Characterization of the lucinid bivalve-bacteria symbiotic system: the significance of the geochemical habitat on bacterial symbiont diversity and phylogeny

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CHARACTERIZATION OF THE LUCINID BIVALVE-BACTERIA SYMBIOTIC SYSTEM: THE SIGNIFICANCE OF THE GEOCHEMICAL HABITAT ON BACTERIAL SYMBIONT DIVERSITY AND PHYLOGENY

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College
In partial fulfillment of the Requirements for the degree of Master of Science

in

The Department of Geology and Geophysics

by
Angela Marissa Green-Garcia
B.S., University of Houston, 2003
May 2008
DEDICATION

This thesis is dedicated to my father, Rudi, who in life and death, has been a driving force behind my pursuit of an education, and to my family, who has continuously supported me and encouraged me to achieve all of my goals. Thank you and I love you all.
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ABSTRACT

Extensive characterization of a single lucinid bivalve habitat was conducted to characterize the relationship between host bivalve and thiotrophic bacterial endosymbionts. For lucinids, the ecological and evolutionary relationships between hosts and endosymbionts are poorly understood. Reconstructing the evolutionary history of lucinid endosymbiosis, and the geologic significance of the association, has been hampered by insufficient knowledge of endosymbiont ecology and taxonomic diversity. Host organisms (*Lucinisca nassula* and *Phacoides pectinatus*) were collected from Cedar Keys, Florida, within the top 15-20 cm of the sediment in sea grass beds. PCR amplification and sequencing of bacterial 16S rRNA genes from lucinid gills and sediment cores retrieved ~900 sequences. Based on comparative phylogenetic methods, gill endosymbiont sequences were most closely related to uncultured *Gammaproteobacteria* associated with symbiosis, and specifically to lucinid endosymbionts (97-99% sequence similarity) and not to free-living organisms. Not all gill sequences were genetically identical, with intra- and inter-gill sequence diversity. Sediment diversity was high, represented by 13 major taxonomic groups, including equally dominant *Chloroflexi*, and *Delta- and Gammaproteobacteria*. Other organisms included the *Bacteroidetes*, *Acidobacteria*, *Spirochetes*, and *Firmicutes*. Rare (<0.3%) sequences from the sediment were related to lucinid gill endosymbionts. Results support the hypothesis that recruitment of free-living organisms is likely. Based on habitat geochemistry, however, the bacteria are constrained to reducing conditions, and this may be reflected in the habitat types colonized by the host. Habitat-host-symbiont diversity was evaluated from other locations from Florida and The Bahamas. 16S rRNA gene sequences retrieved from those hosts revealed that not all lucinid endosymbionts belong to the *Gammaproteobacteria*, because some sequences were most closely related to
*Alphaproteobacteria*. One sequence was most closely related to *Methylobacterium* spp., which may indicate that dual symbiosis (thiotrophy and methanotrophy) in lucinid bivalves may be possible. Together, these results are significant to paleoecological and evolutionary studies using lucinids in the fossil record (e.g. isotope studies).
INTRODUCTION

Symbiosis is considered to be one of the most important driving factors for evolution (Gil et al., 2004; Stewart et al., 2005). Symbiosis is a strictly inter-species association (Savazzi et al., 2001), and symbiotic associations between bacteria and eukaryotes are wide-spread, from mammalian or insect digestive systems to trophosome tissues in tubeworms (e.g., Fullarton et al., 1995; Gil et al., 2004; Goffredi et al., 2004). The exact functional and evolutionary nature of many symbiotic relationships (e.g., species dependency, host specificity, toxicity effects within the environment, etc.) remains unclear (Taylor and Glover, 1997; Distel, 1998). Symbiosis among bacteria and eukaryotes may have allowed eukaryotes an evolutionary strategy to overcome limited metabolic capabilities in extreme habitats (Gil et al., 2004). Therefore, it is not surprising that symbiotic bacteria-eukaryote associations have been widely studied, especially these associations relying on chemosymbiosis (e.g., Wiley and Felbeck, 1995; Gros et al., 1996; Krueger et al., 1996; Distel, 1998; Savazzi, 2001; Gros et al., 2003a; 2003b; Imhoff et al., 2003; Duperron et al., 2005; Gil et al., 2004; Stewart et al., 2005; Suzuki et al., 2005; Duperron et al., 2006; Taylor and Glover, 2006; Caro et al., 2007; Duperron et al., 2007). In a chemosymbiotic relationship, bacteria live inside of a host and metabolize reduced compounds, such as hydrogen sulfide, and fix inorganic carbon to organic carbon that may or not be used by the host (Distel, 1998; Stewart et al., 2005). Symbiosis between invertebrates and chemolithoautotrophic bacteria was first discovered in the late 1970’s when productive ecosystems were found at the deep-sea hydrothermal vents, a place originally considered too extreme for life to thrive (Distel, 1998; Arndt et al., 2001; Goffredi et al., 2004).

One symbiotic association that has received attention recently is between members of the Lucinidae family of the Bivalvia and sulfur-oxidizing (thiotrophic) endosymbiotic bacteria (Dando and Southward, 1986; CoBabe, 1991a; 1991b; Distel, 1998; Barnes and Hickman, 1999;
Chemosymbiosis in the Lucinidae Bivalvia was first reported in the 1980’s (Taylor and Glover, 2006). In this thesis, I continue to investigate this association by examining the genetic diversity and ecology of a variety of lucinid hosts and their bacterial endosymbionts from spatially separated locations, as well as the bacterial diversity of the host habitat (marine siliciclastic sediments) to understand symbiont ecology. The results from this study provide unique genetic evidence for bacterial endosymbiont diversity, as well as indicate that geography may play a role in the diversity of the bacteria-lucinid association.

**Background**

Chemosymbiosis is an ancient evolutionary mechanism found in some Bivalvia that has been suggested as a pervasive evolutionary mechanism to allowing this group to thrive for >400 million years (Watson and Pollack, 1999, Fortey, 2000; Bailey et al., 2006). Chemosymbiosis appears ubiquitous in the Lucinidae family. All lucinid species studied to date (>25) have thiotrophic bacteria that symbiotically colonize the host gills (Taylor and Glover, 1997); no other symbiotic association has been described for the lucinids (e.g., methanotrophy). Lucinid bivalves live in a wide variety of habitats including fjords, shallow marine sea grass beds, mangrove swamps, and deep-sea cold seeps and hydrothermal vent sites (e.g., Wiley and Felbeck, 1995; Gros et al., 1996; Imhoff et al., 2003; Duperron et al., 2005), being situated at or near the oxic-anoxic interface.

The antiquity of lucinid symbioses, widespread geographic distribution of the lucinids, habitat variety, and the fact that symbiosis has been found in all lucinid species make the symbiotic relationship an intriguing system for study. As such, physiological, biochemical, ecological, morphological, isotopic, enzymatic, microscopic, molecular and phylogenetic studies have been conducted for more than two decades on these bivalves and their endosymbionts (Wiley and Felbeck, 1994; Gros et al., 1996; Imhoff et al., 2003; Duperron et al., 2005; Stewart
et al., 2005; Suzuki et al., 2005; Duperron et al., 2006; Taylor and Glover, 2006; Caro et al., 2007; Duperron et al., 2007).

**Morphology and Antiquity**

Lucinids are eulamellibranch bivalve mollusks, having a heterodont shell hinge, leaf-like mantle gills, well-developed siphons, reduced guts, and large thick, fleshy, colored (yellow, black, grey) gills colonized by the endosymbionts (Allen, 1960; Distel and Felbeck, 1987; Wiley and Felbeck, 1995). The thiotrophic endosymbionts are housed in specialized cells called bacteriocytes (Duperron et al., 2005; Caro et al., 2007). Modern Lucinidae shells are aragonitic (Taylor et al., 1969, Falini et al., 1996), having discoidal to subpherical shapes, and ranging in size from a few millimeters to >100 mm (Taylor and Layman, 1972; Taylor and Glover, 2007). Lucinids exhibit cell types which are indicative of their symbiotic nature, including bacteriocytes, membrane whorls, but also intercalary cells (Distel, 1998; Savazzi, 2001). The shells contain lipids and stable isotope ratios that have been identified to indicate dietary preference, growth rate, sexual maturity, and ecological and environmental conditions (e.g., temperature and seawater chemistry) (Jones et al., 1988; CoBabe and Pratt, 1995; Langlet et al., 2002). In addition, internal features of their valves contain scars representing features linked to symbiosis (e.g., detached pallial blood vessel) (Taylor and Glover, 2006).

The earliest member of the Lucinidae, *Iliona prisca*, dates back to the Silurian (Taylor and Glover, 2006). Several other Silurian chemosymbiotic bivalve species (thyasirid, mytilid, and solemyid) are from habitats interpreted to be cold-seep sediments (Distel, 1998). Because all modern symbiont-bearing lucinids share similar morphological characteristics and living positions with *I. prisca*, this species is inferred to have also been a host for thiotrophic bacteria (Taylor and Glover, 2006). Although only well-preserved shells, whose matrix was intact
(Distel, 1998), have been studied, $\delta^{13}C$ isotope analyses of the protein matrix from the fossilized shells suggest chemosymbiosis did exist in ancient bivalves (CoBabe, 1991a; 1991b).

