MICROBIAL ECOLOGY OF *LOPHELIA PERTUSA* IN THE NORTHERN GULF OF MEXICO

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ABSTRACT

Microbes, including bacteria, archaea and fungi, are recognized to be an important part of the total biology of shallow-water corals. Deep-sea corals have a fundamentally different ecology due to their adaptation to cold, dark, high-pressure environments, and as such have novel microbiota. The goal of this study was to characterize the microbial associates of *Lophelia pertusa* in the Gulf of Mexico. This is the first study to include both culture-based and molecular data on deep-sea coral-associated bacterial communities. It is also the first study to collect the coral samples in individual insulated containers and to preserve coral samples at depth in an effort to maintain *in situ* microbial diversity by minimizing contamination and thermal shock.

There are a few links between *Lophelia*-associated bacteria and bacteria from shallow-water corals and deep-sea octocorals, but both cultured isolates and clone libraries revealed many novel bacteria associated with *Lophelia*. There are many bacteria and clone sequences that are similar to symbionts of fish, squid, and methane seep clams. In particular, there is a sequence, VKLP1, present in all *Lophelia* colonies analyzed to date (n=6), which is related to a sulfide-oxidizing gill symbiont of a seep clam. This microbe may be a *Lophelia*-specific bacterium and links the coral to cold seep communities. Molecular analysis of bacterial diversity showed a marked difference between the two sites, Visoca Knoll 906/862 and Visoca Knoll 826. The 16S rRNA bacterial clone libraries from VK826 were dominated by a variety of unknown *Firmicutes*. The dissimilarity between the dominant members of the bacterial communities at these two sites may be evidence of diseased *Lophelia* or thermal stress at one site, or may indicate biogeographical differences.

There was no overlap between the bacteria identified in this study and those from a recent study of *Lophelia* in the Mediterranean. This may indicate biogeographical differences, however, it is more likely due to the significant methodological differences in collection,

extraction, and analysis of the *Lophelia* samples. No archaea have been detected to date, however, a fungus similar to marine species of *Paecilomyces* and *Acremonium* was found.

INTRODUCTION

Coral microbial ecology is the study of the relationship and interactions between coral-associated microorganisms and (1) each other, (2) the coral host, and (3) their environment. Just as humans have beneficial bacteria living on our skin and in our intestines, corals also have co-habitating non-pathogenic (non-disease-causing) microbes. These microbes include bacteria, archaea, and fungi—representing all three of the major domains of life (Fig. 6.1).

EXAMPLES FROM SHALLOW-WATER CORAL STUDIES – What can we learn from studying coral microbial ecology? Why is it important to resource management? One reason is that, as an integral part of the coral's biology, understanding the microbial ecology will lead to insights related to coral health, disease, and overall resiliency. Coral reefs in the Caribbean have been in decline for several decades (Hughes 1994; Shinn et al. 2000). One of the most visible causes is disease. Coral diseases are not well understood, but microorganisms have been found to cause the few that have been characterized (Richardson 1998). In order to understand the diseased state, we must first understand the healthy state. Coral microbial ecology studies contribute missing information to the holistic study of coral biology.

Our understanding of the roles filled by coral-associated microbes is limited. It has been speculated that coral-associated bacteria benefit the coral by fixing nitrogen, breaking down waste products, and cycling basic nutrients back to the zooxanthellae (Lesser et al. 2004; Rohwer et al. 2001; Shashar et al. 1994). Bacteria may also ward off other potentially harmful microbes by producing antibiotics or just by occupying the available space (Dobretsov and Qian 2004; Ritchie 2006). Studies have also found that coral-associated bacterial populations are closely attuned to host metabolism and may change in number or composition in response to a change in coral health (Ducklow and Mitchell 1979; Pantos et al. 2003). Archaea and fungi are associated with tropical corals, but their functions remain unknown (Bentis et al. 2000; Kellogg 2004; Priess et al. 2000; Ravindran et al. 2001; Wegley et al. 2004).

Another important aspect of coral microbial ecology studies is that they contribute to our knowledge of microbial diversity. This is important to our understanding of microbial species richness, biogeography, and specificity for certain environments (Hedlund and Staley 2003; Papke and Ward 2004). Additionally, the discovery of novel microorganisms is of interest to pharmaceutical and biotechnology companies for the development of unusual natural products (Maxwell 2005; Pomponi 2001). Recent studies of shallow-water corals have found that most of

the associated bacteria were novel species, not present in the surrounding seawater (Bourne and Munn 2005; Frias-Lopez et al. 2002; Ritchie and Smith 1995b; Rohwer et al. 2001; Rohwer et al. 2002). Additionally, there is some evidence of specific bacteria-coral interactions; where a particular bacterial 16S rDNA sequence has been repeatedly found in association with multiple individuals within a coral species (Bourne and Munn 2005; Rohwer et al. 2001).

DEEP-SEA CORALS. – The ecology of deep-sea corals is fundamentally different from that of shallow-water corals due to the environmental parameters surrounding them (e.g., darkness, low temperature, pressure) and the absence of symbiotic zooxanthellae. The microbial communities of these cold-adapted corals are likely to contain novel organisms also adapted to the deep-sea environment. In spite of lacking photosynthetic algal symbionts, growth rates for *Lophelia pertusa* have been estimated as high as 26 mm/yr (Bell and Smith 1999). This makes the potential role of coral-associated microbes even more interesting. An hypothesis, yet to be tested, is that the microbes help to feed these corals, either by a mechanism similar to the chemosynthetic bacterial symbionts that nourish hydrothermal-vent worms, or more simply by serving as food particles.

Because of the difficulty and expense of collecting samples, very little work has been conducted on the microbial ecology of deep-sea or cold-water corals. There is one publication based on a limited sample set of black coral and bamboo coral (Penn et al. 2006) and one study that investigated *Lophelia pertusa* in the Mediterranean basin (Yakimov et al. 2006). Both studies concentrated on bacterial associates. There are no published data for archaea associated with deep-sea corals. Yakimov et al. (2006) attempted to amplify archaeal sequences from *L. pertusa* but without success. The only mention of fungi associated with deep-sea corals is that of endolithic species (e.g., *Dodgella priscus*) detected on dead *Lophelia pertusa*, as part of the microbial complex that alters the coral after death (Freiwald et al. 1997; Freiwald and Wilson 1998; Wisshak et al. 2005).

METHODOLOGY ISSUES – Classical microbiology is based on an ability to culture (grow) the organism(s) being studied in the laboratory. Not only is culturing relatively easy and inexpensive, but it also allows for many additional assays to characterize the microbe's biochemical and physiological capabilities. The downside to culture work is that each type of nutrient agar creates a slightly different selection bias. Bacterial studies from marine and soil environments that compared total counts (by microscopy) to viable counts on agar plates have

shown that only 1-10% of the bacterial community are capable of growing on a general nutrient medium (Eilers et al. 2000; Torsvik et al. 1990). The majority of environmental archaea also do not grow on culture media.

In contrast, culture-independent techniques, e.g., those based on direct DNA-extraction from the microbial community, provide access to the 90-99% of bacteria that are not able to grow on a given medium. These molecular techniques also permit detection of cryptic fungi and archaea. Amplifying target-specific genes and then sequencing them can uncover enormous diversity that culture techniques do not detect. However, molecular techniques also have limitations. The end result is typically a list of DNA sequences that are compared to a genetic database. Organisms that are novel or very dissimilar to known entries in the database are of great interest: however, without having the microbe in culture, there is a limit to how much information about the microbe's biochemical capabilities, physiological state, or interactions can be determined by additional molecular work. As such, the best approach for investigating unknown microbiota is to apply a combination of both culture-dependant and culture-independent methodologies to obtain as much information as possible about microbial communities. In the case of *Lophelia*, these initial results can direct future culture-dependant work using appropriate media.

Another methodological issue is the care with which samples need to be collected. Deep-sea coral samples are typically collected by a trawl, dredge, or by a submersible/ROV. With these methods many corals may be combined in a single container. This is not acceptable for microbiological studies since one coral's microbial community could contaminate another's when they touch. Similarly, contact with sediment, other invertebrates, etc., could contaminate the coral samples. Additionally, there is concern that changes in temperature and pressure such as those that exist between the sea floor and the surface could affect the microbial community associated with *Lophelia* (i.e., the community may shift in response to these factors). A special sampling device was designed and built to minimize these concerns while collecting samples for this study (see methods section for a more detailed description).

SCIENTIFIC QUESTIONS DIRECTING THIS STUDY – The objectives of this study were to address the following questions:

1. <u>What kinds of microbes are associated with *Lophelia*?</u>

Diverse communities of bacteria, archaea, and fungi have recently been shown to be

associated with shallow-water scleractinian corals (Bentis et al. 2000; Bourne and Munn 2005; Frias-Lopez et al. 2002; Kellogg 2004; Rohwer et al. 2002; Wegley et al. 2004). Are comparable communities associated with *Lophelia*?

