

**MOLECULAR ANALYSIS OF MICROBIAL 16S rRNA, *mcrA*, *dsrAB* AND *pmoA*
GENES FROM DEEP-SEA HYDROTHERMAL VENT AND COLD SEEP SITES**

by

ANDREW JAY REED

A Dissertation submitted to the
Graduate School-New Brunswick
Rutgers, The State University of New Jersey
in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in Ecology, Evolutionary Biology and Natural Resources

written under the direction of

Dr. Richard A. Lutz

and approved by

Richard A. Lutz

Costantino Vetriani

Timothy M. Shank

Michael J. Kennish

New Brunswick, New Jersey

October 2008

ABSTRACT OF THE DISSERTATION

MOLECULAR ANALYSIS OF MICROBIAL 16S rRNA, *mcrA*, *dsrAB* AND *pmoA* GENES FROM DEEP-SEA HYDROTHERMAL VENT AND COLD SEEP SITES

By ANDREW JAY REED

Dissertation Director:
Dr. Richard A. Lutz

Methane and sulfide are primary sources of energy supporting the dense macrobiological communities often found in cold seep areas and both free-living and symbiotic microorganisms depend on energy provided by the sharp gradient of sulfide and the reservoir of methane in the underlying sediments. In deep-sea hydrothermal vent areas, sulfide is typically the primary source of energy for microbial primary production. To investigate microbial populations and community structure in these areas, microbial communities from both deep-sea hydrothermal vent (Rainbow and Logatchev hydrothermal vent fields, Mid-Atlantic Ridge, Atlantic Ocean; 9°N hydrothermal vent area, East Pacific Rise, Pacific Ocean) and cold seep areas (Blake Ridge, western Atlantic Ocean; Florida Escarpment, Gulf of Mexico) were evaluated by molecular phylogenetic analysis of 16S rRNA, *mcrA*, *dsrAB* and *pmoA* gene sequences. Twenty-one clone libraries were obtained using DNA from friable vent chimney material (hydrothermal vent areas) and from vertically subsampled (top, middle and bottom) sediment core samples (cold seep areas), screened by RFLP and sequenced.

At the Florida Escarpment, phylogenetic analysis of bacterial 16S rDNA 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. Archaeal diversity was low throughout, but increased with depth. Cold seep *mcrA* sequences were distributed among the ANME-2c, -2d and -2e groups. Clone library *dsrAB* sequences grouped primarily within the orders *Desulfobacteriales*, *Syntrophobacteriales* and the gram-positive order Clostridiales. Clone library *pmoA* sequences grouped among the Type I methanotrophs (γ -proteobacteria) within the order *Methylococcales*. Most sequences recovered represented as-yet-uncultivated phylotypes distinct from any other cultivated or environmental clones. In addition, this investigation presents for the first time environmental *mcrA* sequences detected from chimney samples collected from a bare basalt, high-temperature deep-sea hydrothermal vent area that are related to ANME-2e sequences previously shown to be associated with the anaerobic oxidation of methane.

PREFACE

This dissertation was written and is presented as three stand-alone research papers.

Chapter 1 was published with minor changes:

Reed, A.J., Lutz, R.A., and Vetriani, C. (2006) Vertical distribution and diversity of bacteria and archaea in sulfide and methane-rich cold seep sediments located at the base of the Florida Escarpment. *Extremophiles* **10**: 199-211.

Chapter 2 will be submitted to Microbial Ecology with some minor changes. The journal of publication for Chapter 3 has yet to be decided.

ACKNOWLEDGEMENTS

I would like to thank the members of my committee for their support and guidance. I'd also like to thank Linda Ciak and all members past and present of the Lutz and Vetriani labs for making Rutgers such an enjoyable place to work.

TABLE OF CONTENTS

| | |
|--|---------------|
| ABSTRACT OF THE DISSERTATION | ii |
| PREFACE | iv |
| ACKNOWLEDGEMENTS | v |
| LIST OF TABLES | ix |
| LIST OF FIGURES | x |
| CHAPTER 1: Vertical Distribution and Diversity of Bacteria and Archaea in Sulfide and Methane-Rich Cold Seep Sediments Located at the Base of the Florida Escarpment | 1 |
| Abstract | 1 |
| Introduction | 2 |
| Materials and Methods | 5 |
| Sample Collection | 5 |
| DNA Extraction | 6 |
| DNA Amplification by PCR | 6 |
| Construction of Clonal Libraries | 7 |
| Restriction Fragment Length Polymorphism (RFLP) Screening | 7 |
| Sequencing and Phylogenetic Analysis | 7 |
| Nucleotide Sequence Accession Numbers | 8 |
| Application of Ecological Indices to Measure Microbial Diversity | 9 |

| | |
|--|--------|
| Operational Taxonomic Unit (OTU) Diversity | 9 |
| OTU Richness | 9 |
| Species Evenness | 9 |
| Results | 10 |
| Diversity, Community Structure and Vertical Zonation of Bacteria | 10 |
| Phylogenetic Analysis of Bacterial 16S rDNA Sequences | 16 |
| Diversity, Community Structure and Vertical Zonation of Archaea | 25 |
| Phylogenetic Analysis of Archaeal 16S rDNA Sequences | 26 |
| Discussion | 29 |
| Shift in Microbiological Community Structure with Depth | 29 |
| CHAPTER 2: Phylogenetic Diversity of <i>mcrA</i> and <i>dsrAB</i> from Deep-Sea Hydrothermal Vent and Cold Seep Sites | 35 |
| Abstract | 35 |
| Introduction | 36 |
| Materials and Methods | 42 |
| Sample Collection | 42 |
| DNA Extraction and DNA Amplification by PCR | 43 |
| Construction of Clonal Libraries | 44 |
| Restriction Length Polymorphism (RFLP) Screening | 45 |
| Sequencing and Phylogenetic Analysis | 45 |
| Nucleotide Sequence Accession Numbers | 46 |
| Results | 46 |

| | |
|--|----|
| Diversity of <i>mcrA</i> and Phylogenetic Analysis | 46 |
| Diversity of <i>dsrAB</i> and Phylogenetic Analysis | 50 |
| Discussion | 52 |
| | |
| CHAPTER 3: Diversity of Sedimentary Aerobic Methanotrophs by Molecular Analysis of Particulate Methane Monooxygenase (<i>pmoA</i>) Genes from Cold Seeps | 58 |
| Abstract | 58 |
| Introduction | 59 |
| Materials and Methods | 60 |
| Sample Collection | 60 |
| DNA Extraction and DNA Amplification by PCR | 61 |
| Construction of <i>pmoA</i> Clonal Libraries | 61 |
| Restriction Length Polymorphism (RFLP) Screening | 62 |
| Sequencing and Phylogenetic Analysis of <i>pmoA</i> Genes | 62 |
| Nucleotide Sequence Accession Numbers | 63 |
| Results | 63 |
| Diversity of <i>pmoA</i> and Phylogenetic Analysis | 64 |
| Discussion | 66 |
| References | 69 |
| Cirriculum Vita | 82 |

LIST OF TABLES

| | |
|---|----|
| Table 1. Sampling locations, sample type and gene library information for cold seep and hydrothermal vent 16S rRNA and functional gene libraries. | 11 |
| Table 2. Frequency of bacterial 16S rDNA clones in the top (a), middle (b), and bottom (c) sections of a sediment core from the Florida Escarpment. | 14 |

LIST OF FIGURES

- Figure 1. Community structure of top (A), middle (B) and bottom (C) Bacterial 16S rDNA clones. 13
- Figure 2a. Phylogenetic tree of δ -Proteobacterial clones 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. *Escherichia coli* was used as the outgroup. The scale bar represents 4.9 substitutions per 100 sequence positions. Percentages greater than 50% of bootstrap resampling (of 1000 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. 17
- Figure 2b. Phylogenetic tree of ϵ -Proteobacterial clones 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. *Escherichia coli* was used as the outgroup. The scale bar represents 12.0 substitutions per 100 sequence positions. Percentages greater than 50% of bootstrap resampling (of 1000 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. 18
- Figure 2c. Phylogenetic tree of γ -Proteobacterial clones 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. *Aquifex pyrophilus* was used as the outgroup. The scale bar represents 5.5 substitutions per 100 sequence positions. Percentages greater than 50% of bootstrap resampling (of 1000 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. 19
- Figure 2d. Phylogenetic tree of Cytophaga-Flavobacterium-Bacteroidetes (CFB) group and *Chloroflexi*/Green non-sulfur clones 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. *Escherichia coli* was used as the outgroup. The scale bar represents 6.2 substitutions per 100 sequence positions. Percentages greater than 50% of bootstrap resampling (of 1000 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. 20
- Figure 2e. Phylogenetic tree of *Fusobacterium* and Actinomycete clones 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.

Escherichia coli was used as the outgroup. The scale bar represents 4.9 substitutions per 100 sequence positions. Percentages greater than 50% of bootstrap resampling (of 1000 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.

21

Figure 3. Phylogenetic tree of archaeal clones 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 1000 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.

27

Figure 4. Phylogenetic tree of *mcrA* sequences from the base of the Florida Escarpment, the Blake Ridge and the deep sea hydrothermal vent areas Logatchev and Rainbow. The trees were constructed using the neighbor-joining method with translated amino acid sequences. Percentages greater than 50% of bootstrap resampling (of 1000 resamplings) that support each topological element are indicated near the nodes. Representative sequences of *mcrA* sequences from this study are shown in bold letters. Group name nomenclature in for *mcrA* sequences follows that of (Hallam *et al.*, 2003). The scale bar represents 5% estimated distance. The symbol ▲ designates sequences from this study taken from deep-sea hydrothermal vents and the symbol ● designates sequences from this study taken from cold seep areas.

48

Figure 5. Vertical distribution and community structure of *mcrA* clones in the Florida Escarpment and Blake Ridge sediment cores.

49

Figure 6. Phylogenetic tree of *dsrAB* sequences detected from the base of the Florida Escarpment, the Blake Ridge and the deep sea hydrothermal vent areas Rainbow and 9° North on the EPR. The tree was constructed using the neighbor-joining method with translated amino acid sequences. The scale bar represents 10% estimated distance. Percentages greater than 50% of bootstrap resampling (of 1000 resamplings) that support each topological element are indicated near the nodes. Representative sequences of *dsrAB* clones from this study are shown in bold letters. The symbol ▲ designates sequences from this study taken from deep-sea hydrothermal vents and the symbol ● designates sequences from this study taken from cold seep areas.

53

Figure 7. Phylogenetic tree of particulate methane monooxygenase subunit A (*pmoA*) protein sequences detected in the sediment at the base of the Florida Escarpment

and from the Blake Ridge. The tree was constructed using the neighbor-joining method with translated amino acid sequences. Percentages greater than 50% of bootstrap resampling (of 1000 resamplings) that support each topological element are indicated near the nodes. Sequences from this study are shown in bold letters. The scale bar represents 10% estimated distance.

65

Chapter 1: Vertical Distribution and Diversity of Bacteria and Archaea in Sulfide and Methane-Rich Sediments Located at the Base of the Florida Escarpment

Chapter 1: 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. 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 (CFB) 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.

Chapter 1: 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 degrees) which rises ~2000m from the seafloor at a depth of 3270m 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 ($\delta^{13}\text{C} = -61$ 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.* (1999a) 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. Craig *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, 2001b, 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.

Chapter 1: Materials and Methods

Sample Collection. An acrylic tube sediment core (22 cm length x 6 cm width) was collected from sediments at the base of the Florida Escarpment, Gulf of Mexico (latitude 26° 01.8 north, longitude 84° 54.9 west) during the AT-03, leg 58 cruise of RV *Atlantis* and dive 3637 of DSV *Alvin* at a depth of 3288 meters in October of 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 H₂S (indicative of oxygen depletion) 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) 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'-AGGCCTCAATTGCAGGCAGGC-3') and the forward primer 16F (5'-ATAACTGTACTCAGAGCTGAGCCTA-3') were used to selectively amplify bacterial and archaeal clones, respectively. The reverse primer 1517R (5'-CTGGTTGATCCTGCCAG-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) 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) following the manufacturers' recommendations. Amplified 16S rRNA gene fragments were cloned in pCRII plasmid vector (Invitrogen, Inc., Carlsbad, CA) and the ligation products were used to transform competent *E.coli* INV α F' 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) as described in the manufacturers' instructions. Six 16S rDNA libraries (1 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 Length Polymorphism (RFLP) Screening. Clonal inserts of 16S rDNA fragments were digested for 2 hours at 37°C on a thermocycler using the tandem tetrameric restriction endonucleases *Hae*III and *Msp*I (Promega, Inc. Madison, WI). The restriction products were run for 1.5 hours at 75V and 4°C on a 2.5% (weight/volume) Methaphor agarose gel containing ethidium bromide and visualized under UV light.

Sequencing and Phylogenetic Analysis. Representative bacterial and archaeal 16S rDNA clones from unique RFLP patterns were sequenced utilizing an automated DNA

sequencer (310 Avant Genetic Analyzer; Applied Biosystems, Foster City, CA). 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 (NCBI) (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 1000 resamplings.

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

Application of Ecological Indices to Measure Microbial Diversity

Operational Taxonomic Unit (OTU) diversity. OTU diversity was estimated by a measure of the relationship between species richness (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' (Shannon and Weaver, 1949; Pielou, 1966):

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

where s is the number of Operational Taxonomic Units (OTUs), n_i is the number of individuals of the i th OTU, N is the total number of individuals and $p_i = n_i / N$, for the i th OTU.

OTU Richness. The OTU richness was estimated using Margalef's species richness index, (SR), and was calculated following (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 et al. (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.

Chapter 1: 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-specific and bacterial-specific primers for a total of six clonal libraries. The sampling locations, sample type and gene library information for cold seep and hydrothermal vent 16S rRNA and functional gene libraries used in this dissertation are shown in Table 1.

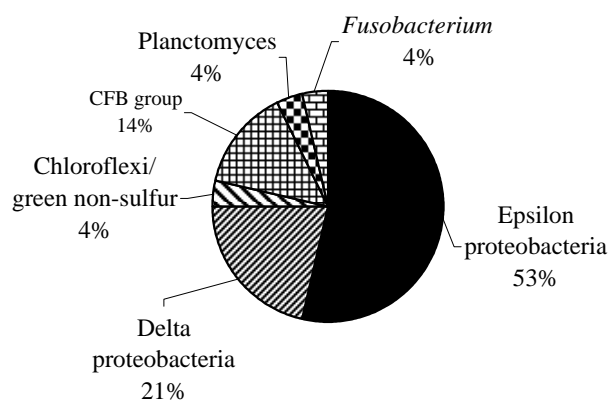
Diversity, Community Structure and Vertical Zonation of Bacteria. A total of 120 bacterial clones (42 clones from the top zone, 39 clones from the middle zone and 39 clones from the bottom zone) were screened by RFLP, grouped together based on their RFLP profiles and representative clones were sequenced. 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

Table 1. Sampling locations and information for cold seep and hydrothermal vent 16S rRNA and functional gene libraries.

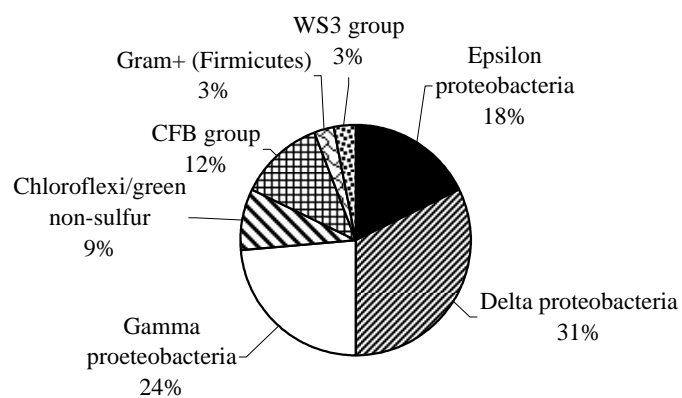
| Location | Cruise/Dive # | Latitude Longitude | Depth (m) | Sample Type | Gene Library |
|--|---|----------------------------|--------------|---|---|
| Florida Escarpment Gulf of Mexico | R/V <i>Atlantis</i> Cruise AT 03-58 DSV <i>Alvin</i> Dive 3637 | 26° 01.80 N 84° 54.9 W | 3288 | sediment core sample vertically sectioned into three zones (top, middle and bottom) | (top) <i>mcrA</i> , <i>dsrAB</i> , <i>pmoA</i> , 16S rRNA (middle) <i>mcrA</i> , <i>dsrAB</i> , 16S rRNA (bottom) <i>mcrA</i> , <i>dsrAB</i> , 16S rRNA |
| Blake Ridge Atlantic Ocean | R/V <i>Atlantis</i> Cruise AT 07-01 DSV <i>Alvin</i> Dive 3709 | 32° 29.41 N 76° 11.09 W | 2178 | sediment core sample vertically sectioned into three zones (top, middle and bottom) | (top) <i>mcrA</i> , <i>dsrAB</i> , <i>pmoA</i> (middle) <i>mcrA</i> , <i>dsrAB</i> (bottom) <i>mcrA</i> , <i>dsrAB</i> |
| Rainbow Hydrothermal Vent Area Mid-Atlantic Ridge | R/V <i>Atlantis</i> Cruise AT 05-03 DSV <i>Alvin</i> Dive 3678 | 36° 13.50 N 33° 53.90 W | 2305 | slurry sample | <i>mcrA</i> , <i>dsrAB</i> |
| Logatchev Hydrothermal Vent Area Mid-Atlantic Ridge | R/V <i>Atlantis</i> Cruise AT 05-03 DSV <i>Alvin</i> Dive 3667 | 14° 45.27 N 44° 58.70 W | 3027 | slurry sample | <i>mcrA</i> |
| 9N Biotranssect, East Pacific Rise | R/V <i>Atlantis</i> Cruise AT 03-50 DSV <i>Alvin</i> Dive 3542 | 9° 49.69 N 104° 17.37 W | 2505 | slurry sample | <i>dsrAB</i> |

numerous other bacterial phyla also represented. Overall, members of the Proteobacteria dominated the clonal libraries, with 33% belonging to the δ -Proteobacteria, 32% 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 11% 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 Figure 1. A table showing the Shannon-Weaver diversity indices, the Margalef's species richness measures and the evenness measures for all six 16S rRNA clonal libraries is shown in Table 2.