**Host-Endosymbiont Relationship**

**Relationship to Environment**

Host bivalves orient themselves in their habitat to ensure optimal substrate attainment for the endosymbionts, and to minimize the amount of sulfate lost to abiotic oxidation (Stewart et al., 2005). Consequently, lucinids predominantly occupy the oxic-anoxic interface allowing them to burrow into anoxic sediments so that the release of hydrogen sulfide from deeper sediment layers is enhanced (Felbeck et al., 1983; Savazzi, 2001). The hydrogen sulfide in the lucinid habitats is produced by free-living sulfate-reducing bacteria (Stewart et al., 2005).

Because lucinid endosymbionts are metabolically dependent on both sulfide content and dissolved oxygen concentrations, Arndt et al. (2001) show that an increase in the level of sulfide under periods of anoxia was due to a decrease in endosymbiotic metabolism. The movement and water pumping of the hosts within the habitat are more important to maintaining slightly oxygenated conditions so that the endosymbionts can be metabolically active. Heme proteins are associated with the gill cytoplasm in symbiotic bivalves (Frenkiel et al., 1995; Kraus, 1995). The purpose of these proteins is not fully understood, but they are inferred to bind oxygen to prevent spontaneous oxidation of sulfide (Frenkiel et al., 1995; Kraus, 1995). Sulfide oxidation by endosymbionts is vital to geochemical sulfur cycling within marine environments (Jorgenson and Bak, 1991; Craddock et al. 1995; Arndt et al., 2001). Thioautotrophic endosymbionts oxidize the sulfide for energy and fix CO$_2$ into organic carbon compounds that can be used by the host (Krueger et al., 1996; Stewart et al., 2005). Organic nutrients are thought to be transmitted to the host via translocation, or by direct ingestion of the endosymbionts (Stewart et al., 2005); fatty acid profiles of the host that mimic those of the endosymbionts suggests that
latter (Pond, 1998; Suzuki et al., 2005), although previous carbon isotope studies could not conclusively show this (CoBabe, 1991a; 1995).

**Endosymbiont Acquisition**

The acquisition of the bacterial endosymbionts by the bivalve hosts has been intensely studied (Gros et al., 1996; Distel, 1998; Peek et al., 1998; Fortey, 2000; Savazzi, 2001; Gros et al., 2003a). Two mechanisms have been explained by Distel (1998) for the transmission of bacteria to host. The first is reproductive, from parent to offspring during gametogenesis (Distel, 1998). The second mechanism is through environmental acquisition; this type of acquisition indicates that the endosymbionts have the ability to live independent of their host (Distel, 1998). For lucinids, previous research determined that the bacterial endosymbionts were free-living in the host habitat (Gros et al., 1996; Peek et al., 1998; Fortey, 2000; Savazzi, 2001; Gros et al., 2003a; Gros et al., 2003b; Caro et al., 2007), confounding the understanding of the degree to which the host and endosymbionts depend on one another. Specifically, Gros et al. (1996) found the environmental form of transmission for the host species, *Codakia orbicularis*, as the symbionts were only found within the host after the larval growth stage. This starkly contrasts what is known about other bacterial symbionts for other marine bivalves and with vertical transmission where no free-living bacterial symbionts have been identified to date (Imhoff et al., 2003).

**Endosymbiont Identity**

The thiotrophic endosymbionts of Lucinidae belong to a monophyletic group of class within the *Proteobacteria* phylum, the *Gammaproteobacteria* (Distel, 1998; Gros et al., Gros et al., 2003b). Distel et al. (1988) suggest that genetically identical thiotrophic species are found in multiple lucinid hosts, regardless of geographic location. However, these results are questionable because hosts and symbionts from the same locale were not examined (e.g., Gros et
al., 1996; Gros et al. 2003a; 2003b), and much of the species-level research for the bacteria had been done with a limited number (8) of partial 16S rRNA gene sequences acquired from different hosts (Distel et al., 1988; Distel et al, 1993; Durand et al., 1996; Distel et al, 1998). Hence, endosymbiont genetic diversity has recently been questioned. Duperron et al. (2007) identified multiple phylotypes of bacterial endosymbionts from the gills of the cold-seep clam, Lucinoma aff. kazani. The dominant 16S rRNA gene phylotypes belonged to the Gammaproteobacteria, and were most closely related to an endosymbiont sequence from ‘Parvilucina’ costata, a shallow tropical lucinid. A second phylotype was most closely related to Spirochetes coccoides, an autotrophic spirochete found in the hindgut of a termite. This work was the first to suggest that lucinid endosymbionts may be genetically diverse.

Moreover, endosymbiont diversity was studied from the nucleic acid content of cells separated in a flow cytometry study of Codakia orbicularis (Caro et al., 2007). Caro et al. (2007) propose that the host controls the entry and distribution of bacteria into and within the bacteriocytes. They also determined that bacteria cell maturation, based on size and sulfur content, differed from the apical to basal tip of the cells within the bacteriocytes. This work suggests that up to seven genetically distinct subpopulations of varying size and intracellular nucleic acid content can exist within individual host specimens.

Objectives and Hypotheses

Symbiosis in the lucinid bivalves is one of the oldest identified symbiotic relationships currently known. Unfortunately, given the recent research on endosymbiont diversity, the degree of dependence between the endosymbiont and hosts is not well understood (Gros et al., 1996. Gros et al.2003b; Duplessis et al., 2004). For example, characterizing the diversity and distribution of free-living bacteria within a lucinid habitat will lead to a better understanding of the degree of dependence between the host and bacterial species. Due to the high number of
lucinid species worldwide, and because at least some endosymbionts can exist outside of the host (Gros et al., 1996; Gros et al., 2002a), it is possible that previous work overlooked host endosymbiont diversity and specificity of bacteria to specific hosts. Therefore, a detailed evaluation of the diversity and ecology of both endosymbionts and hosts, from one location, is needed before the evolutionary significance, and possible cophylogeny, of the thiotrophic symbionts and the bivalve species can be explored.

For my thesis, I had three main goals:

1) Define lucinid and bacteria habitat solid- and aqueous-phase geochemistry;
2) Characterize the bacterial diversity of the sediments where host organisms are found, as well as from the gills of host taxa collected at one site;
3) Compare sediment and endosymbiont bacterial diversity to bacterial endosymbiont diversity from different lucinid species collected from geographically separate locations.

For this work, I choose a siliciclastic sediment site off the coast of Florida, at Cedar Key, Gulf of Mexico. Other comparative sites where lucinids were collected included Lemon Bay, Florida, Jack Island, Florida, and San Salvador, The Bahamas.

The three hypotheses that I tested in the study were:

1) Hypothesis 1: Free-living symbiont diversity will be unique to specific sediment and water geochemical conditions.
2) Hypothesis 2: Bacterial endosymbionts from lucinids at Cedar Key, Florida, will be closely related to free-living bacteria from the same habitat.
3) Hypothesis 3: Bacterial endosymbionts from different species of lucinid hosts collected at geographically separated locations will be genetically distinct from one another.
Importance of Research

Results from this research will lead to a more thorough understanding of the evolutionary and ecological relationship between lucinid bivalves and their thiotrophic symbionts. As this research is the first to characterize the bacterial diversity in the host habitat sediment, nearly exhaustive characterization of a single site will provide a much needed baseline for future research that should study different lucinid geochemical and geological habitats. This research will also provide a background for modern day symbiotic relationships that can be applied to ancient symbiotic relationships.
MATERIALS AND METHODS

Sample Collection

Sediment, water, and lucinid samples were collected from Cedar Key, Florida (29° 7.457’ N; 803° 18.483’ W), located on the Gulf of Mexico side of the state (Figure 1). Some outer islands and sand bars are just off of the coast at Cedar Key. This shallow water habitat supports dense seagrass beds that are biologically active, especially with lucinid species (personal communication, Harry G. Lee, Jacksonville Shell Club), and the targeted lucinid species were expected to be well represented within the first 10 cm of the seagrass beds. Species previously retrieved from the area include *Lucinisca nassula*, *Phacoides pectinatus*, and *Stewartia floridana* (http://www.jaxshells.org/cedarkey.html).

Ten total individual lucinid specimens, including *Lucinisca nassula* and *Phacoides pectinatus*, were collected from *Thalassia* seagrass beds off of Atsena Otie Key near Cedar Key during a three day sampling period in June, 2006. Two sediment cores that were ~30 cm in length and ~4 cm in diameter were collected in PVC tubes. The cores were collected from within 1 m of the lucinid collection site, and were separated from each other by 1m. Cores were stored at -20 °C until they were aseptically cut into 3 depth intervals each,~10 cm thick.

