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Characterization of Microbial Community Structure in the Octocoral *Leptogorgia virgulata*

by

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Honors Thesis

in

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Abstract

The process of spicule formation in *L. virgulata* results in a drop in pH, which, if left unregulated, could lead to demineralization and subsequent animal death. Carbonic anhydrase has been suggested as a possible pH regulatory mechanism in *L. virgulata* tissues. This study focuses on an additional hypothetical mechanism of pH regulation involving the production of urease by endosymbiotic bacteria living within *L. virgulata* tissue. PCR and DGGE are used as culture-independent methods to characterize facets of microbial community structure on *L. virgulata* in order to identify one or many urease-producing endosymbionts. DGGE analysis shows high diversity among the microbial community within *L. virgulata* and varying community structure on different tissue types. Future sequencing of bacterial 16S rDNA fragments will yield significant clues about the possible bacterial relationship involved in *L. virgulata* spicule development.

Introduction

Leptogorgia virgulata is a member of a group of organisms in the phylum Cnidaria called octocorals. Commonly found in sub-tidal waters of the mid-Atlantic United States, *L. virgulata* is significantly involved in shallow water ecosystem inter-organismal interactions. *L. virgulata* contains an internal central axis, a coenenchyme, and polyps that extend into the environment which take in and expel nutrients and other material. Within the coenenchyme are small polycrystalline aggregates of calcite spicules (Ruppert and Fox, 1988). Spicules function as skeletal structures that support and protect the colonial structure of the animal polyps, and are therefore essential to the survival of the animal (Bayer, et al., 1983). In summary, spicule formation is initiated within the spicule-forming vacuole of a scleroblast. During spicule

production, the vacuole enlarges due to the incorporation of proteinaceous matrix and calcium carbonate crystals. The vacuole then fuses with the scleroblast membrane, releasing to the extracellular environment a fully-developed spicule (Kingsley and Watabe, 1984).

All organisms that calcify, including *L. virgulata*, experience mineral formation and a subsequent drop in pH due to acid production (equation 1). If the acid is not removed, demineralization occurs, resulting in malformation or destruction of spicules. The enzyme carbonic anhydrase as well as proton pumps within coenenchyme cell cytoplasm and membranes, have been shown to aid in the regulation of cellular pH during the calcification process in octocorals (Kingsley and Watabe, 1987). It is thought that other mechanisms, such as the function of an enzyme, facilitate pH balance during *L. virgulata* development (Kingsley and Watabe, 1987).



The enzyme urease is known to directly control pH through the protonation of produced ammonia (Stingl et al., 2002). Bacterial production of urease has been shown to aid in pH regulation within eukaryotic cell systems (Stingl, et al., 2002). When urease interacts with its substrate (i.e., urea), ammonia is produced. Ammonia is then able to bind to free-floating H⁺ ions created during spicule formation. Several species of bacteria and some species of plants are known to produce urease (Sumner, 1926 and Mobely, et al., 1995); it would be very unusual for *L. virgulata*, an animal, to independently produce this enzyme. Therefore, it is hypothesized that a bacterial symbiont residing within *L. virgulata* is producing sufficient quantities of urease to aid in spicule development. Identification of a urease-producing bacterial endosymbiont of *L. virgulata* would be the first documented for the animal.

Bacteria are known to reside within *L. virgulata* tissue (Fig. 1). In fact, it has been shown that *L. virgulata* produce homarine as an antimicrobial compound (Shapo, 2007), most likely to evade bacterial disease. This suggests that *L. virgulata* developed an evolutionary response through long-term interaction with one or more parasitic microbes. Similarly, it is entirely possible that *L. virgulata*'s continuing interaction with surrounding microbes has fostered one or

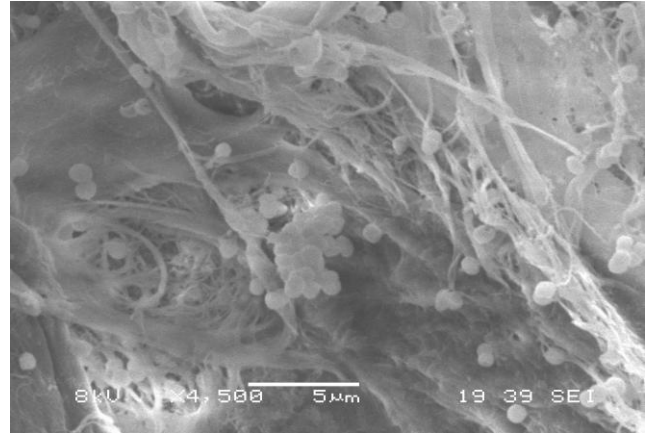


Figure 1: Scanning electron micrograph of bacteria in the gastroventricular cavity of *L. virgulata* (Roni J. Kingsley, unpublished)

