications in reaction volumes. PCR amplification of the near-full-length bacterial 16S rRNA genes from the community DNA was done using the universal primers pA (AGA GTT TGA TCC TGG CTC AG) and pH (AAG GAG GTG ATC CAG CCG CA) [37]. PCR products were purified using a QIAquick PCR purification kit (Qiagen, Chatsworth, CA, U.S.A.). Purified PCR amplicons were ligated into the pCR2.1-TOPO cloning vector and were transformed into chemically competent *E. coli* TOP10F' cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, U.S.A.). Randomly selected clones were sequenced using an ABI PRISM Big Dye Terminator Cycle-Sequencing Ready Reaction kit (PE-Applied Biosystems, Foster, CA, U.S.A.) and an automatic sequence analyzer (ABI 3700). All sequences (600 bp from 5'-end) were tested for chimeras by the Ribosomal Database Project (RDP-II) CHECK_CHIMERA program and compared to sequences in the RDP-II database (http://rdp.cme.msu.edu) [36]. Forty-two randomly selected sequences were aligned with 16S rRNA genes of their closest relatives in the RDP-II database using Clustal W software [56] and subjected to phylogenetic analyses using MEGA software [29]. The evolutionary distances were calculated according to Kimura's 2-parameter model [27]. Phylogenetic trees were inferred using the neighbor-joining algorithm, and the tree topology was statistically evaluated by 1,000 bootstrap resamplings. All sequences produced in this study have been deposited in GenBank under accession numbers AY279036 to AY279077 (Table 1).

Results and Discussion

Cloned bacterial 16S rRNA gene sequences were primarily affiliated with one of four groups: the γ-, δ-, ε-subdivision of *Proteobacteria*, and *Cytophaga-Flavobacterium-Bacteroides* (CFB) (Fig. 2). There were 28 (66.7%) 16S rRNA gene sequences belonging to the *Proteobacteria*. Of these sequences, 13 (31.0%) fell under the γ-*Proteobacteria*, 5 (11.9%) under the δ-*Proteobacteria*, and 10 (23.8%) under the ε-*Proteobacteria*. There were 12 (28.6%) sequences belonging to the CFB group and 2 (4.8%) to Gram-positive bacteria. Clones CS027 and CS031 were assigned to the Gram-positive cluster, and their closest relative was deep-sea sediment clone BD2-4, which was recovered from Suruga Bay and most closely related to *Clostridium felsineum* [31]. No phylotypes were detected from cyanobacteria, prochlorophytes, or α-*Proteobacteria* (e.g., the SAR 11 cluster), which are known to occur in great abundance within the surface mixed layer of the Atlantic and Pacific Oceans [11, 19, 20, 52]. Similarly, α-*Proteobacteria*, which are mainly comprised of oligotrophic taxa, were not recovered from deep-sea sediments in the Guaymas Basin [55], Mid-Atlantic Ridge [49], and Loihi Seamount [38].

CFB Group

Second in abundance to the γ-*Proteobacterial* sequences were those related to the CFB group. The closest relative of clones CS011 and CS045 was the deep-sediment clone BD2-17, which was recovered from Suruga Bay [31], and was most closely related to *Gelidbacter algens*, a psychrophilic bacterium isolated from the Arctic marine habitat [3]. The rest of 10 CFB-related clones were most similar to environmental clone JTB132, which was recovered from the cold-seep area in the Japan Trench and most closely related to *Cytophaga fermantans* [32]. Similarly, Ravenschlag *et*

Table 1. Summary of the 16S rRNA gene sequences identified in cold-seep sediment.