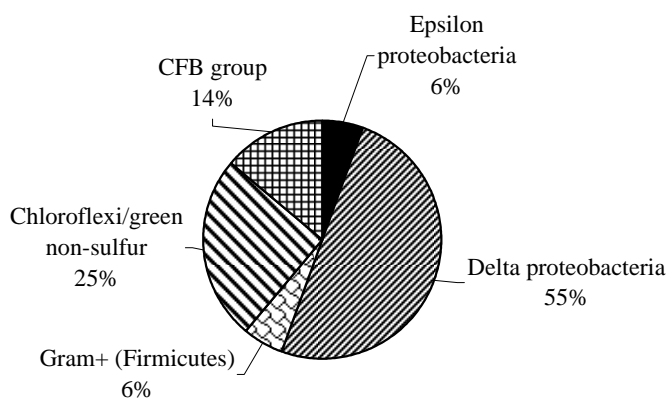
Twenty-nine unique clones were sequenced from the top zone of the core. Of these sequences, the majority (71%) were members of the Proteobacteria, with 53% belonging to the ϵ -Proteobacteria and 21% belonging to the δ -Proteobacteria (Fig. 1A). 14% were members of the *Cytophaga-Flexibacter-Bacteroidetes* group (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 (57% of all top clones) were most closely related to ϵ -Proteobacterial clones previously sequenced from cold-seep areas of the Nankai Trough (Li *et al.*, 1999c) and the Japan Trench (Inagaki *et al.*, 2002). The Shannon-Weaver



A



B



C

Table 2. Shannon-Weaver diversity indices, Margalef's species richness measure and evenness measure estimates for both Bacterial and Archaeal 16S rDNA clones from the Florida Escarpment.

| | Shannon-Weaver Diversity | Margalef's Richness | Evenness |
|------------------|-----------------------------|------------------------|----------|
| Top Archaeal | 0.76 | 0.45 | 0.69 |
| Middle Archaeal | 1.59 | 1.33 | 0.82 |
| Bottom Archaeal | 1.89 | 1.99 | 0.82 |
| Top Bacterial | 3.12 | 5.91 | 0.88 |
| Middle Bacterial | 2.73 | 3.71 | 0.94 |
| Bottom Bacterial | 3.36 | 5.73 | 0.96 |

diversity measure was 2.99, the Margalef's richness measure was 5.38, and the evenness measure was 0.88 (Table 2).

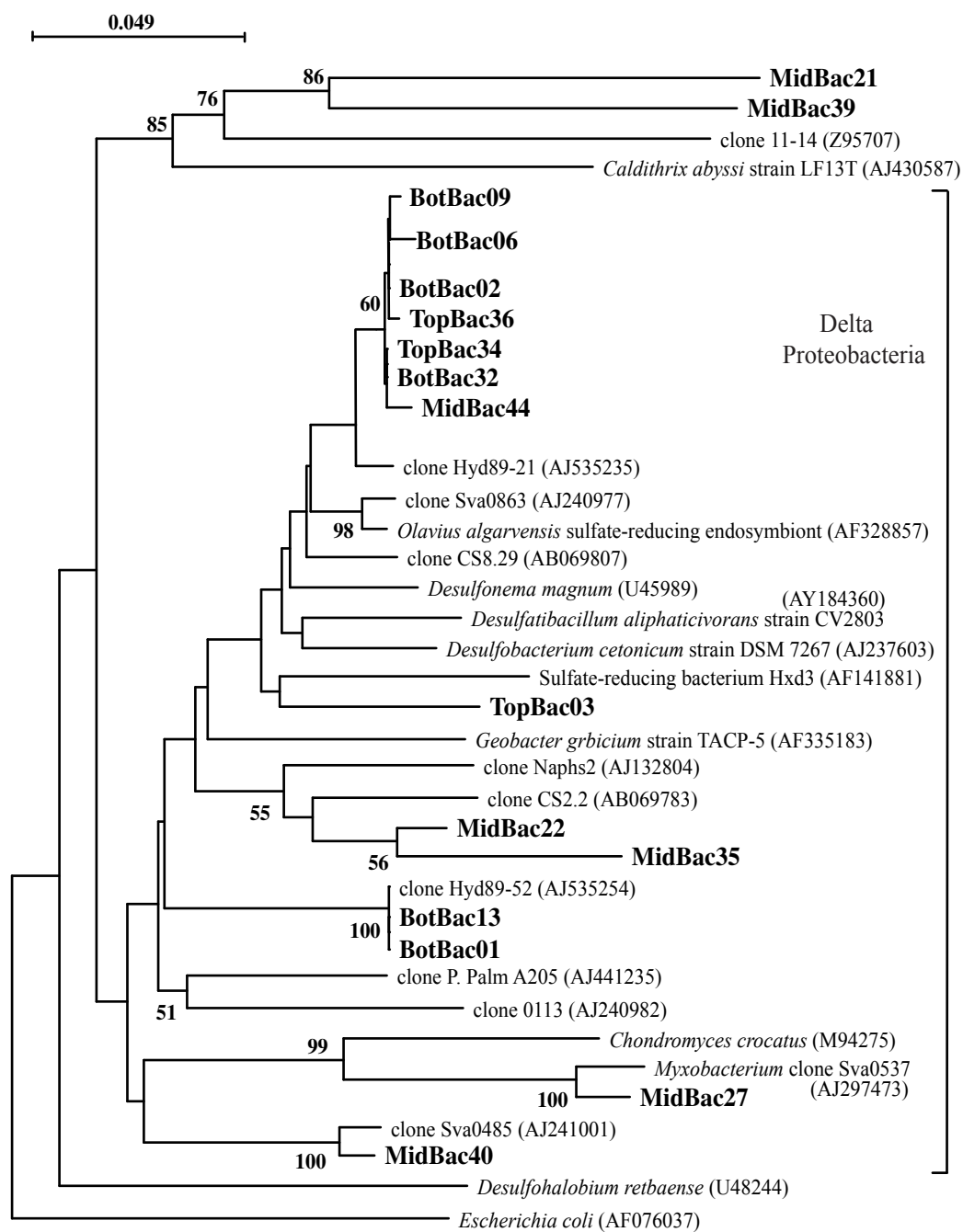
The bacterial clone library from the middle zone of the core was composed of 34 unique clones out of a total of 39 clones. Members of the Proteobacteria accounted for 73% of all sequences, with δ -Proteobacterial clones representing 31% of all sequences, γ -Proteobacterial sequences representing 24% and ε -Proteobacterial sequences representing 18% of the library (Figure 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-positive (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 (Figure 1A-C). Three clones (two from the top zone and one from the middle zone) were found to have 100% sequence identity to ε -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 measure, the Margalef's richness measure and the evenness measure were the highest of any library, with values of 3.42, 6.24 and 0.88, respectively (Table 2).

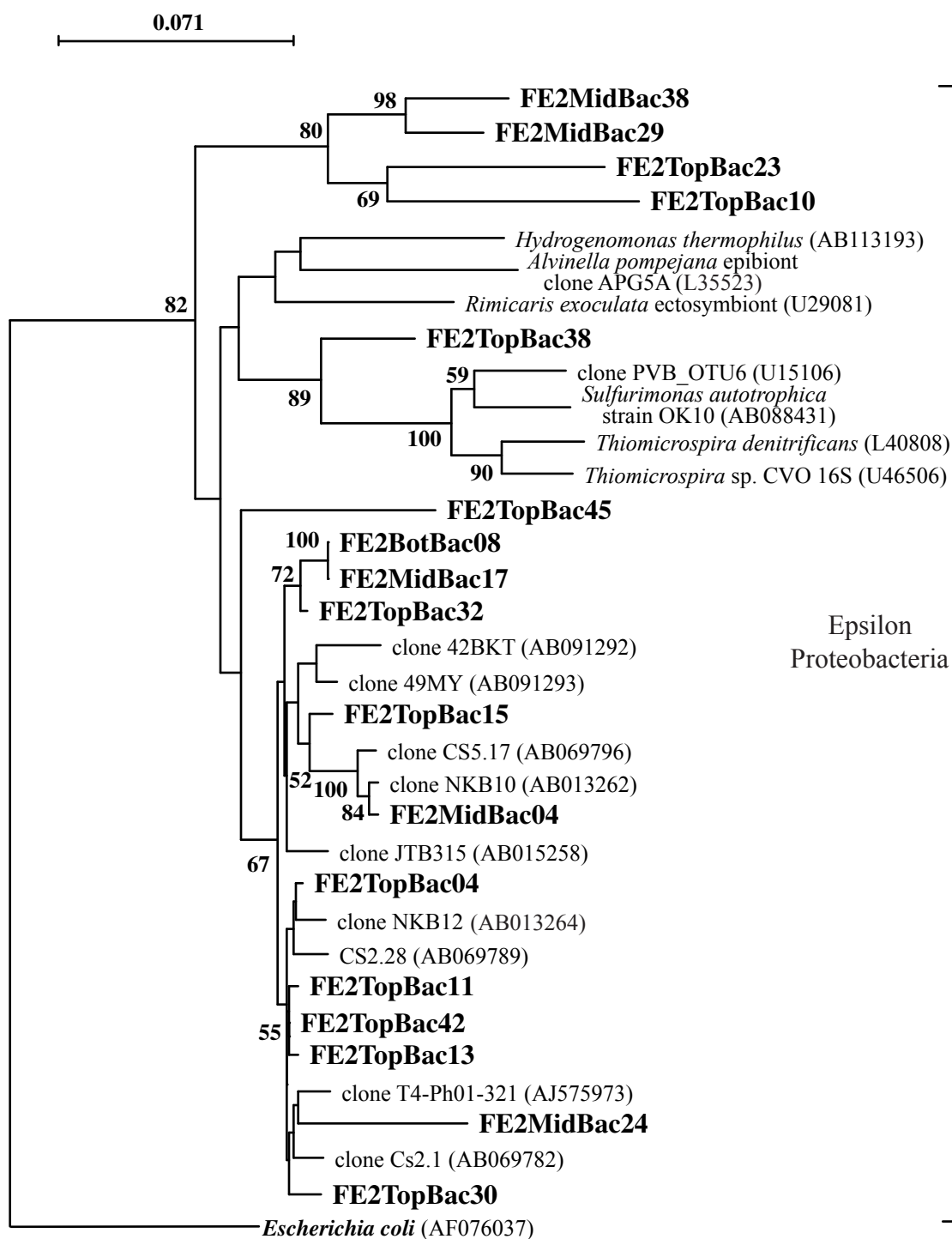
The bacterial clone library constructed from the bottom zone of the core consisted of 23 unique clones (out of 39 clones screened by RFLP). The majority (55%) of the library was affiliated with the Proteobacteria, with 49% belonging to the δ -Proteobacteria and 6% belonging to the ε -Proteobacteria. *Chloroflexi*/green non-sulfur sequences accounted

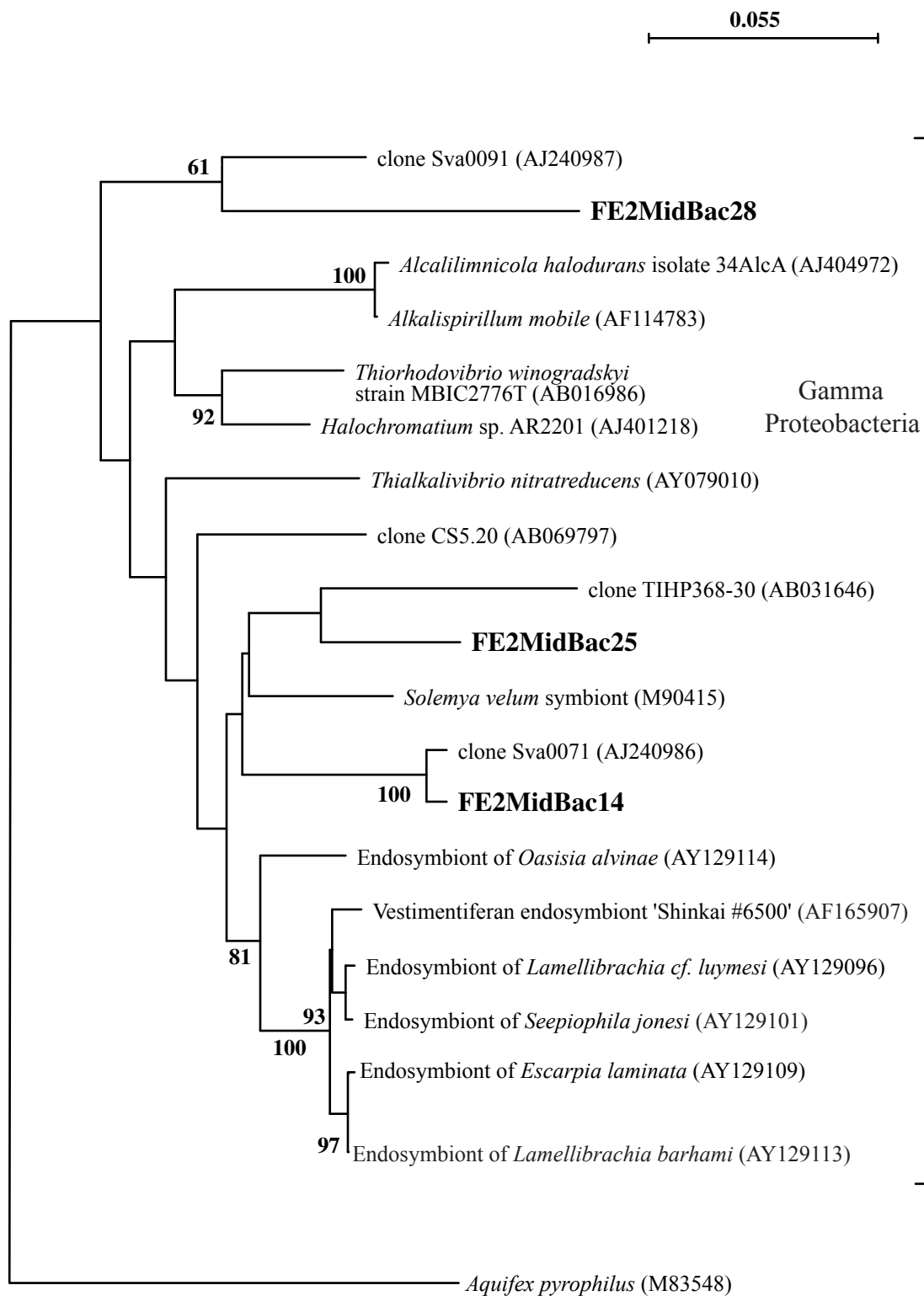
for 25% of the library with CFB group sequences and Gram-positive (Firmicutes) sequences accounting for 14% and 6% of the sequences, respectively (Figure 1C). Sequences related to *Chloroflexi*/green non-sulfur bacteria increased significantly in the bottom zone over the number found in either the top 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 *Caldothrix abyssi*, which remains to date unclassified at the phylum level (Miroshnichenko *et al.*, 1995).

