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Andrew J. Reed · Richard A. Lutz · Costantino Vetriani

Vertical distribution and diversity of bacteria and archaea in sulfide and methane-rich cold seep sediments located at the base of the Florida Escarpment

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Abstract The bacterial and archaeal communities of the sediments at the base of the Florida Escarpment (Gulf of Mexico, USA) were investigated using molecular phylogenetic analysis. The total microbial community DNA of each of three vertical zones (top, middle and bottom) of a sediment core was extracted and the 16S rRNA genes were amplified by PCR, cloned and sequenced. Shannon–Weaver Diversity measures of bacteria were high in all three zones. For the archaea, diversity was generally low, but increased with depth. The archaeal clonal libraries were dominated by representatives of four groups of organisms involved in the anaerobic oxidation of methane (ANME groups). Phylogenetic analysis of bacteria suggests the dominance of ε-proteobacteria in the top zone, the ε-, δ- and γ-proteobacteria in the middle zone and the δ-proteobacteria in the bottom zone of the core. Members of the Cytophaga–Flexibacter–Bacteroidetes group, the *Chloroflexi*/green non-sulfur bacteria, the Gram+ (Firmicutes), the Planctomyces, candidate division WS3 and *Fusobacterium* were also detected. Our data suggest that the community structure and diversity of microorganisms can shift greatly within small vertical distances, possibly in response to changes in the physical and chemical conditions.

Keywords Florida Escarpment · Methane · Cold seep · Shannon–Weaver · Microbial diversity · ANME

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A. J. Reed · R. A. Lutz · C. Vetriani
Institute of Marine and Coastal Sciences, Rutgers University, New Brunswick, NJ 08901, USA

C. Vetriani (✉)
Department of Biochemistry and Microbiology, Rutgers University, New Brunswick, NJ 08901, USA
E-mail: vetriani@imcs.rutgers.edu
Tel.: +1-732-9326555
Fax: +1-732-9326557

Introduction

Cold seeps are characterized by the seepage of fluids, which have an elevated methane and/or sulfide concentration over that of ambient seawater, into surficial sediments. Methane in cold seep fluids can have a biogenic origin or a thermogenic origin. Methane with a biogenic origin is derived from the microbial degradation of organic matter in anoxic sediments, and methane with a thermogenic origin is derived from transformation of organic matter caused by high temperatures (Martens et al. 1991). In addition to methane, a limited number of cold seeps have an increased concentration of hydrogen sulfide in the sediment porewater that is produced by microbial sulfate reduction. Both methane and sulfide play a major role in sustaining the highly productive cold seep biological communities.

The Florida Escarpment is a sharply sloping limestone edifice (average tilt >35°), which rises ~2,000 m from the seafloor at a depth of 3,270 m and extends for hundreds of kilometers. High salinity seawater that is enriched in sulfide, methane and ammonia seeps out of the face of the Florida Escarpment at the junction between the scarp and the sediment (Paull et al. 1984). It has been suggested that the high salinity fluids are the result of hypersaline brines that form in the center of the Florida Platform and mix with lateral intrusions of ambient seawater before exiting along the base of the scarp (Chanton et al. 1991). It is also thought that the microbial reduction of sulfate may be the primary source of sulfide, although the importance of the thermochemical reduction of sulfate within the Florida Platform is unclear. Based on carbon isotope analysis ($\delta^{13}C = -61$ to -94 ppt), the methane in Florida Escarpment seep fluids is the result of microbial production (Martens et al. 1991). Both microbial sulfate reduction and methanogenesis are hypothesized to be important processes within the platform (Martens et al. 1991).

Molecular approaches to the investigation of microbial communities, based on the PCR amplification and cloning of diagnostic molecules, such as the small subunit ribosomal RNA gene (16S rDNA), have led not only to insights into the community diversity and structure of microbial systems, but have revealed new phylogenetic lineages of microorganisms, some of which serve as the dominant constituent in a given microbial community. Numerous studies in the recent past have focused on the identification and community diversity of microorganisms based on 16S rDNA analysis of naturally occurring microbial communities (reviewed in Hugenholtz and Pace 1996; Rappé and Giovannoni 2003). However, our present knowledge regarding the microbial population structure and function of deep-sea cold seeps is limited to a few studies, while most of the recent investigations of low-temperature, reducing marine sediments have been focusing on methane hydrates.

Li et al. (1999c) carried out a phylogenetic analysis of 16S rDNA clones associated with sediments from a cold seep in the Japan Trench, and showed that δ- and ε-proteobacteria, along with archaeal sequences related to the marine group I cluster, occurred frequently in this environment. Inagaki et al. (2002) confirmed these findings by detecting 16S rRNA transcripts from δ- and ε-proteobacteria in similar sediments from the Japan Trench. Cragg et al. (1996) and Knittel et al. (2003, 2005) quantified microbial populations and activity of methane hydrates in the Cascadia Margin accretionary wedge, identifying the concomitant occurrence of anaerobic methane oxidation and sulfate reduction in subsurface sediments, while Marchesi et al. (2001) carried out a phylogenetic analysis of 16S rDNA clones retrieved from the same site, identifying members of the α, β and γ subdivision of the proteobacteria along with members of both the *Methanosarcinales* and *Methanobacteriales*. A similar phylogenetic analysis of a methane hydrate in the Gulf of Mexico also revealed the occurrence of members of several subclasses of the proteobacteria along with the *Methanosarcinales* (Lanoil et al. 2001), while a study of methane hydrate-bearing deep sediments revealed a complex bacterial community which included members of the green non-sulfur bacteria, *Bacteroidetes*, *Planctomyces* and *Actinobacteria*, among other groups (Reed et al. 2002).

By combining lipid biomarker analyses with a rDNA survey (Hinrichs et al. 1999) found that ^{13}C-depleted lipids occurred in methane seep sediments dominated by a new group of archaea. This correlation suggested that the newly discovered archaea (designated as ANME) were oxidizing methane anaerobically. Follow-up studies of the anaerobic methane-oxidizing communities revealed that the ANME archaea operate in a syntrophic consortium with sulfate-reducing bacteria (SRB) (Boetius et al. 2000; Orphan et al. 2001a, b, 2002) and most likely they oxidize methane by reverse methanogenesis (Hallam et al. 2003, 2004). A further proof that anaerobic methane oxidation may be a relevant process in reduced marine sediments came from a recent survey of 16S rRNA transcripts of cold seep sediments from the Gulf of Mexico (Mills et al. 2003). This study revealed that the presumptive metabolically active archaeal community was dominated by the ANME group, while δ-proteobacteria dominated the mid and bottom regions of the sediment cores. Finally, Inagaki et al. (2004) investigated the microbial community of a cold seep located in the Southern Ryukyu Arc (Japan), using both 16S rDNA and functional gene markers. Results from this study indicate that methanogenesis, anaerobic methane oxidation and aerobic methane oxidation occur in close proximity in these sediments.

Here we describe, for the first time, the community structure and vertical zonation of the microbial communities inhabiting the sediments at the base of the Florida Escarpment, and we elucidate their diversity using ecological indices that facilitate direct comparisons between vertical zones in the sediment.