many commensal or mutualistic symbiotic relationships—relationships that could involve urease exchange. A number of symbiotic relationships with eucaryotes are well known in *L. virgulata*. Although the octocoral produces prostaglandin-like toxins to protect itself from predators such as the mollusc *Simnialena uniplacata*, (Gerhart, 1991), the sea whip barnacle *Conopea galeata* and the Atlantic pearl oyster *Pteria colymbus*, organisms are still able to attach to *L. virgulata* individuals (DeVictor, 2009). Additionally, several species of bryozoans, including *Alcyonidium hauffi* and *Membranipora arborescens*, have been observed to use the *L. virgulata* epidermal layer as a substrate for colonization (DeVictor, 2009). In general, little is known about the specific benefits or drawbacks that are obtained from *L. virgulata* attachment (Fox and Ruppert, 1985). However, the very presence of other organisms on *L. virgulata* tissue suggests that the animal has the potential to engage in commensal or mutualistic relationships with other intertidal organisms

This study aims to characterize the microbial community structure within *L. virgulata* tissue in order to identify possible urease-producing endosymbionts. In the past, the description

of marine microbial diversity has presented challenge. Obtaining pure cultures of bacterial communities present in oligotrophic marine environments is often hindered by unknown nutrient requirements needed for the bacteria to grow *in vitro*. As a result, phenotypic characterization of many marine bacteria remains unknown (Schut, 1997). To overcome this limitation, we used a culture-independent polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) method in an attempt to describe *L. virgulata* microbial community structure. The amplification and analysis of 16S rDNA fragments has proven to be invaluable in the attempts to completely characterize microbial communities (Woese, 1987). DGGE is a method used to separate bands of DNA based on their guanine and cytosine content, not fragment size (unlike traditional polyacrylamide gel electrophoresis methods). DGGE is known to have a high sensitivity for separating sequence differences, which gives it the ability to illustrate genetic diversity within a microbial community (Muyzer, et al., 1993). A distinct band on a DGGE theoretically represents the genetic material of one colony of bacteria (Muyzer and Smalla, 1998). A pattern of bands obtained from bacteria on *L. virgulata* tissue indicates the microbial diversity and general community structure within *L. virgulata*. Subsequent sequencing of the genetic fragments in specific bands can lead to genomic and phenotypic characterization of bacterial community members.

Genotypic data obtained from this method was supported through a urease test, which detects the urease-producing phenotype among culturable isolates. Detection of urease production among culturable strains could aid in the process of matching phenotypic urease-producing traits to genotypic characteristics of bacterial strains unable to be obtained in culture. Additionally, finding urease production among bacterial strains would strongly support the hypothesis that *L. virgulata* utilize bacterial-supported pH regulation during spicule development.

The characterization of the microbial community structure within *L. virgulata* tissue can yield significant clues about possible undescribed pH regulation mechanisms that *L. virgulata* use during spicule development. Such a discovery would greatly contribute to the known anatomical and physiological features of *L. virgulata* and other species of octocoral. Additionally, description of the microbial ecosystem within *L. virgulata* could facilitate general hypotheses concerning the specific ecological roles of intertidal marine bacteria.

Methods

Specimen Collection

Individual colonies of *Leptogorgia virgulata* were collected from shores off of Beaufort, and Moorehead City, North Carolina. Individual octocorals were identified based on characteristic size, color (orange and yellow), and polypoid structures. The octocorals were transported in aerated seawater collected from the collection site. A few octocoral tissue samples were placed in 2% gluteraldehyde fixative and stored at -20°C. The remaining octocoral individuals were placed in a marine tank at the University of Richmond animal facility.