Filtered and raw pore fluids and ocean water were collected for geochemical analysis. Pore fluids were sampled from the seagrass bed sediments at a depth of ~30 cm by low-flow pumping through a mini-piezometer. Physiochemical parameters were determined on site immediately using standard electrode methods (American Public Health Association [APHA], 1998), including temperature and pH on an Accumet AP62 meter with a double junction electrode (Accumet, Fisher Scientific, USA), total dissolved solids (TDS) and temperature on a YSI-85 meter (YSI, Inc., Yellow Springs, OH, USA), and other parameters (e.g., oxidation-reduction potential on an Ultrameter).
Dissolved sulfide and trace-level dissolved oxygen (DO) was measured using the Methylene Blue and Rhodazine D CHEMetrics (Calverton, VA) colorimetric methods, respectively, on a portable V-2000 multi-analyte photometer (APHA, 1998). Anions were measured by ion chromatography (IC) from 0.2 μm-filtered samples on an ICS-90 (Dionex, USA). Cations were measured from an acid-preserved 0.2μm-filtered sample by inductively coupled plasma mass spectrometry (ICP-MS) at the University of Texas at Austin (EPA method 9056; Manual SW-846, Test Methods for Evaluating Solid Waste, Physical/Chemical Methods; http://www.epa.gov/epaoswer/hazwaste/test/main.html).

Additional lucinid specimens, sampled by Dr. L. Anderson and Dr. A. Aronowsky for another project, were used as comparison species in this study (Figure 1). Three *Lucinisca nassula* host organisms were collected from Lemon Bay (26 º 55.952’ N; 82 º 21.254’ W), located on the Gulf of Mexico side of Florida, ~163 km from Cedar Key. One of these specimens was collected from nearshore sediments under *Syringodium* sea grass. The other two specimens were collected in an intertidal flat of *Thalassia* grass that was separated from the shore by a muddy channel. Several *Phacoides pectinatus* host specimens were collected from the Atlantic side of Florida (Figure 1), including one host from Jack Island (27 º 30.095’ N; 80 º 18.500’ W) in *Ruppia maritima* sea grass beds and macroalgae, and another specimen from *Thalassia* sea grass beds at the Mouth of Pigeon Creek, San Salvador, The Bahamas (23 º 57.810’ N 74 º 29.331’ W).

Taxonomic designations at the genus level in this thesis are after Taylor and Glover (2000).

**Sediment Mineralogy by X-Ray Diffraction (XRD)**

Two grams of sediment from each core interval were homogenized by mixing with a Micronizing Mill (McCron, USA) equipped with corundum micronizers. Six total sediment
samples were processed. Samples were homogenized for 3 min with 10 mL of 100% ethanol, and then centrifuged for 30 minutes to separate solid and suspended particle phases.

Figure 1: Maps of sample collections sites. Cedar Key and Lemon Bay, Florida, located on the Gulf of Mexico side, Jack Island on the Atlantic side of Florida, and San Salvador, located on the Atlantic side of The Bahamas. Maps were acquired and modified from www.awesomeflorida.com and www.fishforfun.com.
The supernatant was discarded and the remaining sediment slurries were dried overnight at 60 °C. Each dried sample was ground to a fine powder using a mortar and pestle. Samples were analyzed by a BRÜKER Siemens D-5000 XRD using the operating setting of 40 kV and 30 milliamps. The XRD diffractograms of 27 of the most common sedimentary minerals were compared for identification. XRD pattern analysis were processed using JADE pattern processing software version 6.1 (MDI, USA). Minerals were identified based on diffraction peak position; peak identification was checked by hand.

**Gill Acquisition and DNA Extraction**

Ten lucinid bivalve hosts were aseptically dissected to obtain their gills using a Heerburg WILD M5A steereozoom microscope set at a magnification of 500x. One gill and one foot were removed from each specimen. The second gill was frozen as reserve tissue. For each lucinid host sample an entire gill, measuring between 5 and 10 mm in length (depending on individual) and half of each foot sample were used.

DNA was extracted from each tissue sample using the Qiagen DNeasy extraction kit, following manufacturer’s instructions. Briefly, each tissue sample was placed in 180 µL of tissue lysis buffer and 20µL of concentrated proteinase K solution. The tissue was incubated at 55 °C overnight on a shaker table at 225 rpm. In order to completely digest the tissue, 200 µl of AL buffer was added and samples were incubated at 70 °C in a water bath for 10 minutes. Two hundred µl of 100% ethanol was added to each digest and the mixture was pipetted into the DNeasy mini-spin column. DNA was eluted from the columns, following several steps of centrifugation and the addition of DNeasy solutions. The quality and quantity of the DNA was checked by running each extraction on a 1% TBE gel with ethidium bromide staining and using a Nano Drop ND-1000 spectrophotometer. The amount of nucleic acids per sample ranged from 30 to 100 ng/µl. Resulting DNA was stored at -20°C in TE.
Sediment DNA Extraction

Each of the sea grass sediment cores from Cedar Key were divided into three depth intervals (0-10 cm, 10-20 cm, and 20-30 cm). Approximately 3 g of sediment were aseptically removed from the center of the core for each sample. Two samples were used from each depth interval. The sediment was suspended into 5 ml sterile cell lysis buffer (10 mM Tris and 100 mM EDTA, pH 8.0) and shaken on a hand-shaker for 2 hr to disaggregate the material and wash cells from sediment surface. After shaking, 20µl of proteinase K were added to the sediments and the samples continued to shake at room temperature for 2 hr. Large particles in the suspensions were allowed to settle for 10 minutes, then ~3 mL of cloudy supernatant was taken and nucleic acids were extracted based on methods previously described by Engel et al. (2004) and modified from the DNeasy extraction kit (Qiagen). Modifications included adding 5 mL of fresh extraction buffer and 40 µl of additional proteinase K to the supernatant; samples were shaken at 225 rpm overnight at 55 °C; 750 µl of the sediment solutions were separated into smaller tubes and 300µl of a protein precipitation solution (7.5 M NH₄-acetate) was added; the mixture was incubated on ice for 15 min and centrifuged; the supernatant was transferred to 95% isopropanol and incubated on ice for 30 min to precipitate the nucleic acids. The pellet was cleaned with 80% ethanol and eluted in 50µl of TE (10 mM Tris/ 0.5 mM EDTA) buffer, then stored at -20°C until use. The quality and quantity of the nucleic acids was checked by running on a 1% TBE gel with ethidium bromide staining and using a NanoDrop ND-1000 spectrophotometer. The amount of nucleic acids per sample ranged from 100 to 1500 ng/µl.

Polymerase Chain Reaction (PCR) Amplification, Cloning, and Sequencing

Nearly full length 16S rRNA gene sequences from gills, core sediments, and foot tissues were PCR amplified using the bacterial universal primers 8F (5’-AGAGTTTG ATCCTGGCTC-AG-3’) and 1510R (5’-GGTTACCTTGTTACGACTT-3’) (Ausbel et al., 1990).
Amplification was performed with a MJ Research Dyad Disciple thermal cycler (Biorad, USA) with 5 U/µl ABGene Taq DNA polymerase (ABGene, Thermo Fisher Scientific, USA), 10 mg/ml bovine serum albumin (BSA, Fisher Scientific), 10 mM dNTPs, and 10 mM MgCl₂. Optimal PCR conditions included an initial hot start at 94°C for 4 min; denaturation at 94°C for 1 min, primer annealing at 47°C for 1 min, chain extension at 72 °C for 3 min, this process was repeated for 29 more cycles; and a final extension step of 20 min at 72 °C. Amplified PCR products of the correct size (~1500 bp) from each sample were purified by using a 0.7% TAE low-melt agarose gel with a Wizard PCR prep DNA purification kit (Promega Corp., USA), following manufacturer’s recommendations. Concentrations of PCR products and purity were determined by spectrophotometry (NanoDrop).

Purified PCR products were cloned using TOPO Cloning Kit with the PCR2.1-TOPO® vector, according to manufacturer’s instructions (Invitrogen Corp., USA). Optimal ligation reactions were achieved by overnight incubation at 14 °C. Resulting clones were screened by PCR with the M13 forward (5’- GTAAAACGACGGCCAG-3’) and M13 reverse (5’- CAGGAAACAGCTATGAC -3’) vector primers (Invitrogen Corp., USA). Clones were checked for the correct size insert using 1% TBE gels with ethidium bromide staining.

M13 amplified clone sequences that were the correct length were diluted to between 80-100 ng/µl and sequenced in both directions by capillary sequencing at the High-Throughput Sequencing Solutions facility at the High-Throughput Genomics Unit (HTGU), Department of Genome Science (http://www.htseq.org), University of Washington, Seattle, Washington.

**Sequence Analysis**

Resulting sequences were assembled using Contig Express, a component of Vector NTI Advance 10.3.0 (Invitrogen Corp., USA) (http://www.invitrogen.com/). Sequences were
subjected to BLAST Searches using GenBank (http://www.ncbi.nlm.nih.gov/) to determine sequence similarities to cultured and not yet cultured organisms.