Phylogenetic affiliation[a]	Clone no.	GenBank accession no.	Closest relatives	% Sequence similarity[b]	Origin	Reference
γ-*Proteobacteria*	CS016	AY279055	Gill symbiont clone	90.6	Codakia costata	14
γ-*Proteobacteria*	CS020	AY279057	Gill symbiont clone	91.2	Codakia costata	14
γ-*Proteobacteria*	CS038	AY279074	Gill symbiont clone	91.4	Codakia costata	14
γ-*Proteobacteria*	CS050	AY279037	Gill symbiont clone	91.1	Codakia costata	14
γ-*Proteobacteria*	CS047	AY279070	Gill symbiont clone	91.6	Codakia costata	14
γ-*Proteobacteria*	CS013	AY279039	Gill symbiont clone	89.8	Solemya velum	16
γ-*Proteobacteria*	CS049	AY279071	Clone BPC036	93.1	Hydrocarbon seep	42
γ-*Proteobacteria*	CS043	AY279067	Clone BPC036	96.1	Hydrocarbon seep	42
γ-*Proteobacteria*	CS051	AY279072	Clone BPC023	86.0	Hydrocarbon seep	42
γ-*Proteobacteria*	CS028	AY279061	Clone BD6-6	89.2	Deep-sea sediment (RyuKyu Trench)	31
γ-*Proteobacteria*	CS052	AY279073	Clone Sva0318	98.9	Marine sediment (Arctic ocean)	47
γ-*Proteobacteria*	CS040	AY279066	Leucothrix mucor	89.0		35, 57
γ-*Proteobacteria*	CS004	AY279049	Clone NKB17	93.9	Trough sediment (Nankai Trough)	30
δ- *Proteobacteria*	CS054	AY279075	Clone Sva0566	89.6	Marine sediment (Arctic ocean)	47
δ- *Proteobacteria*	CS046	AY279069	Clone NKB13	95.6	Trough sediment (Nankai Trough)	30
δ- *Proteobacteria*	CS002	AY279042	Clone JTB20	99.7	Cold-seep sediment (Japan Trench)	32
δ- *Proteobacteria*	CS026	AY279059	Clone BD7-15	97.0	Deep-sea sediment (Japan Trench)	31
δ- *Proteobacteria*	CS032	AY279044	Clone Sva1036	96.5	Marine sediment (Arctic ocean)	47
ε-*Proteobacteria*	CS039	AY279047	Clone VC2-1 Bac32	89.0	Hydrothermal vent (Mid-Atlantic Ridge)	49
ε-*Proteobacteria*	CS027	AY279060	Clone BD2-1	99.8	Deep-sea sediment (Suruga Bay)	31
ε-*Proteobacteria*	CS031	AY279062	Clone NKB8	99.0	Trough sediment (Nankai Trough)	30
ε-*Proteobacteria*	CS029	AY279065	Clone NKB9	97.1	Trough sediment (Nankai Trough)	30
ε-*Proteobacteria*	CS037	AY279046	Clone JTB129	97.5	Cold-seep sediment (Japan Trench)	32
ε-*Proteobacteria*	CS006	AY279048	Endosymbiont clone	92.5	Vestimentiferan trophosome	39
ε-*Proteobacteria*	CS060	AY279077	Endosymbiont clone	92.9	Vestimentiferan trophosome	39
ε-*Proteobacteria*	CS007	AY279041	Endosymbiont clone	92.0	Vestimentiferan trophosome	39
ε-*Proteobacteria*	CS033	AY279063	Endosymbiont clone	92.8	Vestimentiferan trophosome	39
ε-*Proteobacteria*	CS058	AY279076	Endosymbiont clone	93.0	Vestimentiferan trophosome	39
Gram-positives	CS018	AY279056	Clone BD2-4	97.8	Deep-sea sediment (Suruga Bay)	31
Gram-positives	CS059	AY279045	Clone BD2-4	90.7	Deep-sea sediment (Suruga Bay)	31
Cytophagales	CS011	AY279053	Clone BD2-17	87.1	Deep-sea sediment (Suruga Bay)	31
Cytophagales	CS045	AY279068	Clone BD2-17	87.1	Deep-sea sediment (Suruga Bay)	31
Cytophagales	CS010	AY279052	Clone JTB132	85.0	Cold-seep sediment (Japan Trench)	32
Cytophagales	CS009	AY279051	Clone JTB132	88.3	Cold-seep sediment (Japan Trench)	32
Cytophagales	CS014	AY279040	Clone JTB132	89.1	Cold-seep sediment (Japan Trench)	32
Cytophagales	CS023	AY279058	Clone JTB132	88.9	Cold-seep sediment (Japan Trench)	32
Cytophagales	CS034	AY279064	Clone JTB132	89.8	Cold-seep sediment (Japan Trench)	32
Cytophagales	CS008	AY279043	Clone JTB132	90.0	Cold-seep sediment (Japan Trench)	32
Cytophagales	CS015	AY279054	Clone JTB132	89.8	Cold-seep sediment (Japan Trench)	32
Cytophagales	CS019	AY279038	Clone JTB132	89.8	Cold-seep sediment (Japan Trench)	32
Cytophagales	CS053	AY279036	Clone JTB132	89.6	Cold-seep sediment (Japan Trench)	32
Cytophagales	CS005	AY279050	Clone JTB132	89.8	Cold-seep sediment (Japan Trench)	32

[a]As described by the RDP-II.
[b]Sequence similarity was calculated by converting the evolutionary distance using the equation, (1-evolutionary distance)×100.

al. [47] reported that a significant proportion of their clone library constructed from permanently cold marine sediment in the Arctic Ocean was assigned to the CFB cluster, and the majority of these clones were most similar to *Cytophaga fermantans*.