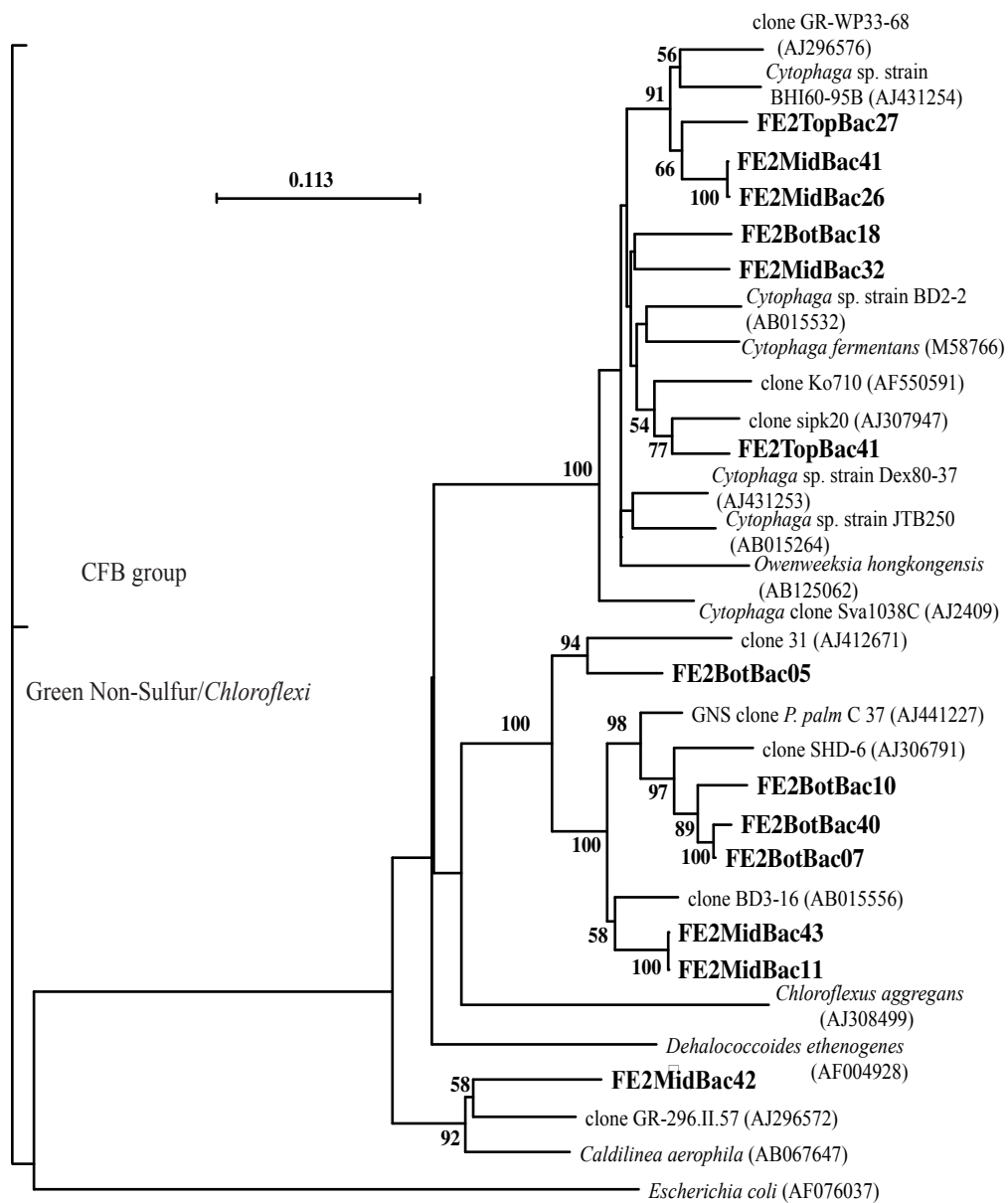
Phylogenetic Analysis of Bacterial 16S rDNA Sequences. Five different phylogenetic trees are shown for the bacterial clones (Figures 2A-E). In general, the clones were most closely related to groups already identified from marine sediments, cold-seep environments or deep-sea hydrothermal vent environments. 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 in 6 clusters) (Figure 2A) were grouped into six clusters. Two distinct clusters (one cluster of seven clones, including BotBac09, TopBac34 and MidBac44, 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 naphthalene









(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 Figure 2A. These clones are related to the deep-sea vent thermophilic bacterium *Caldithrix 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 (Figure 2B) detected from a variety of deep-sea environments. Four clones (MidBac38, MidBac29, TopBac23 and TopBac10) formed a distinct cluster without close relatives from sequence databases. TopBac38 related to *Sulfurimonas autotrophica* strain OK10, a sulfur-oxidizing bacterium isolated from the Okinawa Trough (Inagaki *et al.*, 2003) and clone PVB-OTU6, a sequence detected from Pele hydrothermal vent in Hawaii (unpublished). 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.*, 1999c) 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 (Figure 2B). Sixteen of the seventeen ϵ -Proteobacterial clones detected 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) 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; Ravensschlag *et al.*, 1999) and from coastal marine sediments (clone TIHP368-30, Urakawa *et al.*, unpublished)(Figure 2C).

Phylogenetic analysis indicated that a cluster of clones, represented by MidBac26, MidBac41 and TopBac27, were related to members of the CFB previously detected in association with the deep-sea vent tubeworm *Alvinella 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) (Figure 2D). 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 (Figure 2D). Several clones were classified as members of the green non-sulfur/*Chloroflexi* group (Figure 2D) 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 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) while BotBac05 was related to a clone isolated from a denitrifying reactor (Etchebehere *et al.*, 2002).

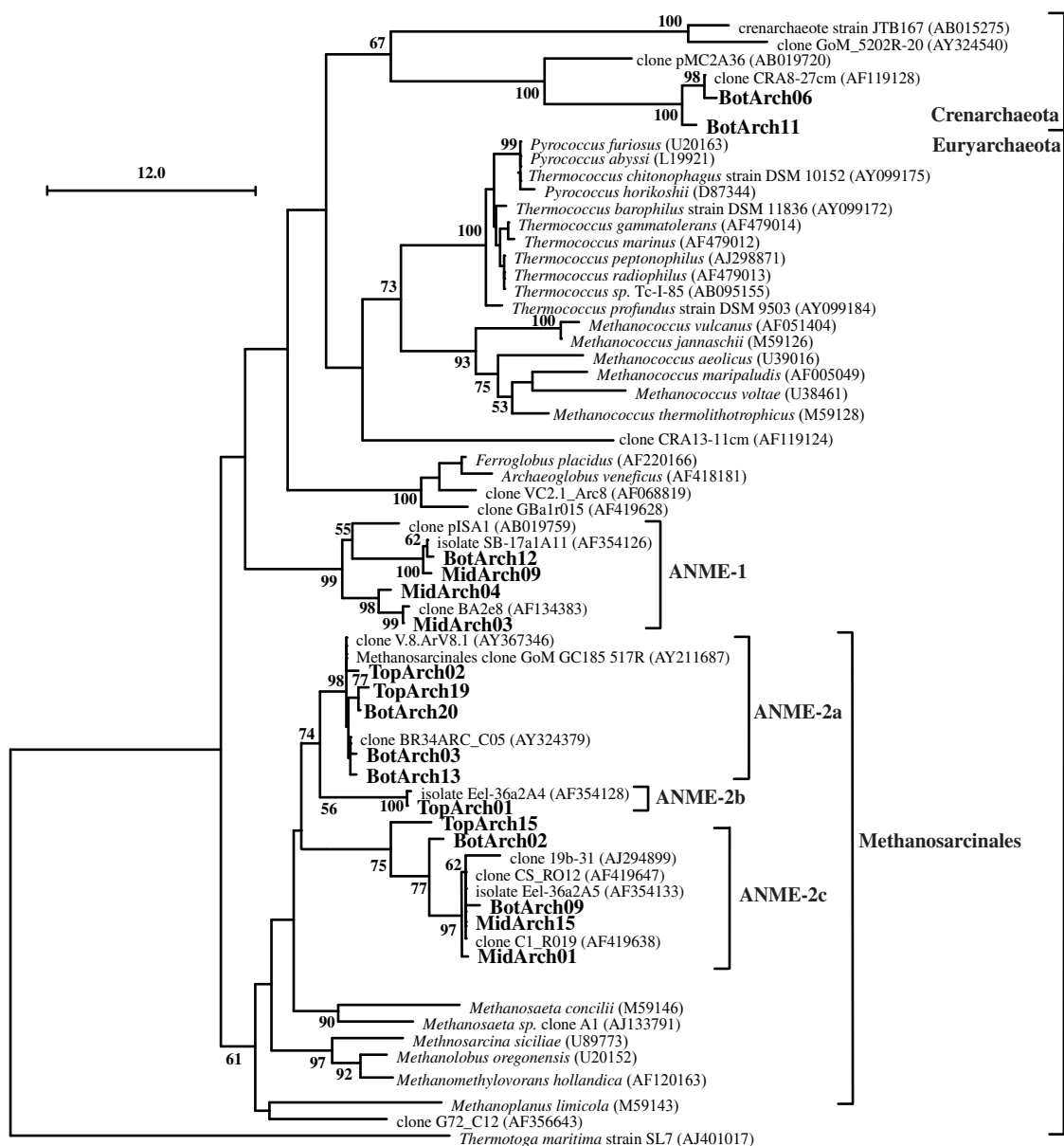
CFB group clones remained a steady 11-14% of all clones throughout the depth of the sediment core. *Chloroflexi*/green non-sulfur clones increased with depth, representing 4% of all bacterial clones in the top zone, 9% in the middle zone and 26% in the bottom zone. Several phyla of microorganisms were represented only infrequently in this study. A single clone, MidBac45, was classified as a member of candidate division WS3, a novel phylogenetic classification containing no cultured members (Figure 2E). 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 22m in the Santa Barbara Channel, California (LaMontagne *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.*, 1999c). The gram-positive Firmicutes phylum was represented by 3 clones (BotBac20, BotBac24 and MidBac07) and these clones 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 (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 species richness, as measured by Margalef's species richness 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 (Figure 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 *Methanosarcinales*, Orphan et al. (2001b) 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% of 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 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.*, 2001b). Six 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.*, 2001b).

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 (isolate SB-17a1A11; Orphan *et al.*, 2001b), 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.*, 2001b; 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 CRA8-27cm; Vetriani *et al.*, 1999) and were more distantly related to a sequence detected in a black smoker chimney sample from Myojin Knoll, Izu-Ogasawara arc (clone pMC2A36; Takai and Horikoshi, 1999).

Chapter 1: 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 (Figure 1). 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 (ϵ -CSG) from cold seep areas of the Nankai Trough (Li *et al.*, 1999c) and the Sanriku Escarpment of the Japan Trench (Inagaki *et al.*, 2002) (Figure 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 (Polz and Cavanaugh, 1995; Reysenbach *et al.*, 2000; Longnecker and Reysenbach, 2001; 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; Urakawa *et al.*, 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 55% of the clones, respectively (Figure 1). Many of the δ -Proteobacteria in cultivation are sulfate-reducing bacteria (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.

Sulfate-reducing bacteria generally outcompete methanogenic Archaea for sulfate 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, H₂S-rich and increasingly anoxic zones of the Florida Escarpment core is consistent with the requirement that SRBs grow only under strictly anaerobic conditions.

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

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 (Figure 1). Further, γ -Proteobacteria are known to

quickly colonize all available surfaces, both biological and mineral, in hydrothermal areas (López-García *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.*, 1999a). The fact that no γ -Proteobacterial clones were detected in the top zone of the sediment and 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 (Mullins *et al.*, 1995; Fuhrman and Davis, 1997). 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.*, 1999c).

The *Chloroflexi*/green non-sulfur bacteria have been recognized as a division-level bacterial group for almost twenty years (Woese, 1987), yet this division is still represented by only a very few isolates. The clones detected in this study are related to members of the *Chloroflexi*/green non-sulfur group that were 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 in the microbial community.

Archaea of the ANME-1 and ANME-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 sulfate-reducing bacteria to oxidize methane in a number of methane-rich sedimentary environments (Boetius *et al.*, 2000; Orphan *et al.*, 2001b; Orphan *et al.*, 2001a; Girguis *et al.*, 2003). 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.

Chapter 2: Phylogenetic Diversity of *mcrA* and *dsrAB* Genes from Deep-Sea Hydrothermal Vent and Cold Seep Sites

Chapter 2: Abstract

Microbial communities from both deep-sea hydrothermal vent (Rainbow and Logatchev hydrothermal vent fields, Mid-Atlantic Ridge, Atlantic Ocean; 9°N hydrothermal vent area, East Pacific Rise, Pacific Ocean) and cold seep areas (Blake Ridge, western Atlantic Ocean; Florida Escarpment, Gulf of Mexico) were investigated by molecular phylogenetic analysis of *mcrA* and *dsrAB* gene sequences. Clone libraries were obtained using DNA from vent chimney material (hydrothermal vent areas) and from vertically subsampled (top, middle and bottom) sediment core samples (cold seep areas) and screened by RFLP. Representative sequences encoding proteins involved in the transformation of the two most common substrates of marine microbial chemosynthesis, methane and sulfide, were sequenced. Clone library *dsrAB* sequences grouped primarily within the orders *Desulfobacteriales*, *Syntrophobacteriales* and the gram-positive order Clostridiales. Cold seep *mcrA* sequences were distributed among the ANME-2c, -2d and -2e groups. Most hydrothermal vent *mcrA* sequences were primarily related to thermophilic members of the anaerobic, methanogenic order *Methanococcales*. In addition, this paper presents for the first time environmental *mcrA* sequences detected from chimney samples collected from a bare basalt, high-temperature deep-sea hydrothermal vent area that are related to ANME-2e sequences previously shown to be associated with the anaerobic oxidation of methane.

Chapter 2: Introduction

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. A number of 16S rDNA-based studies in the recent past have focused on the identification and community diversity of microorganisms at cold seeps (e.g., Li *et al.*, 1999a; Lanoil *et al.*, 2001; Mills *et al.*, 2003; Reed *et al.*, 2006; Heijs *et al.*, 2007) and deep-sea hydrothermal vents (e.g., Takai and Horikoshi, 1999; Reysenbach *et al.*, 2000; Corre *et al.*, 2001; Teske *et al.*, 2002). These 16S rDNA-based surveys can serve as an important step in understanding total microbial diversity and community structure, but they have limitations. Among them is the fact that unless these newly-identified lineages are closely related to cultivated microorganisms, it is difficult to discern much information about the physiological capabilities of that microorganism. The phylogenetic analysis of functional genes that encode important enzymes of characteristic metabolic pathways (e.g. *mcrA* for methanogenesis, *dsrAB* for sulfate reduction, *pmoA* for methane oxidation) have been shown to be useful in detecting microorganisms with specific metabolic capabilities in complex microbial populations in marine environments (e.g., Marchesi *et al.*, 2001; Dhillon *et al.*, 2003; Hallam *et al.*, 2003; Mehta *et al.*, 2003; Nakagawa *et al.*, 2004; Knittel *et al.*, 2005; Lloyd *et al.*, 2006). However, little is known about the metabolic potential of microbial populations from cold seep and deep-sea hydrothermal vent areas.

Methane and sulfide are primary sources of energy supporting the dense macrobiological communities found in many cold seep areas and both free-living and symbiotic microorganisms depend on energy enabled by the presence of the sharp gradient of sulfide and the reservoir of methane in the underlying sediments. Methane has been shown to be consumed anaerobically by a microbial consortium of methane-oxidizing Archaea and sulfate-reducing Bacteria (Hinrichs *et al.*, 1999; Boetius *et al.*, 2000; Orphan *et al.*, 2001b; Orphan *et al.*, 2002; Teske *et al.*, 2002) and fluorescent *in situ* hybridization and isotopic analysis (FISH-SIMS) have identified the various subgroups of this consortium, such as the ANME-1 and ANME-2 groups, as the key microorganisms in the anaerobic oxidation of methane in a variety of marine systems (Orphan *et al.*, 2001a). The upper boundary of the Sulfate-Methane Interface (SMI) in marine sediment is established by the penetration depth of available oxygen (Barnes and Goldberg, 1976; Borowski *et al.*, 1996). Any methane that leaks out of the methane reservoir is oxidized as soon as there is available sulfate. ANME microorganisms are strict anaerobes and therefore will not grow in oxygenated sediments, although they may grow in anaerobic interstitial spaces in otherwise oxygenated sediments. The lower vertical limit for the anaerobic oxidation of methane (AOM) is determined by the penetration and resultant concentration of sulfate with depth. The mediating SRB vital in the AOM process depend upon the availability of sulfate. Sulfate decreases with depth (and sulfide increases with depth) limiting the growth of ANME-related microorganisms.

Cold seeps are found at both active and passive margins and are characterized by the seepage of cold fluids, which have an elevated methane and/or sulfide concentration over that of ambient seawater, into surficial sediments. Cold seeps are often associated with gas hydrate deposits that form under conditions of low temperature, high hydrostatic pressure and gas concentrations in excess of solubility (Kvenvolden, 1988; Buffett, 2000). 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). Methane at high temperature hydrothermal vents originates from H_2/CO_2 reactions at high temperature as well as mantle outgassing (Claypool and Kvenvolden, 1983). In addition to methane, a limited number of cold seeps have an increased concentration of hydrogen sulfide in the porewater of the sediments that is produced by microbial sulfate reduction. Chemosynthetic microorganisms primarily utilize sulfur compounds in vent fluid to produce organic material and sustain the high macrobiological productivity at deep-sea hydrothermal vents. Sulfate-reducing bacteria (SRB) constitute a functional group of strictly anaerobic microorganisms unified by their ability to utilize sulfate as the terminal electron acceptor in their respiratory metabolism. The end product of dissimilatory sulfate reduction is hydrogen sulfide. Both methane and sulfide can play a major role in sustaining the highly productive cold seep and deep-sea hydrothermal vent biological communities.