Nucleotide sequences were aligned using NAST (Nearest Alignment Space Termination) in greengenes (DeSantis et al., 2006), and adjusted manually in BioEdit 7.0.4.1 (Hall, 1999). A neighbor joining phylogeny with branch support from 500 bootstrap replicates was constructed with the program MEGA (ver. 3.1) (Kumar et al., 2004) using the Jukes-Cantor model based on concepts presented in Tamura et al. (2004). Distance matrix files were assembled from nucleotide sequences in Phylip3.67 (Felsenstein, 2005) using the Jukes-Cantor model for nucleotide substitution. Operational taxonomic units (OTUs) were defined and community comparisons were made using DOTUR (Distance Based Operational Taxonomic Units and Richness Determination) (Schloss and Handelsman, 2005). OTUs (phylotypes) for the sequences were determined based on nearest neighbor distance to 99% sequence identity. A sequence identity of 99% was used to conservatively assign species level affinities.

To determine the coverage of the clone library, rarefaction curves were generated using the approximation algorithm aRarefactWin (Analytic Rarefaction version 1.3, S. Holland; http://www.uga.edu/strata/software/Software.html).

Accession numbers for 848 representative gene sequences (full and partial sequences) can be found in GenBank (http://www.ncbi.nlm.nih.gov/) from EU487786 to EU488633.
RESULTS

Aqueous Geochemical Analysis

Physicochemical conditions evaluated in the field for the ocean water and sediment pore fluids included temperature, pH, oxidation reduction potential (ORP), total dissolved solids (TDS), dissolved oxygen concentration, and dissolved sulfide concentration (Table 1). The sediment pore fluids were more reducing, with lower DO and measurable sulfide. Cation and anion concentrations also were measured for the pore water and the ocean water (Table 2). All species were more concentrated in the pore water, except Na⁺ that was more concentrated in seawater. The pore fluids had higher sulfate concentrations than seawater.

| Table 1: Field geochemical data from Cedar Key, Florida. |
|-----------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Geochemical Parameters                      | Ocean Water     | Core Fluids     |
|-----------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Temperature (°C)                              | 29.6            | 30.1            |
| pH                                            | 8.07            | 7.65            |
| Oxidation Reduction Potential (mV)            | +67             | -227            |
| Total Dissolved Solids (ppt)                  | 44.97           | 45.01           |
| Dissolved Oxygen (mg/L)                       | 7.60            | 2.03            |
| Dissolved Sulfide (mg/L)                      | 0               | 1.57            |

Core Mineral Analysis

Cores were collected from the lucinid site, and were separated from each other by 1m. The core sediments consisted predominantly of sand-sized grains. Bulk XRD analysis of the sediments from both cores indicated the dominance of quartz, with rarer undifferentiated clays, pyrite, calcite, and silicate minerals (Table 3). The two cores had slight mineralogical variations with depth. For example, core 2 had more pyrite at the 0-10 cm interval compared to Core 1, which had more calcite.
Table 2: Cation concentrations (ppm) and anion concentrations (ppm) of ocean and pore water at Cedar Key, Florida. Chloride concentrations were within seawater concentrations, but were removed from the waters prior to anion analysis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Description</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
<th>Sr²⁺</th>
<th>Fe</th>
<th>Mn</th>
<th>Al</th>
<th>Si</th>
<th>SO₄²⁻</th>
<th>NO₃⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDK-06-001</td>
<td>Ocean water</td>
<td>9086.9</td>
<td>303.7</td>
<td>279.3</td>
<td>669.3</td>
<td>4.2</td>
<td>0.005</td>
<td>0.006</td>
<td>0.005</td>
<td>0.6</td>
<td>3493.4</td>
<td>11.0</td>
</tr>
<tr>
<td>CDK-06-002</td>
<td>Pore Fluids</td>
<td>8763.7</td>
<td>336.4</td>
<td>544.6</td>
<td>1215.2</td>
<td>9.4</td>
<td>0.5</td>
<td>0.14</td>
<td>0.2</td>
<td>6.1</td>
<td>3635.2</td>
<td>9.2</td>
</tr>
</tbody>
</table>

Table 3: Percentage abundances of minerals in core sediments, Cedar Key, Florida

<table>
<thead>
<tr>
<th>Site</th>
<th>Depth (cm)</th>
<th>Quartz</th>
<th>Clay</th>
<th>Calcite</th>
<th>Pyrite</th>
<th>Plagioclase</th>
<th>Dolomite</th>
<th>K-feldspar</th>
<th>Anhydrite</th>
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<tbody>
<tr>
<td>Core #1</td>
<td>0-10.5</td>
<td>95.4</td>
<td>2.33</td>
<td>0.61</td>
<td>0.55</td>
<td>0.18</td>
<td>0.6</td>
<td>0.6</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>10.5-21</td>
<td>96</td>
<td>1.84</td>
<td>0.71</td>
<td>0.62</td>
<td>0.13</td>
<td>0.086</td>
<td>0.56</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>21-31.5</td>
<td>95.4</td>
<td>1.2</td>
<td>1.03</td>
<td>0.91</td>
<td>0.12</td>
<td>0.52</td>
<td>0.64</td>
<td>0.12</td>
</tr>
<tr>
<td>Core #2</td>
<td>0-10.5</td>
<td>95.5</td>
<td>1.44</td>
<td>0.67</td>
<td>0.8</td>
<td>0.12</td>
<td>0.98</td>
<td>0.4</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>10.5-21</td>
<td>94.5</td>
<td>2.27</td>
<td>0.09</td>
<td>1.6</td>
<td>0.19</td>
<td>0.13</td>
<td>0.97</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>21-31.5</td>
<td>95.1</td>
<td>2.34</td>
<td>0.09</td>
<td>1.44</td>
<td>0.24</td>
<td>0.08</td>
<td>0.59</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Gill Endosymbiont Diversity

The 16S rRNA gene sequences of bacterial populations from lucinid gills were evaluated at three sampling levels to test my research hypotheses. First, a single individual, as well as multiple lucinid individuals of the same species, were evaluated from one site (Cedar Key) to assess intra-species diversity. Second, lucinids from different species at the same location were evaluated to assess inter-species diversity. Third, various lucinid taxa from multiple geographic areas were compared to assess if endosymbiotic bacterial species varied by geography (Cedar Key, Lemon Bay, and Jack Island, Florida, and San Salvador, The Bahamas) (Figure 1). To investigate if the bacterial endosymbionts were also free-living (e.g., Gros et al., 1996; Distel, 1998), the 16S rRNA gene sequence diversity of sediments from the lucinid habitat were examined from Cedar Key, Florida. Sediment mineralogy and fluid geochemistry were also compared from each sediment sample to understand possible ecological relationships to bacterial community structure and metabolic activities. No amplified bacterial 16S rRNA gene sequences were retrieved from lucinid foot samples.

DOTUR analysis of all full-length 16S rRNA endosymbiont sequences from all hosts (214 total ) grouped in 169 phylotypes at 99% relatedness. At 97% relatedness, all sequences grouped into 12 OTUs. According to Stackebrandt and Goebel (1994), 95% sequence identity could roughly correspond to genus-level relationships; 97% sequence identity could correspond to species-level relationships. Assigning OTUs based on 99% sequence identity was done to be conservative of species-level relationships. The DOTUR results imply that there is greater diversity among the lucinid endosymbionts than previously known (Distel et al., 1998), and that there are possibly more than one species of bacteria capable of being a lucinid endosymbiont.
Rarefaction curves estimated if the bacterial diversity retrieved in the clone libraries was representative of the full diversity for the samples. Results from all of the gill sequences show that the coverage of the clone libraries was nearing saturation with respect to the number of phylotypes defined at 99% sequence identity after screening >200 clones from 9 individuals (Figure 2, blue curve). If sequence diversity was low, then a curve would be a nearly horizontal line, as additional clones would represent previously sampled phylotypes. Alternatively, the somewhat steep curves for the separate Cedar Key libraries (Figure 2, pink curve) and the gill clone libraries from the other sites from all sequences (including non-gammaproteoabcterial sequences; Figure 2, orange curve) indicate that phylotype diversity for the gill endosymbionts is much higher than previously considered. These curves indicate that diversity was not sampled completely. More clones may need to be screened in order to achieve full coverage of phylotype diversity.

A total of 65 gill endosymbiont 16S rRNA gene sequences, retrieved from three lucinid *L.nassula* host individuals collected at Cedar Key. All of the bacteria belonged to the *Gammaproteobacteria* (*γ-Proteobacteria*) (Figure 3). Symbiont sequences were distantly related to previously published symbiont sequences from other lucinid hosts (Distel et al., 1988; 1993, Durand and Gros, 1996; Arkawa et al., 2006), including *Codakia orbicularis*, *L. nassula*, Stewartia *floridana*, and ‘Parvilucina’ *costata* (95-99% sequence identity). DOTUR results indicated that all 65 endosymbiont sequences grouped into one phylotype at 95% similarity; at 99%, there were 19 phylotypes.