In general, *Cytophagales* are known for their ability to associate and glide on surfaces and to degrade a variety of polymeric substances [48], and members of this CFB group have been found in various marine habitats, Mid-Atlantic Ridge hydrothermal vents [49], hydrothermal sediments in the Guaymas Basin (Gulf of California, Mexico) [55], and marine aggregates [12, 46]. When investigating Wadden Sea sediment by fluorescent *in situ* hybridization, Llobet-Brossa *et al.* [33] found members of the CFB group to be

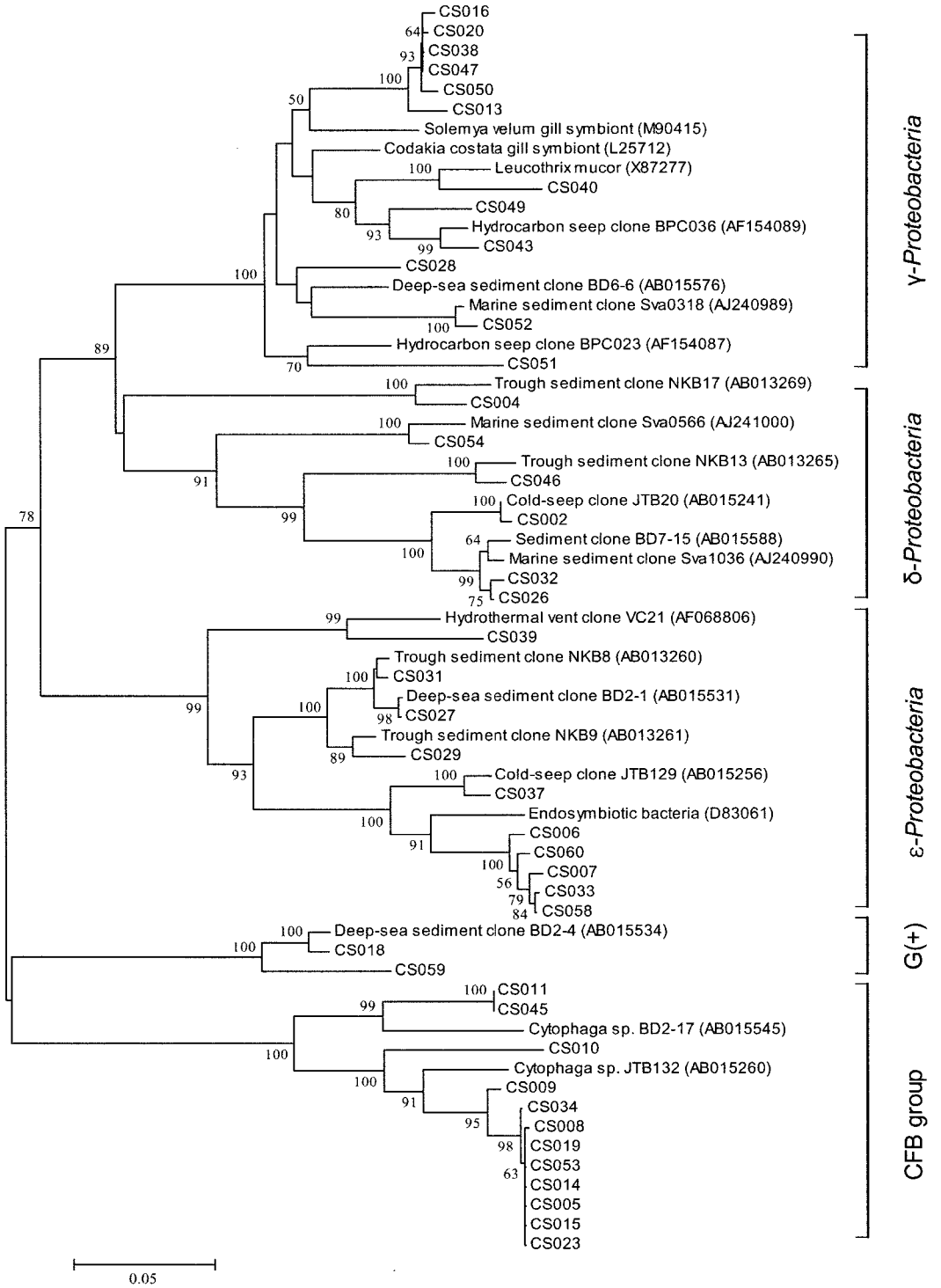

Fig. 2. Phylogenetic relationships of 16S rDNA clone sequences and their closest relatives.
The tree was constructed using the neighbor-joining algorithm. Numbers at nodes represent the bootstrap score (as a percentage). The scale bar represents the expected number of changes per nucleotide position. Bootstrap scores are shown for frequencies at or above the threshold of 50%.

more abundant than δ-*Proteobacteria*. Also, a significant constituent of this study's clone library was CFB-related sequences, indicating that the CFB group might be a common member of marine sediment microbial communities.