Materials and methods

Sample collection

An acrylic tube sediment core (22 cm length×6 cm width) was collected from sediments at the base of the Florida Escarpment, Gulf of Mexico (latitude 26°01.8′N, longitude 84°54.9′W) during voyage AT-03, leg 58 of RV *Atlantis* and dive 3637 of DSV *Alvin* at a depth of 3,288 m in October 2000. Bottom water temperatures in this area were 2–3°C. The sediment core was then frozen whole on board at −80°C until it could be processed in the laboratory at Rutgers University. The sediment core was sectioned into three zones and labeled top, middle and bottom. The top of the core consisted of lightly colored, soft and loosely packed sand-sized sediment. The middle layer of the core consisted of darker, almost black sediment (indicative of reduced oxygen availability) and more densely packed sediment with a smaller grain size. The bottom layer of the core also contained black sediment. Both the middle and bottom zones had a noticeable odor of H_2S and densely packed clay-sized particles.

DNA extraction

Total genomic DNA was extracted from ~1.5 g of sediment from each of the three vertical zones of the core using an UltraClean™ Soil DNA extraction kit (Mo Bio Laboratories, Solana Beach, CA, USA) according to the protocol supplied with the kit. The DNA was resuspended in sterile water and the DNA concentration was measured using a spectrophotometer.

DNA amplification by PCR

The 16S rDNA genes were PCR amplified (Erlich et al. 1991; Steffan and Atlas 1991) using oligonucleotide

primers annealing to highly conserved regions of prokaryotic 16S rDNA. The forward primer 8F (5′-AGAG TTTGATCCTGGCTCAG-3′) and the forward primer 16F (5′-CTGGTTGATCCTGCCAG-3′) were used to selectively amplify bacterial and archaeal clones, respectively. Reverse 1517R (5′-ACGGCTACCTTGTT ACGACTT-3′) was used as the reverse primer for both bacterial and archaeal clones (Weisburg et al. 1991). Reaction mixtures were incubated in a thermal cycler (Perkin-Elmer, Norwalk, CT, USA) and programmed for 35 cycles as follows: denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and chain extension at 72°C for 30 s with a final extension time of 7 min on the last cycle.

Construction of clonal libraries

Clone libraries from PCR products were constructed using the TA cloning kit (Invitrogen Inc., Carlsbad, CA, USA) following the manufacturers' recommendations. Amplified 16S rRNA gene fragments were cloned in pCRII plasmid vector (Invitrogen Inc., Carlsbad, CA, USA) and the ligation products were used to transform competent *E. coli* INVαF′ cells. Clones were grown in Luria-Bertani media at 37°C overnight and kept in long-term storage at −80°C in 96-well plates. Recombinant plasmids were extracted using the QIAprep spin miniprep kit (Qiagen, Santa Clarita, CA, USA) as described in the manufacturers' instructions. Six 16S rDNA libraries (one library each for both bacteria and archaea from all three core layers) were constructed and a total of 216 randomly selected colonies were analyzed. The clones are designated XXXYYY#, where XXX identifies the vertical zone of the core (''Top'' for top, ''Mid'' for middle and ''Bot'' for bottom), YYY identifies the kingdom affiliation (''Bac'' for bacterial and ''Arch'' for archaeal) and # represents the clone number.

Restriction fragment length polymorphism screening

Clonal inserts of 16S rDNA fragments were digested for 2 h at 37°C on a thermocycler using the tandem tetrameric restriction endonucleases *Hae*III and *Msp*I (Promega Inc., Madison, WI, USA). The restriction products were run for 1.5 h at 75 V and 4°C on a 2.5% (w/v) Methaphor agarose gel containing ethidium bromide and visualized under UV light.

Sequencing and phylogenetic analysis

Representative bacterial and archaeal 16S rDNA clones from unique restriction fragment length polymorphism (RFLP) patterns were sequenced utilizing an automated DNA sequencer (310 Avant Genetic Analyzer; Applied Biosystems, Foster City, CA, USA). The sequences obtained in this study were compared with an existing database of rDNA sequences from cultivated microorganisms and environmental clones using the BLAST search program of the National Center for Biotechnology Information (Altschul et al. 1997). For this study, we defined that >97% similarity in sequences represented the same rDNA clone type.

All sequences were aligned in a two-stage process. Multiple alignments in ClustalX v1.8 (Thompson et al. 1997) were followed by manual adjustment using Seaview (Galtier et al. 1996). 16S rDNA sequences of approximately 750 bp were used in the analysis. For the detection of putative chimeric sequences, the Check_Chimera program of the Ribosomal Database Project was used (http://rdp.cme.msu.edu/html/index.html (Cole et al. 2003). We calculated phylogenetic distances using the Jukes–Cantor model. Tree topologies were evaluated using the neighbor-joining method and Phylo_win was utilized to plot the tree topologies (Galtier et al. 1996). The robustness of phylogenetic trees was tested by bootstrap analysis with 1,000 resamplings.

Nucleotide sequence accession numbers

The sequences reported here have been deposited in the Genbank database under the accession numbers AY768961 through AY769060.

Operational taxonomic unit diversity

Operational taxonomic unit (OTU) diversity was estimated by a measure of the relationship between species richness (SR; a function of the number of species in a community) and the distribution of individuals among species was estimated by calculating the Shannon–Weaver diversity index, H′ (Pielou 1966; Shannon and Weaver 1949):

$$H' = -\sum_{i=1}^{s} p_i \log p_i$$

where s is the number of OTUs, n_i is the number of individuals of the ith OTU, N is the total number of individuals and $p_i = n_i/N$, for the ith OTU.

OTU richness

The OTU richness was estimated using Margalef's Species Richness (SR) index, and was calculated (Margalef 1958) using the equation: $SR = (S - 1)/\ln N$, where S is the number of OTUs and N is the number of individuals in the sample.

Species evenness

A species evenness (J') index was calculated after (Pielou 1966) as: $J' = H'/\log S$ where H' is the Shannon–

Weaver diversity index and S is the number of OTUs. For the purposes of this study, two 16S rDNA sequences that show a 97% or higher sequence identity are considered to be members of the same OTU. Stackebrandt and Goebel (1994) demonstrated that strains that are more than 3% divergent in 16S rRNA are almost always members of different species (based on 70% DNA–DNA hybridization), whereas strains that are less than 3% divergent may or may not be members of different species. A cutoff of 3% divergence was therefore recommended as a conservative criterion for delineating between microbial species.

Results

Genomic DNA was extracted from three depth intervals (top, middle and bottom) of the sectioned sediment core. The concentration of total extracted DNA was the highest in the top zone of the core, decreased in the middle zone and was the lowest in bottom zone (data not shown). The decrease in DNA yield with depth suggested a decrease in overall microbial biomass with depth. A 16S rDNA clone library was constructed for each of the three vertical zones of the core using both archaeal- and bacterial-specific primers for a total of six clonal libraries.