All of the endosymbiont sequences from Lemon Bay (LB) were closely related to endosymbiont sequences previously retrieved from *C. orbicularis* at 99% sequence similarity (93% sequence coverage) (Distel et al., 1988) (Figure 4). Mean sequence distances calculated for the LB sequences using MEGA showed that endosymbiont sequences were genetically identical (Table 4).
Forty-one full length 16S rRNA sequences were retrieved from the host *P. pectinatus* from the Mouth of Pigeon Creek (MPC). Of the sequences, 39 grouped into a clade closely related (99% sequence identity, 93% coverage) to gammaproteobacterial endosymbiont sequences previously retrieved from *P. pectinatus* [AJ581863] (Williams et al., 2004) (Figure 4). One sequence (MPC2-46) was closely related to the *α-Proteobacteria*, *Methylobacterium* sp. [AB220088] (99% sequence identity, 93% coverage) (Figure 5). The remaining sequence (MPC2-26) was closely related to the *Bacteroidetes*, *Muricea elongota* [DQ917900] (Figure 5) and (84%). Mean distances were calculated for all of the *γ-Proteobacteria* sequences using MEGA the MPC endosymbionts were 98% related (Table 4).

Fourteen 16S rRNA gene endosymbiont sequences retrieved from the host bivalve species *P. pectinatus* collected at Jack Island (JI), had the most distance of the lucinid individuals sampled. BLAST indicated that half of the bacterial sequences belonged to the *α-Proteobacteria* class.
Figure 3: Phylogenetic relationship based on Jukes-Cantor neighbor-joining analysis of 16S rRNA gene sequences of endosymbionts sequences retrieved from lucinid gills from Cedar Key and 16S rRNA sequences from sediments, representing the free-living habitat. *Beggiatoa alba* was used as an outgroup. Bootstrap percentages were obtained using 500 replicates and values >50% are indicated. Sequences labeled “G” are gill sequences and “C” are sequences retrieved from the cores. Modified published lucinid genera names to follow Taylor and Glover (2000).
(Figure 5). Five of the remaining sequences were identified as \(\gamma\)-Proteobacteria, and two sequences were affiliated with Spirochetes. Distances calculated for only the \(\gamma\)-Proteobacteria sequences using MEGA however, showed that the JI endosymbionts had 99% similarity within group (Table 4).

Endosymbiont 16S rRNA sequences from host bivalves collected on the Gulf of Mexico side of Florida (LB and CK) formed a separate clade from the endosymbiont sequences collected on the Atlantic side of Florida (JI and MPC), with strong bootstrap support (Figure 4). Mean distances calculated between the sequence groups using MEGA indicated that LB endosymbiont sequences shared 97% sequence similarity with sequences from CK and only 94% sequence similarity with endosymbiont sequences from the MPC and JI (Table 5). Endosymbiont sequences from MPC were 98% similar to JI sequences and only 93% similar to CK sequences (Table 5).

<table>
<thead>
<tr>
<th>Table 4: Average genetic distance within host species for (\gamma)-proteobacterial endosymbiont 16S rRNA gene sequences.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Host Specimen Collection Site</strong></td>
</tr>
<tr>
<td>Lemon Bay, Gulf of Mexico Coast, Florida</td>
</tr>
<tr>
<td>Mouth of Pigeon Creek, The Bahamas</td>
</tr>
<tr>
<td>Jack Island, Atlantic, Florida</td>
</tr>
<tr>
<td>Cedar Key, Gulf of Mexico coast, Florida</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 5: Average genetic distance between (\gamma)-proteobacterial endosymbiont 16S rRNA gene sequences from different host taxa</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Site</strong></td>
</tr>
<tr>
<td>Lemon Bay, Florida</td>
</tr>
<tr>
<td>Mouth of Pigeon Creek, Bahamas</td>
</tr>
<tr>
<td>Jack Island, Florida</td>
</tr>
<tr>
<td>Cedar Key, Florida</td>
</tr>
</tbody>
</table>
Figure 4: Phylogenetic reconstruction based on Jukes-Cantor neighbor-joining analysis of gill 16S rRNA bacterial sequences belonging to previously identified γ-proteobacterial groups. Sequences retrieved from hosts were collected for Cedar Key (CK), Lemon Bay (LB), and Jack Island (JI), Florida, and the Mouth of Pigeon Creek (MPC), The Bahamas. *Beggiatoa alba* was used as an outgroup. Bootstrap percentages obtained using 500 replicates and values >50% are indicated. LB sequences not shown in the tree are represented by accession numbers EU488499-EU488592. Modified published lucinid genera names to follow Taylor and Glover (2000).
Representative gill sequences from 3 host bivalves, *Lucina nassula*. Lemon Bay, Gulf of Mexico coast of Florida

- **Cedar Key, Florida; *L. nassula***
  - Stewartia floridiana gill symbiont [AB238979]
  - 'Paryluca' costata gill symbiont [L25712]
  - Lucinisa nassula gill symbiont [X95229]
  - Lucinoma aequizonata gill symbiont [AY536232]
  - Codakia orbicularis gill symbiont [M99447]

- **Mouth of Pigeon Creek, Bahamas; *P. pectinatus***
  - Phacoides pectinatus symbiont [X84980]

- **Jack Island, Florida; *P. pectinatus***
  - Olavias algarvensis sulfate-reducing endosymbiont [AF328857]

0.02 Estimated 94 clone sequences
14 clone sequences
14 clone sequences
38 clone sequences
25 clone sequences
25 clone sequences
38 clone sequences
14 clone sequences

*Beggiatoua alba* [L40994]
Figure 5: Phylogenetic relationship based on Jukes-Cantor neighbor-joining analysis of 16S rRNA gene sequences of endosymbionts sequences retrieved from lucinid gills from Jack Island, Florida, and the Mouth of Pigeon Creek, San Salvador, Bahamas. *Beggiatoa alba* was used as an outgroup. Bootstrap percentages were obtained using 500 replicates and values >50% are indicated. Modified published lucinid genera names to follow Taylor and Glover (2000).
A total of 634 clones, comprising 13 major taxonomic groups, were retrieved from all of the core sediment samples at Cedar Key (Table 6). Sixty-one percent of the sequences were affiliated with the Proteobacteria division (Table 6). $\alpha$-Proteobacteria were the dominant class (43%) followed by $\delta$-Proteobacteria (11%), $\gamma$-Proteobacteria (3%), $\beta$-Proteobacteria (2%), $\varepsilon$-Proteobacteria (2%). Other major phyla represented were the Chloroflexi (17%), Acidobacterium (4.2%) Firmicutes (3.7%), Planctomycetes (3.7%), Candidate divisions (2.6%), Actinobacteria (2.3%), Bacteroidetes (2.0%), Spirochete (1%), Deferribacteres (1.1%), Nitrospirae (0.31%), and Verromicrobiun and Thermotogales (0.15%). Figure 6 shows the rarefaction curves for both of the core sediment sequence datasets; Core 1 had four clone libraries and Core 2 had five. The combined datasets represent 648 full length 16S rRNA gene sequences. Phylotypes (OTUs) were defined at 99% sequence identity. The steep curves for Core 1 show that the diversity of bacteria is higher than retrieved in Core 2 (having a less steep curve). The curves also show that diversity was not fully sampled, and future work should increase the number of sequences per sample in order to reach full coverage.

**Taxonomic Descriptions**

**Alphaproteobacteria**

Most of the 16S rRNA from the sediment sequences were affiliated with the $\alpha$-Proteobacteria (Table 6). The largest clade consisted of 209 sequences and was most closely related to *Rhizobium* spp. [DQ873663], free-living gram-negative soil bacteria that are usually associated with infection of legume roots and nitrogen fixation (Long, 1996). Free-living rhizobia subsist on dead organic matter and do not fix nitrogen (Burdass, 2002). Other $\alpha$-proteobacterial
sequences were closely related to sequences represented by the families *Hyphomonadaceae* and *Rhodobacteraceae*. These groups are typically found in marine environments and exhibit a wide variety of morphological, physiological, and biological features (Lee et al., 2005). Three sequences were related to *Hyphomonas polymorpha* [DSM2665]. Cultured hyphomicrobia are facultative methylotrophs.

Eight sequences formed a clade with *Rhodopseudomonas rhodobacensis* [AB087719], a nitrate-reducing, non-sulfur bacterium isolated from a eutrophic pond in Germany (Hougardy et al., 2000). These bacteria are rod-shaped cells that are capable of growth both photosynthetically under anaerobic conditions and non-photosynthetically under aerobic conditions using a variety of organic compounds as metabolic substrates (e.g., formate, acetate, pyruvate) (Hougardy et al., 2000).

**Deltaproteobacteria**

Eleven percent of the sediment sequences were affiliated with the δ–*Proteobacteria* (Table 6). All of these sequences clustered most closely with anaerobic sulfate-reducing, iron-reducing, or
syntrophic bacterial species. Eighteen sequences were related to *Syntrophus aciditrophicus* [NC_00759], a rod-shaped, gram-negative bacterium that anaerobically recycles organic substrates to carbon and methane (McInerney et al., 2007). Thirteen sequences were most closely related to an uncultured δ–Proteobacteria isolated from an intertidal mud flat in the Wadden Sea, Germany [AY771946] (Mussman et al., 2005). Five sequences formed a clade with an uncultured δ–Proteobacteria from Gulf of Mexico seafloor sediments from a hydrate system in the northern Gulf of Mexico continental slope [AY542227] (Mills et al., 2005).