γ-*Proteobacteria*

The most abundant group of clones (31%) was affiliated with the γ-subdivision of *Proteobacteria*. All clones in this group, except clone CS040, were most closely related to

environmental clones from deep-sea sediments. The closest relative of clone CS040 was *Leucothrix mucor*, a bacterium associated with brine shrimp [35, 54]. Clones CS049, CS043, and CS051 were most closely related to environmental clones BPC036 and BPC023 from a hydrocarbon seep [42], clone CS028 to the clone BD6-6 from the RyuKyu Trench [29], and clone CS052 to clone Sva0318 from permanently cold marine sediment in the Arctic Ocean [47]. The closest relative of clone CS046 was environmental clone NKB17, which was recovered from cold-seep sediment in the Nankai Trough [30], and which showed unclear phylogenetic affiliation. Although the clone CS046 branches from near the root of the γ-*Proteobacteria* cluster, the bootstrap score did not significantly support that this clone was a member of γ-*Proteobacteria*. The rest of the 6 clones belonging to γ-*Proteobacteria* were most closely related to the 16S rRNA gene sequences of sulfur-oxidizing chemoautotrophic symbionts of marine invertebrates *Solemya velum* and *Cadakia costata* [15, 17]. *Solemya velum* symbiont 16S rDNA was originally found in a gill sample from *Solemya velum*, and is considered to be widespread in marine sediments [17]. Many investigators [14, 31, 32, 47] reported that γ-*Proteobacterial* 16S rDNA sequences found in deep-sea sediments were closely related to the symbionts of the bivalves, and Teske *et al.* [55] reported that most γ-*Proteobacterial* clones recovered from hydrothermal sediment in the Guaymas Basin were related to sulfur-oxidizing bacterial endosymbionts of bivalves at hydrothermal vents and sulfidic mud-flats [6]. Similarly, ecto- and endosymbionts of the animals from sulfidic habitats, including hydrothermal vents, were all clustered within the γ-*Proteobacteria* [14, 45].

Symbiosis between chemoautotrophic bacteria and invertebrate animals was first described for Vestimentiferan tubeworms from hydrothermal vents [7, 18], and other symbioses subsequently were reported for invertebrate animals from a variety of sulfide- or methane-rich marine environments [40]. These symbiotic bacteria are thought to provide the hosts with autotrophically fixed carbon, and the energy source for these symbionts appears to be derived from the oxidation of reduced inorganic substrates such as sulfide and thiosulfate. However, since no pure culture representatives for this group have been isolated, it can only be speculated that they might be involved in the oxidation part of the sulfur cycle. Such bacterial symbionts might form the primary base of the food chain of the dense populations of invertebrates at the deep-sea hydrothermal vents and cold-seeps.

δ-*Proteobacteria*

Five sequences fell in the δ-subdivision of *Proteobacteria*. The closest relatives of the clones CS002, CS026, CS032, CS046, and CS054 were cold-seep clone JTB20, deep-sea sediment clone BD7-15, marine sediment clone Sva1036, trough sediment clone NKB13, and marine sediment clone Sva0566, respectively. Clones JTB20, BD7-15, Sva0566, and Sva1036 were identified as relatives of *Desulforhopalus vacuolatu*, *Desulfocapsa thiozymogene*, *Desulfuromonas* sp., and *Desulfotalea* sp., respectively, in other studies [31, 32, 47], and they are all sulfate- and sulfur-reducing bacteria which can utilize sulfate as an electron acceptor for energy-generating processes.