Diversity, community structure and vertical zonation of bacteria

A total of 120 bacterial clones (42 clones from the top zone, 39 clones from the middle zone and 39 clones from the bottom zone) were screened by RFLP, grouped together based on their RFLP profiles and representative clones were sequenced. The bacterial clone libraries from the Florida Escarpment sediments were very diverse and included relatives of numerous cultured and uncultured lineages primarily within the proteobacteria, with numerous other bacterial phyla also represented. Considering all sediment layers together, members of the proteobacteria dominated the clonal libraries, with 33% belonging to the δ-proteobacteria, 26% belonging to the ε-proteobacteria and 8% belonging to the γ-proteobacteria. Clones related to *Chloroflexi*/green non-sulfur bacteria accounted for 12% of all clones and *Cytophaga–Flexibacter–Bacteroidetes* group (CFB group) sequences accounted for 13% of all clones. Sequences related to the Gram+ (Firmicutes), the Planctomyces, the *Fusobacterium* and the candidate division WS3 each accounted for 1% of all clones. Fifty-three of 86 unique sequences were found to be $\leq$ 95% similar to environmental and cultured 16S rDNA sequences from the databases and 16 sequences were $\leq$ 90% similar. The community structure of bacterial 16S rDNA clones from the Florida Escarpment libraries is summarized in Fig. 1. A table showing the Shannon–Weaver diversity indices, the Margalef's SR measures and the evenness measures for all six clonal libraries is shown in Table 1.

Twenty-nine unique clones were sequenced from the top zone of the core. The majority of the clones retrieved from the top zone (74%) were members of the proteobacteria, with 53% belonging to the ε-proteobacteria and 21% belonging to the δ-proteobacteria (Fig. 1a). Fourteen percent were members of the CFB group. The *Chloroflexi*/green non-sulfur bacteria, the Planctomyces and the *Fusobacterium* were each represented by 4% of the sequences from the top of the sediment core. The most abundant clones in the top zone were most closely related to ε-proteobacterial clones previously sequenced from cold seep areas of the Nankai Trough (Li et al. 1999a) and the Japan Trench (Inagaki et al. 2002). The Shannon–Weaver diversity measure was 2.99, the Margalef's richness measure was 5.38 and the evenness measure was 0.88 (Table 1).

The bacterial clone library from the middle zone of the core was composed of 34 unique clones out of a total of 39 clones. Within this zone, members of the proteobacteria accounted for 73% of all sequences, with δ-proteobacterial clones representing 31% of all sequences, γ-proteobacterial sequences representing 24% and ε-proteobacterial sequences representing 18% of the library (Fig. 1b). Clones associated with the CFB group accounted for 12% of the sequences and *Chloroflexi*/green non-sulfur sequences accounted for 9% of the library. Gram-+ (Firmicutes) and candidate division WS3 group clones each represented 3% of the clones from the middle zone of the core. Sequences related to the γ-proteobacteria were detected in only the middle zone and were not detected in either the top or bottom zones (Fig. 1a–c). Three clones (two from the top zone and one from the middle zone) were found to have 100% sequence identity to δ-proteobacterial clones recently sampled from a deep-sea hydrothermal vent located at 13° North on the East Pacific Rise (Alain et al. 2004). The Shannon–Weaver diversity value, the Margalef's richness value and the evenness value were 3.42, 6.24 and 0.88, respectively (Table 1).

The bacterial clone library constructed from the bottom zone of the core consisted of 23 unique clones (out of 39 clones screened by RFLP). Within this zone, the majority (55%) of the library was affiliated with the proteobacteria, with 49% belonging to the δ-proteobacteria and 6% belonging to the ε-proteobacteria. *Chloroflexi*/green non-sulfur sequences accounted for 25% of the library with CFB group sequences and Gram+ (Firmicutes) sequences accounting for 14 and 6% of the sequences, respectively (Fig. 1c). Sequences related to *Chloroflexi*/green non-sulfur bacteria increased significantly in the bottom zone over the number found in either the top or middle zones. Nine of the 23 unique sequences showed only $\leq$ 90% sequence identity to their closest relatives in 16S rDNA databases. The Shannon–Weaver diversity measure was 2.97, the Margalef's richness measure was 4.16 and the evenness measure was 0.95 (Table 1).

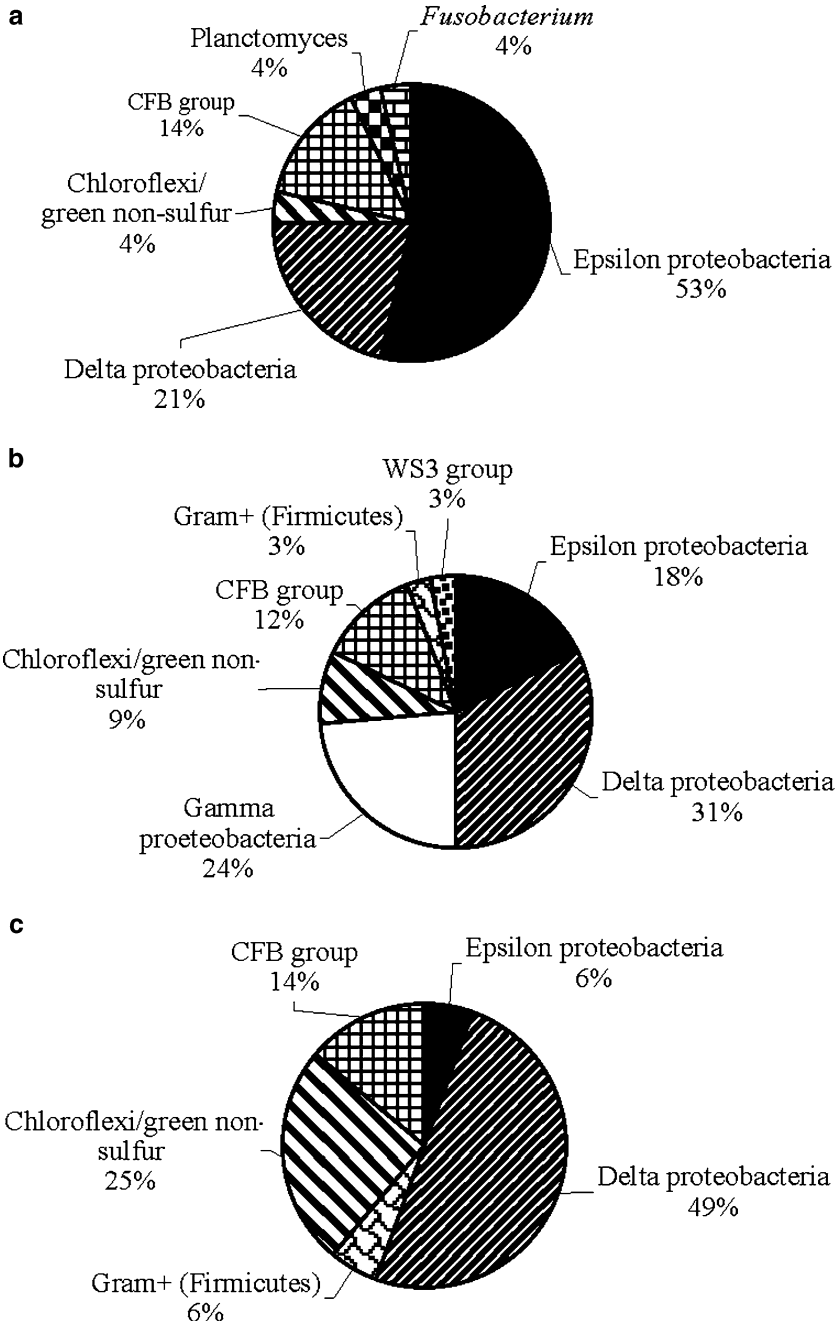

Fig. 1 Frequency of bacterial 16S rDNA clones in the top (**a**), middle (**b**), and bottom (**c**) regions of a sediment core from the Florida Escarpment