**Gammaproteobacteria**

Approximately 3% of the sequences were affiliated with the γ–Proteobacteria. Sequences grouped into two distinct clades related to *Thialkalivibrio thiocyanadenitrificans* [AY360060], an obligate chemolithoautotrophic sulfur-oxidizing bacteria (Sorokin et al., 2004), and to *Thiothrix* spp. [EF641919], a filamentous gram-negative bacterium that oxidizes reduced sulfur compounds and is commonly found in sulfide-containing natural waters (Meyer et al., 2007). Nine of the sequences were closely related to lucinid or other metazoan symbionts within the γ-Proteobacteria. Only two of the sequences were identical to the CK 16S rRNA gene sequences retrieved in this study (Figure 3), representing 0.3% of the sediment sequences. The other sequences were related to previously retrieved sequences from Arctic sediments (Ravenschlag et al., 2000).

**Epsilonproteobacteria**

Two percent of the sequences were affiliated with the ε–Proteobacteria. Two distinct clades were represented. Four of the sequences grouped with *Sulfurimonas denitrificans* [NC_007575], a sulfur-oxidizing chemolithoautotrophic bacterium that oxidizes sulfide and thiosulfate and commonly associated with hydrothermal vent communities (Madigan et al., 2002). The remaining two sequences grouped with *Sulfurovum lithotrophicum* [AB091292], an obligately aerobic, sulfur-
and thiosulfate-oxidizing bacterium commonly associated with deep sea cold seeps and hydrothermal vents (Inagakai et al., 2004; Suzuki et al, 2005).

**Betaproteobacteria**

One clone sequence was most closely related to *Leptothrix* sp. str S11 [DQ241397], a filamentous bacterium typically found in iron-rich waters (Madigan et al., 2005).

**Acidobacteria**

The *Acidobacteria* comprise a poorly understood division of bacteria (Sabree et al., 2006). Approximately 4.2% of the core sediment sequences were related to the *Acidobacteria*. *Acidobacteria* are abundant in soil and alkaline conditions, and some are thought to be photosynthetic in the presence of oxygen (Sabree et al., 2006).

**Actinobacteria**

About 2.3% of the sequences were related to *Actinobacteria*. These bacteria are common in soil, and are almost all anaerobic (Ventura et al., 2007). *Actinobacteria* are gram-positive and are morphologically, physiologically, and metabolically diverse (Ventura et al., 2007). They are common decomposing bacteria that play an integral part in the carbon cycle (Conn, 2005). These bacteria produce many different enzymes and secondary metabolites, such as antibiotics, which are used for drugs (Conn, 2005).

**Bacteroidetes**

*Bacteroidetes* are rod-shaped, gram-negative bacteria found in a variety of habitats, including marine sediment and sea water. These bacteria are predominantly anaerobes (Madigan and Martinko, 2005). *Bacteroidetes* comprise 2.0% of the core sediment sequences. About half of the sequences grouped with bacterial sequences belonging to *Cytophaga* spp. [AB015532, AJ431254], deep sea sediment clones. *Cytophaga* are gliding bacterial group found in soils rich in
organic matter at neutral pH, such as aerobic bottom sediments and algal mats. The remaining sequences grouped with environmental bacterial sequences associated with mangrove soils collected from the Natural Nature Reserve, Hainan Island, China [DQ811911, DQ811905] (Yan et al., 2006).

**Chloroflexi**

Seventeen percent of the sequences retrieved from the core sediments belonged to the *Chloroflexi* phylum (Table 5). *Chloroflexi*, or green non-sulfur bacteria, are filamentous prokaryotes typically found in marine microbial mats (Madigan et al., 2005). They are anoxygenic phototrophs capable of photoheterotrophy and chemoorganotrophy (Madigan et al., 2002). Most sequences were closely related to environmental sediment clones, including the mangrove soil clone MSB-5G10, isolated from mangrove soil in China [DQ811879].

**Planctomycetes**

Sequences represented by the *Planctomycetes* accounted for 3.7% of the bacterial sequences retrieved from the core sediments. *Planctomycetes* are budding bacteria with a protein stalk lacking peptidoglycan (Fuerst, 2001). These chemoorganotrophic bacteria are found in diverse environments, including marine and soil habitats (Fuerst, 2001).

**Firmicutes**

*Firmicutes* are also gram-positive bacteria that have rod- or cocci-shaped cells. Nearly 4% of the sediment sequences were related to the *Firmicutes*. Sequences grouped into three clades, represented by two groups of *Firmicutes*, the clostridia and the bacilli. *Clostridium litorale* DSM 5388 [X77845] is a strictly anaerobic fermentative bacteria that forms endospores. It is commonly associated with extreme conditions, such as the human gut or sulfate-reducing marine biofilms (Zhang and Fang, 2001; Ley et al., 2006). The second clade was represented by the *Ruminococcus*. 
sp. str. 14531 [AJ315979], a bacterium within the bacilli. Bacilli are obligate or facultative aerobes commonly found free-living in soil, as well as natural flora in the human gut (Ley et al., 2006). The third clade was associated with *Alkakiphilus crotonatoxidans* [AF467248], a strictly anaerobic, chemoorganotroph isolated from a methanogenic environment (Coa et al., 2003).

**Spirochetes**

*Spirochetes* are slender, mobile, flexible and coiled, gram-negative bacteria found in aquatic environments (Madigan et al., 2005). Less than 2% of the clone sequences from the sediment were associated with the spirochetes. These bacteria have been previously found in marine, hydrogen sulfide-containing anaerobic habitats (Madigan and Martinko, 2005). They can also be found free living as anaerobic or facultative aerobic bacteria, and pathogens. Most of the sequences grouped in a clade represented by the environmental spirochete [AY605139] retrieved from a microbial mat in the Ebro and Camargue delta, Spain (Gurrero et al., unpublished). The remaining sequences grouped in a clade with *Spirochaeta halophila* [M88722], a facultative anaerobe found in Hunter Hot Spring, Oregon (Paster et al., 1991).

**Nitrospirae, Defferribacteres, Verrucomicrobia, and Thermotogales**

Both *Nitrospirae* and *Defferribacteres* are newly identified phyla within the bacterial domain that little is known about these groups (Madigan et al., 2005). *Nitrospirae* and *Defferribacteres* each accounted for <1% of the clones. Nitrospirae are nitrogen-oxidizing bacteria capable of chemolithoautotrophy or chemoorganotrophy. These bacteria are commonly found in a variety of habitats, (marine and fresh water, deltas, and aquariums) and temperatures (Daims et al., 2001; Madigan and Martinko, 2005). *Defferribacteres* are obligate anaerobes that utilize various electron acceptors, such as sulfur, nitrate, and fumurate (Janssen, 2002). Rare clones from the sediment were affiliated with the *Verrucomicrobia* (0.15%) and *Thermotogales* (0.15%). *Verrucomicrobia*
are warty-shaped bacteria that are aerobic to facultative aerobic, and are found in marine environments (Madigan et al., 2005). *Thermotogales* are strict anaerobes, commonly characterized as sulfate-reducing bacteria (Madigan et al., 2005).

**Candidate Divisions**

Seventeen core sediment sequences were identified as belonging to the proposed bacterial lineages within the Candidate Divisions OP8, OP11, and OD1. The sequences grouped with sequences from the Ocean Drilling Program (ODP), collected from methane hydrate bearing sub seafloor sediments from Peru and the Cascadia Margins (Inagaki et al., 2006).

**Microbial Diversity with Core Depth**

Core sediment samples were compared at ~10.5 cm intervals to determine changes in diversity with depth (Figure 7). Core 1 was dominated by δ-Proteobacteria and Chloroflexi in the first ~30 cm. Between 10-20 cm, ε-Proteobacteria and Planctomycetes were more prevalent. Between 30-40 cm, Actinobacteria and Planctomycetes were dominant, while the relative abundance of Chloroflexi decreased. At 40-50 cm depth, the relative abundance of δ-Proteobacteria decreased with an increase in Chloroflexi, γ-Proteobacteria, and α-Proteobacteria. α-Proteobacteria were the dominant bacteria at all depths from Core 2 (Figure 7), especially at ~40 cm where this group accounted for ~75% of total diversity. Chloroflexi and δ-Proteobacteria were abundant in the top ~20 cm. At 20-40 cm, the abundance of δ-Proteobacteria decreased but at 40-50 cm it increased again. ε-Proteobacteria were not present at any depth in Core 2. Firmicutes were not retrieved at the shallow depth in Core 2, but increased in abundance between 30-40 cm.
### Table 6: Distribution of 16S rRNA gene bacterial sequences from sediment cores at Cedar Key, Florida