2. How do these microbial communities compare to those of shallow-water corals or deep-sea gorgonians?

   Are there scleractinian-specific microbes, deep-sea coral-specific microbes, or do *Lophelia* colonies have unique microbiota?

3. Is there a difference in the microbial communities between coral samples preserved at depth and those brought to the surface alive?

   Do we need special sampling tools to correctly study deepwater microbial communities? These questions are posed to evaluate the collection methods used here as well as to help evaluate previous, more traditional collection methods.

4. Are these microbes acting as symbionts (since there are no algal symbionts)?

   Studies of shallow-water corals suggest that associated bacteria may benefit the corals by cycling carbon, fixing nitrogen, chelating iron, and producing antibiotics that protect the coral from other microbes. The health of *Lophelia* and other deep-sea corals may be dependent upon symbiotic microbes. Many *Lophelia* thickets are located near natural hydrocarbon seeps in the ocean floor, so it is of particular interest to determine if hydrocarbon-utilizing microorganisms are a link between the coral and the seeps.

MATERIALS AND METHODS

Samples of *Lophelia pertusa* were collected by submersible during two cruises in the northern Gulf of Mexico: USGS-GM-2004-03 (July 29-August 5, 2004) and USGS-GM-2005-04 (September 15-21, 2005). The sites sampled during both years were Viosca Knoll 906/862 (the topographic feature crosses both lease blocks; Fig. 6.2) and Viosca Knoll 826 (Fig. 6.3). The specific latitude and longitude and other data for each sample are listed in Table 6.1.

SAMPLE COLLECTION – None of the standard sample containers available on the Johnson-Sea-Link submersible were adequate for microbiological sampling. A custom sampling device was designed and constructed specifically for this study. The 'Kellogg Sampler' (KS) is an insulated container that has 10 separate sample compartments, each with a sliding door and o-ring seal. Syringes of preservative solution are connected to five of the sample compartments by one-way valves. The preservative solution is added at depth by pressing down a plunger with the submersible's manipulator arm. The KS can be mounted on the front of the Johnson-Sea-Link submersible and filled using the manipulator arm or suction tube (Fig. 6.4). The sealed separate compartments keep the individual coral samples from becoming contaminated by contact with other coral samples, sediment, or various depths of water column during the return to the surface. The insulated container keeps the corals at near-collection temperature. The ability to add fixative to half of the samples at depth makes it possible to test the hypothesis that coral-associated microbial communities shift during sample retrieval in response to changes in light, temperature, pressure or other factors.

SAMPLING PROTOCOL – Branches were removed from *Lophelia* colonies using either the submersible's manipulator claw or suction tube (with a screen to prevent the coral from being sucked up into the hose) and then placed into individual sample compartments in the 'Kellogg Sampler'. Coral samples were collected in duplicate from each colony; one sample was brought to the surface alive and the other was fixed at depth by the addition of preservative solution [20% dimethylsulfoxide, 0.25 mM EDTA, saturated salt (Dawson et al. 1998; Seutin et al. 1991)]. Metadata including time of collection, location, depth, temperature, and salinity were recorded (Table 6.1). On deck, *Lophelia* samples were aseptically transferred from the 'Kellogg Sampler' compartments into sterile containers and brought into the lab for immediate processing. Any unused *Lophelia* was provided to the USGS genetics group or preserved for histology (Table 6.1).

CULTURE-BASED ANALYSES – Flame-sterilized needle-nose pliers were used to snip off small pieces of live *Lophelia* (skeleton with one polyp) into sterile aluminum weigh dishes. The pieces were crushed using a sterile hammer and made into slurry by adding two milliliters each of sterile phosphate-buffered saline. The slurry was then spread onto two types of nutrient agar: glycerol artificial seawater agar (GASWA), a general medium, and thiosulfate citrate bile sucrose agar (TCBS), a medium specific for *Vibrio* species (Kobayashi et al. 1963; Smith and

Hayasaka 1982). The GASWA medium was chosen because it has been used to culture bacteria from many species of shallow-water corals (Ritchie 2006; Ritchie and Smith 1995a; Ritchie and Smith 1995b; Ritchie and Smith 1997) and therefore would allow the most direct comparison between studies. A vibrio-specific medium (TCBS) was included because of the recent interest in the roles *Vibrio* sp. may play in coral diseases (Ben-Haim and Rosenberg 2002; Ben-Haim et al. 2003; Cervino et al. 2004; Ritchie 2006; Rosenberg and Falkovitz 2004). The agar plates were incubated at 30˚C (TCBS) and 5˚C (GASWA), respectively.

MOLECULAR ANALYSES – The following culture-independent techniques were used to assay the microbial diversity that is not detectable by culture methods.

DNA EXTRACTION - Flame-sterilized needle-nose pliers were used to snip off small pieces of all *Lophelia* samples (live and preserved at depth) into sterile aluminum weigh dishes. Each piece was crushed using a sterile hammer and the mixture of coral skeleton fragments, polyp tissue, and mucus was transferred to a 2 ml microcentrifuge tube. Microbial community DNA was extracted from the *Lophelia* samples using the PowerSoil DNA Extraction Kit (MoBio Laboratories, Inc., Solana Beach, CA), following the manufacturer's protocol. All samples were processed in triplicate. DNA extracts were frozen (-20°C) for transport back to the U.S. Geological Survey's microbiology laboratory in St. Petersburg, FL.

DENATURING GEL GRADIENT ELECTROPHORESIS (DGGE) – DGGE is a method of "DNA fingerprinting' microbial communities so that comparisons can be made at the community level rather than that of the individual sequence. It involves using polymerase chain reaction (PCR) to amplify target genes, for example the 16S rRNA genes of bacteria, and then running the PCR products through a gradient gel. The gradient denatures or 'pulls apart' the double-stranded DNA in the PCR products at different rates depending on the nucleic acid ratio of the sequences (e.g., sequences that have more GC pairs than AT pairs will take longer to denature and will therefore move through the gel at a different rate). This produces a series of bands in each lane of the gel (each lane represents one sample, i.e., one bacterial community). The pattern of bands in each lane can be compared to determine if the bacterial communities are identical, similar, or very different between samples (Fig. 6.5).

Clone libraries. – In order to separate individual sequences from a microbial community DNA extraction, it is necessary to create clone libraries. This multi-step method uses specific primers (for example, 16S rDNA primers for bacteria) and PCR to amplify a specific subset of

the DNA (in this example, sequence fragments diagnostic for bacterial species present in the community). These fragments are all the same size and are mixed together in the PCR product. To distinguish individual sequences, the PCR product is combined with plasmid vectors, and enzymes are used to incorporate one piece of DNA into each plasmid. The plasmids are then put into *E.coli* cells. The *E. coli* cells are plated onto a selective medium so that cells that have taken up a plasmid containing a piece of DNA are detectable. Each cell has one plasmid and each plasmid has one sequence from the community mixture. This sequence can now be extracted from the *E.coli* 'clone' and the sequence determined. By determining the sequences of many clones (a 'clone library'), the sequences of the dominant members of the microbial community become known.

SEQUENCE ANALYSES – The following software programs were used to process the raw sequence data: Phred, to base-call and add quality scores; Greengenes, to trim poor quality sections, and Blast, to compare the sequences against the GenBank database.

Percent similarity was interpreted as follows: a 97-100% match to a GenBank entry was considered to be within the same species, a 93-96% match was considered to be within the same genus, and an 86-92% match was considered to be a related organism (Stackebrandt and Goebel 1994). However, note that there can be significant genetic and physiological differences between two bacteria that have 99% similarity in their 16S rRNA genes (Jaspers and Overmann 2004), so all estimates of diversity are actually minimum approximations.

RESULTS

Five submersible dives were devoted to *Lophelia* collection for microbial analyses; two in 2004 and three in 2005, with two on Viosca Knoll 906/862 and three on Viosca Knoll 826 (Table 6.1, Figs. 6.2, 6.3).

CULTURE-BASED ANALYSES – Over 200 *Lophelia*-associated bacteria were cultured during this study. Some of these strains could not be maintained in the laboratory, reducing the total number of bacteria to 174, representing 16 genera that were successfully isolated, identified by their 16S rDNA sequences, and archived for future research (Table 6.2). Three *Vibrio* sp. were isolated on TCBS agar from a single coral sample (4753K6). No other growth was observed on this agar medium during either cruise. All other bacteria were isolated from GASWA plates: 39 from the 2004 cruise and 132 from the 2005 cruise.

Many of the cultured bacteria were psychrophiles ('cold-loving') and included close matches (97-99% similarity) to bacteria previously detected in polar waters and pack ice. There were also close matches to bacteria previously cultured from deep-sea sediments and a deep-sea sponge.

It is notable that one sample, 4878K2 from VK906/826, yielded solely *Photobacterium* spp. (62/62 isolates). *Photobacterium* spp. were absent or a minority in all the other cultured samples. This suggests that something was different about the physiology of that particular *Lophelia* colony.