Table 1 Shannon–Weaver diversity indices, Margalef's species richness measure, and evenness measure estimates for both bacterial and archaeal clones

	Shannon–Weaver diversity	Margalef's richness	Evenness
Top archaeal	0.63	0.22	0.69
Middle archaeal	1.30	1.31	0.82
Bottom archaeal	1.76	1.77	0.82
Top bacterial	2.99	5.38	0.88
Middle bacterial	3.42	6.24	0.88
Bottom bacterial	2.97	4.16	0.95

Phylogenetic analysis of bacterial 16S rDNA sequences

Three different phylogenic trees are shown for the bacterial clones (Fig. 2a–c). In general, our clones were most closely related to groups already identified from marine sediments, cold seep environments or deep-sea hydrothermal vent environments. No clones related to the α- or β-proteobacteria were detected in this study.

The majority of δ-proteobacterial phylotypes detected in this study (14 clones; Fig. 2a) were grouped into six clusters. Two distinct clusters (one cluster of seven clones, including BotBac09, TopBac34 and Mid-

Bac44, and one cluster of two clones, BotBac13 and BotBac01) were most closely related to phylotypes detected in Cascadia Margin Sediments (Hyd89-21 and Hyd89-52, respectively; Knittel et al. 2003). TopBac03 clustered with a sulfate-reducer that anaerobically degrades alkanes (Hxd3; So and Young 1999), while clones MidBac22 and MidBac35 were related to both a sulfate-reducer that anaerobically degrades napthalene (Naphs2; Galushko et al. 1999) and a sequence from the Japan Trench (CS2.2; Inagaki et al. 2002). MidBac27 and MidBac40 were related to clones detected in marine sediments from Svalbard, Norway (Ravenschlag et al. 1999). Additionally, two unclassified clones (MidBac21 and MidBac39) are shown in Fig. 2a. These clones are related to *Calditrix abyssi*, a deep-sea vent thermophilic bacterium that grows by fermentation of proteinaceous substrates and by anaerobic respiration of nitrate to ammonium. This organism remains to date unclassified at the phylum level (Miroshnichenko et al. 1995).

Seventeen ε-proteobacterial phylotypes formed three distinct clusters grouped with a number of environmental clones (Fig. 2b) detected from a variety of deep-sea environments. Four clones (MidBac38, MidBac29, TopBac23 and TopBac10) formed a distinct cluster

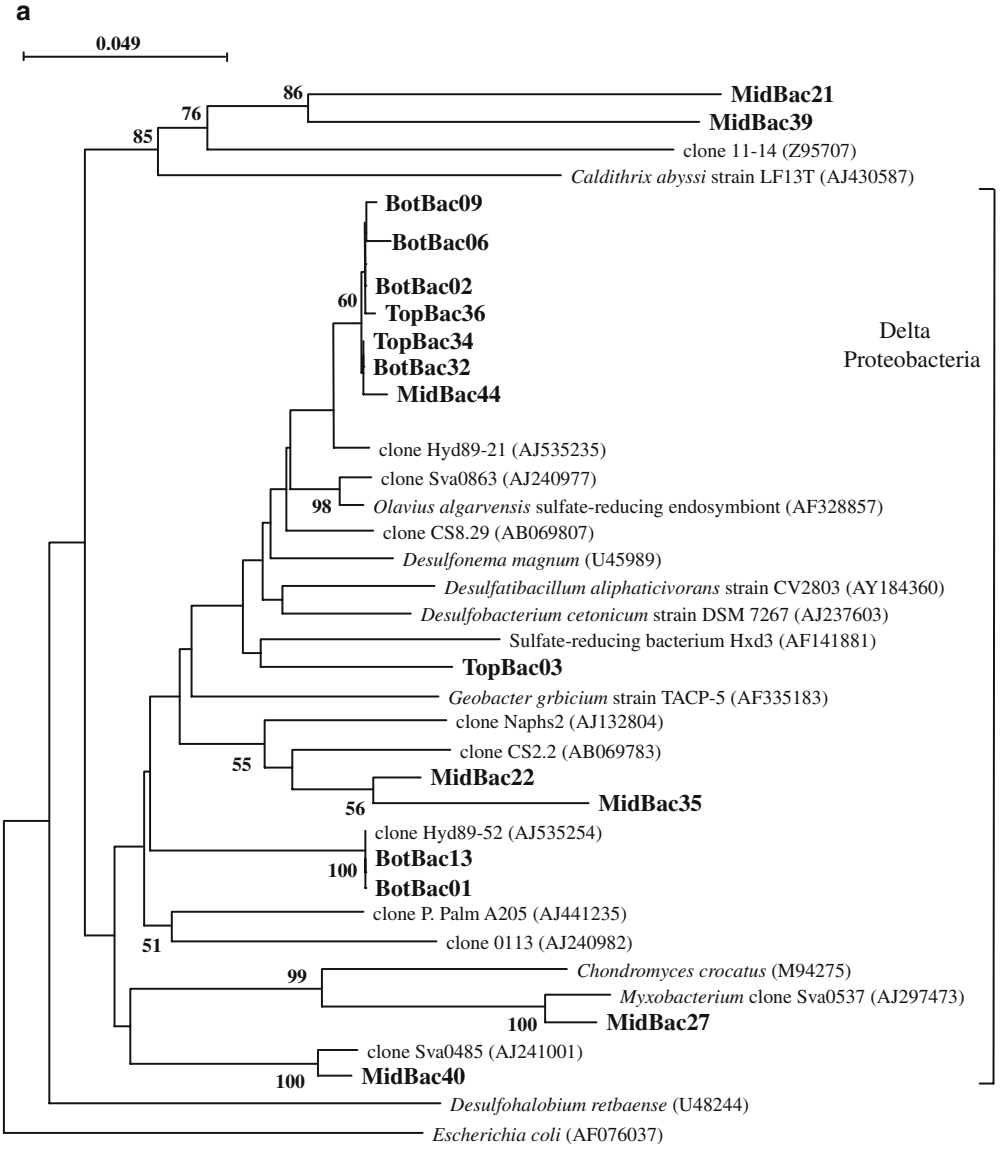

Fig. 2 Phylogenetic analyses of bacterial 16S rDNA sequences from the base of the Florida Escarpment. The trees were constructed using the neighbor-joining method from a similarity matrix based on the Jukes–Cantor distance model. Percentages greater than 50% of bootstrap resampling (of 1,000 resamplings) that support each topological element are indicated near the nodes. Representative sequences of rDNA clones obtained from Florida Escarpment sediment in this study are represented by *bold* letters. δ-Proteobacteria; the *scale bar* represents 4.9 substitutions per 100 sequence positions (**a**). ε-Proteobacteria; the *scale bar* represents 7.1 substitutions per 100 sequence positions (**b**). *Cytophaga–Flavobacterium–Bacteroidetes* group and *Chloroflexi*/green non-sulfur bacteria; the *scale bar* represents 11.3 substitutions per 100 sequence positions (**c**)

