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UNIVERSITY OF CALIIFORNIA, SAN DIEGO

Marine Invertebrate-Microbial Bioactive Metabolite Symbioses

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Marine Biology

by

Koty Sharp

Committee in charge:

Professor Margo G. Haygood, Chair
Professor Nicholas D. Holland
Professor Douglas Bartlett
Professor William Fenical
Professor Kit Pogliano

2006

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Chair

University of California, San Diego

2006

For DJF

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I was fortunate to have a committee that supported a thesis proposal with quite diverse goals. Nick Holland was especially helpful at teaching me how to work "smart," not just hard. His dedication to quick and brutal critique of my writing was extraordinary. He was always available for assistance, and he spent a great deal of time teaching me basic embedding and microscopy techniques. Doug Bartlett invited me to rotate through his lab my first year, and he and his lab taught me a great deal about general microbiology techniques and the exciting challenges of deep-sea

research. Kit Pogliano was incredibly supportive and always brought new perspective and her expertise with fluorescent microscopy to our committee meetings.

Still three years after his death, I cannot express how the loss of John Faulkner has impacted me. While I was taking a chemical ecology class with John, he recruited me to work on sponges with him, and I knew after I showed him my first unsuccessful gel that it was the right choice. It was always exciting to show John new results, even if they were no good. He was an intuitive mentor who managed to challenge, stimulate, and motivate all of us. When I finally mastered the technique of FISH, he congratulated me and proudly introduced me to collaborators as his "icthyology expert." John threatened to leave us for years, and I am thankful that he remained here long enough to be part of my education. He was an awesome scientist, an inspiring mentor, and a dear friend. And I know he's watching whenever I am not being productive.

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Linda Holland was an amazing resource during my time at Scripps. There is no in situ hybridization troubleshooting that Linda cannot do, and like Nick, she was constantly willing to dedicate time to help me, even on Sunday evenings. I was a

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Penland of the Palau International Coral Research Center provided her lab space and use of the island's only epifluorescent microscope on multiple trips.

Working in the Tebo-Haygood Lab sometimes felt like being part of a circus, but it was the greatest show on earth. I enjoyed working with Brad Tebo and his lab group during a first year rotation; when I decided that my heart was really in symbiosis research, I was happy to be staying in the same lab. Over the years, I have been thankful for being part of a lab group with incredible combined expertise. Chris Francis was an important mentor to me in my first year, and I thank him for being a fine example, completely incapable of mincing words, and along with Eric Allen and Richard Long, pouring me a Jose's margarita, my first legal drink in the US. Meriah Arias and Grace Lim taught me the careful art of DGGE, and Meriah also introduced me to the clever tradition of holiday-time wine tasting parties in our lab group.

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Bourdignon arrived in our lab with infectious Italian enthusiasm and spirit, we all savored it, and our lab will never be the same.

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ABSTRACT OF THE DISSERTATION

Marine Invertebrate-Microbial Bioactive Metabolite Symbioses

by

Koty Sharp

Doctor of Philosophy in Marine Biology

University of California, San Diego, 2006

Margo G. Haygood, Chair

Conspicuous and soft-bodied planktonic marine invertebrate larvae often use bioactive compounds for defense against predators. Bioactive compounds play a significant role in shaping the host ecology, and in some cases, the molecules are synthesized by microbial symbionts. This dissertation addresses fundamental questions of bioactive metabolite symbioses between metazoans and microbes. The bryozoan *Bugula neritina* transmits the symbiotic proteobacterium "*Candidatus* Endobugula sertula," via its larvae. *E. sertula* synthesizes the bryostatins, which protect *B. neritina* larvae from predation in the environment. Development of a method for simultaneous *in situ* bryostatin and *E. sertula* localization demonstrates that embryos are coated with bryostatins before release from adult colonies, and there is an ontogenetic shift in bryostatin concentration and localization throughout the host life cycle. *E. sertula* is present in all host life stages of the host, suggesting that bryostatin biosynthesis may somehow be regulated, reserved for specific events in the life history, such as the protection of larvae.

The second invertebrate studied in this dissertation is the sponge *Corticium candelabrum*. *C. candelabrum* possesses tedanolides, anti-tumor polyketides that might possibly be produced by bacteria in the sponge. Two chapters in this dissertation are dedicated to microbial assemblages associated with developing *C. candelabrum* embryos. Both Archaea and Bacteria are present throughout *C. candelabrum* embryonic development. Here we identify three specific bacterial taxa, and show that there are likely dozens more, that are vertically transmitted in *C. candelabrum* and consistently associated with it across space and time. One of these species, a strain of alpha-proteobacterium α-CC01, is present throughout embryogenesis and is also present in swimming larvae. These studies show that *C. candelabrum* inoculates vertically transmits a specific, diverse microbial community.

A main theme linking the studies in this dissertation is vertical transmission of microbes in marine invertebrates. Embryo and larva isolation allows identification of specific microbial associates in marine invertebrates by decreasing the complexity of microbes in a sample. In addition, understanding of microbial assemblages in marine invertebrate embryos and larvae offers valuable insight into how species-specific invertebrate-microbe associations are maintained in the environment.

CHAPTER 1

Introduction:
Bioactive Metabolite Symbiosis

Symbiosis between metazoans and Bacteria or Archaea is common throughout diverse environments. Prokaryotes possess a diverse array of metabolic capabilities not found in the eukaryotic kingdom, and these specialized adaptations allow them to exploit a wide range of chemical habitats and substrates for nutrition and produce metabolites that regulate complex biological processes. In associations with microbes, animals can harness the biosynthetic and nutritional versatility of prokaryotes, with the beneficial result of habitat range expansion, increased fitness for specific environments. Symbiotic associations, in which multiple genomes function together to form a unique evolutionary unit, allow each partner to occupy niches in which they would otherwise be unable to survive.

Bacterial-invertebrate symbiosis occurs across many marine habitats, including deep-sea hydrothermal vents, tropical sand flats, midwater pelagic ecosytems, and coral reefs. It is probable that symbiotic microbes play a significantly larger role in marine ecology than is currently acknowledged. Some of the best known examples of marine symbiosis include the associations between invertebrates and microbes from deep-sea hydrothermal vents and methane seeps (Stahl *et al.* 1984). The biomass of metazoans at deep-sea vents is much greater than would be expected, particularly because the surrounding seawater tends to consist of reduced compounds and heavy metals at concentrations toxic to most animal phyla. In many of the associations from the deep-sea habitats that have been characterized, chemoautotrophic symbionts provide nutrition, converting inorganic carbon sources to useful organic carbon compounds for their animal hosts. Several vestimentiferan worm species have reduced digestive systems and instead harbor sulfide-oxidizing symbionts in

trophosomes (Fisher 1990). Throughout the animal, there are specialized hemes that transport high amounts of sulfide to the symbionts and prevent toxic effects on the host (Fisher *et al.* 1988). Many bivalves, including mussels and vesicomyid clams host sulfide-oxidizing and methanotrophic symbionts in their gills (Fisher 1990). Analogous to the role of the photosynthetic symbiotic microorganisms as the base of the food chain in coral reefs, chemosynthetic symbionts are the basis for hydrothermal vent ecosystems (Cavanaugh 1983).

Another example of nutritional symbiosis in the marine environment occurs in a different habitat, which receives significant attention because of its worldwide detrimental impact on marine industry. Wood-boring shipworms, which live on an all-wood diet, harbor nitrogen-fixing symbiotic bacteria that provide the host with essential nitrogenous compounds, in addition to the enzymes necessary for digestion of their cellulose- and lignin-rich diet (Waterbury *et al.* 1983; Distel *et al.* 1991; Distel *et al.* 2002).

Other marine invertebrates engage in symbiosis with bioluminescent bacteria that mediate interactions between predators and prey. The subtropical bobtail squid *Euprymna scolopes* is certainly the most well-studied example of symbiosis between a marine invertebrate and bioluminescent bacteria and demonstrates the potential for the elegant molecular conversations that can be shared between microbes and their animal hosts. Bioluminescent *Vibrio fischeri* exist both as free-living bacteria in the seawater and within light organs in *E. scolopes*, which uses its light organs for counter-illumination and protection against larger predators. *E. scolopes* utilizes a series of highly evolved biochemical and behavioral mechanisms to acquire a daily inoculum of

a specific strain of *V. fischeri* symbiont from the seawater (McFall-Ngai and Ruby 1991). The symbionts have the capability to induce developmental changes in juvenile hosts (Koropatnick *et al.* 2004; Nyholm and McFall-Ngai 2004). Species from several fish families also harbor bioluminescent gamma-proteobacteria, including *Vibrio* species and *Photobacterium* species (Nealson and Hasting 1992; Haygood 1993).

Though prospecting the terrestrial environment for natural products with pharmaceutical benefit to humans has been occurring for centuries, only in the past three decades have researchers turned to marine organisms as sources of bioactive compounds. Many marine natural products that have demonstrated therapeutic potential for humans have been shown to function in protection against predation, microbial or larval fouling, or competition. Other bioactive compounds in marine invertebrates appear to be larval settlement cues. Chemical defense against potential consumers has been shown to be a common trait across many sessile marine taxa, including tunicates, sponges, bryozoans, and molluscs (Paul 1992). As more has been discovered about global and taxonomic distribution of bioactive compounds in terrestrial and marine organisms, and because many marine natural products possess structural similarity to known microbial metabolites, many compounds isolated from invertebrates are suspected to be synthesized by the microbial symbionts they house (Kobayashi and Ishibashi 1993; Piel 2004).

Though often suspected, a symbiotic origin has only been demonstrated in very few cases of marine natural products. Chemical analysis of cell fractions successfully identified the source of natural products in some sponges. In the sponge *Theonella swinhoei*, cell separation was effective for identifying symbiotic sources of bioactivity.

Bewley *et al*. (1996) examined the microbial role in swinholide and theopalauamide production by separating *T. swinhoei* cells from the unicellular cyanobacteria *Aphanocapsa feldmanni,* large filamentous bacteria – later identified as the delta-proteobacterium "*Candidatus* Entotheonella palauensis" (Schmidt *et al.* 2000) – and small, unicellular bacteria (Bewley *et al.* 1996). Analysis of the cell fractions showed that swinholide was present only in the fraction with unicellular bacteria, and theopalauamide was present only in the fraction with the *E. palauensis*. Similarly, Flowers *et al*. showed that chlorinated diketopiperazines were localized exclusively with cyanobacteria from *Dysidea herbacea,* though the terpene spirodysin was found only in sponge archaeocytes and choanocytes (Flowers *et al.* 1998).

Though cell separation has been a successful approach in some sponges, in other invertebrates, localization of bioactive compounds is not as straightforward. Recent research in two different marine natural products symbioses has demonstrated the importance of utilizing the tools of molecular biology. Recent characterization of the genetics of natural products biosynthesis has led to the identification of symbiotic bioactive metabolite synthesis genes. The tropical ascidian *Lissoclinum patella*, which contains the patellamides, a group of cyclic peptides, is packed densely with cyanobacteria of the genus *Prochloron*. Though cyclic peptides, traditionally known to be microbial metabolites, were suspected to be produced by *Prochloron* sp., it was only possible to identify the patellamide biosynthetic gene cluster once the entire genome of the *L. patella*-associated *Prochloron* was sequenced. Once the genes were identified, they were cloned and heterologously expressed, conclusively demonstrating that the *Prochloron* symbiont in *L. patella* synthesizes the patellamides (Schmidt *et al.*

2005). This was the first expression of an entire symbiotic natural product biosynthetic pathway. Other species of *Prochloron* occur in symbiosis with ascidians across the Pacific, and the chemistry and biosynthetic genes in the symbionts are currently being explored. The temperate bryozoan *Bugula neritina* contains the bryostatins, cytotoxic complex polyketides, and through the use of a homology-based approach, an extremely large (>60kb) biosynthetic gene cluster was isolated from *B. neritina* (Hildebrand *et al.* 2004). Portions of this cluster are expressed by symbiotic bacteria within *B. neritina*, and based on its sequence, it has been shown to be responsible for the synthesis of the bryostatins (Davidson *et al.* 2001).

Bacterial symbionts are associated with larvae and eggs of many marine invertebrates, and is often taken to indicate that the microbe and animal have evolved an obligate association that employs intergenerational transmission to ensure inoculation of symbiont into future host offpsring. However, symbiotic microbes may also play an important role in the ecology of eggs or larvae themselves. Previous research demonstrates that larvae of marine invertebrates contain bioactive compounds that make larvae unpalatable to predators (Lindquist and Hay 1995). Though many of those protective compounds are hypothesized to be microbial in origin, bacterial production of bioactive metabolites for larval defense has been most thoroughly tested in the bryozoan *Bugula neritina* (Davidson *et al.* 2001). Moreover, Gil-Turnes *et al.* (1989) and Gil-Turnes and Fenical (1992) demonstrated that episymbiotic bacteria produce chemical defense to protect developing embryos of the shrimp *Palaemon macrodactylus* and the lobster *Homarus americanus* from marine pathogenic fungus. It is widely accepted that defense compounds significantly impact the evolution of

invertebrate larval morphology and behavior (Lindquist and Hay 1995; McClintock and Baker 1997; Iyengar and Harvell 2001). Biggs and Epel suggested that bacteria associated with egg cases of the squid *Loligo opalescens* protect the eggs from predation or microbial infection (Biggs and Epel 1991). The bacteria colonize the accessory nidamental gland in the adult before egg-laying (Kaufman *et al.* 1998), and Nishiguchi *et al*. suggest that this association is a primitive version of the symbiosis between *Euprymna scolopes* and its bioluminescent symbiont *V. fischeri,* which colonizes the host light organs, thought to be specialized nidamental accessory glands (Nishiguchi *et al.* 2004).

Bugula neritina

Bugula neritina (Gymnolaemota; Cheilostomata; Anasca; Cellularia; Buguliidae) is an intertidal temperate bryozoan that often extensively fouls docks and boat hulls across the globe. *B. neritina* forms upright, branching, colonies that are covered by a chitinous cuticle that is only lightly calcified (Woollacott and Zimmer 1977). Each individual of the colony is termed a zooid, and the zooecium includes the soft animal (polypide) within the cuticle. Autozooids, the feeding zooids, consist of a lophophore – a crown of ciliated tentacles surrounding the mouth –leading to a fully developed gut tract. The lophophore is used for filter feeding when extended from the cuticle, but it can also retract in response to physical or chemical disturbance (Woollacott and Zimmer 1977). *B. neritina* lacks the mechanical defense of specialized zooids called avicularia and vibracula, which are present in other bryozoan species. Other specialized zooids include ovicells – slightly calcified chambers in which embryos are

brooded – and rhizoids – root-like structures at the base of the colonies that are used for attachment to substrate (Woollacott and Zimmer 1977). Nutrients from the feeding zooids are distributed throughout the colony via a network of tubes known as the funicular system (Lutaud 1965; Lutaud 1969; Woollacott and Zimmer 1975).

In the 1970's and the 1980's, bacteria were documented in the larvae of the bryozoan *Bugula neritina* and within the funicular cords, channels of tissue that connect individuals of an adult colony (Woollacott and Zimmer 1975; Woollacott 1981). Soon after, the bryostatins, a group of compounds with significant anti-cancer activity, were isolated from *B. neritina* (Pettit *et al.* 1982; Pettit 1991). Because the bryostatins are complex polyketides, which are typically produced by bacteria (Rawlings 1997), it was proposed that the bryostatins are produced by symbiotic bacteria in *B. neritina* (Anthoni *et al.* 1990).

The life history and larval behavior of *B. neritina* have been extensively studied during the past twenty years, although it was not until 1997 that the bacteria in *B. neritina* larvae were characterized with molecular techniques. Haygood and Davidson (1997) identified a single gamma-proteobacterium that consistently resides in the pallial epithelium of *B. neritina* larva as "*Candidatus* Endobugula sertula." They have since demonstrated that there are at least two cryptic variants of *B. neritina*, and the symbiont within each population is a distinct species (Davidson 1999). Their study highlighted the importance of species identification for the harvest of bioactive compounds, especially because bryostatin1, the only bryostatin in clinical trials, is exclusively found in one *B. neritina* population in California (Davidson 1999). Since the identification of sibling species on the west coast, an additional sibling species of

B. neritina was identified in the Gulf of Mexico (McGovern and Hellberg 2003). A population of *B. neritina* that appears to be aposymbiotic and lacking bryostatins was identified off the coast of Delaware (*N. Targett and N. Lopanik, pers. comm.*), consistent with the hypothesis of a symbiotic source of bryostatins.

Further work demonstrated conclusively that the symbiont *E. sertula* has the biosynthetic capability to make the bryostatins. A large polyketide synthase gene cluster (>60kb) was isolated from a bacterial DNA preparation, and a portion of that cluster, now known as *bry*, is expressed in *E. sertula* cells in *B. neritina* larvae (Davidson *et al.* 2001; Hildebrand *et al.* 2004). Antibiotic treatment of wild *B. neritina* colonies resulted in lower yields of bryostatin (Davidson *et al.* 2001), though the colonies still retained low levels of symbiotic *E. sertula*. Further efforts to test larvae released by antibiotic-treated colonies showed the absence of bryostatins in larvae that had been fully cleared of *E. sertula* (Lopanik *et al.* 2004). Lopanik *et al.* (2004) demonstrated that the bryostatins are the chemical deterrent that protects the larvae from predators (Lindquist 1996). This series of studies established the first confirmed case of symbiotic production of chemical defense in a marine invertebrate.

Because the *Bugula neritina-Endobugula sertula* symbiosis has been so well-characterized, it has been possible to investigate the transport and symbiotic production of bryostatins in *B. neritina*. This dissertation addresses the coordination of symbiotic bryostatin production and localization throughout the life cycle of the host as it progresses from a soft-bodied, singular, lecithotrophic larva to a chitinous, colonial, feeding adult. Lopanik *et al.* (2004) showed that though both adults and larvae contain bryostatins, the larvae contain concentrations of up to 100-fold greater

than bryostatins in adult colonies. Because Lopanik *et al*. (2004) found bryostatins were a hundred times more concentrated in the larvae than in the adults, they proposed that bryostatins are concentrated to protect the vulnerable, soft-bodied larval stage of *B. neritina*, in contrast to the cuticle-encased juvenile and adult stages, which have low bryostatin concentrations. The main questions in the *Bugula neritina* studies of this dissertation addressed 1) bryostatin production and 2) symbiont localization and proliferation throughout the host life cycle. When during the life cycle of the host is bryostatin produced, and in which portions of the host is it most concentrated? Do bryostatin levels change during larval metamorphosis and subsequent development? How does *E. sertula* infect the larva, and ultimately get distributed throughout the adult to ensure defense for the entire colony?