Twelve of the cultured bacteria were novel based on their 16S gene sequences (bottom, Table 6.2). These bacteria were not genetically similar to any currently described bacteria and represent unknown microorganisms. Of particular interest are the four *Entomoplasmataceae* isolates, since they are from the same group (*Firmicutes*) as many of the 16S clone sequences (see CLONE LIBRARIES section below) and their characterization may yield insights into the characteristics of this group and their relationship to the coral. Biochemical and metabolic characterization of these bacteria is planned.

DENATURING GEL GRADIENT ELECTROPHORESIS (DGGE) – One DGGE was successfully completed using samples 4753K1-10 from the 2004 cruise, collected at site VK826 (Table 6.1, Fig. 6.5). These ten samples are actually pairs of samples from five *Lophelia* colonies: K1/K2, K3/K4, K5/K6, K7/K8, and K9/K10. Odd-numbered samples were preserved at depth and even numbered samples were brought up without preservation for comparisons. The banding patterns or 'fingerprints' in Fig. 6.5 represent the dominant bacterial species present in the samples. Similar banding patterns suggest commonality of bacterial communities for these *Lophelia* colonies (e.g., Fig. 6.5a, white arrows mark the two doublets present in most of the samples). However, comparison between odd and even pairs shows that there *is* a difference between samples preserved at depth and those brought up without preservation. For example, Lanes 1 and 2 are sampled from the same coral, but a very prominent band that is present in the preserved sample is missing in the live sample (Fig. 6.5a, white circles). There is also a case where the live sample has many additional bands (Lane 10) relative to the preserved sample (Lane 9). In both cases, we can deduce that there was either a shift in the bacterial community or a qualitative change in the sample that affected the extraction such that the two samples from the same coral individual but preserved differently produced very different results.

Several of the prominent bands on this gel were excised, the DNA extracted, and sequences obtained. The top band in the first doublet (Fig. 6.5b, red box) yielded the same sequence from Lanes 1-9 and was identified as being from a *Shewanella*-like bacterium. The lower band in the first doublet (Fig. 6.5b, blue box) also was consistent across Lanes 1-9, and was identified as a *Mycoplasma*-like bacterium. This further confirms a certain level of similarity between the bacterial communities on each of these five *Lophelia* colonies. All the bands in Lane 10 and one band in several other lanes (Fig. 6.5b, yellow boxes) were similar to an uncultured α-proteobacterium clone sequence, MSB-3. The conspicuous lower band in Lane 1 (Fig. 6.5b, green box) was 99% similar to a bacterial sequence associated with a shallow-water coral (*Oculina patagonica*) in the Mediterranean (Koren and Rosenberg 2006).

CLONE LIBRARIES – Using primer sets specific for bacteria, fungi, and archaea, clone libraries were generated from the *Lophelia* samples and screened by DNA sequencing and subsequent comparison to the GenBank database.

ARCHAEA – Attempts to amplify archaeal 16s rRNA genes from the *Lophelia*-associated microbial community DNA extractions using a standard archaeal primer set, 21F and 958R (DeLong 1992), were unsuccessful. Five alternative archaeal primer sets are currently being tested.

FUNGI – The fungal-specific primer set, nu-SSU-0817-5' and nu-SSU-1536-3' (Borneman and Hartin 2000), was used to generate two clone libraries, one from sample 4873K1 and the other from sample 4881K9. Sixteen percent of the clones in the library generated from sample 4873K1 were 99% similar to both a *Paecilomyces* sp. and an *Acremonium* sp. in the GenBank database. These fungi were both of marine origin: the *Paecilomyces* sp. was isolated from mangroves and the *Acremonium* sp. was isolated from marine sediments. Unfortunately, the primer set was not as specific as claimed in the literature; the remaining clones from both libraries were most similar to 18S sequences from coral.

BACTERIA – Seven clone libraries from the 2004 cruise samples have been screened to date (Table 6.3, Fig. 6.6). Due to methodological problems, the first library (4753K4) had relatively few good quality sequences (i.e., >500 base pairs [bp] in length). This problem has been overcome, and a more extensive library is being generated for that sample. Additionally, clone libraries are being generated and screened for the nine fixed samples collected in 2005 (Table 6.1). Ninety-four percent of the bacterial 16S clones were novel, having less than 97%

(in most cases less than 89%) similarity to previously described sequences. Ten clones were so unusual that they had no real match in the database. The exceptions included a single clone with 99% similarity to a bacterial symbiont of the pink sugarcane mealybug (Franke-Whittle et al. 2004) and a single clone with 99% similarity to a butterfly gut bacterium (GenBank Accession DQ342884; no associated publication). One clone was 99% similar to a sequence from a glacier (Liu et al. 2006) and another single clone was 98% similar to a sequence from deep-sea sediment (Yanagibayashi et al. 1999). Also, there were 30 clones with 97-98% similarity to an uncultured *Colwellia* strain (Wang et al. 2006; Wang et al. 2005). Two clones were 94-95% similar to sequences derived from deep-sea octocorals (Penn et al. 2006).

Fifty-one percent of the clones were unknown *Firmicutes*, 38% were γ-*Proteobacteria*, and 8% were α-*Proteobacteria*. Twenty-seven percent of the clones were related to thiotrophic bacterial symbionts of methane seep clam species (e.g., *Calyptogena* spp. and *Bathymodiolus* sp.). One dominant ribotype (representing one species or a cluster of closely related species) accounted for all but two of these clones. This ribotype, designated VKLP1, was the numerically dominant clone in two libraries (4746K7 and 4746K9) and was present in all the *Lophelia* bacterial 16S libraries except for 4753K4. It's absence from this one library could be due to the small size of the library, but more likely is due to that library being the only one constructed from a sample not fixed at depth. This result corroborates with the DGGE results discussed above where differences were seen among samples fixed at depth versus those brought to the surface alive. Note that VKLP1 was present in the library 4753K3, generated from the same coral colony as sample 4753K4 (Table 6.1).

Unlike the culture-based data, the clone libraries show a definite difference in coral-associated bacterial communities between sites VK906/862 and VK826 (Fig. 6.6). The three libraries from VK906/862 (4746K1, 4746K7, 4746K9) are dominated by psychrophiles, VKLP1 and clones similar to squid symbionts. The four libraries from VK826 (4753K3, 4753K4, 4753K5, 4753K9) are dominated by a variety of unknown *Firmicutes*.

DISCUSSION

WHAT KINDS OF MICROBES ARE ASSOCIATED WITH *LOPHELIA?* – As expected, the majority of the cultured bacteria had very close matches (97-100%) to known bacteria in the genetic database and included many common marine genera (e.g., *Vibrio*, *Photobacterium*). However,

there were 11 cultured isolates whose closest match in the database ranged from 82-90% similarity. This is indicative of novel genera and at the lower end of the range, potentially novel families. These are bacteria unlike any that have been currently characterized using the 16S gene.

Of particular interest among these novel cultured bacteria are four isolates in the phylum *Firmicutes* (family *Entomoplasmataceae*; Table 6.2). These bacteria, within the class *Mollicutes*, lack a cell wall (Tully et al. 1993). Most known *Mollicutes* are primarily parasites, with hosts including plants, insects, animals, and humans. Many of the unusual sequences from the bacterial clone libraries generated from *Lophelia* samples are most similar to *Mollicutes* (family *Mycoplasmatacea*). Having bacteria in culture that are similar to the dominant uncultured bacterial community members provides a rare opportunity to learn more about the biogeochemical capabilities of these unknown microbes. Along the same lines, another cultured *Lophelia*-associated bacterium is 99% similar to clone 131851 (a bacterial DNA sequence) previously extracted from whale bone in the Antarctic at a depth of 560 m (Tringe et al. 2005). That study used a metagenomic approach to look for bacteria specialized for certain environments. Having a very similar cultured bacterium from *Lophelia* makes it possible to characterize the physiological capabilities of this type of microorganism.

In addition to providing insight into previously unknown microbial metabolic capabilities, novel bacteria are of interest as a source of new drugs and chemical compounds. For example, eight of the *Pseudoalteromonas* isolates (Table 6.2) were a close match (97-98% similarity) to *Pseudoalteromonas* sp. 520P1, which produces a purple pigment that is toxic to tumor cells (Enomoto et al. 2005). There may be other unique microbes with medicinal or biotechnological value within those identified in this study.

No obvious differences in the bacterial communities between the sites (VK906/862 and VK826) were discernable based on the culture data. It is difficult to determine if there is any significant temporal difference between the 2004 and 2005 samples, since more isolates (and consequently a larger number of species) were obtained in 2005.

While the cultured bacteria included a relatively large number of representatives of previously unknown or uncultured bacteria, there was still no overlap between the bacteria cultured and the bacteria detected in the clone libraries. This is typical (e.g., Rohwer et al. 2001)

of the two methods, which reveal completely different segments of the total bacterial community. Neither method is exhaustive which is why a combination is the best approach.