Obtaining culturable bacterial isolates

A 1.0 g sample was cut from both orange and yellow octocoral individuals. Samples were then cut in a into 1.0 cm pieces and placed into individual 1.5 mL microcentrifuge tubes containing 1.0 mL filtered seawater (labeled “mucus tube 1”). Tissue samples were shaken (to remove bacteria on epidermal layer) and again transferred into individual microcentrifuge tubes containing 1.0 mL filtered seawater (labeled “mucus tube 2”). After shaking the tissue samples in mucus tube 2, samples were dipped in 70% EtOH for 10s (to remove remaining epidermal bacteria and polypoid bacteria) and placed into microcentrifuge tubes containing 500 µL filtered

seawater (labeled “mucus tube 3). Using a sterile pestle, the tissue in each mucus tube 3 was smashed until the axis layers were exposed, freeing bacteria in the gastroventricular cavity and on the axis. After each mucus tube was centrifuged for 1min at 14,000 rcf, approximately 200 μ L of supernatant was removed, and the the pellet was re-suspended. To isolate bacterial strains in culture, 100 μ L of inoculated fluid from each mucus tube was spread in a lawn on Petri dishes containing 5.5% BD Difco Marine Agar 2116. Cultures were allowed to incubate for 48 h at 37°C. Morphological characteristics of isolates were observed.

Urease test

Twenty-four bacterial isolates were inoculated into individual microcentrifuge tubes containing 1 mL of deionized water. One K650 Urease Tablet (obtained from Key Scientific Products, Inc.) was added to each inoculated test tube, and tubes were allowed to incubate for 8 h. Positive urease production from the bacterial isolate was determined through the appearance of pink media after 8 h incubation.

DNA extraction for culturable and unculturable strains

Mechanical lysis was performed to extract intracellular contents, including genomic material, of cultured isolates. Remaining supernatants from the different mucus tubes underwent DNA purification through the UltraClean[®] Soil DNA Isolation Kit (MoBio). DNA isolation from mucus tube supernatants allowed for the extraction of DNA from unculturable strains residing on various *L. virgulata* tissues.

16s rDNA amplification

DNA from cultured isolates was mixed with 10 μ M concentrations of Universal Primer 518 , EUB341 GC, and GoTaq Green in order to prime for the amplification of a variable region of the 16S rDNA. The samples were then placed in thermocycler and run through the following

program (designated LM2): 95°C= 5min, (94°C=1 min, 60°C= 1 min, 72°C=1 min) x 35 cycles, 72°C= 1 min, 4°C= ∞). Amplified samples were stored at -20°C. DNA obtained through UltraClean[®] Soil DNA Isolation directly from the various mucus tubes was added to 10 µM concentrations 27f primer, 1792r primer, and Takara (obtained from Fisher Scientific). Samples were placed in the thermocycler and run through the following program (designated LM): 95°C=3 min, (95°C=45s, 56°C=45s, 72°C=1 min) x 35 cycles, 72°C=5 min, 4°C= ∞. Amplified samples were stored at -20°C. The amplification of 16S rDNA was verified using 0.8% polyacrylamide gels run on 130V for 25min.

DGGE Method

A 65% denaturing solution (20mL 40% acrylamide/bis, 2mL 50XTAE, 26mL formamide, 27.3g urea) and 35% denaturing solution (20mL 40% acrylamide/bis, 2mL 50X TAE, 14mL formamide, 14.7g urea) were used to cast the gradient gel using a gradient former (Bio-Rad). Amplified 16S rDNA samples, stained with 10X DGGE loading dye, were loaded into individual DGGE wells. The core assembly was set in a tank of 1X TAE buffer for 24 h at 70V. The gel was removed from the cast and stained with EtBr so that bands could be detected using KodakImaging software.

Preparation for genome sequencing

Bands of interest were excised from DGGE gels and placed in 20µL nuclease-free water in order to elute 16S rDNA fragments. Samples were then amplified using Universal 518, EUB341 GC, GoTaq Green and the LM2 temperature cycle. Amplification was verified with 0.8% polyacrylamide gel electrophoresis. Amplified samples were then run through the Wizard[®] PCR Preps DNA Purification System and stored at -20°C. The concentration of purified samples was

determined using a NanoDrop instrument (Thermo Scientific). Samples were diluted to 10 ng μL^{-1} and sent to MCV-VCU Nucleic Acids Research Facilities (Richmond, VA) for sequencing.