The *B. neritina-E. sertula* symbiosis, well-characterized, lends itself to studies aimed at localizing the bioactive compound and its symbiotic producer throughout host metamorphosis and development. However, there are many other potential bioactive metabolite symbioses to be explored. Most intriguing to me are specific sponge-microbe symbioses. From a microbial ecology perspective, “choosing” the right symbiotic microbes, especially if there are multiple microbes functioning in concert within the host, must be an involved and evolutionarily significant process. Using the tropical sponge *Corticium candelabrum* as a model system, I probe basic questions about microbial ecology of sponge-microbe associations in two chapters in this dissertation. How consistent and predictable are sponge-bacterial or sponge-archaeal associations over time, and how are they maintained to persist in the environment?

Sponges – *Corticium candelabrum*

Though sponges are primitive animals, they have several cell types with specialized functions that result in an organism that can carry out complex behaviors and processes. Several examples of sponge-microbe associations appear to be spatiotemporally constant, though how these consistent associations are assembled and maintained from one sponge generation to the next is not well understood.

Of the marine invertebrates examined for bioactive compounds, sponges are notable for being of the most prolific producers of diverse natural products (Faulkner *et al.* 2000; Faulkner 2002). Marine sponges, primitive animals that do not consist of true tissues, may be likened to biofilms – like diverse microbial mats organized by a scaffold of sponge fibers and channels of seawater, that perhaps function to deliver nutrients or organic compounds to the sponge cells and microbes within the animal. Because they consist of highly diverse assemblages of microbes, (*i.e.*, presumably both symbiotic residents and ingested microbes from the seawater), it has been a challenge to identify specific microbial associates in sponges, and subsequently difficult to investigate the symbiotic producers of bioactive compounds in many sponges.

There are few examples in which microbes have been demonstrated to engage in specific, consistent association with sponges, and in some cases, microbial associates have been shown to produce bioactive compounds that are hypothesized to play a role in host defense. However, in general it is very difficult to identify specific symbionts, especially if there one morphologically dominant cell type is not easily separated from

other cells. Many sponges are host to an assortment of microbes reflecting the microbial complexity of the surrounding seawater. In addition, obligate symbionts are notoriously difficult to culture, so large-scale production of symbiotic bioactive compounds is uncommon.

Along with production of bioactive compounds, microbes in sponges may play a variety of other roles as symbionts. Sponges mediate the environmental fate of organic and inorganic nutrients and can affect the cycling of silicon, regulating primary production rates in some ecosystems (Pile 1997; Yahel *et al.* 2003; Hoffmann *et al.* 2005; Maldonado *et al.* 2005). Recent studies have attempted to identify patterns among bacterial communities of diverse sponges (Erpenbeck *et al.* 2002; Hentschel *et al.* 2002; Hentschel *et al.* 2003; Taylor *et al.* 2004), and through the use of advanced molecular techniques, including high-throughput sequencing and metagenome analysis, future studies should be able to unravel some of the functions of complex microbial communities in sponges.

The portion of this dissertation exploring the sponge *Corticium candelabrum* began as a collaboration with the late John Faulkner's laboratory group, most notably Christine Salomon. Because the group was interested in the tedanolides, a family of bioactive compounds in *C. candelabrum*, as an anti-cancer drug candidate, our initial interest was in the possibility of a bioactive metabolite symbiosis based on tedanolides. When I began working on the project, John Faulkner was determined to focus on the "bags of bugs," or "BOBs" that Christine had observed in *C. candelabrum*. John suggested that the sponge harbored large sacs of microbes. We

soon discovered that though they were actually developing embryos, they truly were bags of bugs – filled with diverse microbes.

When my preliminary results of the sponge indicated abundant and diverse microbes in its brooded embryos, I continued to explore this system within the broader perspective of microbial ecology. While a microbial origin of the tedanolides would likely be a valuable discovery, this dissertation focuses on fundamental characterization of the persistence of species-specific interactions within *C. candelabrum* as a model for understanding sponge-microbe associations, and how the associations are maintained in the environment. The concept of vertical transmission of microbes is not a new one to sponge biologists. Many studies over the past few decades have documented the presence of microbial cells associated with embryos, eggs, and even sperm in marine sponges (Sciscioli *et al.* 1994; Boury-Esnault *et al.* 1999; Usher *et al.* 2001; Sciscioli *et al.* 2002; Boury-Esnault *et al.* 2003; Usher *et al.* 2005). However, very few studies have conclusively identified microbes that are passed from one sponge generation to the next.

In this dissertation, the main questions linking the chapters address fundamental aspects of metazoan-microbe symbiosis. Using the *Bugula neritina-Endobugula sertula* association, a known bioactive metabolite symbiosis, I developed localization techniques to discern in which host tissues and in what stages of the host life cycle bryostatins are present. In addition, the fate of the symbiotic bacterium during host metamorphosis and development was explored. The two chapters summarizing the work on the tropical sponge *Corticium candelabrum* highlight the importance of investigating the possibility that some metazoans simultaneously maintain specific

associations with many microbes. The first chapter provides a detailed survey of a diverse but specific vertically transmitted bacterial community in *C. candelabrum*, in addition to exploring the presence of Archaea in the sponge embryos. The second chapter employs microscopy techniques in order to understand the mechanisms of microbial inoculation into sponge embryos.

REFERENCES

Anthoni, U., P. H. Nielsen, M. Pereira and C. Christophersen (1990). Bryozoan Secondary Metabolites - a Chemotaxonomical Challenge. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **96**(3): 431-437.

Bewley, C. A., N. D. Holland and D. J. Faulkner (1996). Two classes of metabolites from Theonella swinhoei are localized in distinct populations of bacterial symbionts. *Experientia (Basel)* **52**(7): 716-722.

Biggs, J. and D. Epel (1991). Egg Capsule Sheath of Loligo-Opalescens Berry - Structure and Association with Bacteria. *Journal of Experimental Zoology* **259**(2): 263-267.

Boury-Esnault, N., S. Efremova, C. Bezac and J. Vacelet (1999). Reproduction of a hexactinellid sponge: first description of gastrulation by cellular determination in the Porifera. *Invertebrate Reproduction & Development* **35**(3): 187-201.

Boury-Esnault, N., A. Ereskovsky, C. Bezac and D. Tokina (2003). Larval development in the Homoscleromorpha (Porifera, Demospongiae). *Invertebrate Biology* **122**(3): 187-202.

Cavanaugh, C. M. (1983). Symbiotic Chemoautotrophic Bacteria in Marine-Invertebrates from Sulfide-Rich Habitats. *Nature* **302**(5903): 58-61.

Davidson, S., S. W. Allen, G. E. Lim, C. M. Anderson and M. G. Haygood (2001). Evidence for the biosynthesis of bryostatins by the bacterial symbiont "*Candidatus* Endobugula sertula" of the bryozoan *Bugula neritina*. *Appl. Env. Microbiol.* **67**: 4531-4537.

Davidson, S. K. (1999). Biology of the Bryostatins in the marine bryozoan *Bugula neritina*: Symbiosis, cryptic speciation and chemical defense. Scripps Institution of Oceanography. La Jolla, University of California San Diego**:** 210.

Distel, D. L., D. J. Beaudoin and W. Morrill (2002). Coexistence of multiple proteobacterial endosymbionts in the gills of the wood-boring bivalve Lyrodus pedicellatus (Bivalvia: Teredinidae). *Applied & Environmental Microbiology* **68**(12): 6292-6299.

Distel, D. L., E. DeLong and J. B. Waterbury (1991). Phylogenetic characterization and *in situ* localization of the bacteria symbiont of shipworms (Teredinidae: Bivalvia) by using 16S rRNA sequence analysis and oligonucleotide probe hybridization. *Appl. Environ. Microbiol.* **57**: 2376-2382.

Erpenbeck, D., J. A. J. Breeuwer, d. V. H. C. van and S. R. W. M. van (2002). Unravelling host and symbiont phylogenies of halichondrid sponges (Demospongiae, Porifera) using a mitochondrial marker. *Marine Biology (Berlin)* **141**(2): 377-386.

Faulkner, D. J. (2002). Marine Natural Products. *Nat. Prod. Rep.* **19**: 1-48.

Faulkner, D. J., M. K. Harper, M. G. Haygood, C. E. Salomon and E. W. Schmidt (2000). Symbiotic Bacteria in Sponges: Sources of Bioactive Substances. New York, Basel, Karger.

Fisher, C. R. (1990). Chemoautotrophic and Methanotrophic Symbioses in Marine Invertebrates. *Reviews in Aquatic Sciences* **2**(3-4): 399-436.

Fisher, C. R., J. J. Childress and N. K. Sanders (1988). The Role of Vestimentiferan Hemoglobin in Providing an Environment Suitable for Chemoautotrophic Sulfide-Oxidizing Endosymbionts. *Symbiosis* **5**(3): 229-246.

Flowers, A. E., M. J. Garson, R. I. Webb, E. J. Dumdei and R. D. Charan (1998). Cellular origin of chlorinated diketopiperazines in the dictyoceratid sponge Dysidea herbacea (Keller). *Cell & Tissue Research* **292**(3): 597-607.

Gil-Turnes, M. S. and W. Fenical (1992). Embryos of *Homarus americanus* are protected by epibiotic bacteria. *Biol. Bull.* **182**: 105-108.

Gil-Turnes, M. S., M. E. Hay and W. Fenical (1989). Symbiotic marine bacteria chemically defend crustacean embryos from a pathogenic fungus. *Science* **246**: 116-118.

Haygood, M. G. (1993). Light organ symbioses in fishes. *Crit Rev Microbiol* **19**(4): 191-216.

Hentschel, U., L. Fieseler, M. Wehrl, C. Gernert, M. Steinert and J. Hacker (2003). Microbial Diversity of Marine Sponges. Marine Molecular Biotechnology. W. E. G. Muller. Berlin Heidelberg, Springer-Verlag. **Sponges (Porifera):** 59-88.

Hentschel, U., J. Hopke, M. Horn, A. B. Friedrich, M. Wagner, J. Hacker and B. S. Moore (2002). Molecular evidence for a uniform microbial community in sponges from different oceans. *Applied and Environmental Microbiology* **68**(9): 4431-4440.

Hildebrand, M., L. Waggoner, H. Liu, S. Sudek, S. Allen, C. Anderson, D. Sherman and M. Haygood (2004). BryA: An unusual modular polyketide synthase gene from the uncultivated bacterial symbiont of the marine bryozoan Bugula neritina. *Chemistry and Biology* **11**: 1543-1552.

Hoffmann, F., O. Larsen, V. Thiel, H. T. Rapp, T. Pape, W. Michaelis and J. Reitner (2005). An anaerobic world in sponges. *Geomicrobiology Journal* **22**(1-2): 1-10.

Iyengar, E. V. and C. D. Harvell (2001). Predator deterrence of early developmental stages of temperate lecithotrophic asteroids and holothuroids. *Journal of Experimental Marine Biology and Ecology* **264**(2): 171-188.

Kaufman, M. R., Y. Ikeda, C. Patton, G. Van Dykhuizen and D. Epel (1998). Bacterial symbionts colonize the accessory nidamental gland of the squid Loligo opalescens via horizontal transmission. *Biological Bulletin* **194**(1): 36-43.

Kobayashi, J. and M. Ishibashi (1993). Bioactive metabolites of symbiotic marine microorganisms. *Chem. Rev.* **93**: 1753.

Koropatnick, T. A., J. T. Engle, M. A. Apicella, E. V. Stabb, W. E. Goldman and M. J. McFall-Ngai (2004). Microbial factor-mediated development in a host-bacterial mutalism. *Science* **306**(5699): 1186-1188.

Lindquist, N. (1996). Palatability of invertebrate larvae to corals and sea anemones. *Mar. Biol.* **126**: 745-755.

Lindquist, N. and M. E. Hay (1995). Can Small Rare Prey Be Chemically Defended - the Case for Marine Larvae. *Ecology* **76**(4): 1347-1358.

Lopanik, N., N. Lindquist and N. Targett (2004). Potent cytotoxins produced by a microbial symbiont protect host larvae from predation. *Oecologia* **139**(1): 131-139.

Lutaud, G. (1965). Sur la présence de microorganismes spécifiques dans le glandes vestibulaires et dans l'aviculaire de *Palmicellaria skenei* (Ellis et Solander). Bryozoaires chilostome. *Cahiers de Biologie Marine* **6**: 181-190.

Lutaud, G. (1969). La nature des corps funiculaires des cellularines, bryozoaires chilostomes. *Arch. de Zool. Exp. et Gen.* **110**: 5-30.

Maldonado, M., C. Carmona, Z. Velasquez, A. Puig, A. Cruzado, A. Lopez and C. M. Young (2005). Siliceous sponges as a silicon sink: An overlooked aspect of benthopelagic coupling in the marine silicon cycle. *Limnology and Oceanography* **50**(3): 799-809.

McClintock, J. B. and B. J. Baker (1997). Palatability and chemical defense of eggs, embryos and larvae of shallow-water Antarctic marine invertebrates. *Marine Ecology-Progress Series* **154**: 121-131.

McFall-Ngai, M. J. and E. G. Ruby (1991). Symbiont Recognition and Subsequent Morphogenesis as Early Events in an Animal-Bacterial Mutualism. *Science* **254**(5037): 1491-1493.

McGovern, T. and M. Hellberg (2003). Cryptic species, cryptic endosymbionts, and geographic variation in chemical defenses in the bryozoan *Bugula neritina. Mol. Ecol.* **12**(12): 1207-1215.

Nealson, K. H. and J. W. Hasting (1992). The luminous bacteria. The Prokaryotes. A. Balows, H. G. Trüper, M. Dworkin, W. Harder and K.-H. Schleifer. New York, Springer-Verlag. **1:** 625-639.

Nishiguchi, M. K., J. E. Lopez and S. von Boletzky (2004). Enlightenment of old ideas from new investigations: more questions regarding the evolution of bacteriogenic light organs in squids. *Evolution & Development* **6**(1): 41-49.

Nyholm, S. V. and M. J. McFall-Ngai (2004). The winnowing: Establishing the squid-Vibrio symbiosis. *Nature Reviews Microbiology* **2**(8): 632-642.

Paul, V. J. (1992). The chemical ecology of marine natural products. Ithaca, NY, Comstock Publishing Associates.

Pettit, G. R. (1991). The bryostatins. *Prog. Chem. Org. Nat. Prod.* **57**: 153-195.

Pettit, G. R., C. L. Herald, D. L. Doubek and D. L. Herald (1982). Isolation and structure of bryostatin 1. *J. Am. Chem. Soc.* **104**: 6846-6847.

Piel, J. (2004). Metabolites from symbiotic bacteria. *Natural Product Reports* **21**(4): 519-538.

Pile, A. J. P., Mark R.; Savarese, Michael; Chernykh, Valeri I.; Fialkov, Vladimir A. (1997). Trophic Effects of Sponge Feeding Within Lake Baikal's Littoral Zone. 2. Sponge Abundance, Diet, Feeding Efficiency, and Carbon Flux. *Limnol. Oceanogr.* **42**(1): 178-184.

Schmidt, E. W., J. T. Nelson, D. A. Rasko, S. Sudek, J. A. Eisen, M. G. Haygood and J. Ravel (2005). Patellamide A and C biosynthesis by a microcin-like pathway in Prochloron didemni, the cyanobacterial symbiont of Lissoclinum patella. *Proceedings of the National Academy of Sciences of the United States of America* **102**(20): 7315-7320.

Schmidt, E. W., A. Y. Obraztsova, S. K. Davidson, D. J. Faulkner and M. G. Haygood (2000). Identification of the antifungal peptide-containing symbiont of the marine sponge Theonella swinhoei as a novel delta-proteobacterium, "Candidatus Entotheonella palauensis". *Marine Biology (Berlin)* **136**(6): 969-977.

Sciscioli, M., E. Lepore, M. Gherardi and L. S. Liaci (1994). Transfer of symbiotic bacteria in the mature oocyte of Geodia cydonium: (Porifera, Demospongiae): an ultrastructural study. *Cah. Biol. Mar.* **35**: 471-478.

Sciscioli, M., E. Lepore, M. Mastrodonato, L. S. Liaci and E. Gaino (2002). Ultrastructural study of the mature oocyte of Tethya aurantium (Porifera: Demospongiae). *Cahiers de Biologie Marine* **43**(1): 1-7.

Stahl, D. A., D. J. Lane, G. J. Olsen and N. R. Pace (1984). Analysis of hydrothermal vent-associated symbionts by ribosomal RNA sequences. *Science* **224**(4647): 409-11.

Taylor, M. W., P. J. Schupp, I. Dahllof, S. Kjelleberg and P. D. Steinberg (2004). Host specificity in marine sponge-associated bacteria, and potential implications for marine microbial diversity. *Environmental Microbiology* **6**(2): 121-130.

Usher, K. M., J. Kuo, J. Fromont and D. C. Sutton (2001). Vertical transmission of cyanobacterial symbionts in the marine sponge Chondrilla australiensis (Demospongiae). *Hydrobiologia* **461**: 15-23.

Usher, K. M., D. C. Sutton, S. Toze, J. Kuo and J. Fromont (2005). Inter-generational transmission of microbial symbionts in the marine sponge Chondrilla australiensis (Demospongiae). *Marine and Freshwater Research* **56**(2): 125-131.

Waterbury, J. B., C. B. Calloway and R. D. Turner (1983). A cellulolytic nitrogen-fixing bacterium cultured from the Gland of Deshayes in shipworms (Bivalvia: Teredinidae). *Science* **221**: 1401-1403.

Woollacott, R. M. (1981). Association of bacteria with bryozoans larvae. *Mar. Biol.* **65**: 155-158.

Woollacott, R. M. and R. L. Zimmer (1975). A simplified placenta-like system for the transport of extraembryonic nutrients during embryogenesis of *Bugula neritina* (Bryozoa). *J. Morph.* **147**: 355-378.

Woollacott, R. M. and R. L. Zimmer (1977). Biology of Bryozoans. R. M. Woollacott and R. L. Zimmer. New York, Academic Press**:** 57-142.