The clone libraries show very different bacterial communities associated with *Lophelia* at the two sites, VK906/862 and VK826 (Fig. 6.6). There are many factors that may influence this disparity, both physical and biological (Table 6.4). Of particular interest is that the libraries from VK826 are dominated by unknown *Firmicutes*, related to mycoplasmas. Most known mycoplasmas are pathogens, parasitizing the animal host's cells for nutrients they lack due to their small genome size and limited biosynthetic capabilities (Pitcher and Nicholas 2005; Rottem 2003). If the mycoplasma-like *Firmicutes* detected in all the clone libraries from VK826 (Table 6.3, Fig. 6.6) are in fact pathogens, this would be the first report of a disease affecting a deep-sea coral. The colonies showed no obvious lesions, but since the typical sign of disease in shallow-water corals is bleaching or discoloration (based on loss or distress of the zooxanthellae), there may not be visible signs in diseased *Lophelia*. Note that VK826 is the site where vibrios were cultured on TCBS medium from one sample (4753K6; Table 6.1). Bacterial communities have been shown to be different in diseased and stressed corals compared to healthy specimens (Pantos et al. 2003). Mycoplasmas are known to infect fish (Kirchhoff and Rosengarten 1984), and there were at least three species of fish that were significantly associated with *Lophelia* at VK826 that were rare or not observed at VK906/862 (Sulak et al., Chapter 2; this report). These include *Conger oceanicus*, which burrows into the *Lophelia* thickets and could transmit bacteria from lesions in the skin or gills, and *Grammicolepis brachiusculus*, which could transmit bacteria from its mouth while nibbling epibionts off of *Lophelia*. Similarly, there are other possible vectors, such as the gastropod, *Coralliophila abbreviata* and the undescribed species of *Periclimenid* shrimp, both found in association with *Lophelia* (Sulak et al., Chapter 3; this report). A marine fireworm has been described as a vector and reservoir for a bacterial pathogen of shallow-water coral (Rosenberg and Falkovitz 2004; Sussman et al. 2003). These are possibilities that remain to be investigated in the future.

Alternately, we must consider that these novel mycoplasma-like *Firmicutes* may not be pathogens, but might in fact be an unknown symbiotic or commensal type of *Firmicute* that makes up a normal part of the *Lophelia*-associated microflora. In support of this possibility is the fact that sequences of unknown *Firmicutes* (including mycoplasma-like sequences) have been found in association with apparently healthy deep-sea octocorals (Kellogg et al. 2005; Penn

et al. 2006). Their presence at VK826 and absence at VK906/862 might be linked to a physical factor; e.g., temperature variability or thermal stress at VK906/862 suppresses their growth. Or perhaps the difference in bacterial communities between the two sites is linked to factors intrinsic to *Lophelia*; e.g., biochemical or metabolic differences that also affect the morphology and degree of calcification (Table 6.4).

Screening additional libraries from different *Lophelia* colonies collected in 2005 at these sites will help to determine whether these mycoplasma-like bacteria are pathogens or normal microflora. If the same patterns seen in 2004 emerge, it suggests a biogeographic effect. Ultimately, it would be valuable to have comparable *Lophelia* samples from other geographic areas, such Green Canyon off the coast of Louisiana (Schroeder et al. 2005), the West Florida slope and the U.S. Atlantic slope (Reed et al. 2006), to more clearly determine what constitutes the "typical" bacterial community of *Lophelia* and how much of an effect geography has on the make-up of those communities.

ARCHAEA – Both this study and another (Yakimov et al. 2006) have tried to detect archaeal associates of *Lophelia* without success. Different methods were applied to the collection, extraction, and amplification of the microbial DNA samples, suggesting the problem is not linked to a particular technique. Archaea have been isolated from shallow-water corals (Kellogg 2004; Wegley et al. 2004), sediments near cold seeps (Knittel et al. 2005), and sediments near *Lophelia* (Yakimov et al. 2006). Archaea have been found to be abundant in cold, pelagic waters (DeLong et al. 1994; Fuhrman and Ouverney 1998). Thus it seems likely that archaea are present on *Lophelia*. The lack of detection may be due to the archaea being so unusual that standard archaeal primers do not amplify them.

FUNGI – This is the first report to use molecular techniques to detect the presence of coral-associated fungi. All previous studies have depended on culturing the fungus for identification by microscopy or histological examinations to look for fungal hyphae in coral sections (Bentis et al. 2000; Freiwald et al. 1997; Freiwald and Wilson 1998; Kölliker 1859; Le Campion-Alsumard et al. 1995; Nagelkerken et al. 1997; Priess et al. 2000; Raghukumar and Raghukumar 1991; Ramos-Flores 1983; Ravindran et al. 2001; Wisshak et al. 2005). The main limitation to employing molecular techniques to detect fungal associates is that the diagnostic gene of choice, the 18S rRNA gene, is present in both the coral and the fungi. Even primers intended to be specific for fungi amplify the coral 18S gene on occasion because the conserved

portions of the 18S gene are similar between these distinct organisms. In spite of this shortcoming, a fungus similar to *Paecilomyces* sp. and an *Acremonium* sp. was revealed to be associated with living *Lophelia*.

HOW DO THESE MICROBIAL COMMUNITIES COMPARE TO THOSE OF SHALLOW-WATER CORALS OR DEEP-SEA GORGONIANS? – A recent study that cultured bacteria from the mucus of the Caribbean elkhorn coral, *Acropora palmata*, employed a novel selection method in an effort to distinguish between 'residents' (bacteria that have a mutually beneficial relationship with the coral) and 'visitors' (bacteria that have become trapped in the coral mucus and may become invasive) (Ritchie 2006). The method involves amending nutrient agar with UV-sterilized coral mucus; the idea being that 'resident' bacteria will be immune to any antibacterial compounds in the mucus while 'visitors' will be inhibited. The 'resident' pool was enriched with *Halomonas* sp., *Alteromonas* sp., and particularly *Photobacterium* sp., including *P. leiognathi* (Ritchie 2006). These genera (and that particular species) were all detected in culture-based analyses of *Lophelia* (Table 6.2), suggesting a coral-specific role for these types of bacteria.

One of the bands excised from the DGGE (Fig. 6.5b, green box) produced a sequence that was 99% similar to a bacterial sequence that had been extracted from a shallow-water coral in the Mediterranean (Koren and Rosenberg 2006). This also lends support to the idea of coral-specific (or scleractinian-specific) bacterial species.

Molecular analyses of shallow-water corals have uncovered species-specific associations between corals and bacteria: an α-*Proteobacterium* (PA1) has been found consistently associated with *Porites astreoides*, a closely related bacterium PF1 with *P. furcata* (Rohwer et al. 2002); and a *Silicibacter* sp. with *Montastraea franksi* (Rohwer et al. 2001). This study has found a γ-*Proteobacterium*, VKLP1, associated with all *Lophelia* samples analyzed to date. VKLP1 appears to be a *Lophelia*-specific bacterium.

Penn et al. (2006) used culture-independent techniques to examine the bacteria associated with black coral and bamboo coral collected from seamounts in the Gulf of Alaska. The study identified 19 phyla, dominated by *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Acidobacteria*. Detailed listings of the similarity between these sequences and known organisms in genetic databases were not offered, but the limited discussion suggests that most of the sequences from these deep-sea soft corals were unfamiliar. The bacterial 16S rDNA clone libraries in my study

were also dominated by γ-*Proteobacteria*, α-*Proteobacteria* and *Firmicutes*, and two clones were similar to clones from Penn et al.'s study: two α-*Proteobacteria* from VK906/862 (94-95% similarity).

The only published study of microbes associated with a deep-sea scleractinian coral focused on *Lophelia pertusa* in the Mediterranean Sea (Yakimov et al. 2006). Employing a different culture-independent technique than Penn et al.'s or my study, these authors found that the majority of the bacterial sequences were related to the *Holophaga-Acidobacterium* and *Nitrospira* but 12% of their sequences were completely novel α-*Proteobacteria*. The bacterial community associated with living *L. pertusa* was significantly different from that of dead coral or nearby sediments. There was no overlap between the bacterial 16S rDNA sequences from the Mediterranean *Lophelia* and my study. This may be due to methodological differences (e.g., coral collected differently, different molecular protocols used) or could indicate biogeographical differences in *Lophelia*-associated bacterial communities.

IS THERE A DIFFERENCE IN THE MICROBIAL COMMUNITIES BETWEEN CORAL SAMPLES PRESERVED AT DEPTH AND THOSE BROUGHT TO THE SURFACE ALIVE? – While is it necessary for culture-based assays to bring the coral samples to the surface alive, there was a question as to whether the entire microbial community (i.e., that detected by cultivation-independent molecular techniques) might be affected as a result of the changes in the environment during collection. The DGGE comparison between pairs of samples from the same coral colony (one brought up alive, one preserved at depth immediately after collection) showed that there can be a significant difference (Fig. 6.5b). Sequencing of some of the bands in the gel further proved this point: All the bands in Lane 10 (a live sample) were different from the dominant bands in the corresponding sample that was preserved at depth (Fig. 6.5b). This is the first study of deep-sea coral microbial ecology that has preserved samples at depth to investigate this issue. Additionally, this is the only deep-sea coral study to use insulated, individual containers to minimize cross-contamination and thermal shock. The two published studies that have examined bacterial diversity associated with deep-sea corals, Alaskan soft corals (Penn et al. 2006) and Mediterranean *Lophelia* (Yakimov et al. 2006), did not take these precautions, and therefore their data may not accurately reflect the *in situ* bacterial community of the corals.