Despite enormous amounts of methane that is stored in methane hydrates, only a relatively small amount of methane escapes from marine sediments due to the Anaerobic Oxidation of Methane (AOM). AOM is the major sink of methane in reducing habitats when methane and sulfate are both available. AOM has been demonstrated in a number of marine environments (Lanoil *et al.*, 2001; Orphan *et al.*, 2001b; Thomsen *et al.*, 2001; e.g., Michaelis *et al.*, 2002; Teske *et al.*, 2002; Krüger *et al.*, 2003; Treude *et al.*, 2003) and is often the primary sulfate-consuming biogeochemical process in these areas. Globally, it is thought that more than 80% of the methane annually produced in the ocean is consumed before it reaches the atmosphere (Reeburgh, 1996) and it is the AOM that serves as a biological barrier to the release of greater amounts of methane into the atmosphere (Hinrichs and Boetius, 2002). AOM is thought to be essentially a reverse methanogenesis coupled to the reduction of sulfate that is mediated by a consortium of anaerobic, methanotrophic archaea (known as ANME) groups and sulfate-reducing bacteria (SRB) from the *Desulfosarcina*-related members of the δ -proteobacteria (Hallam *et al.*, 2003; Hallam *et al.*, 2004). Phylogenetic analysis of three groups of ANME (ANME I, II and III) that are based on the analysis of the gene *mcrA*, a subunit of MCR enzyme methyl coenzyme M reductase (MCR), have shown that they are related to each other and to methanogenic Archaea within the *Methanosarcinales*. Therefore, the phylogenetic study of the gene *mcrA* serves as a tool for studying methanogenic Archaea as well as the anaerobic methane-oxidizing Archaea involved in AOM.

Sulfate reduction is known to be a dominant anaerobic pathway in continental margin sediments and cold seep areas (e.g. Dhillon *et al.*, 2003; Knittel *et al.*, 2003; Lloyd *et al.*,

2006; Kaneko *et al.*, 2007) yet few targeted studies of the distribution and diversity of sulfate reducers have been published. Also, there have been only a few studies of SRB in deep-sea hydrothermal vent and cold seep areas (e.g. Nakagawa *et al.*, 2004; Nercessian *et al.*, 2005; Lloyd *et al.*, 2006). The key diagnostic enzyme in sulfate reduction is dissimilatory sulfite reductase (DSR), which catalyzes sulfite to sulfate, the final step in the dissimilatory sulfate reduction pathway (Odom and Peck, 1984). The DSR enzyme is encoded by α and β subunits of the *dsr* gene (*dsrAB*) and the study of *dsrAB* serves as a tool for studying the community SRB in marine systems.

The Florida Escarpment is a sharply sloping limestone edifice (average tilt > 35 degrees) which rises ~2000m from the seafloor at a depth of 3270m and extends for hundreds of miles. 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. The methane in Florida Escarpment seep fluids is the result of microbial production, based on carbon isotope analysis [$\delta^{13}\text{C}$ = -61 to -94 ppt; (Martens *et al.*, 1991)]. Both microbial sulfate reduction and methanogenesis are hypothesized to be important processes within the platform (Martens *et al.*, 1991).

The Blake Ridge is a sedimentary feature that is 500 km long, between 2000 and 4800 meters deep, and located approximately 400 km east of Charleston, South Carolina in the Atlantic Ocean. The Blake Ridge is one of the most studied occurrences of methane hydrates in the United States (Dillon and Max, 2000). Enormous quantities of methane are stored as methane hydrates with the top of the methane hydrate deposit situated at depths greater than 100 meters below the sediment-water interface in most areas (Paull *et al.*, 1996). However, in some areas gas hydrate and underlying free gas can occur close to the seafloor or actually penetrate the sediment-water interface. The interaction of the hydrate reservoir with geologic, oceanographic, and other processes leads to the development of focused seeps (Van Dover *et al.*, 2003). Carbon isotope readings are characteristic of biogenic (as opposed to thermogenic) methane, implying that the methane present is of microbial origin in this area (Lorenson and Collett, 2000).

The Rainbow and Logatchev hydrothermal vent areas are ultramafic-hosted hydrothermal environments located on a slow-spreading ridge in the Atlantic Ocean. Logatchev hydrothermal field vent fluids show high concentrations of H_2 and CH_4 (up to 19 mM and 3.5 mM, respectively) and H_2S concentrations are 3.5 mM or less (Schmidt *et al.*, 2007). Rainbow field vent fluids show a high concentration of H_2 (16 mM) and CH_4 (2.5 mM) with a lower concentration of H_2S (1.0 mM) when compared with Logatchev endmember fluids (Charlou *et al.*, 1998; Douville, 1999; Charlou *et al.*, 2002). In contrast, the 9° north hydrothermal vent area is a basaltic hydrothermal vent area located on a fast-spreading ridge in the Pacific Ocean. Hydrothermal vents in the 9° North vent field show

low H_2 concentrations of $\leq 0.3\text{mM}$, very low CH_4 concentrations of $\leq 0.0015\text{ mM}$ and higher H_2S concentrations of $\leq 3.3\text{ mM}$ (Von Damm and Lilley, 2004).

Chapter 2: Materials and Methods

Sample Collection. Acrylic tube sediment cores measuring 22 cm were collected from sediments at the base of the Florida Escarpment, Gulf of Mexico (latitude $26^\circ 01.8$ north, longitude $84^\circ 54.9$ west) during the AT-03 cruise, leg 58 of RV *Atlantis* and dive 3637 of DSV *Alvin* at a depth of 3288 meters in October of 2000. Sediment cores were also taken from the Blake Ridge cold seep site in the Atlantic Ocean (latitude $32^\circ 29.41$ north, longitude $76^\circ 11.09$ west) during the AT-07 cruise of RV *Atlantis* and dive 3709 of DSV *Alvin* at a depth of 2178 meters in September of 2001. Bottom water temperatures were about 2°C in the cold seep areas. Two deep-sea hydrothermal vent samples were taken during leg 03 of the AT-05 cruise during July 2001: the Logatchev hydrothermal vent area (*Alvin* dive 3667, $14^\circ 45.27$ north, $44^\circ 58.70$ west, depth 3000 meters) and the Rainbow hydrothermal vent area (*Alvin* dive 3678, $36^\circ 13.50$ north, $33^\circ 53.90$ west, depth 2305 meters) and a third sample was taken during leg 50 of the R/V *Atlantis* cruise AT-03 during April of 2000 ($9^\circ 49.69$ north, $104^\circ 17.37$ west, depth 2505 meters, 9°N Biotransect, East Pacific Rise, Pacific Ocean). Table 2 shows the sample locations and functional gene library information for this study. Hydrothermal vent samples were taken from slurries prepared from the friable chimney material found in the upper portions of high temperature chimneys. The samples were then frozen whole on board at -80°C until it could be processed in the laboratory at Rutgers University. The sediment cores for the

cold seep sites (Florida Escarpment and Blake Ridge) were subsampled by sectioning them into three zones and designated top, middle and bottom. The top of the cores consisted of lightly colored, soft and loosely packed sand-sized sediment. The middle layer of the core consisted of darker and 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 H₂S (indicative of oxygen depletion) and densely packed clay-sized particles.

DNA extraction and DNA amplification by PCR. Total genomic DNA was extracted from ~10 g of sediment from each of the three vertical zones of the cold seep sediment cores using a soil DNA Mega Prep kit UltraClean™ Soil DNA extraction kit (Mo Bio Laboratories, Solana Beach, CA) according to the protocol supplied with the kit. For hydrothermal vent samples, total genomic DNA was extracted from ~2.0 g of slurry using an UltraClean™ Soil DNA extraction kit (Mo Bio Laboratories, Solana Beach, CA) according to the protocol supplied with the kit. The DNA for all samples was resuspended in sterile water and the DNA concentration was measured using a spectrophotometer. DNA fragments encoding the *mcrA* gene were amplified using the forward primer ME1f (5'-CGMATG CARATHGGWATGTC-3') and the reverse primer (5'-TCATKGCRTAGTTDGGRTAGT-3') (Hales *et al.*, 1996). Amplification was performed with Taq PCR Master Mix (Qiagen, Valencia, CA) using a thermocycler (Perkin-Elmer, Norwalk, CT). The PCR conditions were as follows: 35 PCR cycles with denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at

72°C for 30 seconds with a final extension time of 420 seconds on the final cycle. DNA fragments encoding the *dsrAB* gene were amplified using the forward primer dsr1f (5'-ACSCACTGGAAGCACG-3') and the reverse primer dsr4r (5'-GTGTAGCAGTTACCGCA-3') (Wagner *et al.*, 1998). Reaction mixtures were incubated in a thermal cycler (Perkin-Elmer, Norwalk, CT) and programmed for 30 PCR cycles as follows: denaturation at 94°C for 60 seconds, annealing at 54°C for 60 seconds and extension at 72°C for 180 seconds with a final extension time of 60 seconds on the last cycle. PCR amplification without DNA was used as a negative control throughout. Amplified products were subjected to agarose gel electrophoresis and then purified using the Mo Bio Laboratories, Inc. Gel Purification kit according to the manufacturer's instructions.

Construction of Clonal Libraries. Clone libraries from PCR products were constructed using the TA cloning kit (Invitrogen Corporation, San Diego, CA) following the manufacturers' recommendations. Amplified 16S rRNA gene fragments were cloned in pCRII plasmid vector (Invitrogen, Inc., Carlsbad, CA) and the ligation products were used to transform competent *E.coli* INV α F' 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) as described in the manufacturers' instructions. Eight *mcrA* and eight *dsrAB* libraries [3 libraries each (top, middle and bottom) for Blake Ridge and Florida Escarpment] and one library each for Logatchev and Rainbow were constructed. The cold seep clones are designated siteXXX#, where site identifies the sample site of origin

(FE2=Florida Escarpment, BR1=Blake Ridge) and XXX identifies the vertical zone of the core (“Top” for top, “Mid” for middle and “Bot” for bottom for cold seep samples) and # represents the clone number. Hydrothermal vent clones were designated by *Alvin* dive number_name of the vent field_clone number_functional gene name.

Restriction Length Polymorphism (RFLP) Screening. Clonal inserts of *dsrAB* fragments were digested for 2 hours at 37°C on a thermocycler using the tandem tetrameric restriction endonucleases HaeIII and MspI (Promega, Inc. Madison, WI). Clonal inserts for *mcrA* fragments were digested using the endonuclease MnlI. The restriction products were run for 2.5 hours at 75V and 4°C on a 2.5% (weight/volume) Methaphor agarose gel containing ethidium bromide and visualized under UV light.

Sequencing and Phylogenetic Analysis. Representative bacterial and archaeal 16 rDNA clones from unique RFLP patterns were sequenced utilizing an automated DNA sequencer (310 Avant Genetic Analyzer; Applied Biosystems, Foster City, CA). Ribosomal DNA sequences 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 (NCBI) website (www.ncbi.nlm.nih.gov)(Altschul *et al.*, 1997). All sequences were aligned in a two-stage process. Amino acid sequences were translated from nucleotide sequences using the Transeq program from the European Bioinformatics Institute (Rice *et al.*, 2000). Multiple alignments were created using ClustalX v1.8 (Thompson *et al.*, 1997) and was followed by manual adjustment using Seaview (Galtier *et al.*, 1996). 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 1000 resamplings.

Nucleotide sequence accession numbers. The sequences reported here have been deposited in the Genbank database under the accession numbers EU495332 through EU495451.

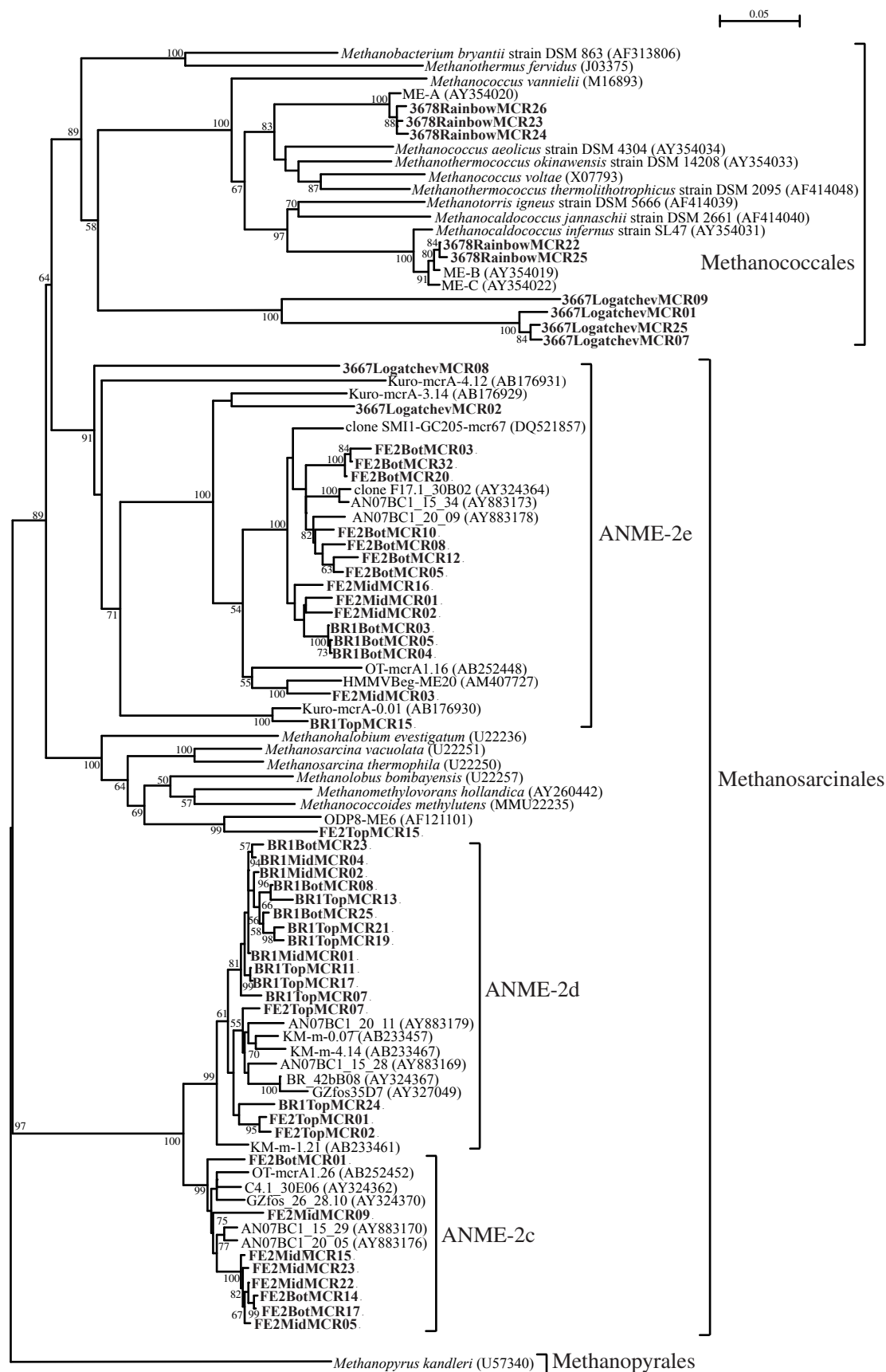
Chapter 2: Results

Genomic DNA was extracted from three depth intervals (top, middle and bottom) of the sectioned sediment cores. 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 *dsrAB* and a *mcrA* clone library was constructed for each of the three vertical zones for cold seep sample cores, thus vertically profiling the distribution and diversity of SRB and *mcrA* gene-containing microorganisms. In addition, clone libraries were created for two deep-sea hydrothermal vent samples for a total of sixteen clonal libraries. The clonal libraries were typed on the basis of restriction fragment banding patterns and unique types were partially sequenced.

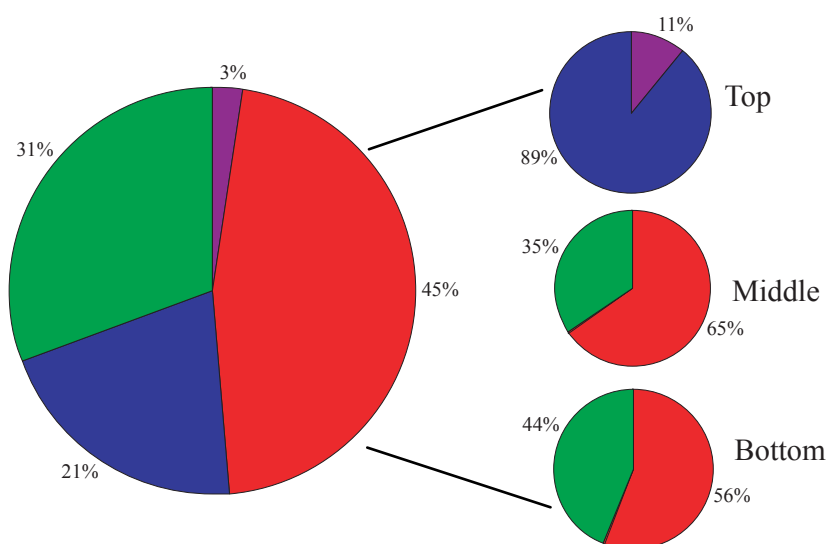
Diversity of *mcrA* and phylogenetic analysis. A total of 215 *mcrA* clones from both deep-sea hydrothermal vent and cold seep samples were screened by RFLP and 51

unique profiles were identified. Phylogenetic analysis of *mcrA* genes indicated that the majority of the sequences from cold seep sites were most closely related to members of ANME-2 groups c, d, and e previously described by (Hallam *et al.*, 2003)(Figure 4). Most cold seep clones clustered within the *Methanosarcinales* group ANME-2d. Figure 5 shows the vertical distribution of *mcrA* clones in the Florida Escarpment and Blake Ridge sediment cores. Close environmental relatives of ANME-2d clones include clone BR_42bB08 detected from the Blake Ridge (Hallam *et al.*, 2003), three clones from mud volcanoes in the eastern Mediterranean Sea (Kormas *et al.*, 2005) and clone KM-m-4.14 detected from the Nankai Trough (Nunoura *et al.*, 2006). Five clones clustered within the ANME-2c group and were most closely related to clone C4.1 30E06 detected from an AMIS microcosm experiment from Monterey Canyon (Hallam *et al.*, 2003) as well as clone ODP8-ME1 which was obtained from sediment near the Cascadia Margin (Bidle *et al.*, 1999).