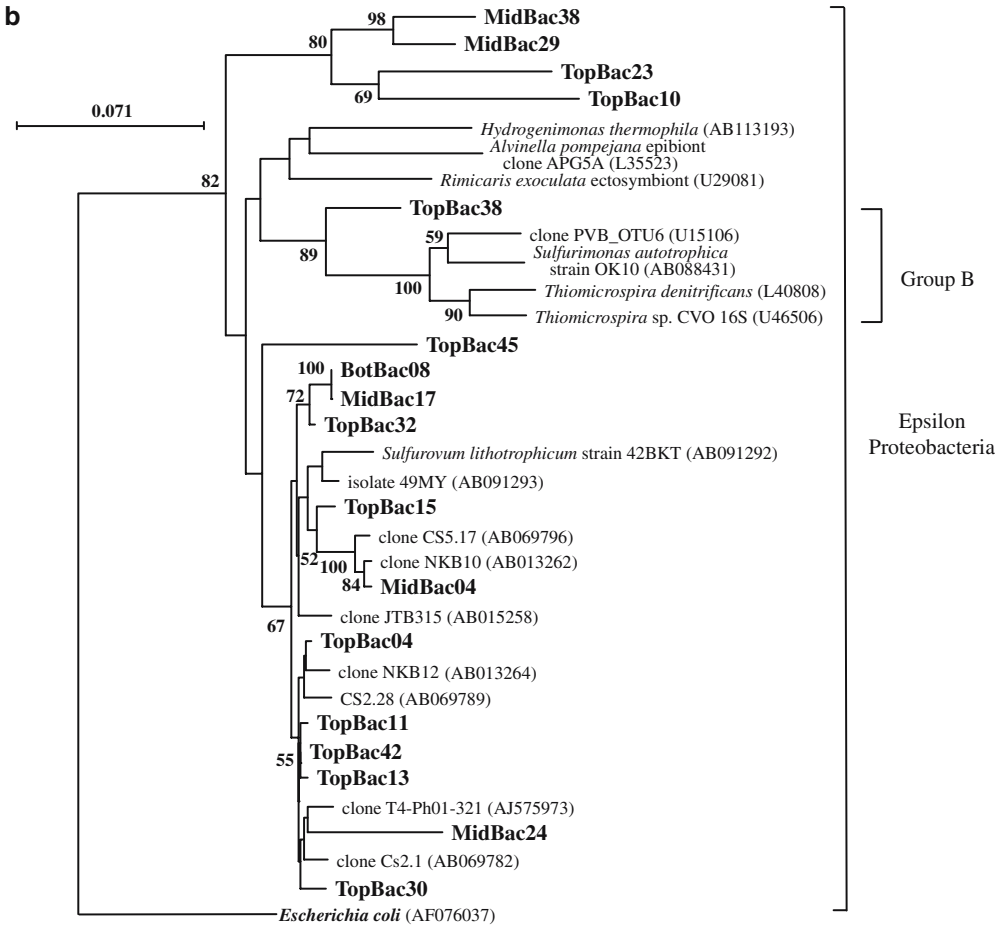

Fig. 2 (Contd)

without close relatives from sequence databases. TopBac38 was related to *Sulfurimonas autotrophica* strain OK10 (94% similarity), a sulfur-oxidizing bacterium isolated from the Okinawa Trough (Inagaki et al. 2003) and to clone PVB-OTU6, a sequence detected from Pele hydrothermal vent in Hawaii. Twelve clones formed a large cluster including clones MidBac04 and TopBac04, 11, 13, 15, 30 and 42, that were most closely related to environmental clones from cold seeps from the Japan Trench (clones CS5.17, CS2.1 and CS2.28; Inagaki et al. 2002), the Nankai Trough (clones NKB10 and NKB12; Li et al. 1999a) as well as clone MidBac24, that was related to a sequence (T4-Ph01-321) that was found to be physically associated with the vent polychaete tubeworm *Alvinella pompejana* (Alain et al. 2004). Within the same large cluster of sequences, clones TopBac32, MidBac17 and BotBac08 formed a discrete subcluster with 96–97% sequence identity to cold seep clones (Fig. 2b). Sixteen of the 17 ε-proteobacterial clones sequenced in this study were detected in the top or middle zones, with one clone detected in the bottom zone of the core.

All γ-proteobacteria in this study (8% of the total bacterial clones, and 24% of the clones detected in the middle zone of the core) were detected in the middle zone of the sediment core, and were most closely related to sequences retrieved from marine sediments in Norway (clones Sva0071 and Sva0091; Ravenschlag et al. 1999) and from coastal marine sediments (clone TIHP368-30, H. Urakawa et al., unpublished).

Phylogenetic analysis indicated that several clones detected in this study were related to the CFB group. Clones MidBac26, MidBac41 and TopBac27, were related to members of the CFB previously detected in association with the deep-sea vent tubeworm *A. pompejana* (strain BHI60-95B; Alain et al. 2002) and drain water from a uranium waste pile (clone GR-WP33-68; Radeva and Selenska-Pobell 1999) (Fig. 2c). TopBac41 was found to be most closely related to environmental sequences obtained from sulfurous springs (clone sipk20; Rudolph et al. 2001), and from an extinct smoker pipe from Kolbeinsey Ridge (clone Ko710, unpublished). Clones BotBac18 and MidBac32 formed a discrete cluster related to TopBac41 (Fig. 2c). Several clones were classified as members of the green non-sulfur/*Chloroflexi* group (Fig. 2c) BotBac07, 10 and 40 were most closely related to environmental sequences recovered from the mucous secretions of the hydrothermal vent polychaete *Paralvinella palmiformis* (clone P. palm C 37; Alain et al. 2002), while MidBac11 and 43 were related to sequences from cold seep sediments of