Yahel, G., J. H. Sharp, D. Marie, C. Hase and A. Genin (2003). In situ feeding and element removal in the symbiont-bearing sponge Theonella swinhoei: Bulk DOC is the major source for carbon. *Limnology and Oceanography* **48**(1): 141-149.

CHAPTER 2

The *Bugula neritina-Endobugula sertula* Symbiosis: symbiont and bioactive compound localization

ABSTRACT

The cosmopolitan bryozoan *Bugula neritina* harbors a proteobacterial symbiont, "*Candidatus* Endobugula sertula," synthesizing bryostatins, which are effective anti-predatory defense compounds. To investigate the chemical ecology of the defense compounds in the symbiosis, I developed a method for simultaneous *in situ* localization of bryostatins and symbionts in the *B. neritina-E. sertula* association. The results of this study demonstrate an ontogenetic shift in bryostatin levels and localization during larval metamorphosis and throughout the host life cycle. Bryostatins are most concentrated on the surface of the larvae, and the larvae retain a bryostatin coating throughout metamorphosis. When *B. neritina* goes through early development and produces its chitinous exoskeleton, the bryostatins are shed onto the settling substrate. The symbiont *E. sertula* is exclusively associated with a transitory larval tissue, the pallial epithelium, throughout metamorphosis. After metamorphosis, when the pallial epithelium is internalized, histogenic differentiation occurs and gives rise to a preancestrula. The symbionts occur in the cystid, a layer of tissue in the epidermis from which the next zooid in the colony arises. Aggregates of *E. sertula* in buds of ancestrulae (single zooid) appear to be synthesizing new bryostatins, though it is not clear whether the exterior of the colony is associated with bryostatins. The rhizoids, root-like structures which attach the host colony to substratum, are coated with bryostatins and contain *E. sertula* in the funicular cords, strings of tissue that connect rhizoids to zooids and ovicells of a colony.

INTRODUCTION

The bryozoan *Bugula neritina* (Cheilostomata, Cellularoidea) harbors an obligate symbiotic gamma proteobacterium, *Candidatus* "Endobugula sertula," which is transferred to the host larvae prior to release from the adult (Haygood and Davidson 1997). Woolacott (1981) first showed the presence of bacteria located within a deep groove in the larvae, the pallial sinus, which is effectively closed off from the surrounding seawater, and these bacteria were later identified as "*Candidatus* E. sertula" (Haygood and Davidson 1997). *B. neritina* larvae are heavily defended from fish predation; after being ingested and expelled by fish, *B. neritina* larvae are still capable of settling and developing into normally functioning adult colonies (Lindquist and Hay 1996). *B. neritina* is the sole source of the bryostatins, a group of cytotoxic complex polyketides currently in trials for their potential clinical value in treatment against a variety of cancers. Symbiotic *E. sertula* synthesizes the bryostatins (Davidson *et al.* 2001), which have recently been shown to be the bioactive compounds responsible for anti-predation defense in *B. neritina* larvae (Lopanik *et al.* 2004). The symbiont benefits the host by producing the chemical defense for the larvae, and there is no indication thus far that the bacterium plays a role in host nutrition (Davidson *et al.* 2001).

Bugula colonies are sessile, arborescent colonies consisting of multiple soft individual animals, or zooids, with fully developed gut tracts, encased within a chitinous cuticle. Surrounding the mouth of each zooid is a crown of ciliated tentacles, the lophophore, which filters food toward and into the mouth. Modified zooids include the rhizoids, root-like structures for substrate attachment, also covered

by a cuticle, and ovicells, chambers in which embryos develop. The zooids, which appear highly compartmentalized, are actually connected to each other by a funiculus – an organ consisting of ribbons of tissue running throughout the colony. The funicular system connects all of the feeding zooids in a colony to each other, as well as connecting the rhizoids and the ovicells to the rest of the colony. At the junction between two zooids, the funicular cords contain communication plates, which are provided with pores, thought to allow the transport of nutrients and small molecules throughout the individuals in a colony. A bundle of funicular cords converges and enter the ovicell at the junction between the ovicell and the maternal zooid, and these funicular cords are hypothesized to function in the transport of extraplacental nutrients to the developing embryo (Woollacott and Zimmer 1975). Transmission electron microscopy shows the presence of bacteria in channels within the funicular cords (Woollacott and Zimmer 1977) in colonies, but any bacteria in funicular cords of *B. neritina* remain unidentified.

Like many marine invertebrates, *B. neritina* has a complex life cycle. Figure 1 shows the progression from developing embryo to adult colony. In *B. neritina*, sexual reproduction occurs via internal fertilization, and embryos are brooded in the ovicell chamber. After embryonic development is complete in the ovicell, fully developed swimming, soft-bodied lecithotrophic larvae are released into the water column. Once attached to a settlement surface, *B. neritina* larvae undergo drastic morphogenesis within two hours, and histogenic differentiation over a period of two days, resulting in an ancestrula, the first zooid of the colony. The primary ancestrula can subsequently give rise to an entire colony via asexual reproduction. The ancestrular cystid buds into

a new zooid and ultimately continues to form a full adult colony (Woollacott and Zimmer 1977). In addition, new colonies can bud from rhizoids; after senescence or mechanical removal of colonies, colonies can be renewed solely from rhizoids left on the substrate (Gordon 1977).

E. sertula are consistently found in swimming *B. neritina* larvae (Haygood and Davidson 1997), suggesting that developing embryos are inoculated with the symbiont before larval release. However, it is unknown where *E. sertula* occurs in the adult colonies, where it proliferates, how it ultimately inoculates the embryos before larval release, and how adult colonies are inoculated during asexual production of new zooids.

Previous research demonstrates that larvae of marine invertebrates contain bioactive compounds that protect larvae from predation and microbial fouling (Gil-Turnes *et al.* 1989; Gil-Turnes and Fenical 1992; Lindquist and Hay 1995). It is widely accepted that defense compounds impact the evolution of invertebrate larval morphology and behavior (Lindquist and Hay 1995; McClintock and Baker 1997; Iyengar and Harvell 2001). Many chemically defended larvae tend to be larger, visually apparent to predators, and active in the water column during daylight hours. Unlike many marine invertebrates, however, *B. neritina* adults contain only nominal concentrations of the bioactive bryostatins relative to the amounts in larvae (Lopanik *et al.* 2004). *B. neritina* larvae are heavily defended from fish predation by high levels of bryostatins, while the adults, containing undetectable amounts of bryostatins, are not defended from fish predation (Lindquist 1996; Lopanik *et al.* 2004). Lopanik *et al*. (2004) propose that the bryostatins protect the larvae, because they are a highly

vulnerable stage in the life cycle, and bryostatins are undetectable in the chitinous, mechanically defended adult *B. neritina* colonies. Lopanik *et al*. (2004) measured average bryostatin levels from entire colonies, not excluding the possibility of regions of localized high concentrations of bryostatins in adult colonies.

Phorbol dibutyrate displacement (PDBu), which is used at the National Cancer Insititute for screening of crude extracts from marine organisms (deVries *et al.* 1997), is an assay that screens for compounds that bind protein kinase C (PKC). Bryostatins bind to PKC at the same site as phorbol ester (Wender *et al.* 1988; Kazanietz *et al.* 1994), and because it has a high affinity for PKC, it can irreversibly displace the phorbol (DeVries *et al.* 1988). *B. neritina* extracts, along with tritiated phorbol dibutyrate (^{3}H-PDBu), are incubated with rat brain liposomes, which are rich in PKC. Liposomes are collected on a filter, unbound ^{3}H-PDBu is rinsed away, and tritium signal is read. Decreases in the tritium levels relative to a sample without added extract indicate PKC-binding activity.

PDBu assays have been commonly used to assess presence of bryostatins (DeVries *et al.* 1988; Schaufelberger *et al.* 1990; deVries *et al.* 1997), and Davidson used PDBu assays to compare PKC-binding activity of extracts of tissues from various portions of *B. neritina* colonies (Davidson 1999). Because the assay is not followed by a purification step, it does not confirm that the active extract contained bryostatins, it only tests for "bryostatin-like activity." However, other bryozoans without bryostatins – *Scrupocellaria* sp. and *Bowerbankia* sp. – showed no activity in the assays (Davidson 1999). In addition, the NCI has analyzed many *B. neritina* samples and found PDBu displacement was due to bryostatins only (unpublished data).

Using PDBu assays normalized for relative comparison by tissue weight, Davidson (1999) found that levels of bioactivity (PKC-binding) varied across different parts of *B. neritina* adult colonies. The highest levels of bioactivity on adult colonies are on the rhizoids and the growing tips (new buds and top two zooids) of colonies, while the middles of the colonies do not display detectable levels of bioactivity (Davidson 1999).

PDBu assays show that levels of bioactivity are equivalent in both halves of *B. neritina* larvae and are not correlated with the presence of *E. sertula* in the pallial sinus (Davidson 1999). The uniformity of bryostatin distribution in larvae suggests that the symbionts might excrete the bryostatins, which are subsequently moved around in the host tissues. Another possible explanation is that the larval bryostatins originate not from *E. sertula* in the pallial sinus, but from *E. sertula* in the adult, and that *E. sertula* translocates the compounds to the developing embryos during brooding. This bryostatin dose may be enough to supply a young larva with protective compound to last throughout its swimming, settling, and subsequent metamorphosis and development.

The present study focuses on the characterization of ontogenetic fluctuations in bryostatin production and shifts in symbiont proliferation and localization over a time series that includes *B. neritina* embryonic development, larval metamorphosis and early development. Here we address questions regarding two aspects of the symbiosis: 1) the maintenance of symbiont populations throughout the life cycle, with a focus on major morphogenetic events during larval metamorphosis, as well as asexual and sexual reproduction; and 2) the location of the bryostatins throughout the

host life cycle. This study employs conventional methods for *in situ* localization of bacteria in addition to a new method for *in situ* visualization of the bryostatins in *B. neritina*.

B. neritina larvae undergo drastic morphogenesis and histogenesis to develop into adult colonies. This study allows us to answer basic questions about how the association between the host and the symbiotic producer of a defense compound is maintained across a complex host life cycle. Where are the symbionts located in the adult colony? How are symbionts transferred to the larvae before release? How is symbiont inoculation ensured during asexual reproduction? How and when do *B. neritina* larvae obtain their bryostatins? Do metamorphosing *B. neritina* retain bryostatin protection? Are the bryostatins retained after metamorphosis when the protective chitinous cuticle emerges? Are bryostatins detectable in specific parts of adult colonies?

To answer these questions, *B. neritina* larvae were harvested, allowed to settle, and fixed at various timepoints throughout the life cycle. *E. sertula* and the bryostatins were then both simultaneously localized, and the data were used to construct a model of *E. sertula* proliferation and bryostatin biosynthesis throughout the life cycle of the symbiosis.

MATERIALS AND METHODS

***B. neritina* collection and larval harvesting.** *B. neritina* colonies were collected by hand from the SIO Pier, via SCUBA or on snorkel. Adults were maintained in

seawater tanks for larval harvesting. Adult colonies were immediately separated into branches with ovicells, branches without ovicells, and rhizoids. These colony pieces were rinsed with sterile filtered seawater (0.2μm filter) and fixed in 4% paraformaldehyde-20mM sodium phosphate (15mM Na_2HPO_4, 5mM NaH_2PO_4; pH 7.4)-0.5M NaCl overnight at 4°C and then placed in 70% EtOH at –20°C for long-term storage. Adult *B. neritina* were kept in ambient running seawater tanks with a 12hr/12hr light/dark cycle, and larvae were collected in the mornings by Pasteur pipet from the water column in the tanks. Larvae were pipetted into a 15-ml Falcon tube and chilled in seawater in an ice bucket, causing the larvae to fall to the bottom of the tube. Seawater was decanted and larvae were subsequently rinsed three times in sterile filtered seawater (0.2μm filter) to remove any particles or contaminating bacteria. In order to fix the swimming larvae, the seawater was removed and larvae were fixed in 4% paraformaldehyde (recipe above) overnight at 4°C, and stored long-term at –20°C in 70% EtOH.

Larval settlement and metamorphosis. Yool *et al.* (1986) demonstrated that excess potassium concentration in defined seawater medium induces settlement and metamorphosis in larvae of four marine invertebrates. The induction effect is dose-dependent, and levels as high as two times the normal amount of potassium in seawater, in the form of potassium chloride (KCl), induce metamorphosis and development in the invertebrates without negative effects on development or growth. In an adaptation of this protocol, *B. neritina* larval settlement was induced through incubation in artificial seawater with an elevated KCl concentration, 2X that in normal seawater (50mM $MgSO_4$, 10mM $CaCl_2$, 300mM NaCl, 10mM KCl). This eliminated

the variable larval swimming stage, which can take anywhere from 20 minutes to 12 hours, allowing synchronization of settlement, metamorphosis and histogenic differentiation. *B. neritina* larvae harvested from the adults were rinsed in sterile filtered seawater and placed on glass microscope slides (Fisherbrand plain pre-cleaned glass slides) with a Pasteur pipet. Residual seawater was pipetted from the slide and replaced with 500µl artificial sterile seawater containing 20mM KCl. Larvae were placed in the dark for 1.25 hours, after which the number of settled larvae were counted. At this stage, when most larvae (average 80% of starting larvae) had attached and begun settlement, sterile filtered artificial seawater with ambient KCl concentration was added to the slide.

After initiation of settlement by adding high-potassium artificial seawater, metamorphosing larvae were observed on a dissecting microscope. After settlement, metamorphosis occurs over about 35-40 minutes (Woollacott and Zimmer 1977; 1978). The timing of major events during metamorphosis and early development was recorded. The stages investigated in this study included: 1) swimming larvae (before addition of high-potassium seawater); 2) attachment to substrate (the glass slide) and loss of the outer layer of ciliated cells; 3) eversion of the pallial epithelium; 4) reversion of the pallial epithelium, internalization of the apical disc and elongation; 5) further elongation and differentiation into the preancestrula; and 6) the development of the first ancestrula and a bud for a second zooid. Slides were stopped at these different time points and fixed by submerging the slides in 4% paraformaldehyde in Coplin jars for overnight fixation at 4°c and long-term storage in 70% EtOH at 20°C.

Symbiont localization. Fixed *B. neritina* larvae were placed on a slide and examined under various UV microscope filter sets, including those designed to visualize DAPI (Chroma set 31000v2), FITC (Chroma set# 41001), TRITC/Rhodamine/CY3 (Chroma set# 31002), and CY5(Chroma set# 41008). *B. neritina* autofluoresced the least in the far-red wavelengths, and as a result, CY5 (excitation maximum=648nm; emission maximum=668nm) was chosen as the label for oligonucleotide probes in fluorescence in situ hybridization (FISH).

FISH was performed on the samples, either in microfuge tubes (swimming larvae and adult colony pieces) or directly on the glass settlement slides (metamorphosis and early development). CY5-Es1253 (5'-CATCGCTGCTTCGCAACCC-3'), an oligonucleotide specific for *E. sertula* (Haygood and Davidson 1997), was used to localize *E. sertula*. A single base mismatch probe was used as a negative control to confirm the specificity of the *E. sertula* specific probe. The negative probe, CY5-Eg1253, (5'-CATCACTGCTTCGCAACCC-3') specifically hybridizes to the *Bugula simplex* symbiont, *Endobugula glebosa* (Lim and Haygood 2004). The general eubacterial probe EUB338 (5'- GCTGCCTCCCGTAGGAGT -3') was used to localize general bacteria in the samples. FISH was performed in a humidity chamber in hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.4], 0.01% sodium dodecyl sulfate) with 35% percent formamide for 2 h. All probes, the Es1253, Eg1253 (negative), and EUB338 were at a concentration of 5ng/ul final concentration in hybridization buffer. After hybridization, the slides were incubated at 48°C in wash buffer (0.7 M NaCl, 20 mM Tris-HCl [pH 7.4], 50 mM EDTA, 0.01% sodium dodecyl sulfate) for 20 minutes. The wash buffer was removed with a Milli-Q water rinse, and

slides were air-dried and mounted in VectaShield (*Vector Labs*, Burlingame, CA) for visualization and image acquisition on the microscope. Probes used in this study are listed in Table 1.

Bryostatin Localization. As in the FISH procedures, bryostatin localization on samples in metamorphosis and early developmental stages were performed on slides, and localization was performed on adults and swimming larvae in tubes.

The bryostatins are clinically unique, because unlike most other potential anti-cancer drug candidates, the bryostatins interact with protein kinase C (PKC). They bind to the same site of the enzyme as phorbol esters, and they have significantly higher affinities to PKC than their natural phorbol substrates (DeVries *et al.* 1988; Wender *et al.* 1988). Based on their high affinity for binding to the phorbol site in mammalian PKCα, a fluorescence-based bryostatin detection method was developed. Much like the PDBu assay discussed in the introduction, this method only tests for "bryostatin-like" activity and detects PKC binding. However, the NCI has executed multiple tests showing that the bryostatins bind PKC with greater affinity than any other substance in *B. neritina*. Before each use, DTT, BSA, and leupeptin were added to final concentrations of 1mM, 1% (wt/vol), and 40μg/ml, respectively, to a stock "PKC buffer" (150mM NaCl, 20mM HEPES, 0.5mM Na_2EDTA, pH 7.5) for the PKC experiments. Bugula samples were incubated in 200ul of the PKC buffer, which was amended with 100ng mouse PKCα (Upstate Signalling, Charlottesville, VA; 2ul total in 200ul PKC buffer) for 20min. Samples were washed two times (5 min each) in phosphate-buffered saline (PBS; 10mM Na_2HPO_4, 2.7mM KCl, 140mM NaCl, pH 7.4) and then incubated in PBS with mouse M4 monoclonal anti-PKCα (Upstate

Signalling, Charlottesville, VA) at a final concentration of 10μg/ml overnight (approximately 16 hr). After overnight incubation in the primary antibody, the samples were washed in PBS three times (5 min each), and incubated in 200ul of 1% gamma globulins/PBS blocking solution for 5min. Secondary antibody (Alexa 555-conjguated goat anti-mouse IgG_1 (Invitrogen, Carlsbad, CA) was added to the blocking solution at a final concentration of 5μg/ml and incubated in the dark for 2hr. Following the secondary antibody incubation, samples were rinsed (2x 5min) in PBS. All steps were carried out at room temperature. For simultaneous bryostatin and *E. sertula* localization, the samples were subsequently treated as described above for the FISH protocol.