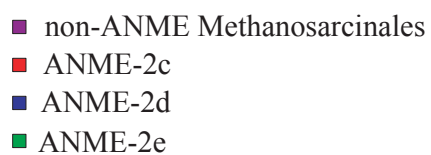
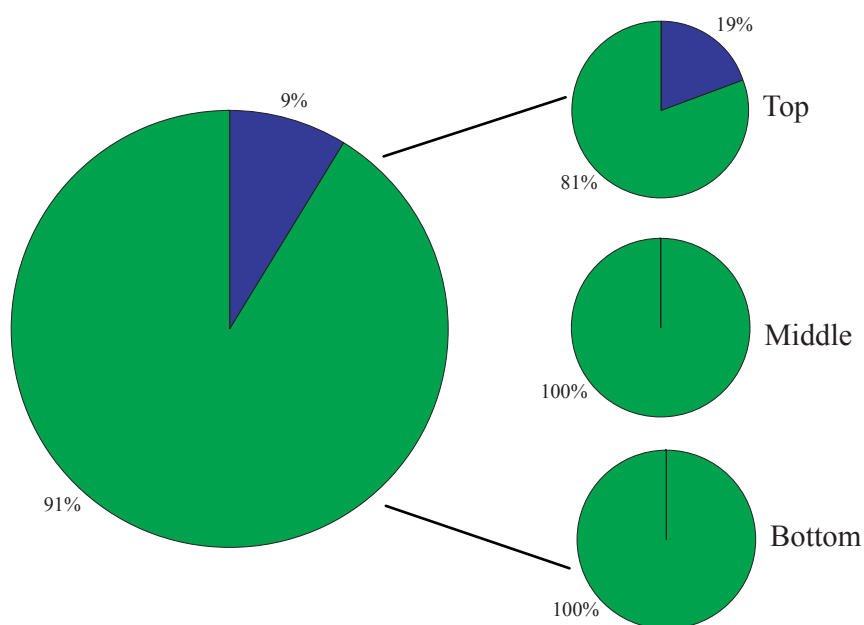
Only one cold seep clone, FE2MCRTop15, was found to be most closely related to non-ANME members of the *Methanosarcinales*. Clone FE2MCRTop15 was most closely related to clone ODP8-ME6, detected in marine sediment near the Cascadia Margin (Bidle *et al.*, 1999). Figure 5 shows the group distribution of the cold seep clones and how the distribution among taxonomic groups changes with depth in the sediment. For the Florida Escarpment, 89% of the *mcrA* clones from the top zone were related to ANME-2d sequences and 11% were related to non-ANME members of the *Methanosarcinales*. No ANME-2d sequences were found in the middle zone; the middle zone was dominated by ANME-2c sequences (65%) and ANME-2e sequences (35%).



Florida Escarpment (FE2) Top, Middle and Bottom *mcrA* Clones



Blake Ridge (BR1) Top, Middle and Bottom *mcrA* Clones



The bottom zone was similar to the middle having 56% ANME-2c clones and 44% ANME-2e clones. For Blake Ridge clones, 19% of the top zone clones were related to ANME-2d sequences and 81% were related to ANME-2e clones. Both middle and bottom zones of the Blake Ridge *mcrA* clonal libraries contained 100% ANME-2e related clones.

Deep-sea hydrothermal vent *mcrA* clones in this study clustered primarily with mesophilic and thermophilic relatives within the *Methanococcales*. Most hydrothermal vent clones clustered in the order *Methanococcales*, with two exceptions: clones 3667LogatchevMCR02 and 3667LogatchevMCR08 and a cluster of four other 3667LogatchevMCR sequences (01, 25, 07 and 09) (Figure 4). Clone 3667LogatchevMCR02 clustered within the ANME-2e group and was found to be most closely related to clone Kuro-mcrA-3.14, detected in cold seep sediments on the Kuroshima Knoll, southern Ryuku Arc (Inagaki *et al.*, 2004). No MCR sequences related to ANME-1 (as described by (Knittel *et al.*, 2005) sequences were detected in this study, although ANME-1 sequences were detected in the related Archaeal 16S rRNA study described in chapter 1 of this dissertation and published previously (Reed *et al.*, 2006).

Diversity of *dsrAB* and phylogenetic analysis. A total of 155 *dsrAB* clones from both deep-sea hydrothermal vent and cold seep samples were screened by RFLP and 67 unique profiles were identified. Diversity was greater in *dsrAB* sequences than *mcrA* sequences at all three depths. There did not appear to be any systematic differences between *dsrAB* taxonomic affiliations between depths. Cold seep sequences were found

to be members of the Bacterial orders *Desulfobacteriales*, *Desulfovibrionales*, *Syntrophobacteriales* as well as the Firmicutes (gram + Bacteria) order *Clostridiales* (Figure 6). Phylogenetic analysis of *dsrAB* genes indicated that the most common groups of *dsrAB* sequences from cold seep sites were most closely related to members of the *Desulfobacteriales*. The *Desulfobacteriales*-related cold seep sequences detected in this study include DSR-J, sampled from a hydrothermal vent at 13° North on the East Pacific Rise (Nercessian *et al.*, 2005), NTd-I07 sampled from the Nankai Trough (Kaneko *et al.*, 2007), S1Adsr_A02.seq sampled from Puget Sound sediment (unpublished) and various characterized species of *Desulfobulbus*. Several sequences from the Blake Ridge core were found to be most closely related to sequences in the order *Syntrophobacteriales*, including NTd-II03 from the Nankai Trough (Kaneko *et al.*, 2007) and *Desulfacinum infernum*, a thermophilic sulfate-reducing bacterium from a petroleum reservoir (Rees *et al.*, 1995). Five clones, representing both FE2 and BR1 samples, were found to be most closely related to sequences within the gram positive order *Clostridiales*, including clones NTd-III05 and NTd-IV05 from the Nankai Trough (Kaneko *et al.*, 2007), clone PIMO2F05 from a New England salt marsh (Bahr *et al.*, 2005), clone AI-DSR 60 from the eastern Mediterranean Sea (unpublished) as well as characterized examples of the genus *Desulfotomaculum*.

Phylogenetic analysis of *dsrAB* sequences (Figure 6) detected from the Rainbow hydrothermal vent site on the MAR (designated 3678MARdsrXX) showed that the sequences grouped into two archaeal clusters and were most closely related to members of the hyperthermophilic archaeal genus *Archaeoglobus*, the environmental clone DSR-M

from 13° North on the EPR (Nercessian *et al.*, 2005) and clone INDO-34 from a deep-sea hydrothermal vent in the Kairei Field, Central Indian Ridge, Indian Ocean (Nakagawa *et al.*, 2004). Phylogenetic analysis of the sequences from the 9°N hydrothermal vent area on the EPR (designated EPRdsrXX) revealed two clusters of sequences, both within the *Desulfobacteriales*. One cluster, consisting of EPR02, 09 and 20, was found to be most closely related to several sequences from cold seep samples in this study as well as *Desulforhopalus singaporensis* and the environmental clone F1SP-03 detected from sediment from the Everglades in Florida (Castro *et al.*, 2002). The second cluster, EPR03, 06, 07 and 13 were found to be most closely related to the environmental clone NTd-III05 from the Nankai Trough (Kaneko *et al.*, 2007), clone S1Adsr_A02.seq detected from the Puget Sound (unpublished) as well as various species of *Desulfolobus*. The distribution of *dsrAB* sequences in the Florida Escarpment and Blake Ridge cores do not appear to shift appreciably with depth.

A number of sequences from both *dsrAB* and *mcrA* libraries were only distantly related to the sequences of cultivated organisms, demonstrating that there are novel lineages within the two functional gene groups included in this study.

Chapter 2: Discussion

In this study, we have investigated the phylogenetic diversity of *mcrA* and *dsrAB* genes from three dissimilar deep-sea hydrothermal vent areas and two cold seep areas. The results reported here lend insight into the potential microbial sulfate reducers,

methanogens and methanotrophic ANME-2-related organisms in these marine environments.

Other studies have confirmed that Archaea affiliated with ANME-2 sequences, in consortium with SRB from the Desulfosarcinales can anaerobically oxidize methane in marine sediments (Boetius et al., 2000; Orphan et al., 2001b; Orphan et al., 2001a) and other studies have detected the presence of ANME-related sequences from a number of areas including other cold seeps (e.g. Treude et al., 2003; Knittel et al., 2005) and hydrothermally-heated sediment from the Guaymas Basin (Teske et al., 2002) and the anoxic basin of the Black Sea (Vetriani et al., 2003). Sequences related to ANME-1 group sequences have been found in hydrothermally heated sediments from the Guaymas Basin area (Teske et al., 2002), and two studies have reported 16S rRNA genes related to ANME groups from the Logatchev hydrothermal vent area (Perner et al., 2007; Voordeckers et al., 2008) but until this study no *mcrA* sequences from high-temperature hydrothermal vents have been shown to group within any of the ANME groups. Two of our clone types, clone 3667LogatchevMCR02 and 3667LogatchevMCR08 clustered the within the ANME-2e subgroup and were found to be most closely related to clone Kuro-mcrA-3.14, detected in cold seep sediments on the Kuroshima Knoll, southern Ryuku Arc (Inagaki et al., 2004). To our knowledge, these two clones are the first *mcrA* sequences clustering within ANME groups to be positively detected in non-sedimented deep-sea hydrothermal vent environments. Our Logatchev chimney sample was taken from friable upper chimney material that was close to the high temperature vent fluid, but not necessarily in direct contact with high temperature fluid. Available sulfate from

intruding seawater combined with anaerobic conditions within the chimney material, methane from the vent fluid and the sharp temperature gradient present within the chimney wall could provide a narrow niche that would allow conditions for the growth of active ANME-related Archaea. In a study also from the Rainbow hydrothermal vent area, Nercessian et al. (2005) found only non-ANME related Methanococcales, with one exception. One sequence detected in a hydrothermal sediment sample, MCR-D, was distantly related to ANME groups but could not be definitively characterized as an ANME-related sequence (Nercessian et al., 2005). No sequences related to ANME-1 sequences were detected in this study, nor were there any ANME-1 16S sequences detected in a 16S survey (Voordeckers *et al.*, 2008). Knittel et al. (2005) proposes that ANME-1 Archaea are more sensitive to aerobic conditions than ANME-2 Archaea and the ephemeral conditions present within a hydrothermal vent chimney may serve to attenuate the growth of ANME-1 microorganisms in our study area.

The Florida Escarpment core revealed a sharp shift in the population structure of the *mcrA* libraries with depth. In addition to the 89% ANME-2d sequences detected, 11% of the *mcrA* sequences detected in the top zone of the Florida Escarpment core fell within the non-ANME related *Methanosarcinales* (Figure 4). This indicates a potential role for *in situ* methanogenesis (rather than biogenically derived methane advected from within the Florida Escarpment itself) in the upper zone of the sediment in this area. Additionally, there is a shift in the dominant ANME-2 group with depth in both Florida Escarpment and Blake Ridge *mcrA* libraries. ANME-2d related clones were detected in the top zones of both cores with the percentages of ANME-2e related clones increasing with depth

(indeed, to saturation in the Blake Ridge libraries). This may indicate that ANME-2e related microorganisms can outcompete other ANME-2 Archaea in layers that are more uniformly anaerobic and where the sulfate concentration is presumably decreased and methane increased.

The distribution of *dsrAB* sequences in the Florida Escarpment and Blake Ridge cores do not appear to shift appreciably with depth, indicating that the SRB community structure does not change with decreasing sulfate and increasing methane with depth. The majority of all seep clones were related to members of the *Desulfobacterales*, with members of the *Syntrophobacterales* also prominent in the Blake Ridge core. These groups are known to contain primarily SRB, and are distributed in all zones of the core samples. The SRB specifically associated with AOM are just a fraction of the SRB community and free-living members of these groups are widespread in marine sediments (Ravenschlag *et al.*, 1999). Further, many SRB can utilize sulfate reduction in their metabolism using various hydrocarbons, including petroleum, as well as a variety of other long and short-chain alkanes and aromatic compounds serving as the carbon source (Spormann and Widdel, 2000). This is known to occur in many Gulf of Mexico sediments (Joye *et al.*, 2004; Orcutt *et al.*, 2004) and serves to allow the decoupling of sulfate reduction and AOM. The chemical and thermal gradients present in the walls of deep-sea hydrothermal chimneys can provide a spectrum of microhabitats that can select for and sustain specifically-adapted microorganisms. Low-temperature, oxygenated seawater with a high sulfate concentration can laterally intrude into a chimney wall and mix with highly reducing hydrothermal fluids from within to create a variety of internal

microenvironments. All *dsr* clones from the MAR hydrothermal vent chimney sample detected in this study were related to various species of the biofilm-forming, hyperthermophilic (optimal growth occurs at $>80^{\circ}\text{C}$), marine sulfate reducer *Archaeoglobus*, suggesting that the sulfate reduction there occurs at high temperature. Samples from the 9°N EPR hydrothermal vent revealed *dsr* clones related only to the Desulfobacterales, with no obvious thermophilic or hypothermophilic relatives. The occurrence of these *dsr* genes in the 9°N EPR sample potentially reveals the presence of low to moderate temperature dissimilatory sulfate reduction within the walls in this deep-sea hydrothermal vent area.

This study has revealed the presence of *dsrAB* and *mcrA* genes in various deep-sea hydrothermal vent and cold seep environments, but does not prove that they are active in these areas. Further investigations into the quantification of *dsr* genes and *dsr* messenger RNA, *mcr* and *mcr* messenger RNA, coupled with geochemical characterizations of the environment, can lend insight into the complex and ephemeral microbial systems found at cold seeps and deep-sea hydrothermal vents. Also, the detection of ANME-2 related sequences in high temperature hydrothermal vent chimney samples opens the door to further study of the groups of microorganisms that mediate AOM in high-temperature habitats.

Chapter 3: Phylogenetic Diversity of Sedimentary Aerobic Methanotrophs by Molecular Analysis of Particulate Methane Monooxygenase (*pmoA*) Genes from Cold Seeps

Chapter 3: ABSTRACT

The aerobic, methanotropic microbial communities from two cold seep areas (Blake Ridge, western Atlantic Ocean; Florida Escarpment, Gulf of Mexico) were investigated by molecular phylogenetic analysis of particulate methane monooxygenase (*pmoA*) genes. Clone libraries were obtained using DNA from sampled from the surface sediment and sequenced. Clone library *pmoA* sequences grouped among the Type I methanotrophs (γ -proteobacteria) within the order *Methylococcales*.

All sequences were found to be Type I Pmo members of the γ -proteobacteria. No Type II Pmo members of the α -proteobacteria were detected, indicating that the methanotrophic communities in these areas are dominated by Type I γ -proteobacteria. Cold seep *pmoA* sequences were primarily distributed among three major clusters of sequences. Sequences from this study were found to be most closely related to sequences from actively venting sediments and chemosynthetic clam beds of the Northern Ridge of the Eel River Basin, the Kuroshima Knoll, southern Ryukyu Arc, the Rainbow hydrothermal vent field, the Puget Sound, a Transbaikalian soda lake in Russia, and three species of the obligate methanotrophic and thermophilic genus *Methylocaldum*. A majority of *pmoA* sequences from this study were only distantly related to clonal sequences from other studies and fewer were closely related to fully characterized species. These sequences are likely as-

yet-uncultivated phylotypes that are distinct from any other cultivated or environmental clones.

Chapter 3: Introduction

Methanotrophic bacteria are ubiquitously distributed in soil, freshwater and marine environments. The bulk of biological methane oxidation occurs in areas overlying anoxic sediment where methane is generated by underlying methanogenic Archaea in the anoxic zone. Aerobic methane-oxidizing bacteria (methanotrophic bacteria) utilize methane as the only source of both carbon and energy (Hanson and Hanson, 1996). The first reaction, the oxidization of methane to methanol is mediated by one of two methane monooxygenase (MMO) enzymes [the membrane-bound MMO (pMMO) and the cytoplasmic MMO (sMMO)] (Murrell *et al.*, 2000).