ARE THESE MICROBES ACTING AS SYMBIONTS? – It is impossible to categorically answer this question without additional research. However, the current data strongly supports the possibility that the *Lophelia*-associated bacteria are in fact acting as symbionts. Several of the cultured *Vibrio* sp., *Shewanella* sp. and most of the *Photobacterium* sp. (Table 6.2) are close matches to species that are symbionts of fish or squid. Nearly a third of the 16S rDNA clones were similar to gill symbionts of seep clam species, and an additional 7% were similar to squid symbionts (Fig. 6.6). There are also two clones that are 99% matches to insect symbionts, plus several clones that are similar to bacteria associated with a deep-sea sponge, a deep-sea shrimp and a sea squirt (ascidian) (Fig.6). In particular, the detection of ribotype VKLP1 (a γ-*Proteobacterium* similar to seep clam symbionts) in all five libraries from fixed corals, dominant in two, suggests that VKLP1 is a *Lophelia*-specific symbiont.

The presence of VKLP1 and other clones that are related to sulfer-oxidizing and methane-oxidizing symbionts of seep invertebrates introduces a microbiological link between *Lophelia* and seep communities. A previously proposed connection between the coral and areas of seepage, known as the 'hydraulic theory,' suggested that microbes in the water column would utilize hydrocarbons (such as methane) in areas of seepage, and cause a localized enrichment, either by providing the corals with a direct source of nutrition, or as the base of a food web that would ultimately benefit the corals (Hovland 1990; Hovland et al. 1998; Hovland and Risk 2003; Hovland and Thomsen 1989; Hovland and Thomsen 1997; Hovland et al. 2002). This theory was intended to explain the distribution patterns of *Lophelia* in Norway, including the frequent co-location of Lophelia reefs and hydrocarbon seeps or hydrocarbon-enriched sediments. However, other studies have suggested that the link is not biological but geological; that *Lophelia* chooses to settle near seeps because the presence of hydrocarbon-associated authigenic carbonate rocks or the shells of seep species provide a substrate for *Lophelia* to colonize (Roberts and Aharon 1994; Rogers 1999). Two studies that measured the $\delta^{13}C$ in *Lophelia* tissue (from corals in Norway and off of Spain) did not find a methane signature (Duineveld et al. 2004; Mikkelsen et al. 1982), which argues that seep carbon is not being incorporated by the corals. However, the presence (and in some cases, numerical dominance) of VKLP1 suggests that there may be other unknown mechanisms connecting the two ecosystems.

Coral microbial ecology is a relatively new field. The first use of molecular techniques to study coral-associated bacteria was just published in 2001(Rohwer et al. 2001). Although the

majority of known coral species occur in deep water (Roberts and Hirshfield 2004), there are only two published reports about bacteria associated with deep-sea corals (Penn et al. 2006; Yakimov et al. 2006). This report documents the first survey of *Lophelia*-associated microbes in the Gulf of Mexico and is the only deep-water study to date to collect the specimens in insulated, individual containers to minimize sample contamination and thermal stress. The results revealed many novel bacteria, including the first example of a species-specific association between a deep-sea coral and a bacterium (VKLP1), and the first identification of a fungal associate of a living deep-sea coral. Additionally, the similarity of many of the *Lophelia*-associated bacteria (including VKLP1) to seep invertebrate symbionts has established a previously unknown link between *Lophelia* and seep communities. The clear difference seen in the clone libraries between the *Lophelia*-associated bacterial communities at VK826 and VK906/862 suggest either a biogeographic difference between the two sites or a stress response at one site. The integrated nature of the larger project has provided critical and necessary information, such as coral genetic data, meio- and macro-faunal associations, and physical parameters, all of which are necessary to connect the microbiological data to the ecology of *Lophelia*.

RECOMMENDATIONS

Future microbial ecology studies on deep-sea corals should incorporate specialized sampling gear (e.g., the Kellogg Sampler) to minimize contamination and reduce thermal shock. At minimum, this should entail insulated separate chambers for each coral sample.

Previous studies on coral-associated microbes have suggested that the best strategy is to employ molecular techniques first and then based on the sequences obtained, optimize culture conditions for the detected groups (Rohwer et al. 2001). Additional culture work using media specific to sulfur-oxidizers and methanotrophs is necessary to understand the dominant *Lophelia*-associated bacteria. Attempts should also be made to culture *Lophelia*-associated fungi.

Additional samples of *Lophelia* from other geographic locations are needed to clarify biogeographic variability of the associated bacterial communities. The West Florida slope in particular has been previously described as having “tremendous potential for unexplored coral and fish habitat” (Reed et al. 2006). Likewise there is a large multi-disciplinary database being processed on the extensive deep coral habitats of the Blake Plateau (S.W. Ross et al., unpubl. data), and comparable microbial studies through this diverse region would be of critical

importance. It is of particular interest to pursue further links between *Lophelia* and seeps, in the context of VKLP1, an apparently *Lophelia*-specific bacterium related to seep clam symbionts.

Future studies of *Lophelia*-associated microbes should remain closely linked to concurrent studies of associated physical and biological parameters. Temperature and depth trends may be major factors in the biogeographic differences seen in the *Lophelia*-associated microbial communities characterized in this study. It is also valuable to have genotype information for the corals sampled for microbial ecology to determine if there is a genetic bias toward certain microbial communities. If a disease agent is present, it may be due to a genetic susceptibility and the evidence may be visible through histological examination.

If a pathogen or symbiont is transmitted by a vector, it is invaluable to understand the interactions of potential vectors (i.e., fish, invertebrates, and microfauna) with *Lophelia*. Because of the possibility that corals may receive some of their microbial communities through contact with other animals, research should be initiated on selected dominant fauna that have direct contact with the corals. Such animals include conger eels, other eels, scorpaenid fishes, galathaeoid squat lobsters, various echinoderms, and hydroids.

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DISCLAIMER

Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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Table 6.1. *Lophelia pertusa* samples collected in the Gulf of Mexico for microbiological analyses.

Collection date[a]	Dive #	General area	Lat/Long	Lophelia samples[b]	Collection time (h)	Depth (m)	Temp (°C)	Salinity (ppt)	Genetics[c]	Histology[d]
July 31,	JSL	Viosca Knoll	N 29:06.4253	4746K1	10:59	311.5	10.7	35.3	✔	
2004	4746	906/862	W 88:23.0651	4746K2	11:01					
			N 29:06.3951	4746K3	10:28	312.4	10.6	35.0	✔	
			W 88:23.0521	4746K4	10:25					
			N 29:06.3800	4746K5	10:09	315.8	10.5	35.3	✔	
			W 88:23.0318	4746K6	10:05					
			N 29:06.4016	4746K7	09:44	310.3	10.7	35.3	✔	
			W 88:23.0418	4746K8	09:40					
			N 29:06.3766	4746K9	08:52	319.4	10.5	35.3	✔	
			W 88:23.0395	4746K10	08:46					
August 3,	JSL	Viosca Knoll	N 29:10.1901	4753K1	18:02	476.4	7.7	35.0	✔	
2004	4753	826	W 88:00.7029	4753K2	18:02					
			N 29:10.2439	4753K3	17:39	460.6	7.8	35.0	✔	
			W 88:00.6962	4753K4	17:39					
			N 29:10.2254	4753K5	17:19	461.2	7.7	35.0	✔	
			W 88:00.7295	4753K6	17:19					
			N 29:10.2148	4753K7	16:53	468.5	7.7	35.0	✔	
			W 88:00.7416	4753K8	16:53					

Table 6.1 (continued)

Collection date[a]	Dive #	General area	Lat/Long	Lophelia samples[b]	Collection time (h)	Depth (m)	Temp (°C)	Salinity (ppt)	Genetics[c]	Histology[d]
			N 29:10.1894	4753K9	16:28	474.3	7.6	35.0	✔	
			W 88:00.7746	4753K10	16:28					
September	JSL	Viosca Knoll	N 29:06.4	4873K1	10:53	314.6	11.0	35.3		✔
16, 2005	4873	906/862	W 88:23.1	4873K2	11:17					
			N 29:06.4	4873K3	11:39	314.6	11.0	35.0		
			W 88:23.0	4873K4	12:01					
			N 29:06.4	4873K5	12:21	314.6	11.0	35.0	✔	
			W 88:23.0							
September	JSL	Viosca Knoll	N 29:10.2139	4878K1	17:02	465.1	8.9	35.1	✔	✔
18, 2005	4878	826	W 88:00:7098	4878K2	17:12					
			N 29:10.2070	4878K3	17:29	461.8	8.9	35.1	✔	✔
			W 88:00.7139	4878K4	17:36					
September	JSL	Viosca Knoll	N 29:09.5829	4881K-1	08:43	453.5	9.0	35.0		✔
20, 2005	4881	826	W 88:01.1313	4881K-2	08:51					
			N 29:09.5972	4881K-3	09:05	453.2	9.0	35.0		
			W 88:01.1716	4881K-4	09:10					

Table 6.1 (continued)

Collection date[a]	Dive #	General area	Lat/Long	Lophelia samples[b]	Collection time (h)	Depth (m)	Temp (°C)	Salinity (ppt)	Genetics[c]	Histology[d]
			N 29:09.6037	4881K-5	09:18	453.2	9.0	35.0		✔
			W 88:01.1570	4881K-6	09:27					
			N 29:09.6219	4881K-7	09:37	453.5	9.0	35.0		✔
			W 88:01.1537	4881K-8	09:45					
			N 29:09.6168	4881K-9	10:03	453.2	9.0	35.0		✔
			W 88:01.1544	4881K-10	10:11					

[a]Cruises: USGS-GM-2004-03, July 29-August 5, 2004; USGS-GM-2005-04, September 15-21, 2005.