Protein kinase C (PKC) antibody(Ab)-based bryostatin detection was performed first, followed by FISH. The samples were subsequently visualized with a Zeiss Axioskop with an LSM Pascal confocal attachment equipped with a krypton/argon laser. Image capture was performed with Zeiss LSM Pascal image acquisition software.

Confocal microscopy uses a mixed krypton/argon laser to excite at wavelengths of 488, 568, and 647nm, and through a variety of filters, different emission wavelengths can be detected. Confocal microscopy permits one to focus on a very fine plane, on the nanometer scale, eliminating all out-of-focus fluorescence, and allowing for precise optical sectioning through millimeter-thick specimens.

In order to confirm that signal observed in the samples was true signal, negative controls (single base mismatch probes for bacterial localization and without the

addition of PKCα) were all taken at exposure times identical to or greater than exposures of the experimental treatments.

RESULTS

Synchronization of settlement and metamorphosis

In this study, larvae were settled onto glass slides in high potassium seawater (t=0 at addition of high-potassium seawater). Table 2 summarizes the timing of 1) attachment to substrate (the glass slide) and loss of the outer layer of ciliated cells; 2) eversion of the pallial epithelium; 3) reversion of the pallial epithelium, internalization of the apical disc and elongation; 4) further elongation and differentiation into the preancestrula; and 5) the development of the first ancestrula and a bud for a second zooid.

Bryostatin Localization

Figure 2 shows bryostatin detection in *Bugula*. In *B. neritina* and *B. simplex* larvae, (panels A, B) both known to contain bryostatins (Lim and Haygood 2004), the bryostatin signal is evenly distributed across the outer layer of the larvae. In the absence of PKCα, there is no signal on the exterior of *B. neritina* larvae (panel C), nor is there signal in or on *B. neritina* rhizoids (panels D,E). In larvae of *B. turrita*, an aposymbiotic *Bugula* species whose larvae do not contain bryostatin-like molecules (Lim, unpublished), the signal on the larval exterior is absent (panel F). When adult structures, including feeding zooids, rhizoids, and ovicells, were sliced open, there was no additional internal bryostatin signal when images were captured exposure time identical to the experimental treamtents (not shown). The results suggest that the

absence of signal reflects the absence of bryostatins, and not the inability of the antibodies to penetrate the tissue or cross the chitinous exoskeleton.

Simultaneous Symbiont and Bryostatin Localization

By confocal microscopy, the fluorophores Alexa555 (excitation=555nm; emission=565nm) and CY5 (excitation maximum=648nm; emission maximum=668nm) were detected with the argon/krypton laser with relatively little background autofluorescence from *B. neritina*. As a result, the *E. sertula*-specific oligonucleotide probe was end-labeled with CY5, and bryostatins were detected with an Alexa-555 conjugated secondary antibody.

Though both FISH and PKC/Ab-based bryostatin detection both yielded intact signal in *B. neritina* samples, when FISH was done on the samples before bryostatin detection, the FISH signal was undetectable. When FISH was performed after the PKC/Ab-based bryostatin detection, both signals were visualized on the confocal microscope, therefore making it possible to localize bryostatins and *E. sertula* simultaneously in *B. neritina*.

Symbiont Inoculation and Bryostatin Loading into Larvae

Figure 3 shows a representative confocal micrograph of *E. sertula* localization near a brooding embryo and in funicular cords leading to an embryo brooding in an ovicell chamber. While the host tissue exhibits strong autofluorescence, the symbiont signal is detectable by the *E. sertula*-specific probe CY3-Es1253 (yellow). The symbiont cells occur in dense aggregates inside the exoskeleton, at the junction

between the ovicell and the feeding zooid. Surrounding the aggregates of the symbionts is a strong bryostatin signal (blue, panel B), spreading into the ovicell and wrapping around the developing larva in the ovicell interior. Within the ovicell (panel C), the symbiont also is in the funicular cords connecting to the ooecial epithelium and embryo. After larval release, neither symbionts nor bryostatins are detectable in the empty ovicell (not shown).

Shifting Bryostatin Levels and the Fate of the Symbiont

Figure 4 shows bryostatin and *E. sertula* localization in *B. neritina* during larval metamorphosis and early development. Simultaneous localization of *E. sertula* and the bryostatins in a *B. neritina* larva (Panel A) shows the symbiont (yellow) is present in the larval pallial sinus, and the bryostatins (blue) appear evenly distributed around the exterior of the larva. The entire swimming larva is coated with bryostatins, and the bryostatin signal does not appear more concentrated around *E. sertula* or the pallial sinus. Panel B shows *B. neritina* localization after attachment to the settlement substrate, during movement of the larval pallial epithelium. This sample is only labeled for general bacteria and *E. sertula* and is not labeled for bryostatins, simply because this result was obtained before the PKC/Ab protocol was developed. The movement of the pallial epithelium is rapid, and the eversion such as shown in this picture was rarely observed. The corona and associated transitory larval tissues involute toward the center of the metamorphosing individual, drawing the pallial epithelium out of its groove and stretching it out and around the larva toward the oral surface. Panel B shows a larva after eversion of the pallial epithelium: *E. sertula*

appear in a tight ring (yellow) around the metamorphosing larva, near the oral surface where the pallial epithelium meets the everted tissue of the metasomal sac near the oral surface. FISH with the general eubacterial probe (CY5-EUB338, red) and the *E. sertula*-specific probe (CY3-Es1253, yellow) shows the only bacteria associated with the *B. neritina* at this stage are *E. sertula*, which are migrating with the pallial epithelium.

After reversion of the pallial epithelium toward the aboral pole, the pallial epithelium contracts around the apical disc and ultimately invaginates into the center of the developing *B. neritina*. Aggregates of *E. sertula* cells (yellow) are internalized with the pallial epithelium (Panel C). The bryostatins (blue) remain around the exterior of the individual through this stage of metamorphosis. Panel D shows *B. neritina* after further elongation. Though the bryostatins are present around most of the developing individual, the aboral end lacks the bryostatin coating (blue). When the preancestrula develops and is covered with a chitinous exoskeleton (Panel E), the bryostatins are no longer on the animal's exterior but on the settlement surface (blue). The bryostatin coating remains on the larva during attachment to the substrate, pallial epithelium eversion and reversion, until the emergence of the cuticle, when they are shed onto the settling surface. At the same stage in which the bryostatins are shed, *E. sertula* aggregates (yellow, Panel F) occur in the apical tip of the preancestrula above the developing lophophore. Panel F shows bacterial localization but was not treated with the PKC/Ab for bryostatin detection.

Figure 5 shows the first ancestrula of a *B. neritina* colony. In a fully developed ancestrula with a feeding lophophore and an early bud for a second zooid, *E. sertula*

cells (yellow) aggregates are present in the cystid surrounding the lophophore, and in the bud forming the second zooid. Panel C shows a layer of bryostatin signal surrounding the aggregates in the cystid. This micrograph is representative of results seen in aggregates of all observed buds. Bryostatin signal was consistently low enough in these samples that detection was challenging.

Figure 6 shows a longitudinal section of a *B. neritina* rhizoid. Dual-labeled *E. sertula* cells (CY3-Es1253, red; FITC-EUB, green) occur in funicular cords in the rhizoids (Panel A). In addition. there are diverse bacterial morphotypes on the exterior of the rhizoid. *E. sertula* (yellow) is inside the rhizoid, associated with the funicular cords, and no other bacterial cells are detected inside the rhizoids. Bryostatin detection method was not used on the sample in panel A. In Panel B, bryostatins and *E. sertula* are both localized in rhizoids. The bryostatins form a contiguous coating on the exterior of the rhizoids (blue), and *E. sertula* aggregates (yellow) are in the funicular cords inside the rhizoid. Panel C displays a cross-section of the rhizoid, showing the bryostatins on the rhizoid exterior and the symbiont *E. sertula* inside the rhizoid funicular cords. Negative control treatment (no added PKC) of a longitudinal view of a rhizoid is shown in panel D, with low signal.

There is no bryostatin signal in the funicular cords of feeding zooids or rhizoids (not shown). While the symbiont is present in small aggregates in the funicular cords throughout rhizoids, feeding zooids, and ovicells, the only portion of the funicular system in which bryostatins are detected is the network of funicular cords leading to developing embryos in the ovicells.

Throughout metamorphosis, EUB338 only hybridizes to *E. sertula* cells. No other bacteria are detected on or in *B. neritina* until after metamorphosis, when the cuticle becomes densely colonized by an assortment of bacteria (not shown). The preancestrula, during cuticle formation, is the first stage in early development in which bacteria other than *E. sertula* are detected on *B. neritina*. FITC-EUB338 also reveals bacteria on the surface of the rhizoid (Fig 6), but no cells on the exterior bind the *E. sertula*-specific probe.

DISCUSSION

In this study, *E. sertula* and the bryostatins were localized in host bryozoan *B. neritina*. During its life cycle, *B. neritina* exists both as a soft-bodied larva and as a sessile, chitinous adult colony. Localization of symbiont and bioactive compound throughout host metamorphosis, development, and reproduction yields a model of the ontogenetic shifts in localization of the symbiont and the bryostatins. This study focuses on the fate of the symbiont and localization of the bryostatins throughout the host life cycle: what happens to the symbionts in the larval pallial sinus as the larvae undergo metamorphosis and development, and how and when are *E. sertula* transmitted to new generations via asexual and sexual reproduction? Are the bryostatins present throughout all stages of the life cycle? If not, when in the life cycle are they made? At what stage do they disappear, and how does that happen?

E. sertula is the only detected bacterial species inside the tissues in many different parts of the host life cycle. In the ovicell and the larvae, throughout metamorphosis, the general eubacterial probe exclusively hybridizes to *E. sertula* cells. While *E.*

sertula is also the only detected bacterial species inside the preancestrula and ancestrula, other bacteria colonize the cuticle, as well as the settlement substrate. This supports previous hypotheses that the *E. sertula-B. neritina* symbiosis is a highly specific relationship, and the host bryozoan does not harbor other bacteria with which it forms persistent associations.

Figure 7 provides a summary of the localization results in this study, indicating symbiont location and sites where bryostatins were observed in embryonic development, larval metamorphosis, and differentiation and development into a new colony. Localization of symbiont and natural product indicate that *E. sertula* are not always producing bryostatins. For example, in the nascent preancestrula with a newly formed cuticle, *E. sertula* cells are present, but bryostatins are not detectable in any tissues. In contrast, during embryonic development in the ovicell, *E. sertula* densely populates the embryo and the inside of the ovicell. In this stage, there are high levels of bryostatins near and inside the ovicell. These results indicate that *E. sertula* in the pallial sinus of swimming larvae do not produce the bryostatins found in the larvae, but rather the symbionts in the ovicell supply the larva in the adult, prior to release. In sum, I hypothesize that the symbiosis with *E. sertula* has evolved for the particular functions of 1) proliferating to numbers dense enough to colonize a developing embryo; and 2) for producing the protective bryostatin coating on the larva.

After the larva is released from the adult colony, *E. sertula* and the bryostatins are undetectable in the abandoned ovicell. The results of this study corroborate the previous findings (Davidson 1999) that in the larvae, bryostatins are evenly distributed around larvae and are not correlated with the aboral localization of *E. sertula*.

Localization of bryostatins to the surface of the larvae is consistent with the findings of Lopanik *et al*., in which they showed that treatments disturbing *B. neritina* larval exterior result in an increase in palatability (loss of bryostatins from the exterior of the larvae) (Lopanik *et al.* 2004). The results illustrate the potential for bryostatins to be transported around the host tissue and do not necessarily remain on *E. sertula* cells.

Once the larvae are released from adult colonies, *E. sertula* occurs in the pallial sinus of the swimming larva and remains persistently associated with the pallial epithelium during metamorphosis. Throughout metamorphosis, the larva is encased by the calyx, a matrix of polysaccharides released from granules in the metasomal sac at the onset of metamorphosis. During metamorphosis, under the calyx, the pallial epithelium migrates around the developing host to the oral surface, contracts back to the aboral surface, and invaginates into the center of the nascent preancestrula. *E. sertula* cells follow the movement of the pallial epithelium during morphogenesis and development, suggesting that the symbionts are specifically associated with a component of the pallial epithelial cells.

The material for the bryostatin "coating" that is evidently put onto the embryo during development persists throughout metamorphosis and early development. When larvae are enveloped by the calyx, the bryostatins are not the outermost layer on the metamorphosing individual. When the developing individual elongates, a portion of the apical region of developing *B. neritina* is no longer coated with bryostatins. *B. neritina* sheds the remaining bryostatin coat onto the settlement surface as the chitinous cuticle develops in the preancestrula, and bryostatins are not detectable in or on any tissues of the *B. neritina* preancestrulae. The *E. sertula* aggregates that are

internalized with the pallial epithelium during metamorphosis ultimately migrate to the epidermis of the preancestrula as polypide tissues differentiate into lophophore and gut. As the preancestrula develops into the ancestrula, *E. sertula* remains in the cystid, ultimately giving rise to a bud that will form a second zooid, inoculated with symbiont, as shown in Fig. 5.

Rhizoids, which have the potential to produce new adult colonies via asexual reproduction, contain small populations of *E. sertula* in the funicular cords. These aggregates may serve as the inoculum for newly forming zooids. Corroborating the bioactivity data (Davidson 1999), the study presented here uses fluorescence localization to demonstrate the presence of bryostatins on the exterior of the rhizoid cuticle. In contrast with the symbiotic bacteria in the host during metamorphosis and early development, it is not yet clear which *E. sertula* cells synthesize the rhizoid bryostatins, or how the bryostatins are transported to the exterior of the rhizoid cuticle.

My results highlight an important aspect of this symbiosis: not only are bryostatins present at varying levels throughout the life cycle, the symbionts do not produce the bryostatins at a constant level. Localization of bryostatins in adult colonies demonstrates that the bioactive compounds appear to be concentrated for protection of the larva during its swimming stage and subsequent metamorphosis until cuticle formation. I hypothesize that the bryostatins are involved in competition for subtrate or defense of substrate. Localization of bryostatins suggest substrate defense as a potential ecological role of the bryostatins: 1) once the preancestrula develops a cuticle, the bryostatins are shed onto the settlement substrate; and 2) the rhizoids are coated with bryostatins. Bryostatin are detected at their highest levels outside of

ovicells, while embryos are developing, and the bryostatins are loaded onto the embryo before its release as a larva. The exterior coating of bryostatins remains on the host larva until the emergence of the cuticle from the preancestrula, perhaps reflecting a shift from chemical to mechanical defense. Both bioactivity assays and fluorescence-based localization indicate that bryostatins levels are high in rhizoids. I hypothesize that the bryostatins are deterrent to predators that potentially would feed on growing colony tips or embryos within ovicells, and that bryostatins confer competitive advantage to *B. neritina* for claiming substrate with their rhizoids. Fluorescent localization used in my study indicates the presence of bryostatins in the rhizoids, but localization on other portions of the adult colonies, especially the tips of the colonies, was inconclusive.

Symbiont populations are maintained throughout different stages of the host, but at densities that fluctuate throughout the life cycle. *E. sertula* densely populates the ovicell funicular system during embryonic development, but symbionts occur in low numbers in other parts of the colony and in other stages of the life cycle. Aggregates of 5-10 *E. sertula* cells occur sparsely in the rhizoid funicular system, presumably retained as inoculum for future asexually growing colonies. *E. sertula* occurs in small, sparse aggregates in *B. neritina* after metamorphosis. However, in the preancestrula, the symbionts seem to have started proliferating and there are numerous microcolonies in the epidermis during histogenic differentiation of the ancestrula. The differences in symbiont density throughout different parts of the adult colony and different life stages suggests that the symbiont proliferates for inoculation of the developing embryo, and then again in the formation of the ancestrula. With the loss of bryostatins

after the formation of the preancestrula, perhaps the newly proliferating *E. sertula* cells produce bryostatins to replace the bryostatins that were shed during development.

Results of this study suggest that ovicell chambers with developing embryos are densely populated with symbionts actively synthesizing bryostatins. Like other obligate symbionts, *E. sertula* has proven to be resistant to conventional culturing techniques. The identification of a specific tissue type during a particular stage in the life cycle, in which symbionts seem to be in high abundance and producing the compound of interest, lays the foundation for future culturing efforts. Further characterization of the biochemical microenvironment in the tissues outside brooding embryos may identify nutrients or signal compounds essential for symbiont growth and bryostatin production.

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Probe Name	Sequence (5'-3')	Reference
EUB338	5'-GCTGCCTCCCGTAGGAGT-3'	(Loy *et al*. 2003)
Es 1253	5'-CATCGCTGCTTCGCAACCC-3'	(Haygood and Davidson 1997)
Egl 1253(EsNON)	5'-CATCACTGCTTCGCAACCC-3'	(Lim and Haygood 2005)

Table 1. Oligonucleotide probes used in this study. The general eubacterial probe, EUB338, was used to detect bacteria. Es1253, a probe designed specifically to target the 16S rRNA gene sequence of *E. sertula*, was used to detect *E. sertula*. A single base mismatch probe, which corresponds to the 16S rRNA gene sequence of the *B. simplex* symbiont *E. glebosa*, was used as a negative control for the specificity of the *E. sertula* oligonucleotide probe.

Time (h)	Stage	Morphological Description
--	Swimming	Ciliated, free-swimming
0	Attachment	Metasomal sac everted onto substrate; attachment disc formed
1.5	Pallial epithelium (pe) eversion	pallial epithelium evagination toward oral surface; still round/spherical shape
2	Pallial epithelium internalization/elongation	elongation from spherical shape to a pillar perpendicular to the substrate; internalization of apical disc and larval pallial epithelium
48	Preancestrula	Further elongation; emergence of a chitinous cuticle; histogenic differentiation beginning
100	First ancestrula, with new bud	Lophophore fully formed and functional; bud for second autozooid starting to develop

Table 2. Timeline of major morphogenetic movement and histogenic differentiation in *Bugula neritina* larval metamorphosis. Larvae induced to settle by addition of high-potassium seawater undergo metamorphosis and development into the first ancestrula within 100 hours. Individuals were fixed at each of the stages described above; observations and morphological changes are listed for each stage.