The δ-subdivision of *Proteobacteria* comprises mainly anaerobic bacteria, which are expected to be dominant in marine sediments. Li *et al.* [32] and Ravenschlag *et al.* [47] reported that clone libraries constructed from cold-seep areas in the Japan Trench and permanently cold marine sediments in the Arctic Ocean were dominated by sequences related to δ-*Proteobacteria*. However, clone libraries constructed from deep-sea sediments in the Nankai Trough by Li *et al.* [30, 31], at the Mid-Atlantic Ridge by Reysenbach *et al.* [49], and in the Guaymas Basin by Teske *et al.* [55] showed that δ-*Proteobacteria* were a minority group. Also, according to the clone library of this study, only 11.9% of clones were δ-*Proteobacteria*. It was considered that anaerobic respiration in this cold-seep sediment is not limited to classical δ-*Proteobacterial* sulfate-reducing bacteria, and that alternative sulfate-reducing bacteria might fill this niche.

ε-*Proteobacteria*

ε-*Proteobacteria* constitute 23.8% of the clone library. The ε-*Proteobacterial* sequences formed two deep branches, and each branch was supported by a bootstrap score of >93. The first clade included a clone, CS039, of which the closest relative was an environmental clone, VC Bac3.2, found at a Mid-Atlantic Ridge hydrothermal vent [49]. The second clade comprised the rest of the ε-*Proteobacterial* sequences, which were closely related to the symbiotic bacteria of deep-sea sediment benthic fauna. In this clade, there were two monophyletic subgroups supported by a bootstrap score of 100. One subgroup included environmental clones, BD2-1, NKB8, and NKB9, which were recovered from Suruga Bay and the Nankai Trough, and the closest relative of them was an *Alvinella pompejana* epibiont clone [30, 31]. The other subgroup included the environmental clone JTB129, and a 16S rRNA gene sequence from an endosymbiotic bacterium. Clone CS031 showed 99.0% sequence similarity to clone JTB129, which was recovered from the Japan Trench [32]. The closest relative to the clones in this subgroup, which included clone JTB129, was an *Arcobacter*-related endosymbiont in a Vestimentiferan tube worm [39].

It was observed that a significant portion of the clone library corresponded to ε-*Proteobacterial* 16S rRNA gene sequences, and most of the clones were closely related to symbiotic bacteria of benthic fauna. This is a distinctly different result compared to previous molecular phylogenetic

analyses of ecto- and endosymbionts of animals from sulfidic habitats, including hydrothermal vents, which all clustered within the γ-*Proteobacteria* [15, 44, 47]. In the clone library of this study, it was found that only 6 clones (14.3%) were closely related to γ-*Proteobacterial* symbionts of deep-sea marine bivalves. Association with animals is the best-known mode of living in the ε-*Proteobacteria*, although the majority of examples are parasitic. The significance of the ε-*Proteobacteria* in deep-sea sediments has only recently been realized [4, 23, 49, 55]. Many appear to be symbionts of the deep-sea invertebrates, although their association remains speculative. Polz and Cavanaugh [44], Reysenbach *et al.* [49], and Cary *et al.* [4] reported that the microbial communities at a Mid-Atlantic Ridge hydrothermal vent and a East Pacific Rise hydrothermal vent were dominated by symbiotic and free-living ε-*Proteobacteria*. They postulated that this group might be prevalent in low-temperature mats rich in iron and sulfur precipitates and that these organisms might be important mesophilic or moderately thermophilic microorganisms involved in iron or sulfur cycling at deep-sea hydrothermal vents. Teske *et al.* [55] also recovered ε-*Proteobacterial* 16S rRNA gene sequences from hydrothermal sediments in the Guaymas Basin, and concluded that these ε-*Proteobacteria* could be involved in sulfur oxidation. Similarly, the majority of clones in the 16S rRNA gene library constructed from a microbial mat at Pele's Vent on Loihi Seamount, Hawaii, were ε-*Proteobacteria* [38]. Although there were no known representatives of these microorganisms in culture and their exact role is not yet clear, these findings suggest that in addition to the better-known chemoautotrophic symbiotic γ-*Proteobacteria* aligned with the ε-*Proteobacteria*, they likely play an important role in the ecology of deep-sea sediment ecosystems, including hydrothermal vents and cold-seeps.

In conclusion, the cold-seep sediment in this study was dominated by the γ-, δ-, ε-subdivisions of *Proteobacteria*, and by the CFB group. The majority of cloned γ- and ε-*Proteobacterial* sequences were closely related to chemoautotrophic sulfur-oxidizing symbionts of marine benthic fauna, and the cloned δ-*Proteobacterial* sequences to sulfate- and sulfur-reducing bacteria, indicating that those δ-*Proteobacteria* might play an important role in chemoautotrophic primary production and the sulfur cycle in cold-seep areas. These results demonstrate that cold-seep sediment harbors highly diverse and as-yet-uncultured bacteria, and substantially expand our knowledge of the extent of bacterial diversity in this unique habitat.

Acknowledgments

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