The functional gene *pmoA* encodes the active site of the pMMO enzyme. The *pmoA* gene is present in all currently known methanotrophs with the exception of the genus *Methylocella* (Dedysh *et al.*, 2002) making it an effective marker for detecting aerobic methanotrophs in the environment. The majority of microbiological studies in hydrocarbon seep environments have focused on the methane-oxidizing archaea and sulfate-reducing bacteria performing the anaerobic oxidation of methane (Hinrichs *et al.*, 1999; Boetius *et al.*, 2000; Lanoil *et al.*, 2001; Orphan *et al.*, 2001b; Orphan *et al.*, 2001a; Orphan *et al.*, 2002; Mills *et al.*, 2003; Joye *et al.*, 2004; Orcutt *et al.*, 2004). In

comparison, the aerobic methanotrophic microbiological community in cold seep and gas hydrate areas has been little studied.

Chapter 3: Materials and Methods

Sample Collection. Acrylic tube sediment cores measuring 22 cm were collected from sediments at the base of the Florida Escarpment, Gulf of Mexico (latitude 26° 01.8 north, longitude 84° 54.9 west) during the AT-03 cruise, leg 58 of RV *Atlantis* and dive 3637 of DSV *Alvin* at a depth of 3288 meters in October of 2000. Sediment cores were also taken from the Blake Ridge cold seep site in the Atlantic Ocean (latitude 32° 29.41 north, longitude 76° 11.09 west) during the AT-07 cruise of RV *Atlantis* and dive 3709 of DSV *Alvin* at a depth of 2178 meters in September of 2001. Bottom water temperatures were about 2° C in both cold seep areas.

Table 1 shows the sampling locations and information for cold seep *pmoA* libraries. The sediment cores for the cold seep sites were subsampled by sectioning them into three zones and designated top, middle and bottom and then frozen whole on board at -80°C until they could be processed in the laboratory at Rutgers University. The top of the cores consisted of lightly colored, oxygenated and loosely packed sand-sized sediment. There was no noticeable odor of H₂S (indicative of oxygen depletion) in the top layer of the sediment core.

DNA Extraction and DNA Amplification by PCR. Total genomic DNA was extracted from ~10 g of sediment from top layer of the cold seep sediment cores using a soil DNA Mega Prep kit UltraClean™ Soil DNA extraction kit (Mo Bio Laboratories, Solana Beach, CA) according to the protocol supplied with the kit. The DNA for all samples was resuspended in sterile water and the DNA concentration was measured using a spectrophotometer. DNA fragments encoding the *pmoA* gene were amplified using the forward primer A189 (5'-GGNGACTGGGACTTCTGG-3') and the reverse primer A682 (5'-GAASGCNGAGAAGAASGC-3') (Holmes *et al.*, 1995; Semrau *et al.*, 1995). This set of primers amplifies only the *pmoA* gene and not the *amoA* gene (Costello and Lidstrom, 1999).

Amplification was performed with Taq PCR Master Mix (Qiagen, Valencia, CA) using a thermocycler (Perkin-Elmer, Norwalk, CT). The PCR conditions were as follows: 25 PCR cycles with denaturation at 92°C for 60 seconds, annealing at 55°C for 90 seconds and extension at 72°C for 60 seconds with a final extension time of 300 seconds on the final cycle. PCR amplification without DNA was used as a negative control throughout. Amplified products were subjected to agarose gel electrophoresis and then purified using the Mo Bio Laboratories, Inc. Gel Purification kit according to the manufacturer's instructions.

Construction of *pmoA* Clonal Libraries. Clone libraries from PCR products were constructed using the TA cloning kit (Invitrogen Corporation, San Diego, CA) following the manufacturers' recommendations. Amplified *pmoA* gene fragments were cloned in

pCRII plasmid vector (Invitrogen, Inc., Carlsbad, CA) and the ligation products were used to transform competent *E.coli* INV α F' 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) as described in the manufacturers' instructions. One *pmoA* library was created from surficial sediment from the Blake Ridge and one *pmoA* library was created from Florida Escarpment surficial sediment. The seep clones are designated BRpmoXXX (for Blake Ridge) and FEpmoXXX (for Florida Escarpment), where XX indicates the clone number.

Restriction Length Polymorphism (RFLP) Screening. Clonal inserts of *pmoA* fragments were digested for 2 hours at 37°C on a thermocycler using the restriction endonuclease RsaI (Promega, Inc. Madison, WI). The restriction products were run for 2.5 hours at 75V and 4°C on a 2.5% (weight/volume) Methaphor agarose gel containing ethidium bromide and visualized under UV light.

Sequencing and Phylogenetic Analysis of *pmoA* Genes. Clones were sequenced utilizing an automated DNA sequencer (310 Avant Genetic Analyzer; Applied Biosystems, Foster City, CA). Resulting sequences were compared with cultivated microorganisms and environmental clones from the GenBank database using the BLAST search program of the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov)(Altschul *et al.*, 1997). All sequences were aligned in a two-stage process. Amino acid sequences were translated from nucleotide sequences using the

Transeq program from the European Bioinformatics Institute (Rice *et al.*, 2000). Multiple alignments were created using ClustalX v2.0 (Larkin *et al.*, 2007). The distance matrix of amino acids between sequences was created using PHYLIP version 3.6 (Felsenstein, 1989). 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 the phylogenetic tree was tested by bootstrap analysis with 1000 resamplings.

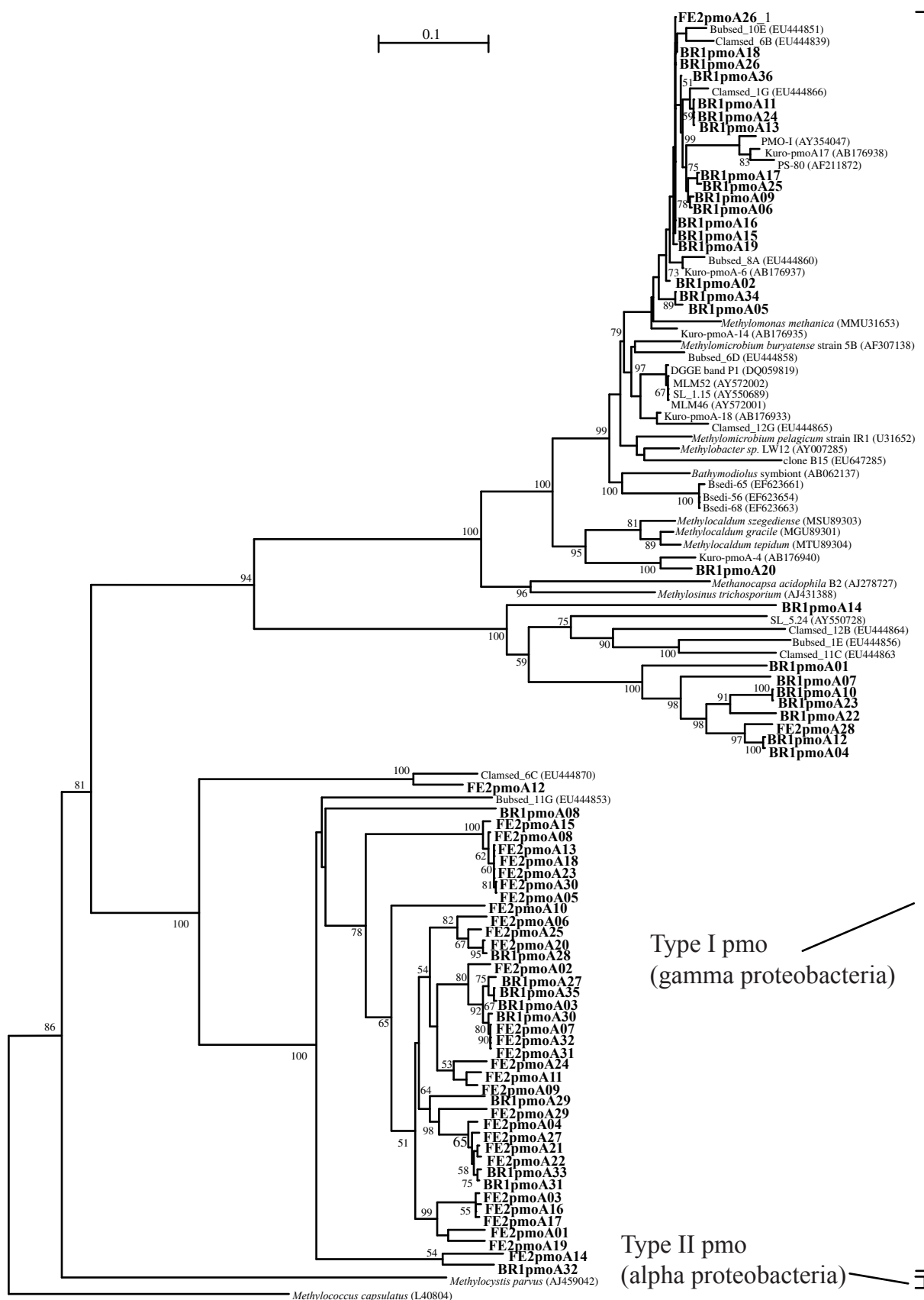
Nucleotide Sequence Accession Numbers. The sequences reported here have been deposited in the Genbank database under the accession numbers EU982943 through EU983009.

Chapter 3: Results

Genomic DNA was extracted from three depth intervals (top, middle and bottom) of the sectioned sediment cores. 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. PCR using *pmoA* primers were attempted on all three depth levels from both locations, but only the top depth intervals yielded successful PCR products. A *pmoA* clone library was constructed for both cold seep sites. The clonal libraries were typed on the basis of restriction fragment banding patterns and clones were partially sequenced.

Diversity of *pmoA* and Phylogenetic Analysis. A total of 68 *pmoA* clones from the Blake Ridge and the Florida Escarpment were screened by RFLP and 35 unique profiles were identified. All sequences were found to be Type I *pmo* members of the γ -proteobacteria. No Type II *pmo* members of the α -proteobacteria were detected. Phylogenetic analysis of *pmoA* genes indicated that the majority of the sequences grouped in three major groups within the γ -proteobacteria. The first group (top of Figure 7), consisted of 17 (25.0% of the total *pmoA* clones) closely related sequences (>97% protein sequence identity) from both the Blake Ridge and one sequence, FE2*pmoA*26, from the Florida Escarpment. This group was found to be most closely related to 4 sequences from actively venting sediments (labeled 'Bubsed') and chemosynthetic clam beds (labeled 'Clamsed') of the Northern Ridge of the Eel River Basin (Tavormina *et al.*, 2008), as well as 2 sequences (Kuro-*pmoA*17 and Kuro-*pmoA*-6) from the Kuroshima Knoll, southern Ryukyu Arc (Inagaki *et al.*, 2004), a single sequence (PMO-I) from the Rainbow hydrothermal vent field (Nercessian *et al.*, 2005), and a single sequence (PS-80) from the Puget Sound (Nold *et al.*, 2000).

The second group of sequences consisted of 8 sequences from the Blake Ridge and a single sequence from the Florida Escarpment (for a total of 13.2% of all *pmoA* sequences). Close environmental relatives of the second group include three clones from the Northern Ridge of the Eel River Basin (Tavormina *et al.*, 2008) and clone SL_5.24 (Lin *et al.*, 2004) sequenced from a Transbaikal soda lake in Russia. The majority (41 sequences; 60.3% of the total) of the *pmoA* clones in this study formed the third major



cluster of sequences. This cluster has few environmental relatives and a relatively low percent identity between the two most divergent protein sequences within the group (71%). The closest relatives of this large group were clones Clamsed_6C and Bubsed_11G, sampled from the Northern Ridge of the Eel River Basin (Tavormina *et al.*, 2008).

One sequence from this study, BR1pmoA20, did not fall into a cluster with other BR1 or FE2 sequences. The closest environmental relative was Kuro-pmoA-4, sampled from the Kuroshima Knoll, southern Ryukyu Arc (Inagaki *et al.*, 2004). BR1pmoA20 was also found to be closely related to the three species of the obligate methanotrophic and thermophilic genus *Methylocaldum* (*M. tepidum*, *M. gracile* and *M. szegediense*).

Chapter 3: Discussion

This study investigates the phylogenetic diversity of *pmoA* genes from two cold seep areas. The results reported here lend insight into the potential aerobic, methanotrophic microorganisms in these marine environments.

There were no successful *pmoA* PCR amplifications from the middle and bottom zones of the cold seep cores in this study. The penetration depth of oxygen is typically very limited in methane-rich sediments in marine systems because the available oxygen is depleted in the uppermost millimeters of sediment as a result of the microbial degradation of organic matter (Wenzhofer and Glud, 2002). As a result, the middle and bottom zones of the cores were only microaerophilic to anaerobic and therefore were inhospitable niches for significant populations of aerobic methanotrophs. On a global scale, this

narrow veneer of aerobic methane oxidation in surficial sediments serves to limit methane oxidation as a sink for methane in the ocean, especially when compared to the anaerobic oxidation of methane in marine sediments (Hinrichs and Boetius, 2002).

There was some distinct geographic separation in the clusters of *pmoA* genes sequenced in this study. Blake Ridge *pmoA* sequences grouped primarily within two clusters (upper portion of Figure 7). Florida Escarpment *pmoA* sequences all grouped within a single cluster of sequences that also included nine Blake Ridge sequences (bottom portion of Figure 7). A primary difference between the two study sites is the concentration of methane in the sediments found at each site. Methane in the Florida Escarpment sediments have been found to be about 10 mM (Chanton *et al.*, 1991) while Blake Ridge methane concentrations range from 1.0 to 3.4 mM (Paull *et al.*, 1996). No Type II *pmo* members of the α -proteobacteria were detected, indicating that the methanotroph communities in these areas are dominated by Type I γ -proteobacteria. It has been shown that environmental conditions, including copper concentration, nitrogen and methane limitation conditions can serve to select for Type I or Type II methanotrophs (Graham *et al.*, 1993).

Knowledge of the diversity and function of aerobic methanotrophs in deep-sea areas is very limited. Future studies should include the molecular screening of other functional genes (e.g. *amoA*), the isolation of some of these organisms and the investigation of their *in situ* population abundance as well as their role in carbon cycling in these environments. This study has revealed the presence of *pmoA* genes in two cold seep

environments, but does not prove that they are active in these areas. Further investigations into the quantification of *pmoA* genes and pmo messenger RNA, coupled with physiological characterizations of the environment, can lend insight into the complex and ephemeral microbial systems found in cold seep areas.

References

- Alain, K., Zbinden, M., Le Bris, N., Lesongeur, F., Querellou, J., Gaill, F., and Cambon-Bonavita, M.A. (2004) Early steps in microbial colonization processes at deep-sea hydrothermal vents. *Environmental Microbiology* **6**: 227-241.
- Alain, K., Olagnon, M., Desbruyeres, D., Page, A., Barbier, G., Juniper, S.K. et al. (2002) Phylogenetic characterization of the bacterial assemblage associated with mucous secretions of the hydrothermal vent polychaete *Paralvinella palmiformis*. *FEMS Microbiology Ecology* **42**: 463-476.
- Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-3402.
- Bahr, M., Crump, B.C., Klepac-Ceraj, V., Teske, A., Sogin, M.L., and Hobbie, J.E. (2005) Molecular characterization of sulfate-reducing bacteria in a New England salt marsh. *Environmental Microbiology* **7**: 1175-1185.
- Barnes, R.O., and Goldberg, E.D. (1976) Methane production and consumption in anaerobic marine sediments. *Geology* **4**: 297-300.
- Bidle, K.A., Kastner, M., and Bartlett, D.H. (1999) Phylogenetic Analysis of Microbial Communities Associated with Methane Hydrate Containing Marine Fluids and Sediments in the Cascadia Margin (ODP Site 892B). *FEMS Microbiology Letters* **177**: 101-108.
- Boetius, A., Ravensschlag, K., Schubert, C.J., Rickert, D., Widdel, F., Gieseke, A. et al. (2000) A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature* **407**: 623-626.
- Borowski, W.S., Paull, C.K., and Ussler III, W. (1996) Marine pore-water sulfate profiles indicate in situ methane flux from underlying gas hydrate. *Geology* **24**: 655-658.
- Buffett, B. (2000) Clathrate hydrates. *Annual Reviews of Earth and Planetary Science* **28**: 477-507.
- Castro, H., Reddy, K.R., and Ogram, A. (2002) Composition and Function of Sulfate-Reducing Prokaryotes in Eutrophic and Pristine Areas of the Florida Everglades. *Applied and Environmental Microbiology* **68**: 6129-6137.