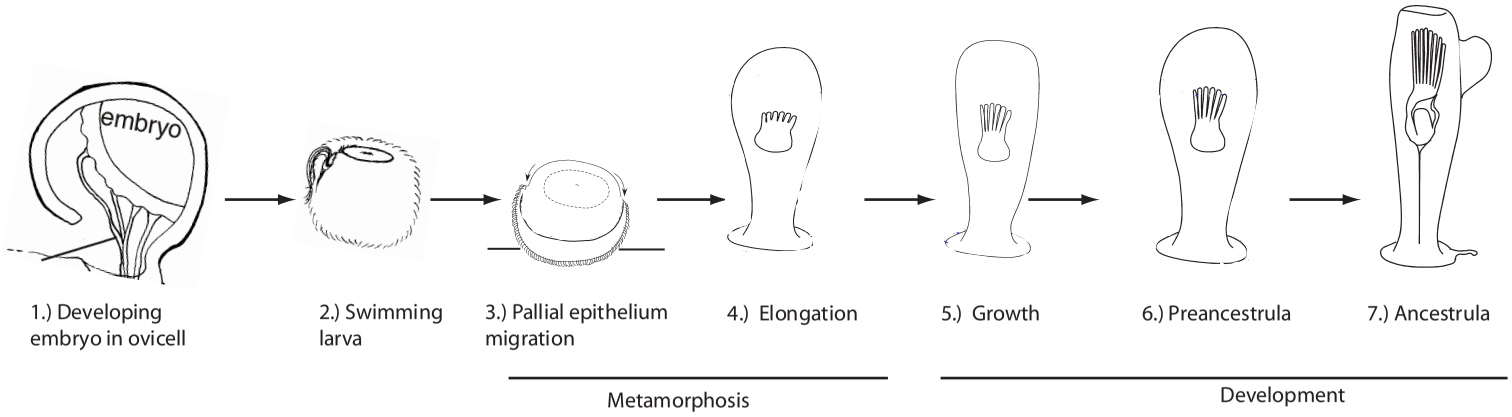


Figure 1. The life cycle of *Bugula neritina*. Embryos (1) develop in the ovicell chamber and are released as swimming larvae. Larvae (2) are released into the water column, settle onto a substrate, and beging metamorphosis. During metamorphosis (3,4), the epithelium of the pallial sinus everts and migrates to the settlement surface, and then reverts back to the aboral pole of the developing individual. *B. neritina* elongates and internal tissues begin differentiation to form a preancestrula (5). The preancestrula develops into an ancestrula (6), the first unit of the colony, which consists of one zooid with a fully functioning feeding structure (lophophore) and digestive system, and a bud of tissue for growth of the next unit of the colony.

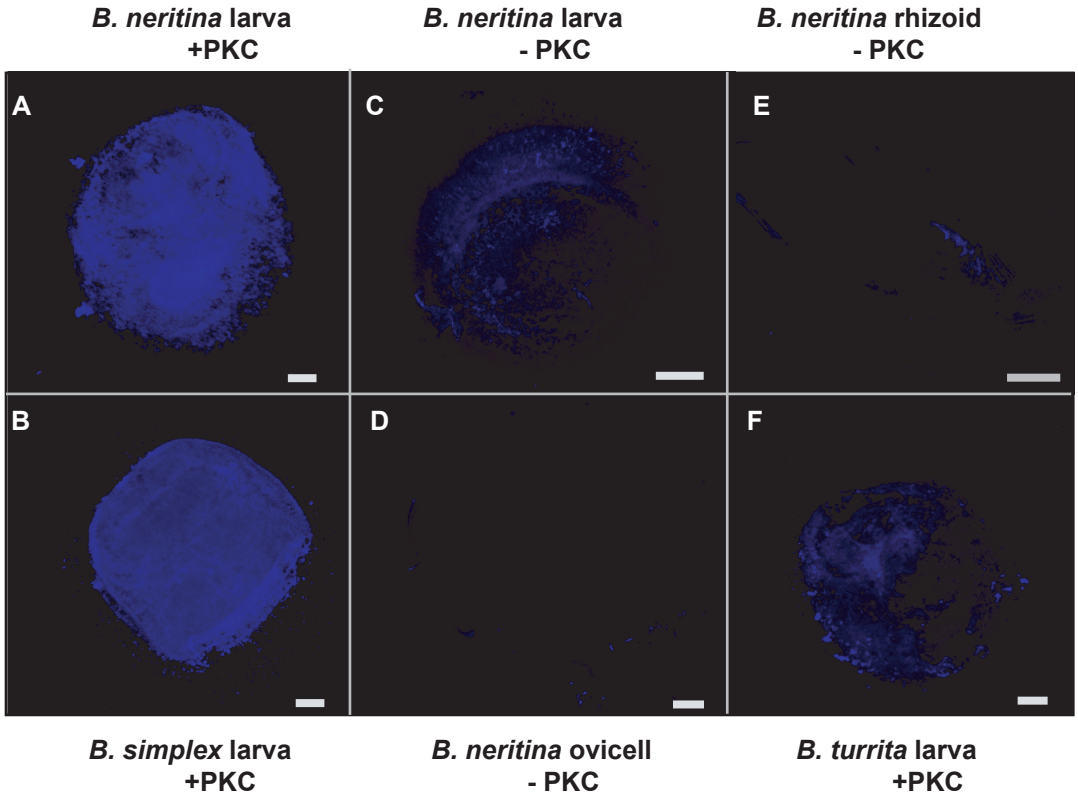


Figure 2. Bryostatin detection in *Bugula* species. (A) *B. neritina* larvae treated with full PKCα-based bryostatin detection. Blue signal indicates bryostatin detection around the exterior of the larva. (B) Bryostatin detection in *B. simplex* larva yields a contiguous blue signal around the exterior of the larva. (C) Negative control: *B. neritina* larva treated for bryostatin detection, but without initial addition of PKCα shows no signal on the larval exterior. Ovicell chamber (D) and rhizoid (E) of adult *B. neritina* colony shows no signal without addition of PKCα shows no signal. (F) Larva of *B. turrita*, an aposymbiotic species without bryostatins in the larvae, shows no signal after application of the bryostatin detection method. Bar= 50um in all micrographs, except (B): Bar=20um.

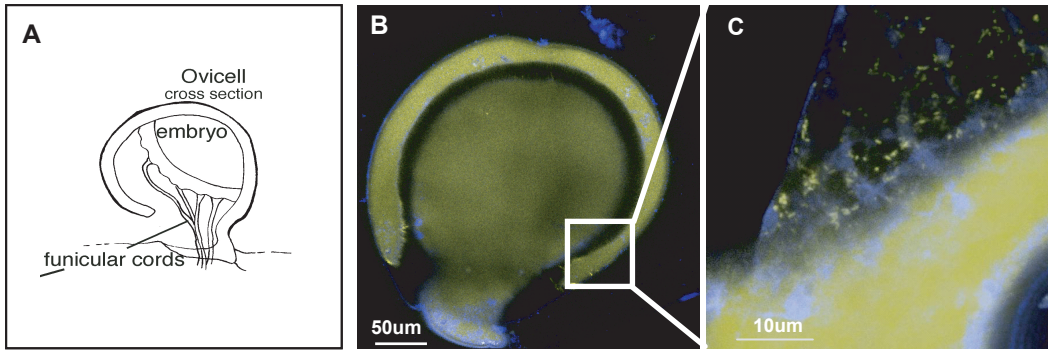


Figure 3. Bryostatin and *E. sertula* Localization in Developing *B. neritina* Embryos in Ovicells. (A) Line drawing of ovicell chamber on adult *B. neritina* colony highlights the location of funicular cords connecting the maternal zooid to the embryo within the ovicell. (B) Bryostatin signal (blue) densely localizes around the base of the ovicell and leads into the embryonic chamber. (C) High magnification image of ovicell reveals *E. sertula* (yellow) and bryostatins (blue) along funicular cords in ovicell.

Figure 4. Bryostatin and *E. sertula* Localization During *B. neritina* Larval Metamorphosis and Early Development.
(A) Localization shows *E. sertula* (yellow) in the pallial sinus of swimming *B. neritina* larva and bryostatins (blue) around the exterior of the larva. (B) FISH detection of *E. sertula* (CY3-Es1253; yellow) and general bacteria (CY5-EUB338; red) shows the symbiont is the only bacterium on the metamorphosing larva. *E. sertula* is associated with the pallial epithelium as it migrates toward the oral surface and meets the evaginated contents of the metasomal sac. (C) Later in metamorphosis once the pallial epithelium and apical disc have invaginated, *E. sertula* (yellow) is localized to the internalized aboral tissues. The developing individual remains covered by a layer of bryostatin. (D) As the individual continues to develop and elongate, the apical portion breaks out of the bryostatin (blue) coating. Symbiotic *E. sertula* remain inside the developing individual at this stage (not shown). (E) As the preancestrula begins to form, the bryostatins (blue) are absent from the individual and present on the settling substrate. (F) During histogenic differentiation and lophophore formation, *E. sertula* aggregates (yellow) occur in the preancestrular cystid.

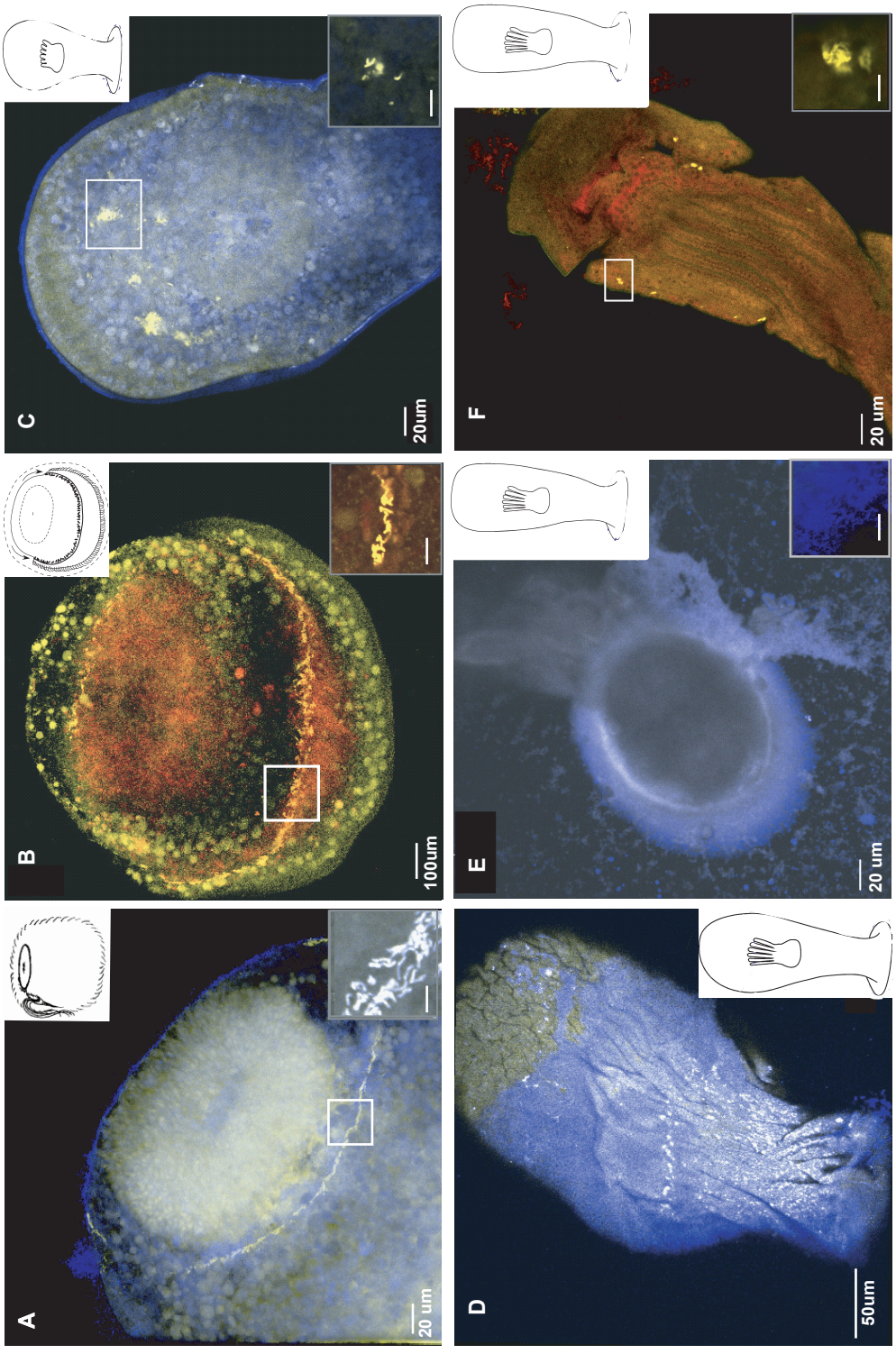


Figure 4. Bryostatin and *E. sertula* Localization in *B. neritina* Larval Metamorphosis and Early Development.

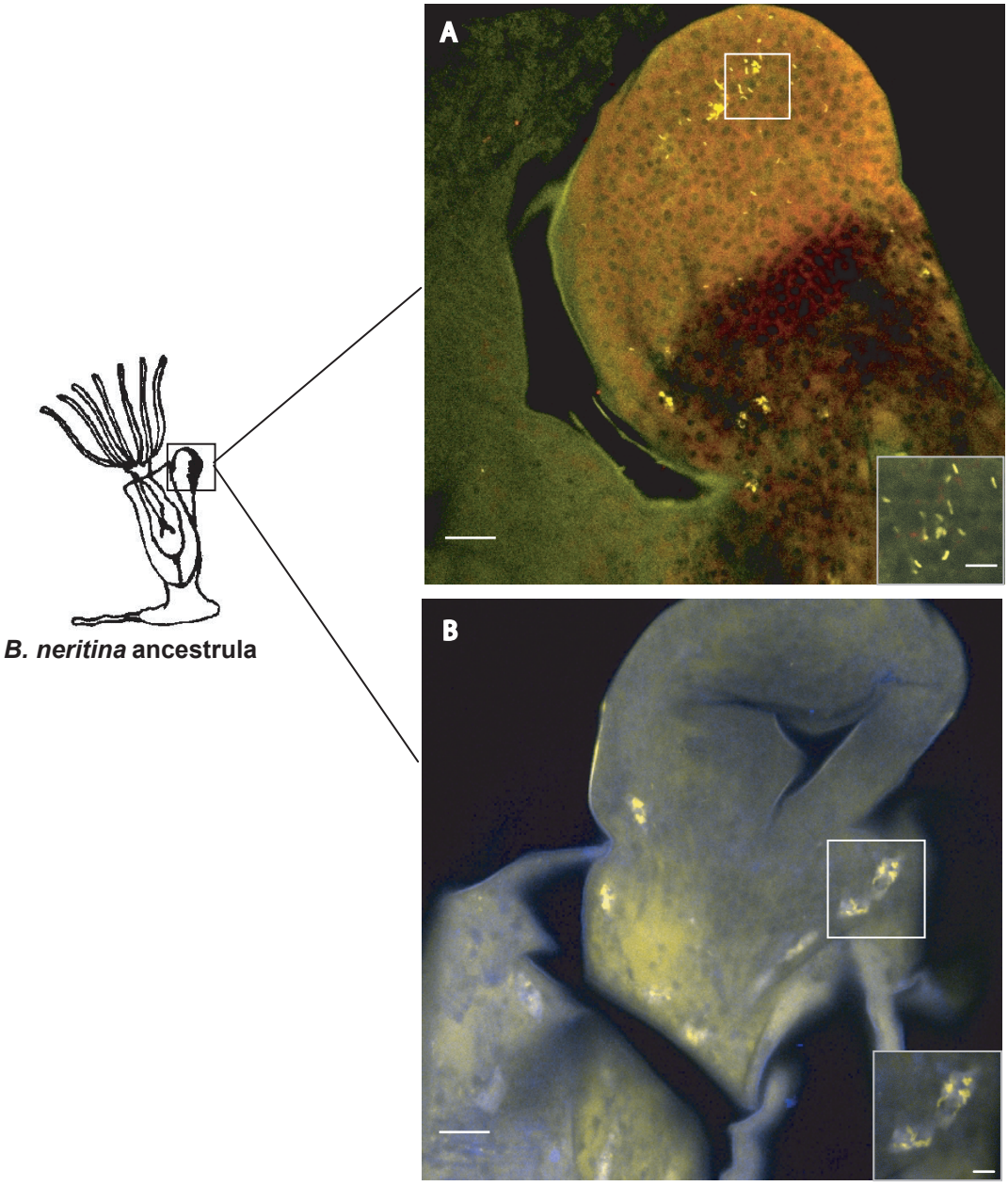


Figure 5. Bryostatin and *E. sertula* Localization in *B. neritina* Ancestrulae. *E. sertula* aggregates and bryostatin surrounding them localize are present in developing buds for second zooids in young *B. neritina* colonies. (A) *E. sertula* localization with CY3-Es1253 (yellow) and CY5-EUB338 (red) shows *E. sertula* as yellow cells throughout the the bud. (B) *E. sertula* aggregates (yellow) are surrounded by a layer of bryostatins (blue) in the first ancestrula, and extending into the bud. A,B: Bar=20um. insets: Bar=5um.