[b]Note: Paired samples were collected from the same *Lophelia* colony; odd-numbered samples were preserved at depth with a fixative solution for later processing by molecular techniques, even-numbered samples were kept alive for culture-based analyses.

[c]*Lophelia* provided to the USGS genetics group (King, Morrison, Schill) for their analyses. During the 2005 cruise a new collection technique was used that, while faster and less prone to damaging the *Lophelia* colony, resulted in much smaller samples. As such there was not always a large enough *Lophelia* sample to divide and share with the genetics group.

[d]*Lophelia* provided to the International Registry of Coral Pathology (IRCP) at the NOAA, NOS, NCCOS, CCEHBR, Cooperative Oxford Laboratory, 904 South Morris Street, Oxford, Maryland, 21654, USA [contact person: Dr. Shawn McLaughlin]. The samples were from healthy specimens and were submitted to provide baseline samples for the registry.

Table 6.2. Summary of cultured *Lophelia*-associated bacterial isolates.

Genus	**Notes**	**# Species**	**# Isolates**
Achromobacter	β-*Proteobacterium*	1	1
Alteromonas	This species makes a melanin-like pigment	1	1
Cobetia	Similar to species isolated from deep-sea sediments	4	8
Colwellia	Typically psychrophiles; some species are barophiles	1	1
Halomonas	Halophilic and psychrophilic, γ-*Proteobacteria*	1	5
Moritella	Similar to an isolate from Atlantic cod	1	1
Photobacterium	Similar to luminous fish symbionts; commonly cultured from shallow-water corals	11	68
Pseudoalteromonas	Psychrophiles, similar to isolates from sea-ice and deep-sea sediments	15	44
Pseudomonas	99% similar to a *Pseudomonas* isolated from hydrocarbon-contaminated soil	1	1
Psychrobacter	Psychrophiles, similar to isolates from polar pack ice	2	5
Shewanella	Psychrophiles, similar to squid symbionts and isolates from a deep-sea sponge	5	8

Table 6.2 (continued)

Genus	**Notes**	**# Species**	**# Isolates**
Vibrio	Includes isolates similar to fish symbionts and isolates associated with marine invertebrates (oysters, clams, sea urchins)	14	19
Previously uncultured bacterial clone 131851	From a metagenomics study of bacteria associated with a whale bone at 560 m depth	1	1
Unknown (Family *Alteromonadaceae*)[a]	Nearest genetic match is a silica regenerating bacterium found associated with diatoms	1	6
Unknown (Family *Entomoplasmataceae*)[a]	*Firmicutes*	1	4
Unknown (Family *Vibrionaceae*)[a]	Most similar to psychrophiles isolated from sea ice	1	1

[a]The closest matches to these sequences ranged from 82-90% similarity. A similarity score in this range indicates that the isolates are related to this group, but they represent undescribed (new) genera.

Table 6.3. Summary of *Lophelia*-associated bacterial 16S rDNA clone libraries.

Sample	Site	Number of clones sequenced	Number of sequences ≥500 bp
4746K1	VK906/862	96	74
4746K7	VK906/862	91	80
4746K9	VK906/862	64	53
4753K3	VK826	109	93
4753K4[a]	VK826	100	31
4753K5	VK826	147	120
4753K9	VK826	96	96

[a]This sample is from the same coral as 4753-K3, however, it was not preserved at depth.

Table 6.4. Comparison of physical and biological factors between sites VK906/862 and VK826.

Factor	Viosca Knoll 906/862	Viosca Knoll 826
Bacterial community[a]	Dominated by symbiont-types	Dominated by *Firmicutes* (mycoplasma-like)
Depth	325m horizon	500m horizon
Temperature	>10˚C	<10˚C
Lophelia architecture	Individual clumps	Extensive thickets
Lophelia morphology	Delicate branches	Thick, heavily calicified
Seep proximity	No seep	Localized seepage
Fish statistically associated with *Lophelia*[b]	None	*Conger oceanicus* *Grammicolepis brachiusculus* *Hoplostethys occidentalis*

[a]Based on clone libraries
[b] Sulak et al. (Chapter 2, this report)

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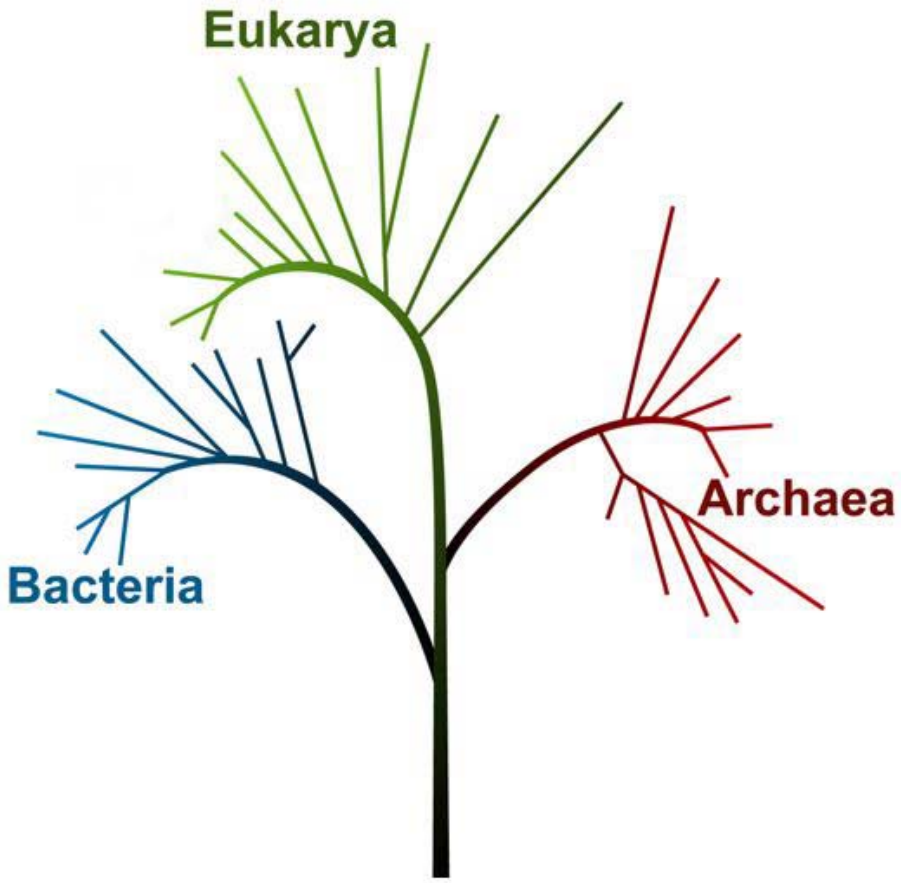

Figure 1. The tree of life, based on the 16S ribosomal RNA (rRNA) gene. The three domains include all the kingdoms of organisms except for viruses (which lack rRNA genes). The domain *Bacteria* includes the true bacteria. *Archaea* are prokaryotes (cells with no nucleus) similar in appearance to bacteria but genetically and biochemically more similar to eukaryotes (organisms with cells that contain a true nucleus). *Eukarya* includes multicelluar organisms such as fungi, plants and animals. Graphic design by Betsy Boynton.