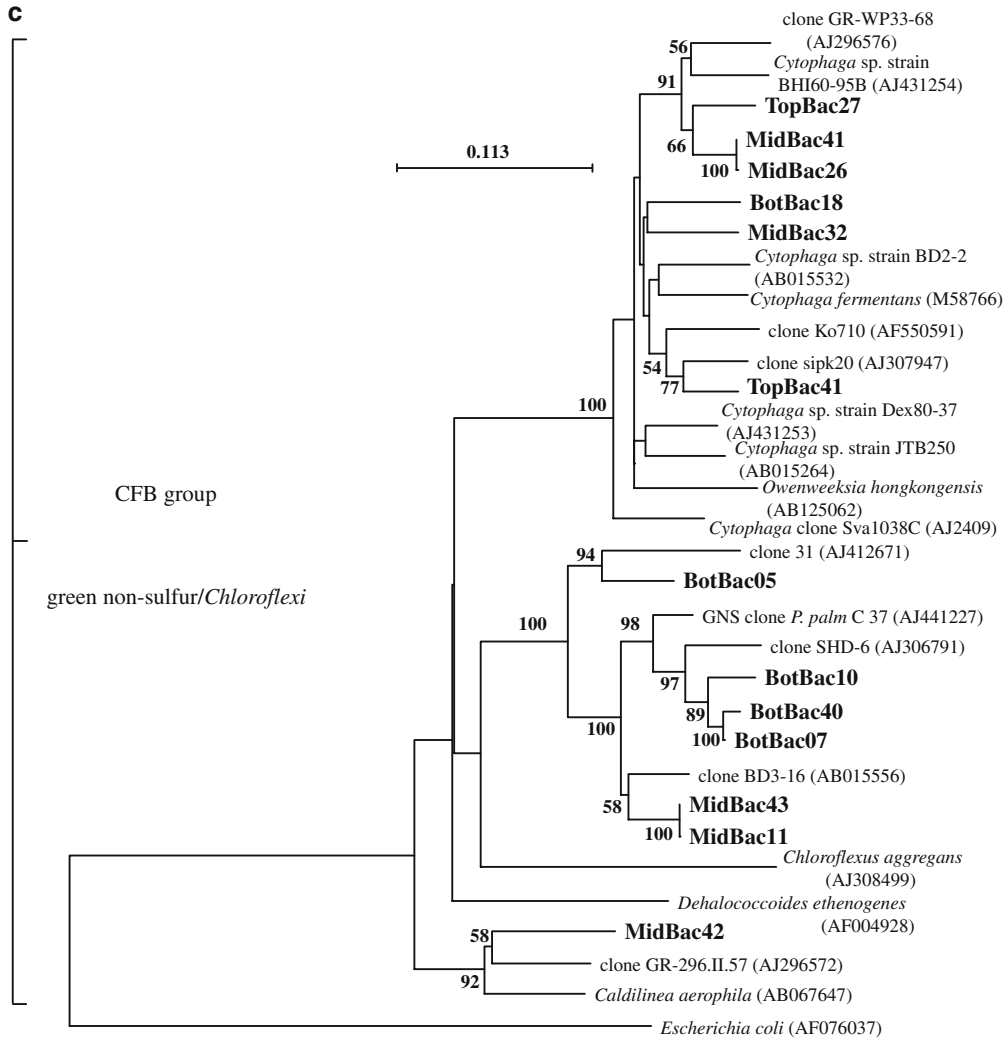

Fig. 2 (Contd)

the Japan Trench (clone BD3-16; Li et al. 1999b). Clone MidBac42 was related to a sequence retrieved from the drain water of a uranium waste pile (clone GR-296.II.57; Radeva and Selenska-Pobell 1999) and to *Caldilinea aerophila* (87% similarity), a thermophilic filamentous green non-sulfur bacterium isolated from a hot spring in Japan, while BotBac05 was related to a clone isolated from a denitrifying reactor (Etchebehere et al. 2002).

Cytophaga–Flexibacter–Bacteroidetes group clones remained a steady 12–14% of the clones throughout the depth of the sediment core (Fig. 1a–c). *Chloroflexi*/green non-sulfur clones increased with depth, representing 4% of the bacterial clones in the top zone, 9% in the middle zone and 25% in the bottom zone (Fig. 1a–c).

Several phyla of microorganisms were represented only infrequently in this study (data not shown). Among these scarcely represented phylotypes, a single clone, MidBac45, was classified as a member of candidate division WS3, a novel phylogenetic group containing no cultured members. Other candidate division WS3 clones have been detected in soil samples and in hydrocarbon seep sediment from Coal Oil Point Seep Field at a depth of 22 m in the Santa Barbara Channel, California (La-Montagne et al. 2004), as well as anoxic marine sediment at Loch Duich, Scotland (Freitag and Prosser 2003). Another clone, TopBac31, was classified as a *Fusobacterium* and was most closely related to an environmental clone detected from a hydrothermal area at the Kolbeinsey Ridge (clone Ko711, unpublished). The phylum Planctomyces was represented by one clone, TopBac09, and was most closely related to an environmental clone from the Nankai Trough (clone BD2-16; Li et al. 1999a). The Gram + Firmicutes phylum was represented by three clones (BotBac20, BotBac24 and MidBac07) that were most closely related to environmental clones from coastal marine sediment (clone TIHP368-03, unpublished).

Diversity, community structure and vertical zonation of archaea

The diversity of the archaeal community was evaluated by the construction of three 16S rDNA clone libraries