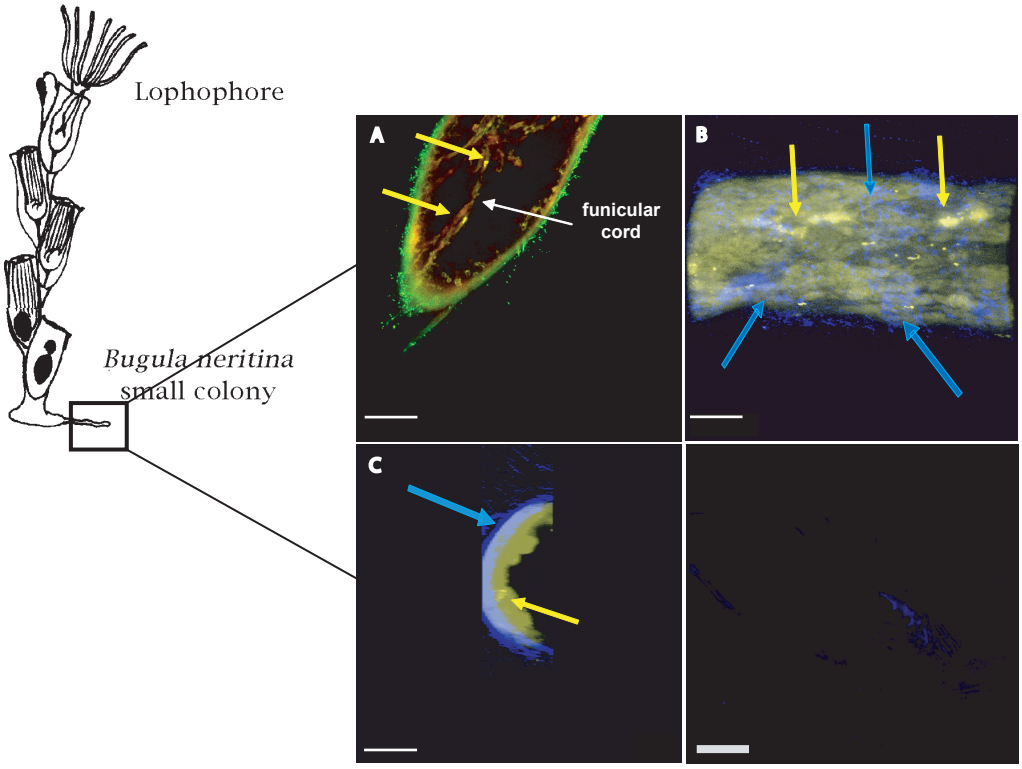


Figure 6. Bryostatin and *E. sertula* Localization in Rhizoids of adult *B. neritina* Colonies. (A) FISH detection of *E. sertula* (CY3-Es1253; green) and general bacteria (CY5-EUB338; red) shows aggregates of the symbiont (yellow) within funicular cords running through the rhizoids. Other eubacteria (green) colonize the cuticle of the rhizoid. (B) Bryostatins (blue) are present in and on the rhizoid cuticle, but not detectable inside. *E. sertula* (yellow) aggregates are present in the funicular cords of the rhizoids. (C) A cross-section of a *B. neritina* rhizoid shows the bryostatin coating (blue) with *E. sertula* (yellow) cells inside. (D) Negative control (no PKC incubation) shows low signal in a *B. neritina* rhizoid. Bar = 50um in all micrographs.

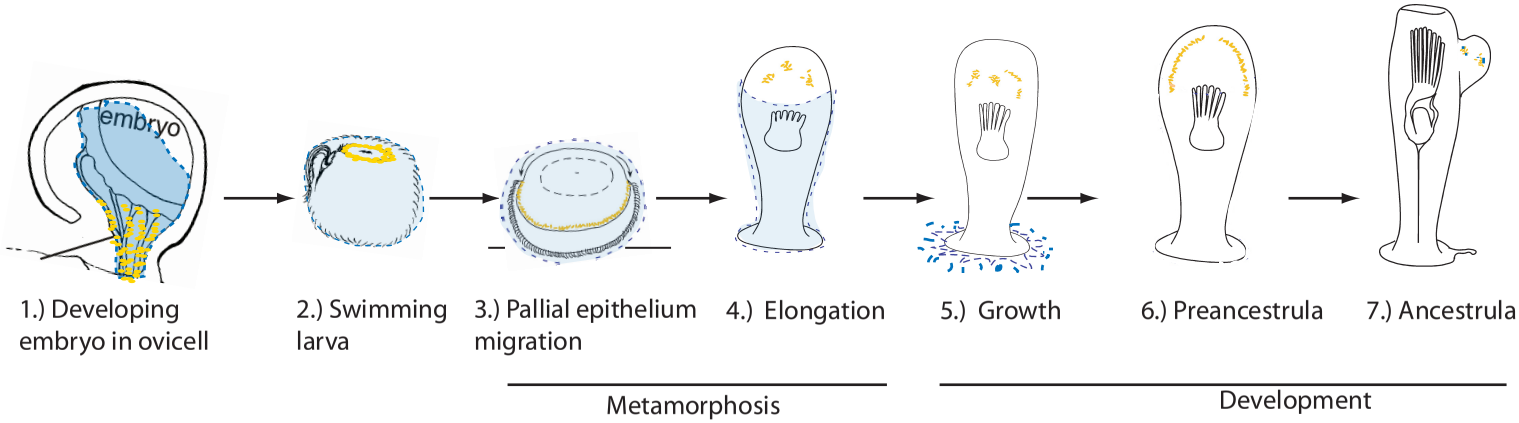


Figure 7. *E. sertula* and bryostatin localization during the life cycle of *Bugula neritina*. Bryostatins are represented by blue dotted line and covering; *E. sertula* cells represented by yellow ovals. Bryostatins are present outside of the ovicell (1) and extend into the embryonary, coating the developing embryo. *E. sertula* cells travel into the embryo via the funicular cords. The swimming larva (2) is coated with bryostatins, and *E. sertula* cells are exclusively in the pallial sinus. *E. sertula* migrates with the pallial epithelium, beneath a bryostatin coating (3), which remains on metamorphosing *B. neritina* until the animal breaks out of the coating at the end of metamorphosis (4) and the coating is shed onto the settlement surface (5). In the preancestrula (6), *E. sertula* cells are present in the cystid, and ultimately are passed to the next zooid through the new bud of the ancestrula (7). At this stage of new zooid formation, *E. sertula* appears to be making bryostatins. All line drawings, Seana K. Davidson.

REFERENCES

Davidson, S., S. W. Allen, G. E. Lim, C. M. Anderson and M. G. Haygood (2001). Evidence for the biosynthesis of bryostatins by the bacterial symbiont "*Candidatus* Endobugula sertula" of the bryozoan *Bugula neritina*. *Appl. Env. Microbiol.* **67**: 4531-4537.

Davidson, S. K. (1999). Biology of the Bryostatins in the marine bryozoan *Bugula neritina*: Symbiosis, cryptic speciation and chemical defense. Scripps Institution of Oceanography. La Jolla, University of California San Diego**:** 210.

deVries, D. J., C. L. Herald, G. R. Pettit and P. M. Blumberg (1988). Demonstration of sub-nanomolar affinity of bryostatin 1 for the phorbol ester receptor in rat brain. *Biochem. Pharmacol.* **37**: 4068-4073.

deVries, D. J., K. S. Rao and R. H. Willis (1997). Application of a radioreceptor assay to the screening and characterisation of compounds from marine organisms with activity at the phorbol ester binding site of protein kinase C. *Toxicon* **35**(3): 347-354.

Gil-Turnes, M. S. and W. Fenical (1992). Embryos of *Homarus americanus* are protected by epibiotic bacteria. *Biol. Bull.* **182**: 105-108.

Gil-Turnes, M. S., M. E. Hay and W. Fenical (1989). Symbiotic marine bacteria chemically defend crustacean embryos from a pathogenic fungus. *Science* **246**: 116-118.

Gordon, D. P. (1977). The aging process in bryozoans. Biology of Bryozoans. R. M. Woollacott and R. L. Zimmer. New York, Academic Press**:** 335-375.

Haygood, M. and S. Davidson (1997). Small-subunit rRNA genes and in situ hybridization with oligonucleotides specific for the bacterial symbionts in the larvae of the bryozoan Bugula neritina and proposal of "Candidatus Endobugula sertula". *Appl. Environ. Microbiol.* **63**(11): 4612-4616.

Haygood, M. G. and S. K. Davidson (1997). Small subunit ribosomal RNA genes and *in situ* hybridization of the bacterial symbionts in the larvae of the bryozoan *Bugula neritina* and proposal of "*Candidatus* Endobugula sertula". *Appl. Env. Microbiol.* **63**: 4612-4616.

Iyengar, E. V. and C. D. Harvell (2001). Predator deterrence of early developmental stages of temperate lecithotrophic asteroids and holothuroids. *Journal of Experimental Marine Biology and Ecology* **264**(2): 171-188.

Kazanietz, M. G., N. E. Lewin, F. Gao, G. R. Pettit and P. M. Blumberg (1994). Binding of [26-3H]bryostatin 1 and analogs to calcium-dependent and calcium-independent protein kinase C isozymes. *Molec. Pharmacol.* **46**(2): 374-9.

Lim, G. E. and M. G. Haygood (2004). "*Candidatus* Endobugula glebosa", a specific symbiont of the marine bryozoan Bugula simplex. *Appl. Env.Microbiol.* **704921–4929**: 4921–4929.

Lindquist, N. (1996). Palatability of invertebrate larvae to corals and sea anemones. *Mar. Biol.* **126**: 745-755.

Lindquist, N. and M. E. Hay (1995). Can Small Rare Prey Be Chemically Defended - the Case for Marine Larvae. *Ecology* **76**(4): 1347-1358.

Lindquist, N. and M. E. Hay (1996). Palatability and chemical defense of marine invertebrate larvae. *Ecol. Monog.* **66**: 431-450.

Lopanik, N., N. Lindquist and N. Targett (2004). Potent cytotoxins produced by a microbial symbiont protect host larvae from predation. *Oecologia* **139**(1): 131-139.

McClintock, J. B. and B. J. Baker (1997). Palatability and chemical defense of eggs, embryos and larvae of shallow-water Antarctic marine invertebrates. *Marine Ecology-Progress Series* **154**: 121-131.

Schaufelberger, D. E., A. B. Alvarado, P. Andrews and J. A. Beutler (1990). Detection and quantitation of bryostatin 1 and 2 in *Bugula neritina* by combined high-performance liquid chromatography and ^{3}H-phorbol dibutyrate displacement. *J. Liquid Chromatogr.* **13**: 583-598.

Wender, P. A., C. M. Cribbs, K. F. Koehler, N. A. Sharkey, C. L. Herald, Y. Kamano, G. R. Pettit and P. M. Blumberg (1988). Modeling of the Bryostatins to the Phorbol Ester Pharmacophore on Protein Kinase-C. *Proceedings of the National Academy of Sciences of the United States of America* **85**(19): 7197-7201.

Woollacott, R. M. and R. L. Zimmer (1975). A simplified placenta-like system for the transport of extraembryonic nutrients during embryogenesis of *Bugula neritina* (Bryozoa). *J. Morph.* **147**: 355-378.

Woollacott, R. M. and R. L. Zimmer (1977). Biology of Bryozoans. R. M. Woollacott and R. L. Zimmer. New York, Academic Press**:** 57-142.

Woollacott, R. M. and R. L. Zimmer (1978). Metamorphosis of cellularioid bryozoans. Settlement and metamorphosis of marine invertebrate larvae. Chia and Rice, Elsevier/North-Holland Biomedical Press**:** 49-63.

Yool, A. J., S. M. Grau, M. G. Hadfield, R. A. Jensen, D. A. Markell and D. E. Morse (1986). Excess Potassium Induces Larval Metamorphosis in 4 Marine Invertebrate Species. *Biological Bulletin* **170**(2): 255-266.

CHAPTER 3

Microbial Ecology of the Tedanolide-Containing Tropical Sponge *Corticium candelabrum*

ABSTRACT

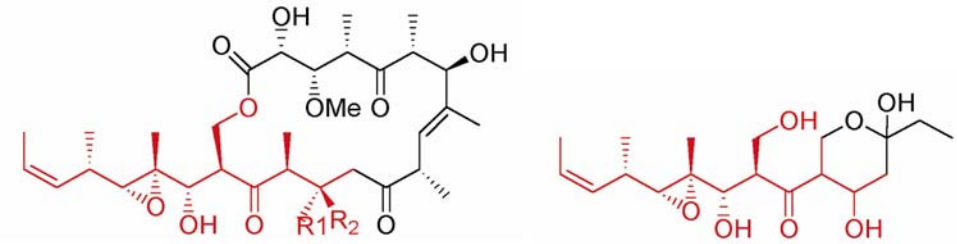
Sponge-microbe associations, which play crucial roles in nutrient and carbon cycling in oligotrophic reefs (Buss and Jackson 1981; Richter and Wunsch 1999; Yahel *et al.* 2003), appear to be spatiotemporally stable. However, little is known about how these consistent associations are assembled and maintained from one sponge generation to the next. Sponges also are a prolific source of diverse bioactive compounds with potential therapeutic and clinical value, many of which have long been suspected to be of microbial origin. Here I investigate *Corticium candelabrum*, a sponge from the tropical Pacific, which contains the tedanolides, complex polyketides with highly potent anti-tumor bioactivity. At the beginning of this project, the ultimate goal was to test the hypothesis that the tedanolides are microbially produced. However, the present study is a necessary prelude that characterizes diversity of microbial associates in *C. candelabrum* and also demonstrates the vertical transmission of a specific subset of microbes (Proteobacteria, Actinobacteria, and an unidentified clade of bacteria) that are consistently present in aggregates within embryos of the sponge. Three of the taxa were present in all adult samples tested over a three-year period. This is the first demonstration of vertical transmission of multiple microorganisms in a marine invertebrate and provides evidence that sponges maintain specific associations with complex assemblages of microbes. Finally, an additional chemotype of *C. candelabrum* was collected, and here I present a preliminary investigation of its associated microbes and suggest approaches for future testing of a microbial origin of the tedanolides.

INTRODUCTION

Sponges are perhaps the most prolific source of bioactive compounds among marine invertebrates. Compounds isolated from sponges fall into a dizzying array of structural categories, but one is particularly relevant to this study. Complex polyketides are secondary metabolites characteristic of microbes. They have been found – and suspected to be of symbiotic origin – not only in sponges, but also in bryozoans and tunicates. Giant multimodular bacterial enzymes (modular polyketide synthases, PKS) with a unique "assembly line" mechanism of polymerization make the complex polyketides (Hopwood and Sherman 1990; Donadio *et al.* 1991). The present study focuses on *Corticium candelabrum*, a sponge that contains tedanolides, members of the complex polyketide class.

Tedanolides and tedanolide derivatives occur in phylogenetically diverse sponge and bryozoan species across the world. Schmidtz *et al*. first identified tedanolide **(1)** in a collection of the Caribbean "fire sponge" *Tedania ignis* collected from Summerland Key in Florida (Schmidtz *et al.* 1984). A highly potent cytotoxin and anti-tumor agent, tedanolide was isolated via bioassay guided fractionation using two cancer cell lines (Schmidtz *et al.* 1984). 13-deoxytedanolide **(2)** was later isolated from *Mycale adhaerens*, collected in Japan (Fusetani *et al.* 1991), and new tedanolide derivatives **(3)** were isolated from the Faulkner lab collections of *Leiosella* cf. *arenifibrosa* from the Philippines, and *Corticium candelabrum* from Palau and the Philippines (Rao, Kantorowski, and Faulkner, unpublished). The myriaporones, isolated from the Mediterranean bryozoan *Myriapora truncata* (Rinehart *et al.* 1996),

includes the highly cytotoxic myriaporone 3 **(4)**, which is closely related to the lower half of the tedanolide molecule. Because tedanolides and tedanolide derivatives have been isolated from diverse invertebrate genera, and because they are classified as complex polyketides, tedanolides have long been suspected to be products of symbiotic microbes.



1: tedanolide $R_1 = R_2 = H$
2: 13-deoxytedanolide $R_1 = OH$; $R_2 = H$
3: new tedanolides (proprietary)

4: myriaporone 3

C. candelabrum (Order Homoscleromorpha) is widespread across shallow reefs in the tropical Pacific, including Guam, Palau, the Philippines, and Fiji. *C. candelabrum* is one of several *Corticium* species in the Pacific known to contain steroidal alkaloids (Ridley and Faulkner 2003), but *C. candelabrum* is the only *Corticium* species from which the tedanolides have been extracted.

Figure 1 shows a cross section of *C. candelabrum*, a sponge collected by members of the Faulkner laboratory in Palau. *C. candelabrum* consists of a highly pigmented, collagenous outer cortex layer. Beneath the cortex is a softer layer, the mesohyl, which is less pigmented and appears gray to light beige when brought to the surface. The mesohyl consists of choanocyte chambers, channels lined by ciliated cells that

filter seawater into the sponge. Within the mesohyl are embryos, which develop within chambers until their release as swimming larvae.

C. candelabrum, like most sponges, can reproduce asexually or sexually. In sexual reproduction, fertilization occurs within flagellated chambers, and the embryo develops into a cinctoblastula larva within the sponge mesohyl (Boury-Esnault *et al.* 2003). The larva is released from the sponge and settles onto substrate where it continues growth into an adult sponge. Persistent presence of microbes in the central larval cavity of *C. candelabrum* has been noted via ultrastructural studies (Boury-Esnault *et al.* 2003), but these microbes have yet to be identified.

The organic extract of *C. candelabrum* collected from Palau is highly potent – detectable at the nanogram level (IC_{50} values $\leq$ 40ng/μl) – against human colon tumor cells (HCT-116). Through bioassay-guided fractionation and NMR spectroscopy, a mixture of tedanolides was isolated as the cytotoxic component in *C. candelabrum* (Rao, Kantorowski, and Faulkner, unpublished results). We also have collected a gray color morph of what we believe is *C. candelabrum* (based on spicule morphology) in the two most recent expeditions. It is rarer than the black version, and to date, we have only collected it in one location, in a small cave outside of Venture Lake, a small marine lake in the rock islands of the Palau archipelago.

The occurrence of four very closely related polyketide metabolites in three unrelated sponges and one bryozoan suggests a microbial source of the tedanolides. Because *C. candelabrum* harbors a diverse assemblage of microbes, identification of a specific microbial source of the tedanolides was not achieved. However, this chapter

identifies microbes associated with developing embryos in *C. candelabrum*, characterizes their spatial distribution within the host sponge, and explores the stability of specific microbe-sponge associations over time and space.

Sponges are ancient metazoans inhabiting diverse benthic environments around the globe, including tropical and temperate marine and freshwater ecosystems. Estimates of sponge species in the world near 10,000 (Brusca and Brusca 1990), but taking cryptic speciation into consideration, this number may be significantly greater. Sponges are dynamic consortia of specialized cell types that form sessile, filter-feeding organisms. There is mounting evidence that sponges mediate the environmental fate of organic and inorganic nutrients, as well as pollutants in the marine environment (Pile 1997; Yahel *et al.* 2003; Hoffmann *et al.* 2005). Much about sponges is still enigmatic; their taxonomy proves challenging because of phenotypic plasticity within species and quickly evolving genomes.