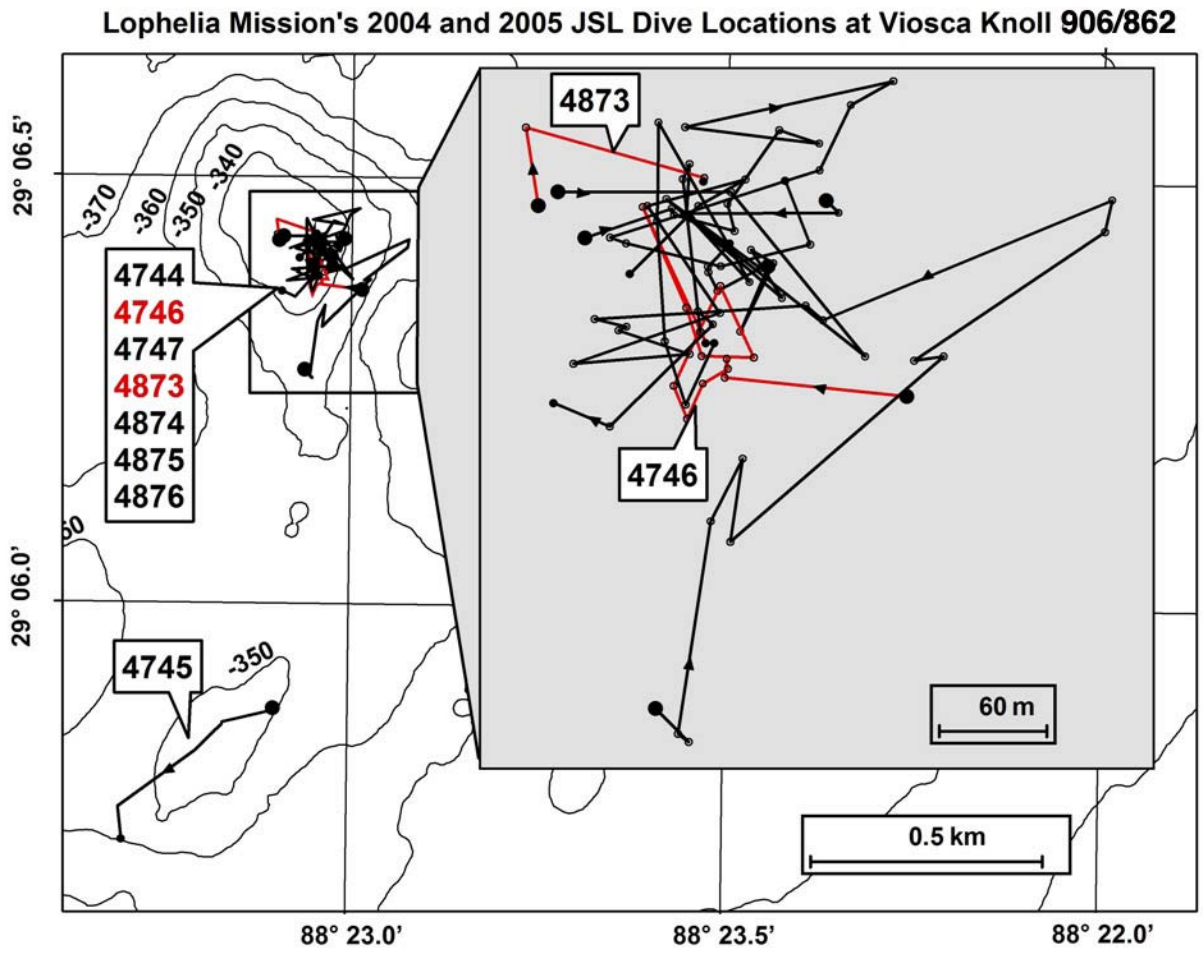

Figure 6.2. 2004 and 2005 submersible dive tracks at Viosca Knoll 906/862 with the microbial ecology dives (4746 in 2004, 4873 in 2005) highlighted in red.

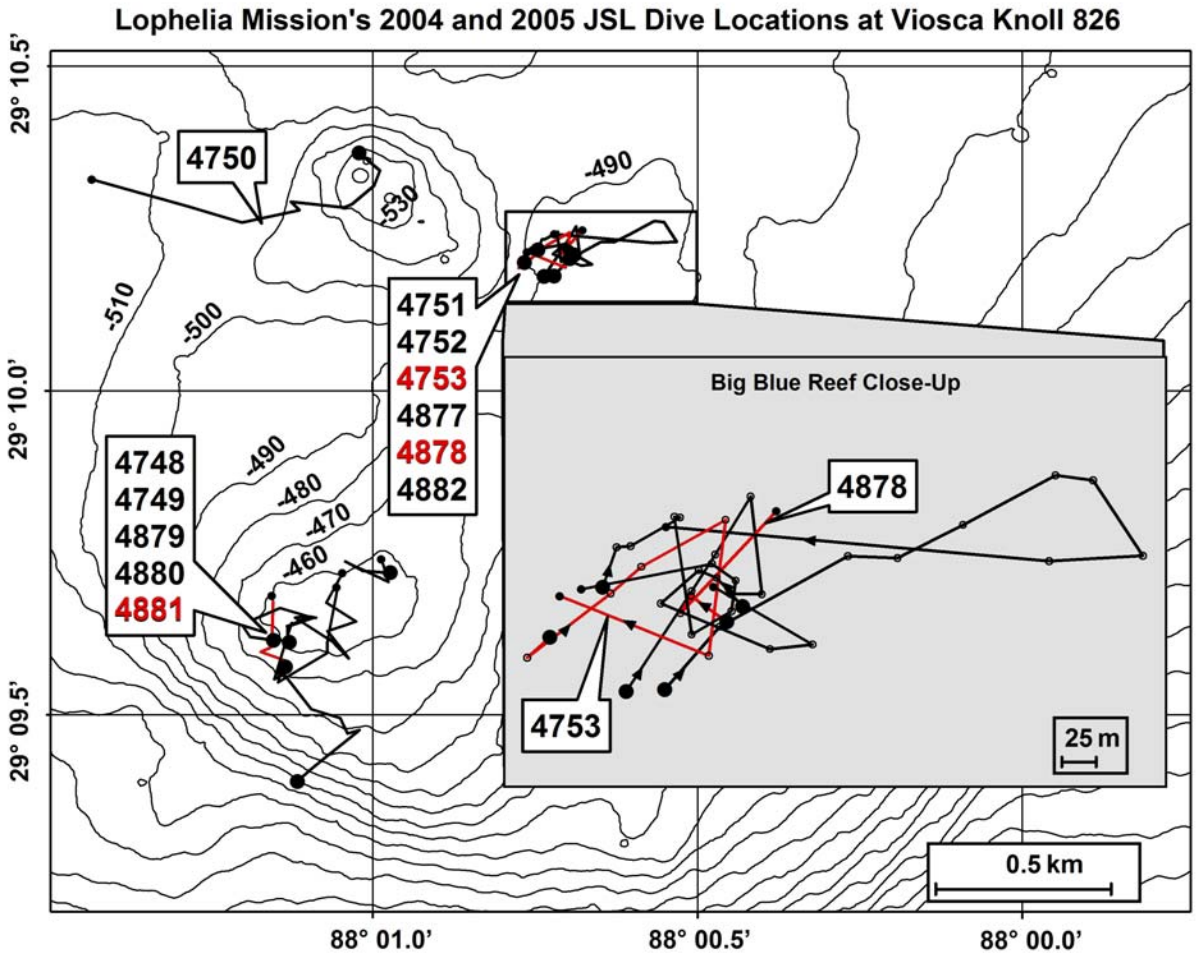

Figure 6.3. 2004 and 2005 submersible dive tracks at Viosca Knoll 826 with the microbial ecology dives (4753 in 2004, 4878 and 4881 in 2005) highlighted in red.

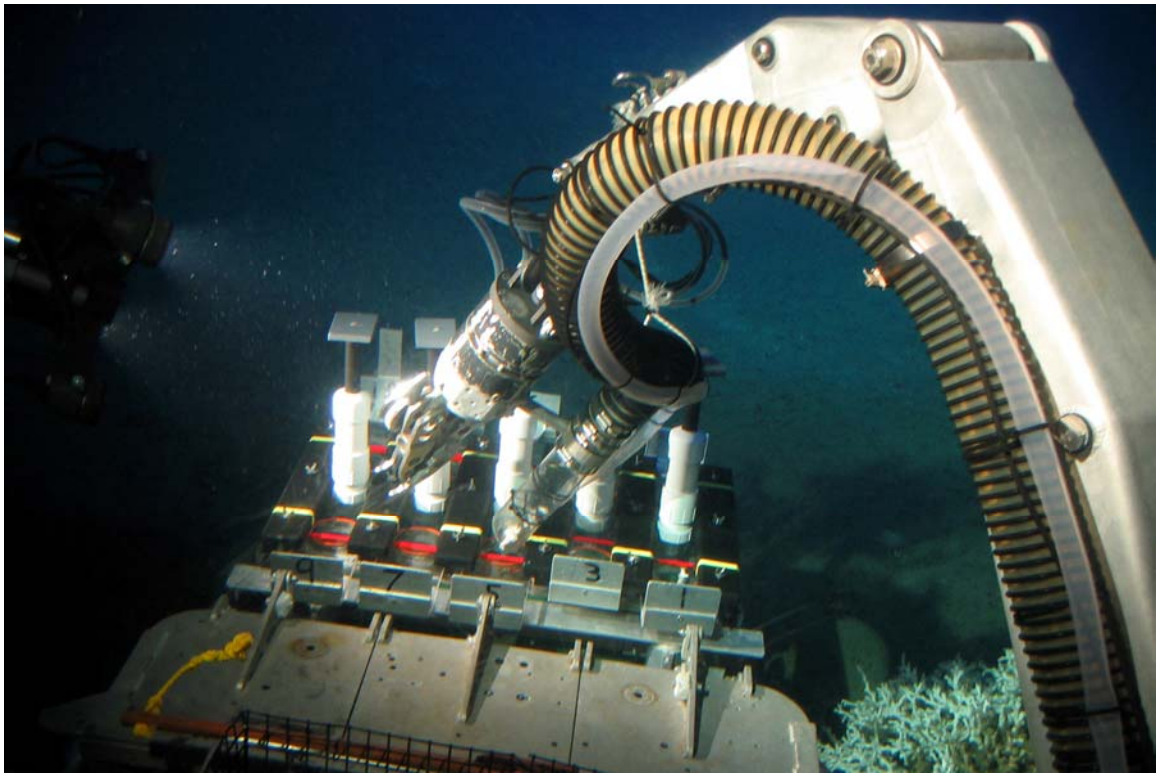

Figure 6.4. The 'Kellogg Sampler'. This specially designed microbial isolation/sterility box with multiple chambers is used to collect discrete *Lophelia* samples for coral microbial ecology experiments.