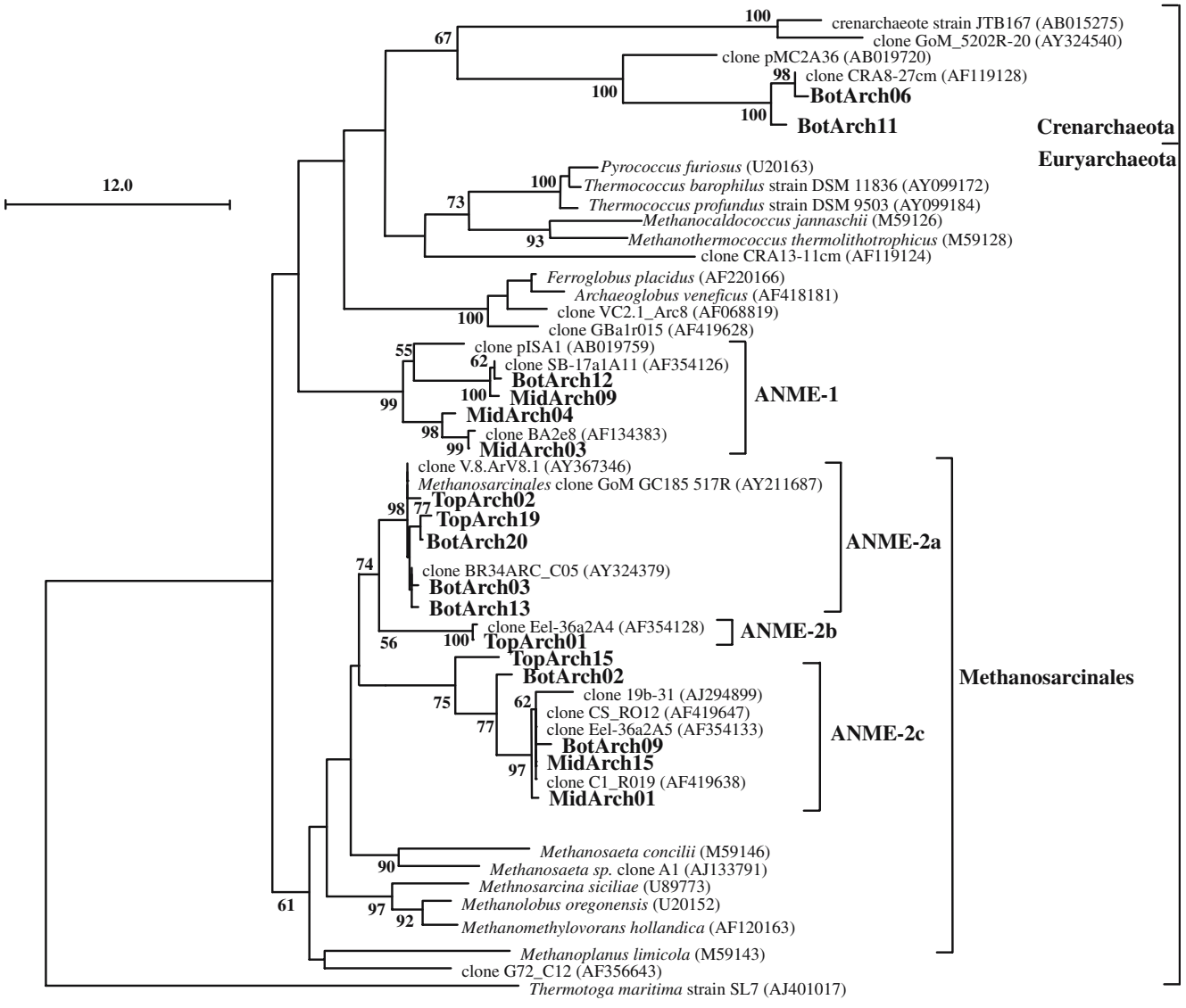

Fig. 3 Phylogenetic tree of archaeal 16S rDNA sequences from the base of the Florida Escarpment. The tree was constructed using the neighbor-joining method from a similarity matrix based on the Jukes–Cantor distance model. *Thermotoga maritima* was used as the outgroup. The *scale bar* represents 12.0 substitutions per 100 sequence positions. Percentages greater than 50% of bootstrap resampling (of 1,000 resamplings) that support each topological element are indicated near the nodes. Representative sequences of rDNA clones obtained from Florida Escarpment sediment in this study are represented by *bold* letters

(top, middle and bottom) and subsequent phylogenetic analysis. A total of 68 archaeal clones (22 clones from the top zone, 23 clones from the middle zone and 23 clones from the bottom zone) were screened by RFLP, grouped together based on their RFLP profiles and representative clones were partially sequenced. The archaeal clone libraries from the Florida Escarpment sediments showed low diversity overall, although there was a significant increase in diversity and richness with depth (Table 1). Overall, archaeal clones sequenced in this study showed relatively low sequence identity with sequences in rDNA databases; 14 of the 17 (82%) unique archaeal phylotypes showed $\leq 95\%$ similarity to cultivated or uncultivated archaea, 2 of the 17 (12%) showed $\leq 90\%$ sequence similarity and just 1 of the 17 (6%) showed $\geq 95\%$ sequence similarity.

Both archaeal diversity, as measured by the Shannon–Weaver diversity index, and SR, as measured by Margalef's SR measure, increased with depth in the sediment core (Table 1). Archaeal diversity in the top zone measured 0.63, increased to 1.30 in the middle zone and to 1.76 in the bottom zone. The Margalef's richness measure was very low at 0.22 in the top zone of the core, increased to 1.31 in the middle zone and increased again to 1.77 in the bottom zone. The estimation of the coverage of our clonal libraries was generally high for the archaeal libraries and lower for the bacterial clone libraries (Table 1).

Phylogentic analysis of archaeal 16S rDNA sequences

To infer phylogenetic affiliations of archaeal rDNA clones, representative phylotypes were sequenced and investigated using phylogenetic analysis. A total of 68 archaeal clones were screened by RFLP analysis and 17 unique phylotypes were identified and sequenced. Phylogenetic analysis of these sequences by the neighbor-joining method revealed the presence of five distinct clusters of sequences in the sediment sampled from the base of the Florida Escarpment (Fig. 3). Of these five clusters, four grouped within the Euryarchaeota and one grouped within the Crenarchaeota.

The archaeal library (combined top, middle and bottom zones) was dominated (90%) by clones that were grouped within the *Methanosarcinales*. Within the *Methanosarcinale*s, Orphan et al. (2001a) showed that the ANME-2 cluster could be separated into three distinct clusters. All three clusters (ANME-2a, AMNE-2b and AMNE-2c) are represented in this study. Twenty-three clones (34% or all archaeal clones) cluster into the ANME-2a group, 15 clones (22%) cluster into the ANME-2b group, and 23 clones (34%) cluster into the ANME-2c group. The ANME-2a group sequences identified in this study (represented by five clones in Fig. 3) were related to environmental sequences from other methane-rich sediments in the Gulf of Mexico (clone GoM GC185 517R; Mills et al. 2003) and from sediment from the Blake Ridge (clone BR34ARC_C05; Hallam et al. 2003). One phylotype (TopArch01), representing 68% of the clones from the top zone of the core and 22% of all archaeal clones, was clustered in the ANME-2b group and was most closely related to an environmental clone sampled from a methane seep at the Eel River Basin (clone Eel-36a2A4; Orphan et al. 2001a). Five clones, representing 31% of all archaeal sequences, were clustered in the ANME-2c group and were related to environmental clones sampled from a shallow water hydrothermal vent in the Aegean Sea (clone 19b-31, unpublished), clones from both sediment and bottom seawater from the Guaymas Basin (clones C1_R019 and CS_RO12; Teske et al. 2002), and clones from a methane seep at the Eel River Basin (clone Eel-36a2A5; Orphan et al. 2001a).

The four clones that clustered within the ANME-1 group accounted for 6% of the total archaeal library and were related to other ANME-1 group clones detected from methane seep environments (clone SB-17a1A11; Orphan et al. 2001a), anaerobic, methane-rich sediments (Hinrichs et al. 1999) and hydrothermally heated sediment from the Okinawa Trough (clone pISA1; Takai and Horikoshi 1999). Both ANME-1 and ANME-2 have been often detected in various methane seep environments (e.g., Orphan et al. 2001a; Reed et al. 2002; Teske et al. 2002) and have been linked in the microbially mediated anaerobic oxidation of methane (AOM; Boetius et al. 2000).

Clones BotArch06 and BotArch11, which represent 3% of all archaeal sequences, were associated with the Crenarchaeota, and were grouped with a sequence detected in the lower section of deep-sea sediments from the NW Atlantic Ocean (clone CRA8-27 cm; Vetriani et al. 1999). These clones were also related to a sequence detected in a black smoker chimney sample from Myojin Knoll, Izu-Ogasawara arc (clone pMC2A36; Takai and Horikoshi 1999).

Discussion

The diversity, community structure and vertical zonation of sedimentary bacteria and archaea in the methane-rich sediments found at the base of the Florida Escarpment were evaluated using a 16S rDNA-based survey. Most sequences recovered represented as-yet-uncultivated phylotypes distinct from any other cultivated or environmental clones.