<table>
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<tr>
<th>PHYLUM</th>
<th>CK 1C-1</th>
<th>CK 1C-2</th>
<th>CK 1C-3</th>
<th>CK 1C-4</th>
<th>CK 1C-5</th>
<th>CK 2C-6</th>
<th>CK 2C-5</th>
<th>CK 2C-4</th>
<th>CK 2C-3</th>
<th>CK 2C-2</th>
<th>Total # Clones</th>
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<tr>
<td>Core Depth (cm)</td>
<td>0-10.5</td>
<td>0-10.5</td>
<td>10.5-21</td>
<td>10.5-21</td>
<td>21-31.5</td>
<td>0-10.5</td>
<td>0-10.5</td>
<td>10.5-21</td>
<td>10.5-21</td>
<td>21-31.5</td>
<td></td>
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<td>23</td>
<td>31</td>
<td>4</td>
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<td>30</td>
<td>105</td>
<td>122</td>
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<td>402</td>
</tr>
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<td>1</td>
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<td>14</td>
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<td>0</td>
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<td>0</td>
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<td>1</td>
<td>1</td>
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<td>0</td>
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<td>0</td>
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<td>0</td>
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<td>2</td>
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<tr>
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<td>51</td>
<td>69</td>
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<td><strong>Total # full length sequences</strong></td>
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<td>51</td>
<td>69</td>
<td>11</td>
<td>23</td>
<td>45</td>
<td>142</td>
<td>145</td>
<td>33</td>
<td></td>
</tr>
</tbody>
</table>
The microbial communities between the two cores had a 0.93 Sorenson Index of Similarity (calculated by EstimateS), meaning that all of the major taxa were represented in both cores, even though the relative abundances of each major taxonomic group were different. Visual inspection of core distribution versus chemistry and mineralogy of the cores shows no statistical difference. Further statistical testing is needed to determine any differences.
Figure 7: Bacterial diversity of each of the core sediment 16S rRNA gene sequence clone libraries, shown grouped with increasing depth (every 10 cm).
DISCUSSION

Despite the numerous investigations describing the symbiotic association between lucinid hosts and their thiotrophic bacterial endosymbionts (e.g., Wiley and Felbeck, 1994; Gros et al., 1996; Krueger et al., 1996; Distel, 1998; Imhoff et al., 2003; Duperron et al., 2005; Stewart et al., 2005; Suzuki et al., 2005; Duperron et al., 2006; Taylor and Glover, 2006; Caro et al., 2007; Duperron et al., 2007), many questions still remain regarding bacterial endosymbiont phylogenetic diversity, host-bacterial species specificity, and the diversity and availability of endosymbionts in host habitats. This study aimed at addressing these issues by examining the diversity among bacterial endosymbionts within hosts of the same species from the same habitat, as well as among different host species from the same habitat, and from different geographically separated host species. To my knowledge, this is the first study to examine the bacterial diversity of a lucinid habitat, which includes the identification of free-living bacteria belonging to the Gammaproteobacteria and possibly related to lucinid endosymbiont sequences. The main study site was the sea grass and silicate sediments habitat at Cedar Key, Florida. For Cedar Key, habitat parameters such as geochemistry and mineralogy were assessed to evaluate possible relationships among the Cedar Key habitat, host taxa, and their endosymbiont species. Results indicate that the bacterial endosymbionts from lucinid bivalves are not all genetically identical across different host species in the same or different habitats, within the same host species from the same or different habitats, or even within an individual host organism.

Habitat Geochemistry and Mineralogy

Shallow marine lucinids require a unique geochemical habitat, not unlike animals living at deep-sea vent systems. Previous work from shallow marine environments did not assess the environmental habitat parameters for lucinids and their symbionts, and determine if there was a
connection between the environmental conditions and the host species or endosymbiont species. Lucinid hosts must position themselves in the sediment to balance dissolved oxygen and dissolved sulfide concentrations (Felbeck et al., 1983; Savazzi, 2001). Because sulfide oxidation can occur spontaneously in the presence of high concentrations of dissolved oxygen, host bivalves live at or near the oxic-anoxic interface (Distel, 1998; Stewart et al., 2005). The dissolved oxygen concentration within the Cedar Key sediment pore waters was 2.03 mg/L (Table 1), which is high enough that some dissolved sulfide could spontaneously oxidize (Frenkiel et al., 1995; Kraus, 1995). However, because the sediment had high concentrations of dissolved sulfide, conditions within the sediment were still conducive for thiotrophy. Slight variations in pyrite abundance with depth (Table 3), but with slightly more pyrite from the deeper sediment, may indicate changing reducing conditions with depth, but may also correlate to the zones where the flux of dissolved sulfide through the sediments is greatest. With little to no iron in the sediment, formation of pyrite would decrease and would increase the amount of dissolved sulfide. Based on the depths that lucinid hosts were collected (within the first 10 cm of the sediment surface), the hosts may position themselves above the depths where the potential for pyrite mineralization is greatest.

The type of bacteria present in any environment, and available for acquisition for symbiosis, is likely controlled by the distinct geochemistry and mineralogy of the sediment habitat. The mineralogy of the Cedar Key sediments showed that the dominant mineral was quartz, as would be expected in a siliciclastic environment (Table 3). Lemon Bay is also a siliciclastic setting, and this could be a reason why the sequence similarities between the CK and LB endosymbiont sequences was higher than those from JI and MPC, whose habitats are dominated by carbonates (Table 5). A habitat study in a carbonate dominated environment would be necessary to determine whether the free-living symbionts grouped with the endosymbionts from host bivalves found there. Until that
work is done, this study of the microbial diversity from a lucinid sea grass siliciclastic sediment habitat (Cedar Key) is unique. A comparative habitat study would also be important to test whether or not the acquisition of endosymbionts depends on the availability of bacteria within the habitat, or if acquisition is related to specificity for the host species. If the free-living symbionts do not exist in carbonate sediments, then this could indicate that the endosymbionts must be acquired from another source, such as free-living cells from seawater.

**Intra- and Inter-Species Endosymbiont Diversity**

Previous research suggested that all lucinid bivalve taxa contained the same species of bacterial endosymbionts (e.g., Gros et al., 2003). Distel et al. (1988; 1994) and Gros et al. (2003) suggest that endosymbionts are species-specific to their hosts. I hypothesized that bacterial endosymbionts from hosts collected at geographically separated locations would be genetically distinct. One way I tested this hypothesis was by evaluating the bacterial diversity of different host taxa from the same habitat. However, because DNA quality and quantity was low from hosts other than *L. nassula*, I was unable to determine whether the endosymbionts found in multiple species from the same habitat were the same or different. With the 16S rRNA gene sequences I retrieved from Cedar Key from three *L. nassula* hosts, DOTUR analysis demonstrated that there was only one phylotype at 95% sequence identity. This indicates that the *L. nassula* hosts from Cedar Key contain endosymbionts from the same genus. Comparing all of the gammaproteobacterial sequences at 99% sequence identity, however, revealed that 19 phylotypes were identified, suggesting that endosymbiont identity varies among host organisms, even within the same habitat (Figure 6).

Similarly, the endosymbionts do not always belong to the same bacterial phylotype within the same host organism (e.g., clones beginning with the label “CK_G8”) (Figure 3) or within multiple hosts of the same species (e.g., clones beginning with the label “CK_G8” versus
“CK_G5”) (Figure 3, Table 4). These results are consistent with the recent study of Caro et al. (2007) that found that up to seven genetically distinct subpopulations of endosymbionts, of varying size and intracellular nucleic acid content, were present within individual host specimens.

When geography was examined as an influencing factor that may control bacterial endosymbiont diversity, sequences from host bivalves taken at geographically similar locations grouped more closely than those sequences from bivalves collected at distant geographic locales (Table 5). Endosymbiont sequences from host bivalves collected on the Gulf of Mexico side of Florida (CK and LB) grouped into a separate clade compared to the endosymbiont sequences collected on the Atlantic side of Florida (JI and MPC), with high bootstrap support (Figure 5). Coincidentally, these clades also correlated to the same host taxa (e.g., *L. nassula* from CK and LB and *P. pectinatus* for MPC and JI). Therefore, it is difficult to determine whether endosymbiont phylotype diversity is controlled more by geography, habitat type (e.g., siliciclastic versus carbonate), or if diversity is associated with host taxa specificity. Further research should be done to determine whether or not it is host species or geography that might have a greater influence on endosymbiont species diversity. It is possible that various metabolic requirements for both the endosymbiont and host, and the variety of habitats that these organisms live in, have caused genetic variations and adaptations among endosymbionts (Caro et al., 2007). Regardless of these limitations, it is clear that bacterial endosymbionts associated with lucinids are not all the same species within a single host, across hosts of the same species, or across different species of hosts.

**Free-living Bacterial Diversity**

One of my research goals was to characterize the bacterial diversity within the lucinid free-living (sediment) habitat. I hypothesized that the free-living bacterial diversity would reveal sequences related to the endosymbionts. Thirteen major taxonomic phyla were retrieved from the
lucinid sea grass bed sediments at Cedar Key. Although a thorough study of the bacterial diversity from seagrass sediments has been done, the overall sequence diversity among bacteria was comparable to the types of microbial groups previously found in marine sediments (e.g., Ravenschlag et al., 2001). Core 1 and 2 were separated by 1m, and the communities in the two cores were very similar (0.93 for the Sorenson Index of Similarity), but the distributions of the group varied. Core 1 was dominated nearly equally by $\delta$-Proteobacteria and Chloroflexi while Core 2 was dominated by $\alpha$-Proteobacteria (Figure 7).