Results and Discussion

The urease test of 24 isolated bacterial strains showed definite urease production in culture (Fig. 2), strongly suggesting a possible bacterial-induced pH regulation mechanism in *L. virgulata* tissue. This data served as a springboard for further investigation into and genetic characterization of members of the bacterial community within *L. virgulata*. In hindsight, DNA sequencing of 16S rDNA of these urease-producing strains would have proven invaluable for the future genotypic comparison of urease-producing bacteria obtained from

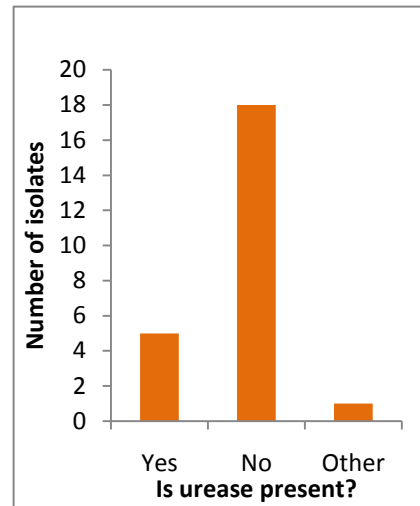


Figure 2: Results of the urease test performed on bacteria isolated from *L. virgulata* tissue on July 7, 2008.

various collection dates. Furthermore, 16S rDNA sequences from unculturable bacteria could have been compared to those of urease-producing bacteria in culture in order to make possible phenotypic associations with genotypic similarity. Future study will most definitely accompany genetic sequencing with the urease test.

DGGE analysis of nearly 100 gels from various specimen collection and 16S rDNA isolation dates (not shown) revealed high diversity within the microbial community structure of *L. virgulata*. Moreover, DGGE analysis detected the presence of over 25 distinct bacterial species present within *L. virgulata* tissue. Additionally, DGGE showed that various parts of the *L. virgulata* animal, such as the epidermis, polyps, and the gastroventricular cavity contained

variable microbial community structure (Fig. 3), suggesting that different tissue parts fulfill distinct ecological niches for resident bacteria. General consistency in band patterns among *L. virgulata* organisms collected at different times suggests the presence of a stable microbial community found within *L. virgulata* in the long-term. This data supports the hypothesis that resident bacteria are involved in a distinct ecological interaction with *L. virgulata*.

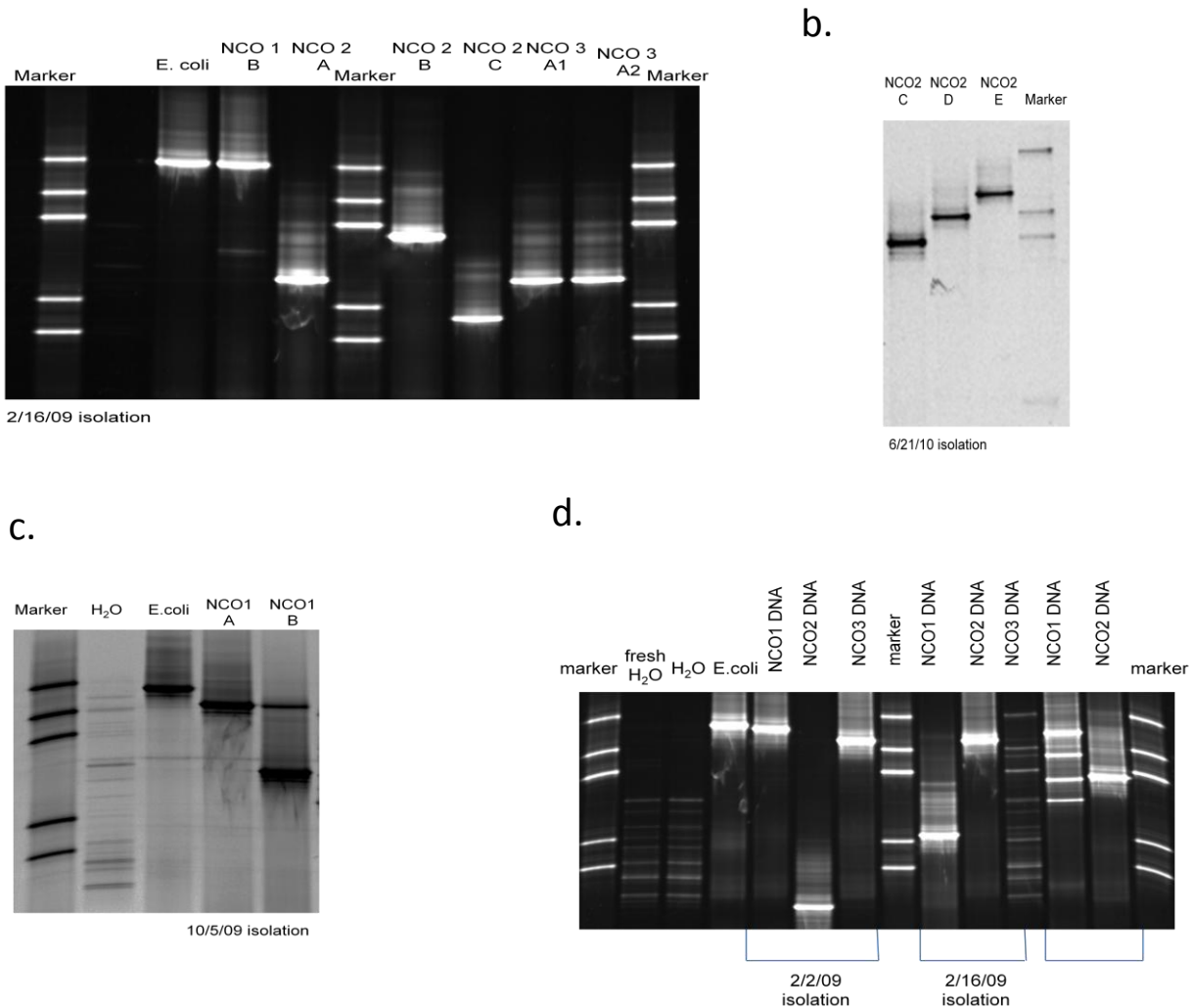
The 16S rDNA of bacterial strains not obtained in culture (through direct DNA amplification from mucus tubes) were successfully represented in DGGE (Figure 3d.). Genetic identification of the unculturable strains could yield significant information about the bacteria's ecological role. Additionally, it is possible that the 16S rDNA belongs to a previously undescribed marine bacterial species.