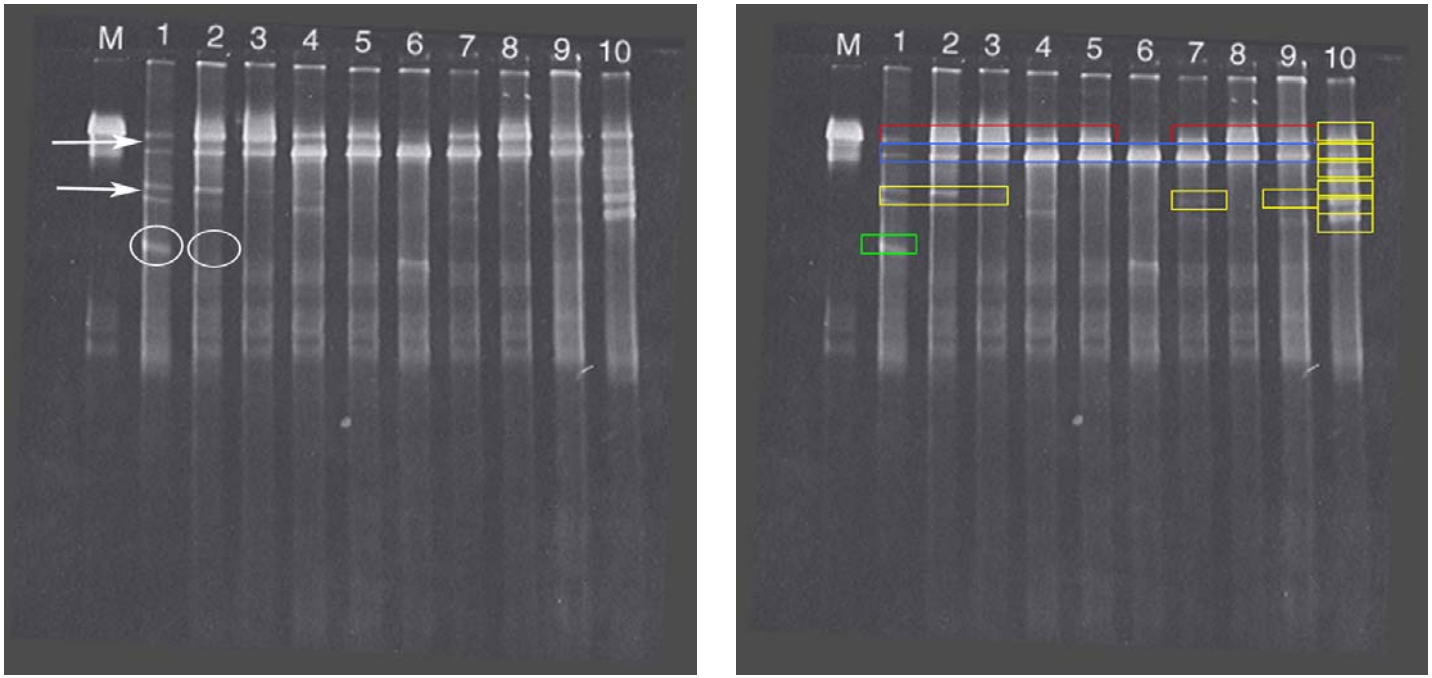

Figure 6.5. Denaturing gel gradient electrophoresis (DGGE) of *Lophelia* bacterial communities. Lane M is a standard marker. The pairs of lanes represent pairs of *Lophelia* samples. The odd-numbered lanes are bacterial communities from *Lophelia* samples preserved at depth; the even-numbered lanes are bacterial communities from the same *Lophelia* colony without preservative. Panel A: The white arrows indicate two prominent doublets that are present in many of the samples, indicating similarity of the bacterial communities between these coral colonies. The white circles highlight the presence of a band in the fixed sample but its absence in the corresponding live sample. Panel B: Bands enclosed in colored boxes were cut out of the gel and sequenced. Red boxes indicate a sequence similar to a *Shewanella* sp., blue boxes indicate a sequence similar to a mycoplasma, yellow boxes indicate a sequence similar to clone MSB, and the green box indicates a sequence 99% similar to a bacterial clone from the shallow-water coral *Oculina patagonica*.

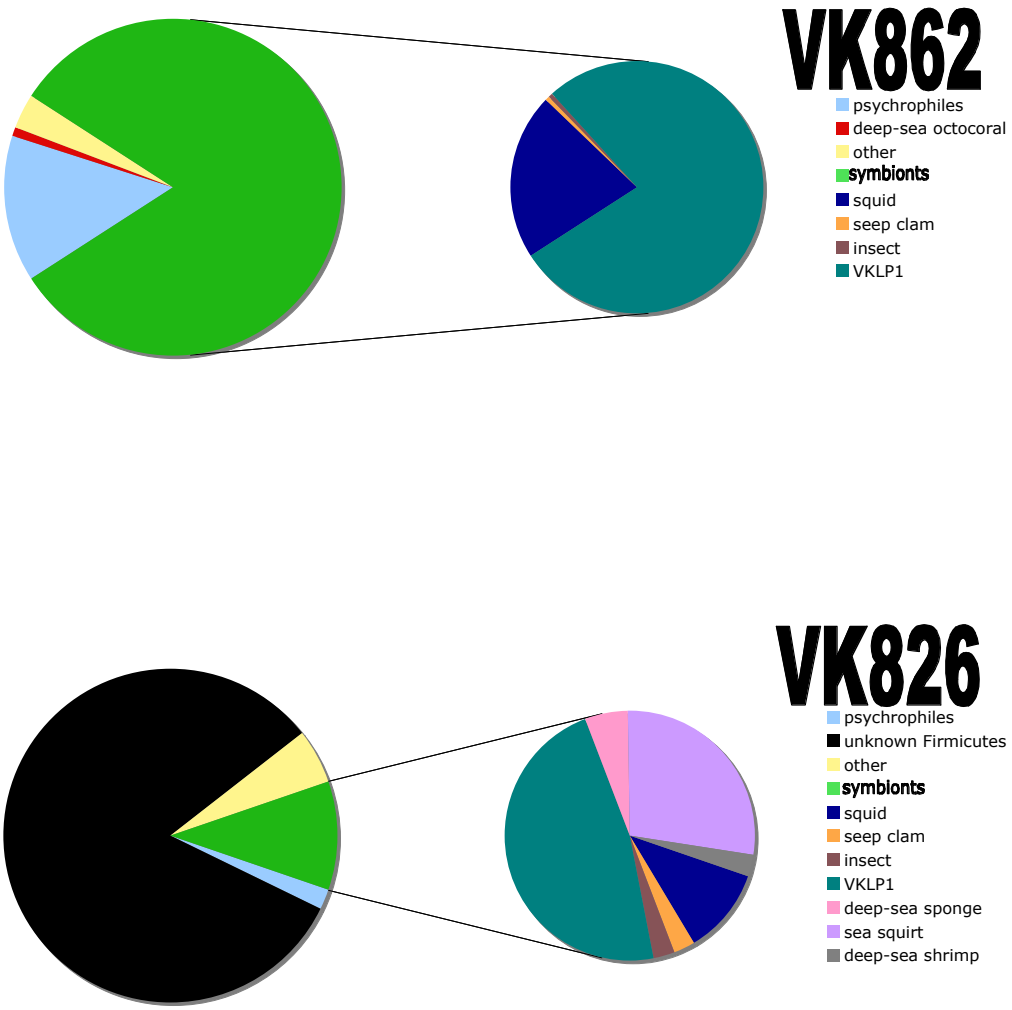

Figure 6.6. Comparison of bacterial 16S clone libraries between the two sites. The pie charts on the left are summaries of all clones from all libraries at each geographic site: VK906/862 (3 libraries, 206 clones) and VK826 (4 libraries, 328 clones). The smaller pie charts on the right are more detailed breakdowns of the "symbiont" portion of the clones (shown in green on the left charts). These are sequences most closely related to bacterial symbionts of other marine invertebrates and insects.