Microbes intimately associated with sponges began to be noticed frequently with the advent of electron microscopy. Although some sponges appear to be sterile except for the exterior and choanocyte chambers (Vacelet and Donadey 1977; Hentschel *et al.* 2003), there are numerous studies of microbes associated with sponges, using both cultivation and cultivation-independent approaches. Nonetheless, the nature of microbial associations with sponges have barely begun to be investigated, and in many cases, the specificity with which the microbes interact with sponges, the biochemical and physiological adaptations that mediate these symbioses, and the evolutionary history of microbial symbioses with sponges remain unclear. As in most other

environments, cultivated microbial isolates are not representative of the natural community. Microbes are common in the interior of the sponge mesohyl, as opposed to the choanocyte chambers where filter feeding occurs, demonstrating that sponges tolerate intimate relations with microbes and are far more promiscuous in their associations than most other metazoans. Indeed, the tremendous microbial biomass and complex communities found in some sponges leads us to view them as a type of stable, macroscopic microbial consortium organized by a scaffold of sponge cells: a "microbial mat with plumbing". Rates of microbial processes such as sulfate reduction and sulfide oxidation can be comparable to those in microbial mats, the most active habitats in nature (Hoffmann *et al.* 2005). Key questions raised by this unusual life style include: What processes are occurring within these associations? How are they organized? How is the composition of the community controlled? From a genomic point of view, these associations are often orders of magnitude more complex than rat, *Drosophila* or human genomes. This complexity creates major challenges in investigating these fascinating creatures.

Many marine invertebrates engage in long-term, specific associations with microorganisms. Sometimes these symbiotic associations are highly evolved, like the association between the squid *Euprymna scolopes* and a single species of light-producing bacterium (*Vibrio fischeri*), where the host utilizes strain-specific acquisition mechanisms, and the symbionts induce developmental changes in the host (Koropatnick *et al.* 2004; Nyholm and McFall-Ngai 2004). Recent studies in the gutless marine oligochaete genus *Olavius* indicate that even multiple bacterial symbionts are consistently associated with a specific animal host (Blazejak *et al.*

2005). In marine sponges, which are constantly exposed to highly diverse and complex assemblages of bacteria and archaea in seawater, the nature of the associations is challenging to understand. Do they maintain obligate associations with multiple species of microbes? If so, how are these maintained over time and space?

Table 1 summarizes recent analyses of microbial communities of sponges. There are some sponges in which one symbiotic bacterial or archaeal species dominates a relatively simple microbial assemblage inside the sponge (Unson and Faulkner 1993; Unson *et al.* 1994; Preston *et al.* 1996; Thacker and Starnes 2003; Ridley *et al.* 2005a; Ridley *et al.* 2005b). Certain groups are characteristic of sponges. For example, *E. palauensis*, the theopalauamide–producing symbiont of *Theonella swinhoei* belongs to a novel, deeply branching group of delta proteobacteria (Schmidt *et al.* 2000). Members of this group have been found in a variety of sponges, and thus far appear to be limited to sponges (Hentschel *et al.* 2002).

Although it has been difficult to identify microbes inside of sponges and determine that they are symbiotic (stably associated) as opposed to randomly acquired environmental bacteria, there are a handful of examples in which functions of specific microbe-sponge associations have been clearly demonstrated. Sponges have been reported to contain heterotrophic and phototrophic symbionts (Unson *et al.* 1994; Bewley *et al.* 1996), as well as psychrophilic and methane-oxidizing archaeal species (Preston *et al.* 1996; Vacelet *et al.* 1996). Biochemical experiments have demonstrated translocation of carbon, nutrients, and antioxidants from photosynthetic symbionts to sponges (Regoli *et al.* 2000; Steindler *et al.* 2001). In addition to cycling

inorganic nutrients and producing fixed carbon, some microbial symbionts produce bioactive compounds in their sponge hosts (Faulkner 2000; 2001).

Most sponges contain assemblages of hundreds if not thousands of diverse types of microbes. The challenge with such large microbial communities is the separation of significant symbionts from incidental environmental microbes. Researchers have used 16S rRNA-based molecular techniques to identify microbes that exist exclusively within sponges and not in surrounding seawater (Hentschel *et al.* 2001; Hentschel *et al.* 2002). Fieseler *et al*. have recently suggested a novel microbial division termed "Poribacteria," a clade of planctomycete-like microbes that may be symbionts in at least a handful of sponge hosts (Fieseler *et al.* 2004). They found these microbes associated with a handful of sponges, but no clear evolutionary pattern to their distribution has been noted yet. In halichondrid sponges, analyses of host and symbiont phylogenies (Erpenbeck *et al.* 2002) have revealed an evolutionary history of obligate species-specific relationships of microbes with some halichondrid hosts. Culture-based research also provides evidence of persistent, specific associations of the sponge *Rhopaloides odorabile* with a clade of novel alpha-proteobacterial species that are, to date, found only in this sponge (Webster and Hill 2001; Webster *et al.* 2001). Though the advancement of studies on microbial symbionts in sponges has been fast-paced in the past few years, there is still much to do to discern how microbes affect their eukaryotic hosts and the reefs they inhabit, and how truly widespread the phenomenon of species-specific microbial-sponge symbiosis is.

The occurrence of microbes in or on sponge reproductive structures has been documented, but the phylogenetic affiliations of the microbes have yet to be identified with modern molecular techniques. Physiological and behavioral adaptations have also been noted in many sponge species. The transmission of maternal bacteriocytes into developing embryos has been found in *Petrosia ficiformis*, *Chondrosia reniformis*, and at least two species of *Oscarella spp*, and there is increasing evidence that vertical symbiont transmission is a common strategy in sponges (Sciscioli *et al.* 1994; Regoli *et al.* 2000; Usher *et al.* 2001; Ereskovksy and Boury-Esnault 2002; Muricy *et al.* 2002; Usher *et al.* 2005).

In obligate symbioses, host and symbiont develop mechanisms for intergenerational symbiont transmission. Symbiont transmission from one host generation to the next can be classified as either vertical (transovarian) or horizontal (environmental). While some obligate symbionts are acquired from the environment, microbes persistently associated with oocytes, embryos, and larvae are likely to be normally present in the animal and therefore play an consistent, and in some cases important, role in the life of the host. For example, the bryozoan *Bugula neritina* transmits a microbial symbiont to its larvae (Haygood and Davidson 1997), and these symbionts biosynthesize bryostatins (Davidson *et al.* 2001), which are effective fish-feeding deterrents defending the larvae (Lopanik *et al.* 2004). Highly evolved mechanisms of vertical symbiont transmission are documented in numerous marine invertebrate-microbe associations, including but not limited to bryozoans (Woollacott 1981; Haygood and Davidson 1997; Davidson *et al.* 2001), ascidians (Hirose 2000), and bivalves (Cary 1994; Krueger *et al.* 1996; Gros *et al.* 2000; Sipe *et al.* 2000; Distel

et al. 2002; Fiala-Medioni *et al.* 2002). Such adaptations suggest that there has been evolutionary selection for the maintenance of these specific bacteria, and recent research suggests that vertical symbiont transmission can be reflected by highly co-evolved host-symbiont associations (Peek *et al.* 1998).

In order to understand how microbes interact with sponges, the abundance, distribution, and species-specificity of their associations with marine sponges must be better understood. Identification of vertically transmitted Bacteria and Archaea in sponges will be a powerful tool for addressing species-specificity of sponge-microbe associations. Staggering species diversity within most sponges has hampered characterization of microbial communities in sponges. Conventional molecular biology techniques on whole sponges yield diverse libraries of 16S rRNA gene sequences, including thousands of sequences from environmental Bacteria and Archaea and sequences from specific symbionts, if any. The work proposed here targets sponge embryos and larvae, minimizing the obstacle of casually associated microbes. Using embryos for these analyses allows us to enrich for DNA from microbes that are most likely to be significant to their hosts: Bacteria and Archaea that are in, on, and around reproductive structures.

In this chapter, bacteria and archaea are localized in *C. candelabrum* embryos. Microbial diversity associated with *C. candelabrum* embryos is characterized, and the persistence of associations of specific bacteria with *C. candelabrum* adults and embryos over time and space is tested. In addition, the hypothesis that the tedanolides are associated with the developing embryos, ultimately for chemical defense of the

larvae, is tested. In addition, morphological features of the gray "*C. candelabrum,"* in addition to chemical analysis and localization of its associated microbes, were studied.

MATERIALS AND METHODS

Sponge Collection. *C. candelabrum* was collected via SCUBA from ten different locations in the Palau archipelago, at depths of 3-20 m in September 2001, September 2002, and March 2004. Sponges were collected together in plastic bags at depth and brought to the surface. Samples were frozen, preserved in 100% ethanol, 2.5% glutaraldehyde, or frozen solid at -20°C until use. For FISH, whole sponges were fixed in 4% paraformaldehyde (4% in buffer: 20mM K_2HPO_4, 0.5M NaCl, pH 7.4) for 2 hours at room temperature and transferred to 70% ethanol for long-term storage at -20°C.

16S rRNA gene library construction. For embryo DNA extractions, approximately 100 embryos were picked with a sterile 23-G syringe needle from each ethanol-preserved sponge sample and rinsed twice in sterile artificial seawater (ASW) (100mM $MgSO_4 \cdot 7H_2O$, 80mM $CaCl_2 \cdot 2H_2O$, 2.4M NaCl, 80mM KCl). Genomic DNA was extracted from embryos of ethanol-preserved sponges using a protocol adapted from Preston *et al.* (Preston *et al.* 1996). ASW was removed and replaced by 1mg/ml lysozyme/TE (10mM Tris-HCl; 1mM EDTA, pH 8.0), and the samples were incubated at 37°C for 30 minutes. Proteinase K was added to final concentration of 0.5mg/ml, and the sample was incubated at 55°C for 1.5hr, until the solution was transparent. To complete lysis, the sample was boiled for 60s. The DNEasy genomic extraction kit (Qiagen) bacterial DNA protocol was used. For whole sponge DNA

extraction, approximately 3g of sponge tissue was minced in ASW and then homogenized in 1mg/ml lysozyme with a glass dounce. This homogenate then underwent lysis and DNEasy extraction as described above.

Polymerase chain reaction (PCR) with general eubacterial primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') was done with the following profile conditions: initial denaturation (3 min at 95°C); 35 cycles of denaturation (30 sec at 95°C), annealing (1 min at 50°C), and elongation (1 min at 72°C); and a final extension step (7 min at 72°C). Product was analyzed by electrophoresis on a 0.8% agarose gel and purified with a kit (Marligen). The purified PCR fragment was cloned into a PCR 2.1 vector (Invitrogen), which was transformed into TOP10 cells (Invitrogen). Transformants were selected using LB-Kanamycin plates topspread with 50ng/ml X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside). Inserts were amplified from white colonies picked from the selective plates in 96-well format PCR, with plasmid-specific primers (M13f: 5'-GTAAAACGACGGCCAG-3'; M13r: 5'-CAGGAAACAGCTATGAC-3', Invitrogen). Resulting PCR products were screened in a restriction digest with the enzymes HhaI and HaeIII. Approximately 3-5 colonies of each unique restriction pattern in the library were sequenced with 2X coverage. Sequence contigs were constructed and aligned in Sequencher 4.2 (*GeneCodes Corp., Ann Arbor, Mich.*) and compared to databases at Ribosomal Database Project and NCBI. (http://rdp.cme.msu.edu/index.jsp; http://www.ncbi.nlm.nih.gov/BLAST/)

FISH. Samples were fixed and stored on the day of collection as described above. Individual sponges embedded in paraffin wax were sectioned to 10µm. Sections were

deparaffinized (2x5min in xylene; 2x5min in 100% ethanol, one rinse in Milli-Q water) and air-dried. FISH was performed in a humidity chamber in hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.4], 0.01% sodium dodecyl sulfate) with 35% percent formamide for 2 h. All probes, including the general eubacterial probe (EUB338: 5'-GCTGCCTCCCGTAGGAGT-3') and the sequence-specific probes designed in this study, were used at a concentration of 5ng/μl final concentration in hybridization buffer. After hybridization, the slides were incubated at 48°C in wash buffer (0.7 M NaCl, 20 mM Tris-HCl [pH 7.4], 50 mM EDTA, 0.01% sodium dodecyl sulfate) for 20 minutes. The wash buffer was rinsed off with Milli-Q water, and slides were air-dried and mounted in VectaShield (*Vector Labs*, Burlingame, CA). Slides were visualized on an Axioskop epifluroescence microscope (Zeiss).

Phylogenetic Analysis. Sequences from each individual clone were edited and assembled in Sequencher 4.2 (GeneCodes Corp, Ann Arbor, Mich.). The 16S rRNA gene sequences obtained from the clones were compared with other bacteria by using BLAST (Altschul *et al.* 1990) and the Ribosomal Database Project (Cole *et al.* 2003). Sequences that matched most closely were used in an alignment with the 16S rRNA sequences. Sequences were combined with alignments downloaded from Ribosomal Database Project (Cole *et al.* 2003) using Sequencher 4.2 (Gene Codes Corp., Ann Arbor, Mich.) and aligned by eye with secondary structure information (Cannone *et al.* 2002) yielding 1,400 bp of aligned sequence. Phylogenetic trees were constructed in PAUP* 4.0b10 (Swofford 2003) using a maximum parsimony (MP) algorithm. Transversions were weighted three times more than transitions (based on ML estimations of the transition-to-transversion ratio), and a heuristic search of 100

repetitions with random addition of sequences was performed. MP bootstrapping was performed with 1,000 replicates.

Design and application of species-specific primers and probes. Probes were designed targeting three groups of 16S rRNA gene sequences from the library. The sequences, representing one α-proteobacterial 16S sequence from the library, one actinobacterial sequence, and a deeply-branching clade of bacteria, were targeted with the oligonucleotide primers CC01-1216 (5'-CGACCTCGCGATCTCGCT-3'), CC08-1245 (5'-CGCTTGACCTCGCGGTGT-3'), and SpC1 (5'-CTACACATTCCACCGCTA-3'), respectively. PCR cycling conditions with the specific primers were: initial denaturation (3 min at 95°C); 35 cycles of denaturation (30 sec at 95°C), annealing (1 min at 65°C), and elongation (1 min at 72°C); and a final extension step (7 min at 72°C). The primers were designed to be specific by targeting a hypervariable region of the 16S molecule, and to be efficient probes for 16SrRNA extremely high accessibility to oligonucleotide probes (Behrens *et al.* 2003). ProbeMatch (RDP; http://rdp.cme.msu.edu/ probematch/search.jsp) suggests that the three probes do not match to 16S sequence of any microbes known in the database.

Transmission electron microscopy. *C. candelabrum* was fixed in 2.5% glutaraldehyde-phosphate/sucrose (2.5% glutaraldehyde in phosphate buffer: 20mM KH2PO4; 60mM K2HPO4; 350mM sucrose, pH 7.3) and stored at -20°C. Samples were stained with 4% uranyl acetate in 50% ethanol, and subsequently dehydrated using a graded series of ethanol solutions followed by propylene oxide and infiltration

with epoxy resin (Scipoxy 812, Energy Beam Sciences, Agawam, MA). After polymerization at 65C overnight, thin sections were cut and stained with uranyl acetate (4% uranyl acetate in 50% ethanol) followed by bismuth subnitrate. Sections were examined at an accelerating voltage of 60kV using a Zeiss EM10C electron microscope.

Spicule mounts. Soft sponge tissue was dissolved in a bleach solution, leaving intact spicules. The spicules were then repeatedly washed in sterile water and mounted in Permount (Biomeda, Foster City, CA) and observed on a compound microscope (Zeiss Axioskop).

Anti-tumor activitiy and bioactivity localization. Relatively large *C. candelabrum* individuals contained high numbers of developing embryos within the mesohyl, but embryos were not observed in smaller (presumably younger) *C. candelabrum* individuals. To test if older, embryo-brooding *C. candelabrum* had higher levels of bioactivity than younger, non-reproductive individuals, both were tested for cytotoxicity against the human colon tumor cell line HCT-116.

HCT-116 cells were plated in 96-well plates and incubated overnight at 37 C in 5% CO_2/air. Extracted were added to the plate and serially diluted. After an additional incubation of 72 h, cell viability was assessed by a CellTiter 96 AQ_{ueous} nonradioactive cell proliferation assay (Promega). Inhibition concentration (IC_{50}) was determined from colorimetric detection of MTS/PMS bioreduction into a formazan product, determined by absorbance of cell solutions (OD_{490nm}) in an Emax microplate reader (Molecular Devices). IC_{50} values were calculated by a SOFTMax analysis program.

Etoposide (Sigma) and DMSO (solvent) were used as positive and negative controls, respectively.

Methanolic extracts from identical masses of reproductive and non-reproductive *C. candelabrum* (40g wet weight) were tested against HCT-116 cells. In addition, *C. candelabrum* cortex, mesohyl, and embryos were isolated from each other, and bioactivity of methanolic extracts against HCT-116 were assayed similarly. Methanolic extract from gray *C. candelabrum* was also tested in the same assay. These were only crude extracts and this procedure, and without subsequent HPLC or NMR analysis, it is impossible to confirm the presence of tedanolides. However, pure tedanolides have been shown to be the only component in *C. candelabrum* that exhibits levels of cytotoxicity in the nanomolar range (Kantorowski, Rao, and Faulkner, unpublished).

RESULTS

Microbes in *C. candelabrum*

Fluorescence in situ hybridization (FISH) with the fluorescently-labeled general eubacterial oligonucleotide probe (CY5-EUB338) on *C. candelabrum* sections (Figure 2) reveals a nearly pure population of a large filamentous bacterium in the cortex, whereas the choanocyte chambers (cc) in the mesohyl are densely lined with diverse bacterial morphotypes (panel A). The filamentous form (f) also resides within the interior of the mesohyl, in the space between choanocyte chambers (panel B). Strong autofluorescence in sponge cells allows visualization of sponge structures without

counterstaining, and probe-conferred signal is readily distinguished from autofluorescence.

Transmission electron microscopy (TEM) reveals remarkable structures within the large, abundant filaments in *C. candelabrum*. Figure 3 shows filaments from one sample of *C. candelabrum* collected in 2000. Most of the volume of the cells in the filament is taken up by electron-transparent inclusions resembling poly-hydroxybutyrate storage vacuoles (*picture, C. Salomon 2001*). In samples collected in 2002, the filamentous cells contain smaller electron-dense inclusions that line the inner periphery of the cells.

Figure 4 shows the results of preliminary attempts to identify the filamentous microbes in *C. candelabrum*. FISH with the general probes, CY5-EUB338 and CY3-ARCH915, demonstrates that the filamentous cells fluoresce after treatment with either the eubacterial and archaeal probes (panels A, B), though the signal is greater when probed with EUB338. In the red channel, used to detect CY3, very low levels of autofluorescence are observed in the filamentous cells without added hybridization buffer (panel C). In a control in which the sponge sections are incubated in hybridization buffer without probe, the filamentous cells fluoresce (panel D) at levels similar to the sponge cells, and at levels similar to those in the ARCH915-probed sections (panel B), suggesting that incubation in hybridization buffer causes autofluorescence to increase.