- Chanton, J.P., Martens, C.S., and Paull, C.K. (1991) Control of pore-water chemistry at the base of the Florida escarpment by processes within the platform. *Nature* **349**: 229-231.
- Charlou, J.L., Donval, J.P., Fouquet, Y., Jean-Baptiste, P., and Holm, N. (2002) Geochemistry of high H₂ and CH₄ vent fluids issuing from ultramafic rocks at the Rainbow hydrothermal field (36°14'N, MAR) *Chemical Geology* **191**: 345-359.
- Charlou, J.L., Donval, J.P., Fouquet, Y., Jean-Baptiste, P., Dapoigny, A., Appriou, P., and Rona, P.A. (1998) Intense CH₄ plumes generated by serpentinization of ultramafic rocks at the intersection of the 15° 20'N fracture zone and the Mid-Atlantic Ridge. *Geochimica et Cosmochimica Acta* **62**: 2323-2333.
- Claypool, G.E., and Kvenvolden, K.A. (1983) Methane and other hydrocarbon gases in marine sediment. *Annual Reviews of Earth and Planetary Science* **11**: 299-327.
- Cole, J.R., Chai, B., Marsh, T.L., Farris, R.J., Wang, Q., Kulam, S.A. et al. (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Research* **31**: 442-443.
- Corre, E., Reysenbach, A.-L., and Prieur, D. (2001) Epsilon-proteobacterial diversity from a deep-sea hydrothermal vent of the Mid-Atlantic Ridge. *FEMS Microbial Ecology* **205**: 329-335.
- Costello, A.M., and Lidstrom, M.E. (1999) Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. *Applied and Environmental Microbiology* **65**: 5066-5074.
- Cragg, B.A., Parkes, R.J., Fry, J.C., Weightman, A.J., Rochelle, P.A., and Maxwell, J.R. (1996) Bacterial populations and processes in sediments containing gas hydrates (ODP Leg 146: Cascadia Margin). *Earth and Planetary Science Letters* **139**: 497-507.
- Dedysh, S.N., Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A., Semrau, J.D., Liesack, W., and Tiedje, J.M. (2002) *Methylocapsa acidiphilia* gen. nov., sp. nov., a novel methane-oxidizing and dinitrogen-fixing acidophilic bacterium from Sphagnum bog. *International Journal of Systematic Bacteriology* **52**: 251-261.
- Dhillon, A., Teske, A., Dillon, J., Stahl, D.A., and Sogin, M.L. (2003) Molecular Characterization of Sulfate-Reducing Bacteria in the Guaymas Basin. *Applied and Environmental Microbiology* **69**: 2765-2772.

Dillon, W.P., and Max, M.D. (2000) U.S. Atlantic continental margin; the best-known gas hydrate locality. In *Natural Gas Hydrate in Oceanic and Polar Environments*. Max, M.D. (ed). Dordrecht: Kluwer Academic Publishers, pp. 245-260.

Distel, D.L., Lane, D.J., Olsen, G.J., Giovannoni, S.J., Pace, B., Pace, N.R. et al. (1988) Sulfur-oxidizing bacterial endosymbionts: analysis of phylogeny and specificity by 16S rRNA sequences. *Journal of Bacteriology* **170**: 2506-2510.

Douville, E. (1999) Les fluides hydrothermaux océaniques: comportement géochimique des éléments traces et des terres rares - Processus associés et modélisation thermodynamique. In. Brest: Université de Bretagne Occidentale.

Erlich, H.A., Gelfand, D., and Sninsky, J.J. (1991) Recent advances in the polymerase chain reaction. *Science* **252**: 1643-1651.

Etchebehere, C., Errazquin, M., Dabert, P., and Muxi, L. (2002) Community analysis of a denitrifying reactor treating landfill leachate. *FEMS Microbial Ecology* **40**: 97-106.

Felsenstein, J. (1989) Phylogeny Inference Package (Version 3.2). *Cladistics* **5**: 164-166.

Finster, K., Liesack, W., and Tindall, B.J. (1997) *Sulfurospirillum arcachonense* sp. nov., a new-microaerophilic sulfur-reducing bacterium. *International Journal of Systematic Bacteriology* **47**: 1212-1217.

Freitag, T.E., and Prosser, J.I. (2003) Community Structure of Ammonia-Oxidizing Bacteria within Anoxic Marine Sediments. *Applied and Environmental Microbiology* **69**: 1359-1371.

Fuhrman, J.A., and Davis, A.A. (1997) Widespread Archaea and novel Bacteria from the deep sea as shown by 16S rRNA gene sequences. *Marine Ecology Progress Series* **150**: 275-285.

Galtier, N., Gouy, M., and Gaultier, C. (1996) SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. *Computer Applications in the Biosciences* **12**: 543-548.

Galushko, A., Minz, D., Schink, B., and Widdel, F. (1999) Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium. *Environmental Microbiology* **1**: 415-420.

- Girguis, P.R., Orphan, V.J., Hallam, S.J., and E., D. (2003) Growth and methane oxidation rates of anaerobic methanotrophic archaea in a continuous-flow bioreactor. *Applied and Environmental Microbiology* **69**: 5472-5482.
- Graham, D., Chaudhary, J., Hanson, R., and Arnold, R. (1993) Factors Affecting Competition Between Type I and Type II Methanotrophs in Two-organism, Continuous-flow Reactors. *Microbial Ecology* **25**: 1-17.
- Hales, B.A., Edwards, C., Ritchie, D.A., Hall, G., Pickup, R.W., and Sanders, J.R. (1996) Isolation and identification of methogen-specific DNA from blanket bog peat by pcr amplification and sequence analysis. *Applied and Environmental Microbiology* **62**: 668-675.
- Hallam, S.J., Girguis, P.R., Preston, C.M., Richardson, P.M., and DeLong, E.F. (2003) Identification of Methyl Coenzyme M Reductase A (mcrA) Genes Associated with Methane-Oxidizing Archaea. *Applied and Environmental Microbiology* **69**: 5483-5491.
- Hallam, S.J., Putnam, N., Preston, C.M., Detter, J.C., Rokhsar, D., Richardson, P.M., and DeLong, E.F. (2004) Reverse Methanogenesis: Testing the Hypothesis Using Environmental Genomics. *Science* **305**: 1457-1462.
- Hanson, J.L., and Hanson, T.E. (1996) Methanotrophic bacteria. *Microbiological Reviews* **60**: 439-471.
- Heijs, S.K., Haese, R.R., van der Wielen, P.W.J.J., Forney, L.J., and van Elsas, J.D. (2007) Use of 16S rRNA Gene Based Clone Libraries to Assess Microbial Communities Potentially Involved in Anaerobic Methane Oxidation in a Mediterranean Cold Seep. *Microbial Ecology* **53**: 384-398.
- Hinrichs, K.-U., and Boetius, A. (2002) The anaerobic oxidation of methane: New insights in microbial ecology and biogeochemistry. In *Ocean MArgin Systems*. Wefer, G., Billett, D., Hebbeln, D., Jorgensen, B.B., Schluter, M., and Van Weering, T. (eds). Berlin: Springer.
- Hinrichs, K.U., Hayes, J.M., Sylva, S.P., Brewer, P.G., and DeLong, E.F. (1999) Methane-consuming archaeobacteria in marine sediments. *Nature* **398**: 802-805.
- Holmes, A., Costello, A., Lidstrom, M., and Murrell, J. (1995) Detection of novel marine methanotrophs using phylogenetic and functional gene probes after methane enrichment. *Microbiology* **141**: 1947-1955.

Hugenholtz, P., and Pace, N.R. (1996) Identifying microbial diversity in the natural environment: a molecular phylogenetic approach. *Trends in Biotechnology* **14**: 190-197.

Inagaki, F., Sakihama, Y., Inoue, A., Kato, C., and Horikoshi, K. (2002) Molecular phylogenetic analyses of reverse-transcribed bacterial rRNA obtained from deep-sea cold seep sediments. *Environmental Microbiology* **4**: 277-286.

Inagaki, F., Takai, K., Kobayashi, H., Nealson, K.H., and Horikoshi, K. (2003) *Sulfurimonas autotrophica* gen. nov., sp. nov., a novel sulfur-oxidizing -proteobacterium isolated from hydrothermal sediments in the Mid-Okinawa Trough. *International Journal of Systematic and Evolutionary Microbiology* **53**: 1801-1805.

Inagaki, F., Tsunogai, U., Suzuki, M., Kosaka, A., Machiyama, H., Takai, K. et al. (2004) Characterization of C₁-metabolizing prokaryotic communities in methane seep habitats at the Kuroshima Knoll, the Southern Ryukyu Arc, by analyzing pmoA, mmoX, mxaF, mcrA, and 16S rRNA genes. *Applied and Environmental Microbiology* **70**: 7445-7455.

Joye, S.B., Boetious, A., Orcutt, B.N., Montoya, J.P., Schulz, H.N., Erickson, M.J., and Lugo, S.K. (2004) The anaerobic oxidation of methane and sulfate reduction in sediments from Gulf of Mexico cold seeps. *Chemical Geology* **205**: 219-238.

Kaneko, R., Hayashi, T., Tanahashi, M., and Naganuma, T. (2007) Phylogenetic diversity and distribution of dissimilatory sulfite reductase genes from deep-sea sediment cores. *Marine Biotechnology* **9**: 429-436.

Knittel, K., Lösekann, T., Boetious, A., Kort, R., and Amann, R. (2005) Diversity and Distribution of Methanotrophic Archaea at Cold Seeps. *Applied and Environmental Microbiology* **71**: 467-479.

Knittel, K., Boetius, A., Lemke, A., Eilers, H., Lochte, K., Pfannkuche, O. et al. (2003) Activity, distribution, and diversity of sulfate reducers and other bacteria in sediments above gas hydrate (Cascadia Margin, Oregon). *Geomicrobiology Journal* **20**: 269-294.

Kormas, K.A., Meziti, A., Dahlman, A., de Lange, G., and Lykousis, V. (2005) Molecular fingerprinting of methanogens in eastern Mediterranean Sea mud volcanoes. *Geochimica et Cosmochimica Acta* **69** (suppl. 1).

Krüger, M., Meyerdierks, A., Glöckner, F.O., Amann, R., Widdel, F., Kube, M. et al. (2003) A conspicuous nickel protein in microbial mats that oxidize methane anaerobically. *Nature* **426**: 878-881.

Kvenvolden, K. (1988) Methane hydrate - a major reservoir of carbon in the shallow geosphere? *Chemical Geology* **71**: 41-51.

LaMontagne, M.G., Leifer, I., Bergmann, S., Van De Werfhorst, L.C., and Holden, P.A. (2004) Bacterial Diversity in Marine Hydrocarbon Seep Sediments. *Environmental Microbiology* **6**: 799-808.

Lanoil, B.D., Sassen, R., La Duc, M.T., Sweet, S.T., and Nealson, K.H. (2001) Bacteria and Archaea physically associated with Gulf of Mexico gas hydrates. *Applied and Environmental Microbiology* **67**: 5143-5153.

Larkin, J.M., and Henk, M.C. (1996) Filamentous sulfide-oxidizing bacteria at hydrocarbon seeps of the Gulf of Mexico. *Microscopy Research and Technique* **33**: 23-31.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H. et al. (2007) Clustal W and Clustal X Version 2.0. *Bioinformatics* **23**: 2947-2948.

Li, L., Kato, C., and Horikoshi, K. (1999a) Microbial Diversity in Sediments Collected from the Deepest Cold-Seep Area, the Japan Trench. *Marine Biotechnology* **1**: 391-400.

Li, L., Kato, C., and Horikoshi, K. (1999b) Bacterial diversity in deep-sea sediments from different depths. *Biodiversity and Conservation* **8**: 659-677.

Li, L., Guezennec, J., Nichols, P., Henry, P., Yanagibayashi, M., and Kato, C. (1999c) Microbial diversity in Nankai Trough sediments at a depth of 3,843m. *Journal of Oceanography* **55**: 635-642.

Lin, L.-L., Radajewski, S., Eshinimaev, B.T., Trotsenko, Y.A., McDonald, I.R., and Murrell, J.C. (2004) Molecular Diversity of Methanotrophs in Transbaikial Soda Lake Sediments and Identification of Potentially Active Populations By Stable Isotope Probing. *Environmental Microbiology* **6**: 1049-1060.

Lloyd, K.G., Lapham, L., and Teske, A. (2006) An Anaerobic Methane-Oxidizing Community of ANME-1b Archaea in Hypersaline Gulf of Mexico Sediments. *Applied and Environmental Microbiology* **72**: 7218-7230.

Longnecker, K., and Reysenbach, A.-L. (2001) Expansion of the geographic distribution of a novel lineage of the epsilon Proteobacteria to a hydrothermal vent on the Southern east Pacific Rise. *FEMS Microbiology Ecology* **35**: 287-293.

López-García, P., López-López, A., Moreira, D., and Rodríguez-Valera, F. (2001) Diversity of free-living prokaryotes from a deep-sea site at the Antarctic Polar Front. *FEMS Microbial Ecology* **36**: 193-202.

Lorenson, T.D., and Collett, T.S. (2000) Gas content and composition of gas hydrate from sediments of the southeastern North American continental margin. In *Proceedings of the Ocean Drilling Program, Scientific Results 164*. Paul, C.K., Matsumoto, R., Wallace, P.J., and Dillon, W.P. (eds). College Station, Texas: Ocean Drilling Program, pp. 37-46.

Marchesi, J.R., Weightman, A.J., Cragg, B.A., Parkes, R.J., and Fry, J.C. (2001) Methanogen and bacterial diversity and distribution in deep gas hydrate sediments from the Cascadia Margin as revealed by 16S rRNA molecular analysis. *FEMS Microbial Ecology* **34**: 221-228.

Margalef, R. (1958) Information theory in ecology. *General Systems* **3**: 36-71.

Martens, C.S., Chanton, J.P., and Paull, C.K. (1991) Biogenic methane from abyssal brine seeps at the base of the Florida Escarpment. *Geology* **19**: 851-854.

Mehta, M.P., Butterfield, D.A., and Baross, J.A. (2003) Phylogenetic diversity of nitrogenase (nifH) genes in deep-sea and hydrothermal vent environments of the Juan de Fuca ridge. *Applied and Environmental Microbiology* **69**: 960-970.

Michaelis, W., Seifert, R., Nauhaus, K., Trede, T., Thiel, V., Blumenberg, M. et al. (2002) Microbial reefs in the Black Sea fueled by anaerobic oxidation of methane. *Science* **297**: 1013-1015.

Mills, H.J., Hodges, C., Wilson, K., MacDonald, I.R., and Sobecky, P. (2003) Microbial diversity in sediments associated with surface-breaching gas hydrate mounds in the Gulf of Mexico. *FEMS Microbiology Ecology* **46**: 39-52.

Miroshnichenko, M.L., Kostrikina, N.A., Chernykh, N.A., Pimenov, N.V., Tourova, T.P., Antipov, A.N. et al. (1995) *Caldithrix abyssi* gen. nov., sp. nov., a nitrate-reducing, thermophilic, anaerobic bacterium isolated from a Mid-Atlantic Ridge hydrothermal vent,

represents a novel bacterial lineage. *International Journal of Systematic and Evolutionary Microbiology* **53**: 323-329.

Mullins, T.D., Britschgi, T.B., Krest, R.L., and Giovannoni, S.J. (1995) Genetic comparisons reveal the same unknown bacterial lineages in Atlantic and Pacific bacterioplankton communities. *Limnology and Oceanography* **40**: 148-158.

Murrell, J.C., Gilbert, B., and McDonald, I.R. (2000) Molecular biology and regulation of methane monooxygenase. *Archives of Microbiology* **173**: 325-332.

Nakagawa, T., Nakagawa, S., Inagaki, F., Takai, K., and Horikoshi, K. (2004) Phylogenetic Diversity of Sulfate-Reducing Prokaryotes in Active Deep-Sea Hydrothermal Vent Chimney Structures. *FEMS Microbiology Letters* **232**: 145-152.

Nercessian, O., Bienvenu, N., Moreira, D., Prieur, D., and Jeanthon, C. (2005) Diversity of functional genes of methanogenes, methanotrophs and sulfate reducers in deep-sea hydrothermal vents. *Environmental Microbiology* **7**: 118-132.

Nold, S.C., Zhou, J., Devol, A.H., and Tiedje, J.M. (2000) Pacific Northwest Marine Sediments Contain Ammonia-Oxidizing Bacteria in the β Subdivision of the Proteobacteria. *Applied and Environmental Microbiology* **66**: 4532-4535.

Nunoura, T., Oida, H., Toki, T., Ashi, J., Takai, K., and Horikoshi, K. (2006) Quantification of *mcrA* by quantitative fluorescent PCR in sediments from methane seep of the Nankai Trough. *FEMS Microbiology Ecology* **57**: 149-157.

Odom, J.M., and Peck, H.D. (1984) Hydrogenase, electron-transfer proteins, and energy coupling in the sulfate-reducing bacteria *Desulfovibrio*. *Annual Review of Microbiology* **38**: 551-592.

Orcutt, B.N., Boetius, A., Lugo, S.K., MacDonald, I.R., Samarkin, V.A., and Joye, S.B. (2004) Life at the edge of methane ice: microbial cycling of carbon and sulfur in Gulf of Mexico gas hydrates. *Chemical Geology* **205**: 239-251.