Shift in microbiological community structure with depth

Clonal libraries were created from three vertical zones with different chemical and physical properties. Environmental characteristics have an impact on the microbial communities and are important in determining the microbial community structure and diversity in a given environment. This study revealed that the microbial community structure shifted significantly with increasing depth in Florida Escarpment sediment. For bacterial clones, the Shannon–Weaver diversity did not change greatly with depth in the sediment, but the community structure shifted significantly. In the top layer of the core, 53% of all top layer bacterial clones were members of the ε-proteobacteria (Fig. 1a). The percentage of ε-proteobacterial clones decreased to 18% in the middle zone of the core and again to 6% in the bottom zone of the core. The ε-proteobacterial sequences clustered with a number of sequences designated as Cold Seep ε-Group from cold seep areas of the Nankai Trough (Li et al. 1999a) and the Sanriku Escarpment of the Japan Trench (Inagaki et al. 2002) (Fig. 2b). All ε-proteobacteria isolated to date have been showed to be involved in the sulfur cycle by acting as sulfur reducers or sulfide oxidizers with some species having the capability to carry out both reactions (Schumacher et al. 1992). Members of the ε-proteobacteria are metabolically diverse and have the ability to utilize a variety of electron acceptors including nitrate, various sulfur species, and oxygen under microaerophilic conditions (Finster et al. 1997; Stolz et al. 1999). Recent microbial diversity studies in hydrothermal vent areas have shown that ε-proteobacteria can be dominant members of the hydrothermal vent areas and may be important mediators of both the sulfur and nitrogen cycling in hydrothermal vent ecosystems (Longnecker and Reysenbach 2001; Polz and Cavanaugh 1995; Reysenbach et al. 2000; Takai et al. 2003; Voordeckers et al. 2005). Also, ε-proteobacterial

sequences have only been rarely detected in typical non-seep marine sediment (e.g., Li et al. 1999b; Urakawa et al. 1999, 2000) and not at all in a microbial community associated with Gulf of Mexico gas hydrates (Lanoil et al. 2001) and gas hydrate sediments from the Cascadia Margin (Marchesi et al. 2001). It is possible that the dominance of ε-proteobacterial sequences in the top zone of the sediment of the Florida Escarpment is driven by the relatively high concentration of hydrogen sulfide (5.7 mM) measured in the porewater (Chanton et al. 1991) coupled with the availability of oxygen in the top zone of the core. As phylogenetic microbial diversity surveys of cold seeps and hydrothermal vent areas expand, the distribution of ε-proteobacteria and their importance in the sulfur cycle in these environments becomes more evident.

The percentage of δ-proteobacterial clones detected in this study increased with depth in the sediment. δ-Proteobacterial clones in the middle and bottom zones of the Florida Escarpment core, dominated the libraries representing 31 and 49% of the clones, respectively (Fig. 1b–c). Many of the δ-proteobacteria in cultivation are SRB. The SRB are strict anaerobes that generate their energy by the anaerobic respiration of a variety of organic compounds, coupled to the reduction of sulfate. SRB generally outcompete methanogenic archaea for hydrogen in sulfate-rich sediments, but some methanogenesis can occur because some methanogens can use non-competitive substrates that are inaccessible to SRB (e.g., methylamine utilization by some members of the Methanosarcinales). The fact that δ-proteobacterial clones dominated our libraries in the deeper, H_2S-rich and increasingly anoxic zones of the Florida Escarpment core is consistent with the general requirement of these organisms for reducing conditions.

Methane and sulfide are primary sources of energy supporting the dense macrobiological communities found at the base of the Florida Escarpment and both free-living and symbiotic microorganisms depend on energy provided by the sulfide and methane in the underlying sediments. Methane has been shown to be consumed by a microbial consortium of methane-oxidizing archaea and SRB (Boetius et al. 2000) and any methane that leaks out of the methane reservoir is oxidized as long as sulfate is available.

The microbial symbionts of a number of cold seep and hydrothermal vent macrofauna are sulfide-oxidizing γ-proteobacteria (Distel et al. 1988). Also, the filamentous sulfur-oxidizing γ-proteobacteria *Beggiatoa* and *Thiothrix* are known to occur in large mats in Gulf of Mexico seep areas (Larkin and Henk 1996). In our study, γ-proteobacterial clones were not detected in the top or bottom zones but accounted for 24% of the bacterial clones in the middle zone (Fig. 1). Further, γ-proteobacteria are known to quickly colonize all available surfaces, both biological and mineral, in hydrothermal areas (López-Garcia et al. 2001). One clone, MidBac25, was most closely related to the symbiont of the Atlantic Coast protobranch bivalve *Solemya velum* and one phylotype, representing five clones, was 97.5% identical to a clone detected from the cold seep area of the Japan Trench (Li et al. 1999c). The fact that no γ-proteobacterial clones were detected in the top zone of the sediment and that no α- or β-proteobacterial sequences were detected in this study at all may indicate that our sediment was not contaminated by bottom water, and that we surveyed only the sediment microbial community. Members of the α-proteobacteria are thought to dominate the water column in some deep-sea environments (Fuhrman and Davis 1997; Mullins et al. 1995), and both α- and β-proteobacteria have been detected in other cold seep environments like the Japan Trench (Inagaki et al. 2002) and the Nankai Trough (Li et al. 1999a).

The *Chloroflexi*/green non-sulfur bacteria have been recognized as a division-level bacterial group for almost 20 years (Woese 1987), yet this division is still represented by only very few isolates. The clones detected in this study are related to members of the *Chloroflexi*/green non-sulfur group that were previously found in a wide range of environments, including hot springs, subsurface environments and marine environments. The percentage of clones of *Chloroflexi*/green non-sulfur bacteria increased with depth in the Florida Escarpment core, suggesting that they may play an important role in deeper, more anoxic microhabitats.

Archaea of the ANME-1 and AMNE-2 groups have no previously cultivated members, but form a distinct cluster within the *Methanosarcinales*, the only archaea that can utilize acetate, methylamines or methanol. Other investigations have demonstrated that archaea associated with the ANME-2 group have been detected in a number of methane-rich marine environments such as another site in the Gulf of Mexico (Lanoil et al. 2001), cold seeps in the Eel River Basin (Hinrichs et al. 1999), and methane hydrate-associated sediments in the Cascadia Margin (Boetius et al. 2000). ANME-2 group archaea have been shown to act in concert with SRB to oxidize methane in a number of methane-rich sedimentary environments (Boetius et al. 2000; Girguis et al. 2003; Orphan et al. 2001a, b). More recently, ANME-1 group archaea have been suggested to have an active role in anaerobic methane oxidation as well, based on molecular, isotopic, and phylogenetic evidence (Orphan et al. 2002; Teske et al. 2002). Furthermore, the identification of most of the genes associated with methanogenesis in the ANME-1 group suggests that these organisms may oxidize methane by reverse methanogenesis (Hallam et al. 2004). The finding of sequences related to both the ANME-1 and ANME-2 groups in this study suggests that they may be associated with the anaerobic oxidation of methane in the sediments at the base of the Florida Escarpment.

The diverse bacterial and archaeal microbial communities of the sediments found at the base of the Florida Escarpment suggest a variety of physiologies, as well as a vast potential for the discovery of novel organisms. The proteobacteria, CFB group, *Chloroflexi*/green non-sulfur and ANME groups 1 and 2 lineages identified in our study have relatives found in other

marine environments including methane seeps, marine sediments and hydrothermal environments. The molecular analysis of functional genes and the concomitant analysis of large genomic fragments from environmental DNA will lead to a better understanding of these complex microbial systems.

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