Most of the bands represented in Figure 3 were successfully amplified (Fig. 4). However, NanoDrop data following the purification of the samples showed nucleotide concentrations lower than $10 \text{ ng } \mu\text{L}^{-1}$, which is too low for successful sequencing. This suggests a possible defect in the Wizard[®] PCR Preps DNA Purification System used in this study. Time proved to be a limiting factor in the re-amplification and re-purification of 16s rDNA. Future amplification and purification of the 16S rDNA present in the bands in Fig. 3 will lead to the identity of bacterial species comprising the microbial community within various parts of the *L. virgulata* tissue.

Future study could attempt to transform species-specific 16S rDNA fragments into a plasmid that can be cloned in a culturable bacterial strain so that species can be identified through DNA sequencing. Further characterization of the bacterial community structure could be accomplished through fluorescence *in situ* hybridization, which could detect the presence of a urease-encoding gene present in unculturable strains of bacteria found on *L. virgulata* tissue.

Additionally, urease genes present among bacterial community members could be detected through the creation of a primer specific to the urease gene and subsequent amplification. Also, stable isotope analysis could provide data proving energy exchange (and subsequent symbiosis) between *L. virgulata* and resident bacteria. Overall, data presented in this study provide a solid ground for further research into the possible bacteria-facilitated mechanism of pH regulation used by *L. virgulata* during spicule development.

Figure 3: a.), b.), c.) DGGE gel of 16S rDNA fragments of culturable bacteria from epidermal (NCO1), polyp (NCO2), and gastroventricular (NCO3) tissue of individual orange *L. virgulata* collected from Beaufort, and Moorehead City, NC. The 16S rDNA fragments from bands have been purified for sequencing. d.) 16S rDNA from unculturable bacteria residing on *L. virgulata* tissue.



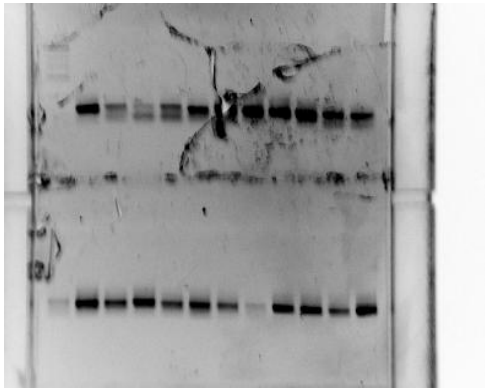


Figure 4: Image of 0.8% polyacrylamide gel showing successful amplification of several 16S rRNA fragments obtained from DGGE bands shown in Figure 3. These 16S rRNA fragments were run through the Wizard[®] PCR Preps DNA Purification System, which resulted in nucleotide concentrations too low for sequencing.

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