The most significant result from studying the sediment was that 0.3% of the sequences retrieved from all 856 clone sediment sequences were related to sequences from the Cedar Key gill gammaproteobacterial endosymbionts (Figure 3). One sequence [CK_1C1_40] was retrieved from 0-10.5 sediment depth, while the other [CK_1C3_51] was retrieved from 10.5-21 cm depth. Lucinid hosts were collected from within the top 10 cm of the sediment surface. Gros et al. (2003a) suggest that this group is free-living in sediments hosting lucinids. Others have suggested that there are free-living bacteria are environmentally acquired (Gros et al., 1996; Peek et al., 1998; Fortey, 2000; Savazzi, 2001; Gros et al., 2003b; Caro et al., 2007). Therefore, the low representation of these bacteria in sediment may be due to a high recruitment rate by host bivalves, which would keep the overall abundance of free-living cells low in the lucinid habitat. But, finding sequences closely related to the gammaproteobacterial endosymbionts in the sediments does not necessarily mean that the endosymbionts are metabolically active while free-living, as their distribution in marine sediments and their metabolic role in sediment have not been established.

The similarity between some free-living symbiont sequences to endosymbiont sequences at CK, as well as the fact that endosymbiont sequences were found most closely related to sequences from hosts collected at the same locale, could suggest that endosymbiont diversity is not regulated
by host specificity, but rather by environmental availability. This could also be supported by the presence of significantly different endosymbionts (e.g., α-Proteobacteria) in host species found in distinctly different habitats (e.g., carbonate or siliciclastic).

Future research could include the characterization of another habitat similar to Cedar Key, but also a distinctly different habitat (e.g., carbonate) to determine the presence of free-living endosymbionts. Future research should also include an investigation of the changes in free-living bacterial diversity through time, as well as bivalve abundance and determine the changes in bacterial diversity with changes in bivalve presence, or even seasonal changes. Disturbance, such as hurricanes, can also redistribute bacteria within sediment, as well as lucinids, and may impact diversity through time.

**Endosymbiont Sequence Diversity and Possible Metabolic Implications**

Retrieving clone sequences from lucinid gills that were not related to *Gammaproteobacteria* was an unexpected result. Although recent research by Caro et al. (2007) suggest that individual lucinid gills can contain up to seven different bacterial subpopulations (from *C. orbicularis*), and Duperron et al. (2007) found non-gammaproteobacterial sequences in the gill of a deep sea clam, no studies have retrieved 16S rRNA gene sequences from shallow marine lucinids (i.e. not deep sea) that were not *Gammaproteobacteria*. In addition to gammaproteobacterial sequences, a bacterial sequence was retrieved from a *P. pectinatus* host collected from the Mouth of Pigeon Creek, The Bahamas [MPC-2_46] that grouped most closely with an *Alphaproteobacteria*, a *Methylobacterium* spp. clone (Figure 5). Also from *P. pectinatus* from Jack Island, FL, 16S rRNA sequences were related to both γ- and α-Proteobacteria (Figure 5). These results are unique to this thesis, as no study has uncovered non-gammaproteobacterial diversity for the shallow marine lucinids. Hosting both γ- and α-Proteobacteria could be due to the environment in which the host lived, or could be a
trait specific to *P. pectinatus*. These results may indicate dual symbiosis (thiotrophy and methanotrophy) in some lucinid bivalves. Dual symbiosis has previously been found in the Mytilidae clams from deep sea vent systems (Distel et al., 1995). Further work is underway to address possible dual symbiosis for the MPC and JI individuals, as well as to sequences more clones from lucinids hosts to achieve more coverage in clone diversity. The presence of dual symbiosis may suggest either an evolutionary shift toward utilization of multiple metabolic pathways, or possibly an ancient metabolic pathway that is being abandoned in other lucinids through genetic alteration in order to have sulfide oxidation be the dominate endosymbiotic pathway.
CONCLUSIONS

Characterization of a single lucinid habitat, including aqueous and sediment geochemistry, as well as the gill endosymbiont and free-living bacterial diversity, has allowed us to better understand the functional and evolutionary relationship between bacterial endosymbiont and their hosts. Previous research indicated that all endosymbiont sequences belonged to a single phylogenetic clade within the $\gamma$–Proteobacteria (Distel, 1998; Gros et al., 2003). With recent evidence suggesting that diversity may be greater than expected (Caro et al., 2007; Duperron et al., 2007), I determined from 16S rRNA gene surveys that lucinid bivalve hosts do not always contain the same species of bacterial endosymbiont, and that endosymbiont sequences are not identical within the same species, across multiple hosts within the same environment, across host species within the same geographic locale, or across geographic locales.

The results from this study indicate that endosymbionts in lucinid bivalves form several distinct phylogenetic clades (Figure 4 and 5). This bacterial diversity may indicate that endosymbionts are not host specific. The presence or absence of free-living symbionts in a lucinid habitat, and especially those symbionts that could potentially become thiotrophic gill endosymbionts, may play an important role for host specificity for symbiont acquisition. However, based on the geochemical variation of the habitat, metabolic requirements (i.e., habitat conditions) of the free-living symbionts will also influence potential symbiont diversity. It is also possible that environmental stresses to the host lucinids could provoke the utilization of multiple metabolic bacterial types within the gills. Therefore, the effects of habitat geochemistry could explain the possible existence of dual symbiosis in one lucinid host, but more research needs to be done to determine conclusively if dual symbiosis in some species of lucinids is actively occurring.
It has been suggested that isotope studies of bivalve shells may be related to changes in environmental parameters, dietary preference, growth rate, and sexual maturity (Jones et al., 1988; CoBabe and Pratt, 1995; Langlet et al., 2002; Leitard and Pierre, 2006). Bacterial metabolism may also be recorded in the stable isotope composition of hosts. However, given that there is significant bacterial endosymbiont diversity, isotopic ratios obtained in ancient bivalve shells may not reflect the true nature of a lucinid’s metabolic history (e.g., Peng et al., 2007).

Lastly, results from this study will lead to a more thorough understanding of the evolutionary and ecological relationships between lucinid bivalves and their thiotrophic endosymbionts. This research is the first to characterize the bacterial diversity of a lucinid host habitat, and specifically a nearly exhaustive study of a single site. My research will also provide a background for modern day symbiotic relationships that can be applied to ancient symbiotic relationships.
REFERENCES


Felsenstein, J., 2005, PHYLIP, (phylogeny inference package) version 3.6: Distributed by the author: Department of Genome Sciences, University of Washington, Seattle.


APPENDIX A
CORE MINERAL ANALYSIS AND XRD

1. XRD Mineral Abundance Reports for All Core Samples
2. Core 1 Mineral Peak Comparison
3. All Core Mineral Peak Comparison
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ARAGONITE 1.5641

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XRD Data File: c:\progra~1\xrdfil\1c-3b.raw

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X-RAY DIFFRACTION : MINERAL CONCENTRATION REPORT

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LSU Geology & Geophysics Dept.  9/24/2007 - 10:25:56.95
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Library File: mineral.lib
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LSU Geology & Geophysics Dept.  12/04/2007 - 10:27:53.45
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X-RAY DIFFRACTION : MINERAL CONCENTRATION REPORT

Library File: mineral.lib
XRD Data File: c:\progra~1\xrdfil\1c-4b.raw

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LSU Geology & Geophysics Dept.  9/24/2007 - 10:26:35.18

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PLAGIOCLASE 0.1239
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CLAY       1.1978

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APPENDIX B
TAXONOMIC DISTRIBUTION OF BACTERIAL CLONES

1. Taxonomic Distribution of Bacterial Clones
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<th>Phylogenetic Affiliation</th>
<th>Representative Sequence [Accession Numbers]</th>
<th>Closest Relative [Accession Numbers]</th>
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<th>No. Clones</th>
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<td>Agrobacterium sp. PB [AF482682]</td>
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<td>Hyphomicrobium sulfonivorans strain CT [AY468372]</td>
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VITA

Angela Marissa Garcia was born in September, 1978, to Jeannie Green and Rudolph (Rudi) Garcia in Idabel, Oklahoma. She is the only girl among four children. She has one older brother, Angel Garcia, and two younger brothers, Anastacio Garcia and Adan Garcia. Angela was raised in Houston, Texas, where she graduated from Bellaire High School. She worked for NASA as a coordinator and director of an Urban Outreach Program in Earth and Space Science while pursuing her bachelor’s in biology at the University of Houston-Downtown. She was a member of the Scholar’s Academy and a recipient of the Office of Naval Research, USFDA, and Shell Mentor scholarships. Angela began her pursuit of a master’s degree at Louisiana State University (LSU) in the Fall of 2005, where she was a member of the Geoscience Alliance to Enhance Minority Participation (GAEMP) program in the Department of Geology and Geophysics. Angela attended her first Geological Society of America conference in October, 2005, where she presented a poster about the GAEMP program titled: “Evolution and Geomicrobiology: An Essential Teaching Tool for GAEMP”. In the summer of 2006, Angela was a field camp instructor at the LSU geology field camp in Colorado Springs, Colorado. She has presented posters about her thesis research at the Geological Society of America’s annual meeting in Denver, Colorado, in October, 2007, as well as the American Geophysical Union’s annual meeting in San Francisco, California, in December, 2007.