Orphan, V.J., House, C.H., Hinrichs, K.-U., McKeegan, K.D., and DeLong, E.F. (2001a) Methane-consuming archaea revealed by directly coupled isotopic and phylogenetic analysis. *Science* **293**: 484-487.

Orphan, V.J., House, C.H., Hinrichs, C.U., McKeegan, K.D., and DeLong, E.F. (2002) Multiple archaeal groups mediate methane oxidation in anoxic cold seep sediments. *Proceedings of the National Academy of Science* **99**: 7663-7668.

Orphan, V.J., Hinrichs, K.U., Ussler III, W., Paull, C.K., Taylor, L.T., Sylva, S.P. et al. (2001b) Comparative analysis of methane-oxidizing archaea and sulfate-reducing bacteria in anoxic marine sediments. *Applied and Environmental Microbiology* **67**: 1922-1934.

Paull, C.K., Matsumoto, R., and Wallace, P.J. (1996) Site 996. Proceedings of the Ocean Drilling Program, Initial Reports. **164**: 241-275.

Paull, C.K., Hecker, B., Commeau, R., Freeman-Lynde, R.P., Neumann, C., Corso, W.P. et al. (1984) Biological communities at the Florida Escarpment resemble hydrothermal vent taxa. *Science* **226**: 965-967.

Perner, M., Kuever, J., Seifert, R., Pape, T., Koschinsky, A., Schmidt, K. et al. (2007) The influence of ultramafic rocks on microbial communities at the Logatchev hydrothermal field, located 15N on the Mid-Atlantic Ridge. *FEMS Microbial Ecology*: 1-13.

Pielou, E. (1966) The measure of diversity in different types of biological collections. *Journal of Theoretical Biology* **13**: 131-144.

Polz, M.F., and Cavanaugh, C.M. (1995) Dominance of one bacterial phylotype at a Mid-Atlantic Ridge hydrothermal vent site. *Proceedings of the National Academy of Science* **92**: 7232-7236.

Radeva, G., and Selenska-Pobell, S. (1999) Bacterial diversity in drain waters of several uranium waste piles. *Annual Report of the Institute of Radiochemistry*: 56.

Rappé, M., and Giovannoni, S. (2003) The uncultured microbial majority. *Annual Review of Microbiology* **57**: 369-394.

Ravenschlag, K., Sahm, K., Pernthaler, J., and Amann, R. (1999) High bacterial diversity in permanently cold marine sediments. *Applied and Environmental Microbiology* **65**: 3982-3989.

Reeburgh, W. (1996) "Soft Spots" in the global methane budget. In *Microbial Growth on C1 Compounds*. Lidstrom, M., and Tabita, F. (eds). Dordrecht: Kluwer Academic Publishers, pp. 334-342.

Reed, A.J., Lutz, R.A., and Vetriani, C. (2006) Vertical distribution and diversity of bacteria and archaea in sulfide and methane-rich cold seep sediments located at the base of the Florida Escarpment. *Extremophiles* **10**: 199-211.

Reed, D.W., Fuji, A.Y., Delwiche, M.E., Blackwelder, D.B., Sheridan, P.P., Uchida, T., and Colwell, F.S. (2002) Microbial communities from methane hydrate-bearing deep marine sediments in a fore-arc basin. *Applied and Environmental Microbiology* **68**: 3759-3770.

Rees, G.N., Grassia, G.S., Sheehy, A.J., Dwivedi, P.P., and Patel, B.K.C. (1995) *Desulfacinum infernum* gen. nov., sp. nov., a thermophilic sulfate-reducing bacterium from a petroleum reservoir. *International Journal of Systematic Bacteriology* **45**: 85-89.

Reysenbach, A.-L., Longnecker, K., and Kirshtein, J. (2000) Novel bacterial and archaeal lineages from an in situ growth chamber deployed at a Mid-Atlantic Ridge hydrothermal vent. *Applied and Environmental Microbiology* **66**: 3798-3806.

Rice, P., Longden, I., and Bleasby, A. (2000) EMBOS: the European Molecular Biology Open Software Suite. *Trends in Genetics* **16**: 276-277.

Rudolph, C., Wanner, G., and Huber, R. (2001) Natural communities of novel archaea and bacteria growing in cold sulfurous springs with a string-of-pearls-like morphology. *Applied and Environmental Microbiology* **67**: 2336-2344.

Schmidt, K., Koschinsky, A., Garbe-Schönberg, D., de Carvalho, L.M., and Seifert, R. (2007) Geochemistry of hydrothermal fluids from the ultramafic-hosted Logatchev hydrothermal field, 15°N on the Mid-Atlantic Ridge: Temporal and spatial investigation. *Chemical Geology* **242**: 1-21.

Schumacher, W., Kroneck, P.M.H., and Pfennig, N. (1992) Comparative systematic study on *Spirillum*-5175, *Campylobacter* and *Wolinella* species - Description of *Spirillum*-5175 as *Sulfurospirillum deleyianum* gen. nov., sp. nov. *Archives of Microbiology* **158**: 287-293.

Semrau, J., Chistoserdov, A., Lebron, J., Costello, A., Davagnino, J., Kenna, E. et al. (1995) Particulate methane monooxygenase genes in methanotrophs. *Journal of Bacteriology* **177**: 3071-3079.

Shannon, C.E., and Weaver, W. (1949) *The mathematical theory of communication*. Urbana: University of Illinois Press.

So, C., and Young, L. (1999) Isolation and characterization of a sulfate-reducing bacterium that anaerobically degrades alkanes. *Applied and Environmental Microbiology* **65**: 2969-2976.

Spormann, A.M., and Widdel, F. (2000) Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. *Biodegradation* **11**: 85-105.

Stackebrandt, E., and Goebel, B. (1994) Taxonomic note: a place for DNA:DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology* **44**: 846-849.

Steffan, R., and Atlas, R. (1991) Polymerase chain reaction: applications in environmental microbiology. *Annual Review of Microbiology* **45**: 137-161.

Stolz, J., Ellis, D., Blum, J., Ahmann, D., Lovley, D., and Oremland, R. (1999) *Sulfurospirillum barnesii* sp. nov. and *Sulfurospirillum arsenophilum* sp. nov., new members of the *Sulfurospirillum* clade of the epsilon Proteobacteria. *International Journal of Systematic and Evolutionary Microbiology* **49**: 1177-1180.

Takai, K., and Horikoshi, K. (1999) Genetic diversity of archaea in deep-sea hydrothermal vent environments. *Genetics* **152**: 1285-1297.

Takai, K., Inagaki, F., Nakagawa, S., Hirayama, H., Nunoura, T., and Sako, Y. (2003) Isolation and phylogenetic diversity of members of previously uncultivated epsilon-Proteobacteria in deep-sea hydrothermal fields. *FEMS Microbial Ecology* **218**: 167-174.

Tavormina, P.L., Ussler III, W., and Orphan, V.J. (2008) Planktonic and Sediment-Associated Aerobic Methanotrophs in Two Seep Systems along the North American Margin. *Applied and Environmental Microbiology* **74**: 3985-3995.

Teske, A., Hinrichs, K., Edgcomb, V., Gomez, A.d.V., Kysela, D., Sylva, S. et al. (2002) Microbial diversity of hydrothermal sediments in the Guaymas Basin: evidence for anaerobic methanotrophic communities. *Applied and Environmental Microbiology* **68**: 1994-2007.

Thompson, J., Gibson, T., Plewniak, F., Jeanmougin, F., and Higgins, D. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**: 4876-4882.

Thomsen, T., Finster, K., and Ramsing, N. (2001) Biochemical and molecular signatures of anaerobic methane oxidation in marine sediments. *Applied and Environmental Microbiology* **67**: 1646-1656.

Treude, T., Boetius, A., Knittel, K., Wallmann, K., and Jørgenson, B.B. (2003) Anaerobic oxidation of methane above gas hydrates (Hydrate Ridge, OR). *Marine Ecology Progress Series* **264**: 1-14.

Urakawa, H., Kita-Tsukamoto, K., and Ohwada, K. (1999) Microbial diversity in marine sediments from Sagami Bay and Tokyo Bay, Japan, as determined by 16S rRNA gene analysis. *Microbiology* **145**: 3305-3315.

Urakawa, H., Yoshida, T., Nishimura, M., and Ohwada, K. (2000) Characterization of depth-related population variation in microbial communities of a coastal marine sediment using 16S rDNA-based approaches and quinone profiling. *Environmental Microbiology* **2**: 542-554.

Van Dover, C., Aharon, P., Bernhard, J., Caylor, E., Doerries, M., Flickinger, W. et al. (2003) Blake Ridge methane seeps: characterization of a soft-sediment, chemosynthetically based ecosystem. *Deep-Sea Research I* **50**: 281-300.

Vetriani, C., Tran, H.V., and Kerkhof, L.J. (2003) Fingerprinting Microbial Assemblages from the Oxic/Anoxic Chemocline of the Black Sea. *Applied and Environmental Microbiology* **69**: 6481-6488.

Vetriani, C., Jannasch, H., MacGregor, B., Stahl, D., and Reysenbach, A.-L. (1999) Population Structure and Phylogenetic Characterization of Marine Benthic Archaea in Deep-Sea Sediments. *Applied and Environmental Microbiology* **65**: 4375-4384.

Von Damm, K.L., and Lilley, M.D. (2004) Diffuse flow hydrothermal fluids from 9° 50'N East Pacific Rise: origin, evolution and biogeochemical controls. In *The*

Subseafloor Biosphere at Mid-Ocean Ridges. Wilcock, W.S.D., DeLong, E.F., Kelley, D., Baross, J.A., and Cary, S.C. (eds). Washington D.C.: American Geophysical Union, pp. 245-268.

Voordeckers, J., Starovoytov, V., and Vetriani, C. (2005) *Caminibacter mediatlanticus* sp. nov., a thermophilic, hemolithoautotrophic, nitrate-ammonifying bacterium isolated from a deep-sea hydrothermal vent on the Mid-Atlantic Ridge. *International Journal of Systematic and Evolutionary Microbiology* **55**: 773-779.

Voordeckers, J., Do, M., Hügler, M., Ko, V., Sievert, S.M., and Vetriani, C. (2008) Culture dependent and independent analysis of 16S rRNA and ATP citrate lyase genes: a comparison of microbial communities from different black smoker chimneys on the Mid-Atlantic Ridge. *Extremophiles* **12**: 627-640.

Wagner, M., Roger, A.J., Flax, J.L., Brusseau, G.A., and Stahl, D.A. (1998) Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. *Journal of Bacteriology* **180**: 2975-2982.

Weisburg, W., Barns, S., Pelletier, D., and Lane, D. (1991) 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**: 697-703.

Wenzhofer, F., and Glud, R. (2002) Benthic carbon mineralization in the Atlantic: a synthesis based on in situ data from the last decade. *Deep-Sea Research Part I: Oceanographic Research Papers* **49**: 1255-1279.

Woese, C. (1987) Bacterial evolution. *Microbiological Reviews* **51**: 221-271.

Andrew J. Reed

Rutgers University
Email: DrewReed@aol.com

EDUCATION

Rutgers University Ph.D. (2008)

Dept. of Ecology and Evolution

Major Professor: R.A. Lutz

Dissertation Title: Molecular Analysis of Microbial 16S
rRNA, *mcrA*, *dsrAB* and *pmoA* Genes From Deep-Sea
Hydrothermal Vent and Cold Seep Sites.

Marine Biological Laboratory, Woods Hole Summer 2001

Microbial Diversity Course

University of Tennessee M.S. (1998)

Dept. of Ecology and Evolutionary Biology

Major Professor: J.M. Grebmeier

Thesis title: Benthic Macrofaunal Invertebrates of the East
Siberian and Chukchi Seas: Benthic Biomass, Community
Structure and Radionuclide Contamination.

University of Washington Summer 1996

Friday Harbor Marine Laboratory

Invertebrate Zoology and Behavior Course

University of Tennessee B.S. (1995)

Major: Organismal and Systems Biology

Minor: Zoology

POSITIONS HELD

Research Assistant (current)

Rutgers University, Center for Deep-Sea Ecology and Biotechnology

Dr. Richard A. Lutz, Major Professor

Partner, Reed Engineering (current)

Graduate Student/Research Assistant (1995 to 1998)

University of Tennessee

Dr. Jacqueline Grebmeier, Major Professor

Research Assistant

Oak Ridge National Laboratory (1995 to 1998)

Dr. Lee Cooper, Supervisor

Undergraduate Research Assistant

University of Tennessee (1992-1995)

Dr. Jacqueline Grebmeier, Supervisor

Undergraduate Research Assistant

University of Tennessee (Spring 1992)

Dr. Walker O. Smith, Supervisor

Resident Assistant

Upward Bound Program, University of Tennessee (Summer 1992)

Oceanographic Cruises:

April 2004, R/V Atlantis and DSRV Alvin, East Pacific Rise, Pacific Ocean

April 2003, R/V Atlantis and DSRV Alvin, East Pacific Rise, Pacific Ocean

August 2001, R/V Atlantis and DSRV Alvin, Mid-Atlantic Ridge, Atlantic Ocean

April 2000, R/V Atlantis and DSRV Alvin, East Pacific Rise, Pacific Ocean

November 1999, R/V Atlantis and DSRV Alvin, East Pacific Rise, Pacific Ocean

May 1999, R/V Atlantis and DSRV Alvin, East Pacific Rise, Pacific Ocean

December 1998, R/V Atlantis and DSRV Alvin, Southern East Pacific Rise, Pacific Ocean

August-September 1995, R/V Alpha Helix, East Siberian and Chukchi Seas

May-June 1994, R/V Alpha Helix, Bering Sea

August-September 1993, R/V Okean, Bering and Chukchi Seas

June 1993, R/V Alpha Helix, Bering and Beaufort Seas

Teaching

Oceanographic Scientific Inquiry: From Biogeochemistry to Genomes: Explorations at Aquatic Interfaces. Spring 2005.

Guest Lecturer, Deep-Sea Hydrothermal Vent Ecology. 2005, 2007.

Publications

Vetriani, C., Crespo-Medina, M., Lutz, R. A., Reed, A. J., Voordeckers, J.W. and Wong, R. (in preparation). Culture-dependant and independent analyses of bacterial assemblages along a temperature gradient at a deep-sea hydrothermal vent.

Reed, A.J., R.A. Lutz, and C. Vetriani. (in preparation). Analysis of *pmoA* Functional Genes from the Blake Ridge and Florida Escarpment Cold Seeps.

Reed, A.J., R.A. Lutz, and C. Vetriani. (in preparation). Phylogenetic Diversity of *dsrAB* and *mcrA* Functional Genes from Deep-Sea Hydrothermal Vent and Cold Seep Sites.

Allen, C.E. A.J. Reed, R.C. Vrijenhoek, R. Hey, J.E Lupton and R.A. Lutz. (in preparation). Deep-sea hydrothermal vent communities of the southern East Pacific Rise south of the Easter Island microplate at 31°09'S and 31°52'S.

Lutz, R.A., T.M. Shank, G.W. Luther, C. Vetriani, M. Tolstoy, D.B. Nuzzio, T.S. Moore, F. Waldhauser, M.Crespo-Medina, A.D. Chatziefthimiou, E.R. Annis, and A.J. Reed. 2008. Interrelationships between vent fluid chemistry, temperature, seismic activity, and biological community structure at a mussel-dominated, deep-sea hydrothermal vent along the East Pacific Rise. *Journal of Shellfish Research* 27: 177-190.

Bennett, K.F., A.J. Reed, and R.A. Lutz. (submitted). Brachidontes (Bivalvia: Mytilidae) from ecologically distinct intertidal habitats in the middle Florida Keys are cryptic species, not ecotypes.

Lutz, R.A., A.G. Collins, E.R. Annis, A.J. Reed, K.F. Bennett, K.M. Halanych and R.C. Vrijenhoek. 2006. Stauromedusan populations inhabiting deep sea hydrothermal vents along the southern East Pacific Rise. *Cahiers de Biologie Marine* 47: 409-413.

Reed, A.J., R.A. Lutz, and C. Vetriani. 2006. Vertical Distribution and Diversity of Bacteria and Archaea in Sulfide and Methane-Rich Cold Seep Sediments Located at the Base of the Florida Escarpment. *Extremophiles*. 10: 199-211.

Lutz, R.A., T. Shank, P. Rona, A. Reed, W. Lange, S. Low, E. Kristof. (2002). Recent advances in imaging deep-sea hydrothermal vents. *Cahiers de Biologie Marine*, 43: 267-269.

Cooper, L.W. J.M. Grebmeier, I.L. Larsen, S.S. Dolvin, A.J. Reed (1998). Inventories and distribution of radiocesium in Arctic marine sediments: Influence of biological and physical processes. *Chemistry and Ecology*. 15: 27-46.

Ph.D. Dissertation: Molecular Analysis of Microbial 16S rRNA, *mcrA*, *dsrAB* and *pmoA* Genes From Deep-Sea Hydrothermal Vent and Cold Seep Sites.

M.S. Thesis: Benthic Macrofaunal Invertebrates of the East Siberian and Chukchi Seas: Benthic Biomass, Community Structure and Radionuclide Contamination.