Figure 5 shows the sponge mesohyl, probed with general bacterial probe CY5-EUB338 and general archaeal probe CY3-ARCH915. According to FISH with

general probes, both eubacteria and archaea are present in *C. candelabrum*, concentrated in the mesohyl just peripheral to the choanocyte chambers. Signals from the eubacterial probe and the archaeal probe do not colocalize in any of the unicellular microbial cells, indicating that the probing was domain-specific. Co-localization would appear yellow in the combined image. Split images were also inspected for cross-hybridization. Bacterial cells appear more abundant in the mesohyl than archaea, and the archaea are most dense among the sponge cells inside the the choanocyte chambers, they also occur outside the choanocyte chambers. Negative control probes for both the eubacterial and archaeal probes show no signal above background (panel B). While the sponge sections contained many "spots" of autofluorescence as shown in panel B, bacterial-shaped signal could be distinguished from the particulate autofluorescence.

FISH was used to test the hypothesis that bacteria and archaea are transmitted through the embryos in *C. candelabrum*. Sections of the sponge were hybridized with a CY5-labelled general eubacterial probe EUB338 in fluorescence in situ hybridization (FISH). Early stage embryos contain conspicuous and regularly arranged clusters of bacteria, resembling beads on a necklace, lining the inside of the embryos (Figure 6A). In later developmental stages, bacteria are in the central cavity of the developing embryo (Figure 6B). Localization of bacteria in the late-stage embryos is consistent with previous ultrastructural studies of *C. candelabrum* swimming larvae, in which bacterial cells were noted in the larval cavity (Boury-Esnault *et al.* 2003). These masses comprise bacterial morphotypes (Figure 6C), including a large, filamentous shape (Figure 6D) resembling the abundant morphotype

observed throughout the sponge. In addition to bacteria, archaea are also present in the embryos, both in the aggregates of the early embryonic developmental stages (Figure 7A) and in later stages of development (Figure 7B). Negative control probes show no hybridization in the embryos.

I constructed five clone libraries, each consisting of bacterial 16S ribosomal RNA genes amplified from embryo DNA from one sponge from each of three different locations, and two libraries, each from one whole sponge. The libraries contained diverse microbial 16S rRNA gene sequences, and none of the libraries was dominated by any one sequence. All sequences in the embryo libraries were present in the whole sponge libraries. In contrast, however, the embryonic DNA libraries lacked some groups of free-living marine microbial groups such as unicellular cyanobacteria and microbes from the SAR cluster (Mullins *et al.* 1995) that were present in the whole sponge extracts. Clone libraries prepared from whole *C. candelabrum* yielded diverse clones and no discernible pattern of distribution across different *C. candelabrum* specimens. 200 clones from embryos from five *C. candelabrum* specimens were screened, and unique restriction types were sequenced (1400 bp, 2x coverage). We identified a suite of microbial 16SrRNA gene sequences that are consistently present in DNA extracts of *C. candelabrum* from different locations in Palau. In Figure 8, a histogram showing the distribution and abundance of the cloned sequences demonstrates that picking out the embryos is effective at enriching the DNA extract for a consistent subset of microbes, simplifying the complex microbial assemblage present in whole sponge. Diverse groups of microbes are represented in the clone

libraries, but in each of the five libraries, the same phylogenetic groups are represented.

Bacterial strains represented in the embryo clone libraries (Figure 9) were all less than 90% similar to any microbial 16S rRNA gene sequences in the Ribosomal Database Project or GenBank (NCBI) databases. Cloned sequences represented members of several groups of bacteria, including the Proteobacteria, the Actinobacteria, and the *Nitrospira* group. One of the most abundant sequences, CC01, is closely related to the terrestrial plant symbiotic *Rhizobium-Agrobacterium* group. Other proteobacterial sequences cluster with sulfur-oxidizing chemoautotrophic γ-proteobacterial symbionts, in addition to *Pseudomonas* and *Shewanella* genera. Several other species in the embryo libraries are distantly related to actinobacterial 16S rRNA sequences known from microorganisms isolated from seawater and marine sponges. Some sequences fall into the *Nitrospira* group, and others are distantly related to the *Chloroflexus* group. Other sequences are close matches to those of unclassified microbes, including the "Poribacteria" clade proposed by Fieseler *et al*. (Fieseler *et al.* 2004). Three clades (SpC1, SpC2, SpC3) of closely related clones group with sequences from other marine sponges but do not match any other sequences currently in RDP or GenBank databases. In sum, these findings suggest that *C. candelabrum* hosts a diverse suite of microbes in its embryos, including bacteria from phylogenetic groups previously observed in other marine sponges (Webster *et al.* 2001; Hentschel *et al.* 2002; Taylor *et al.* 2004).

In Figure 10, a parsimony phylogenetic tree shows one of the cloned sequences, which falls into the alpha-proteobacteria, that was present in the libraries of all of the

tested *C. candelabrum* embryo samples. Though distantly related to any known microbes, α-CC01 is most closely related to pathogenic alpha-proteobacteria genera such as *Bartonella* and symbiotic genera from the Rhizobiales. Interestingly, it is not especially closely related to the α-proteobacterium found in the Great Barrier Reef sponge *Rhopaloeides odorabile*, or to a clade of other "sponge-associated" α-proteobacteria. More closely related is a metal-oxidizing bacterium (SI85-9A1) isolated from the anoxic-oxic interface from sediment in the Saanich Inlet.

Figure 11 shows a parsimony tree of three actinobacterial 16S rRNA gene sequences from *C. candelabrum* embryo libraries. Two of these sequences fall into a clade with another actinobacterial sequence cloned from the Palauan sponge *Theonella swinhoei*.

Because three cloned sequences were similar to 16S rRNA sequences of previously described symbionts or bacteria found in other marine sponges and thus of potentially general significance, the abundance and localization of these bacteria – CC01 (α-proteobacterium), CC08 (actinobacterium), and SpC1 (sponge clade) – were further investigated. Specific reverse primers were paired with general eubacterial primers for PCR amplification. PCR with α-CC01 and actino-CC08 primers both yielded only one 16S sequence from *C. candelabrum* samples, and PCR with the SpC1 primer yielded a group of closely related sequences.

To confirm that the sequences obtained represent bacteria associated with the embryos, oligonucleotide probes specific to α-CC01, actino-CC08, and sponge clade 1 (SpC1) were fluorescently labeled and used to probe the embryos. Each of these probes hybridized to bacterial cells within the peripheral clusters of microbes in early

stage embryos (Figures 12-14), and negative control probes (single-base mismatch) hybridized to no cells in the aggregates.

A PCR survey of twelve samples, using the three specific primers showed that the two bacterial species (α-CC01, actino-CC08) and members of the SpC1 clade persist in *C. candelabrum* populations across broad temporal (3 years) and geographic (~100km) scales (Fig. 15).

Spatial Distribution of Bioactive Compounds

Methanolic extracts from each of the *C. candelabrum* pieces were tested for activity against human colon tumor cell line 116 (HCT-116). Extracts of reproductive (containing embryos) and non-reproductive (no embryos in the mesohyl) *C. candelabrum* did not differ significantly from each other in the HCT-116 bioactivity assay. In addition, there was no detectable difference between the bioactivity of the cortex, the mesohyl, or the embryos (*C. Sincich, pers. comm.*).

Gray *C. candelabrum* Chemotype

Though less pigmented, the gray *C. candelabrum* morph bears morphological similarity to the black *C. candelabrum* – it contains heterolophose calthrop spicules, unique to *C. candelabrum*, and it bears a thick, collagen-rich cortex that is more heavily pigmented than the inner mesohyl. Figure 16 shows light micrographs of spicules from black and gray C. candelabrum collected in Palau, in addition to *Corticium sp*. from Guam. The heterolophose calthrops (A,C,D), characteristic of *C. candelabrum*, are present in all three sponges. The large calthrop spicule is present in

all three samples. Though the calthrop in the grey and the black *C. candelabrum* from Palau are similar sizes, it is at least 10X larger in the Guam sample. All gray specimens tested exhibit very low bioactivity in the HCT-116 assay (IC_{50} levels ≥ 40μg/ml), and organic extracts did not contain any of the NMR peak signals characteristic of tedanolides (data not shown). The spicules of gray "*Corticium,*" which we first collected in 2002, appear to be similar to spicules in the black *C. candelabrum* from Palau. The organic fraction has bioactivity levels three orders of magnitude lower than the organic fraction extracted from black *C. candelabrum.*

FISH with the general eubacterial probe Cy3-EUB338 on the gray *Corticium* from Palau (Figure 17) reveals the presence of both unicellular bacteria as well as filamentous microbes in the sponge.

DISCUSSION

The filamentous microbes appear to have evolved to be in a specific association with *C. candelabrum*: the filamentous morphotype is spatially dominant, and large, filamentous cells are consistently present in the embryos. While it is tempting to propose that the filamentous microbes synthesize the tedanolides for the host sponge, there is no evidence yet to support that hypothesis. The filaments are evenly spread spatially across the host sponge; however, they are the main microbial morphotype that occurs in the highly pigmented sponge cortex in both *C. candelabrum* chemotypes. The filaments are also packed into mesohyl, which they occupy with diverse morphotypes of unicellular bacteria. Perhaps their dominance in the sponge

cortex is an indication of a specific microniche relating to oxygen, light, or other nutrients that are in high concentration in the outer layer of the sponge.

Though they are numerically and spatially dominant in *C. candelabrum*, the filamentous microbes are still not identified. FISH probing was inconclusive, because the filaments fluoresce mildly in the presence of hybridization buffer (see Fig 4). Without the addition of hybridization buffer, the filaments do not autofluoresce, suggesting that the signal is not due to pigments in the microbes. Figure 4 shows that both archaeal and eubacterial probes yield signal; however, the results of this hybridization strongly suggest that the filaments are bacteria. The intensity of the hybridization signal from the eubacterial FISH probe is much greater than that observed with ARCH915, and the signal from the ARCH915 probe is similar to signal from no probe, as well as many other group-specific probes (not shown). From this, one can tentatively conclude that the filaments are bacteria of unknown affiliation.

Mechanical and chemical cell separation techniques failed to separate the filaments from other microbes. In the cell preparations, the filaments tenaciously bound to sponge cells, which also commonly had smaller unicellular microbes attached to them. The filaments are on average 7-10μm wide and tens of microns long, and the current limit of resolution of laser microdissection is 5μm. Isolation of the filaments by laser microdissection and subsequent nucleic acid extraction should be used for 16S rRNA gene-based identification of the microbes.

The results of this study demonstrate that the embryos contain a diverse assortment of microbes, including both bacteria and archaea. Though the microbial diversity of *C. candelabrum* embryos is likely undersampled with a meager collection of only 200

clones and requires additional cloning and sequencing for assessment of the diversity, preliminary analysis of the cloned sequences show 43 unique OTU's (<3% sequence difference) from the 200 cloned 16S rRNA gene sequences cloned from the embryos. Rarefaction data from the Sargasso Sea metagenomic library suggests that accurate coverage of 16S sequences can be obtained from sequencing of approximately 690 sequences (Schloss and Handelsman 2005). Compared to that diverse sample, there is much less complexity in the sponge embryos: at 200 clones, the Sargasso Sea collection contained approximately 60 OTU's (<3%) compared to the 43 OTU's in the 200 clones from *C. candelabrum* embryos. While the diversity of sequences in the embryonic DNA clone libraries does not suggest that the embryos harbor a simple microbial community, it should feasible to get an accurate sampling of the bacterial diversity in the embryos with additional sequencing.

The three sequence-specific probes confirm the presence of sequences from the library in the embryos, but they also indicate that they are only a small portion of the vertically transmitted bacterial community. The FISH images show that none of the three phylotypes tested is a dominant phylotype in the bacterial biomass in the embryos, suggesting that many other bacteria are transmitted in the embryos. Because of PCR bias, abundance of 16S rRNA gene sequences in clone libraries should never be interpreted as an indicator of true abundance in the sample. These localization results demonstrate the importance of using FISH to confirm the presence and abundance of specific microbes: α-CC01, which was one of the most abundant sequences among all of the embryo clone libraries, represents a small portion of the total bacterial biomass in the embryos.

Phylogenetic analysis suggests that vertical transmission mechanisms are often present in highly co-evolved host-microbe associations (Peek *et al.* 1998). While prior work on vertical transmission in sponges has focused on single host-single symbiont associations (Usher *et al.* 2001), the present study for the first time addresses the transmission of a highly diverse microbial assemblage. Such specialized mechanisms of transmission are likely to have general significance, because the composition of this community is strikingly similar to that previously described in many other sponges. Though it is unknown how or if the microbial community functions as part of the host sponge physiology, the community in *C. candelabrum* includes microbes whose closest relatives oxidize sulfur, fix nitrogen, and produce complex bioactive compounds. It thus seems likely that this sponge (and others) has a predictable set of microbial partners that construct chemical microenvironments within the animal host and control microbial fouling or predation via production of bioactive molecules. These results indicate that diverse microbial communities found in filter-feeders may be members of specific, highly evolved associations that are maintained and regulated by reproductive transmission in the host animal.

Localization of Tedanolides

Because both chemotypes of *C. candelabrum* are small and sparsely distributed in Palau, collections were never large enough (> 1kg) to obtain quantitative chemical data. Detection of tedanolides was accomplished by the sensitive HCT-116 assay, and the results from this assay suggest that tedanolides are present in *C. candelabrum* embryos, as well as in the cortex and the mesohyl of the adult. No significant

difference in bioactivity was detected among the three different parts of the sponge, but this assay's detection limit is on the nanogram scale. Picograms of compounds as potent as the tedanolides may be ecologically active against predators; therefore, in order to test the hypothesis that tedanolides are allocated to different portions of the sponge for chemical defense should be done tested with higher sensitivity, with a tool like liquid chromatography - mass spectrometry (LC-MS). This demands more sponge material, but a concerted collection over several sampling sites may yield enough.

The Gray *Corticium*

While the anatomy and the spicule morphology indicate that what we collected as "Gray *Corticium*" suggest that it is *C. candelabrum*, we have no sponge molecular taxonomic data to date. The candelabrum spicules, the heterolophose spicules, have to date only been found in *C. candelabrum* – both in the Mediterranean and the tropical Pacific. The larger spicule type, the triaene calthrop, is similarly sized in the gray and black morphs of *C. candelabrum* from Palau. However, triaene spicules from a Guam collection of *Corticium sp*. are extremely large. Previous research demonstrates that spicule size can vary depending on salinity and temperature of the surrounding seawater (Schonberg and Barthel 1997).

When the gray *C. candelabrum* was first screened for bioactivity, the HCT-116 assay indicated extremely low levels of anti-tumor activity (three orders of magnitude lower than that of black tedanolide-containing C. candelabrum extract). Further investigation using NMR spectroscopy on gray samples collected during two separate

expeditions (2002 and 2004) suggests the presence of steroidal alkaloids, but the absence of any tedanolides.

FISH revealed that the large, filamentous microbes prominent in the tedanolide-containing *C. candelabrum* are also present in gray *C. candelabrum*, which does not contain tedanolides. There are several ways to interpret that result, including: 1) the filaments may not be the producers of the tedanolides in *C. candelabrum* and their presence has nothing to do with the presence of the tedanolides; or 2) the filaments produce the tedanolides, but only in the black morph. Perhaps there is differential expression of the tedanolide biosynthesis genes in the two associations. It is also a possibility that if the filaments are the source of the tedanolides in the black *C. candelabrum*, then maybe the filaments in the gray sponge do not even contain the tedanolide biosynthetic gene cluster. There is a precedence for this type of chemotype variation in sponge-microbial associations. *Dysidea herbacea* harbors the large, filamentous photosynthetic bacterium *Oscillatoria spongeliae,* which produces an assortment of polychlorinated peptides. In collections of *D. herbacea*, in which the polychlorinated peptides are absent, the symbiotic *O. spongeliae* is still present, but it does not contain the biosynthetic gene cluster. (Flatt *et al.* 2005)

Though there are no data to date suggesting that the filaments are the tedanolide producers, data from this study suggest that the filaments consistently populate a large portion of the volume of the sponge *C. candelabrum*. It is thus tempting to speculate that they are the source of the bioactive compounds, and that the gray *Corticium* represents a separate chemotype of the same association. If the difference between the gray and the black *C. candelabrum*-filament associations is simply that one has a

biosynthetic gene cluster for the production of tedanolides and the other does not, comparative genetic analysis of the two *C. candelabrum* types would be valuable for identifying a tedanolide biosynthetic gene cluster. Using sequence from other homologous PKS biosynthetic genes to probe for for potential biosynthetic gene clusters in DNA extracted from the laser-microdissected filaments may be a valuable pursuit, especially with a comparison of filaments in the gray chemotype.

SPONGE	MICROBES	REFERENCES
Theonella swinhoei	*Aphanocapsa feldmanii, "Candidatus* Entotheonella palauensis," alpha-, gamma-, and delta-Proteobacteria, Nitrospira, Bacteroidetes, Cyanobacteria, Spirochaetes, Actinobacteria, Acidobacteria, Chloroflexi	Bewley *et al.* 1997; Schmidt *et al*., 2000; Hentschel *et al.,* 2002
Aplysina aerophoba	alpha-, gamma-, and delta-Proteobacteria, Nitrospira, Bacteroidetes, Cyanobacteria, Spirochaetes, Actinobacteria, Acidobacteria, Chloroflexi	Hentschel *et al*., 2002
Rhopaloeides odorabile	alpha-, gamma-, and delta-Proteobacteria, Chloroflexi, Actinobacteria, Green non-sulfur bacteria	Webster *et al.,* 2001
Halichondria panicea	alpha-, gamma-, and delta-Proteobacteria, *Rhodobacter sp.,*	Althoff *et al.,* 1998
Axinella mexicana	*Cenarchaeum symbiosum*	Preston *et al.,* 1996
Dysidea sp.	*Oscillatoria sp.*	Unson *et al.,* 1993
Cymbastela concentrica	alpha-, beta-, and gamma-Proteobacteria, Cyanobacteria, Actinobacteria	Taylor *et al.,* 2004
Callyspongia sp.	alpha-, beta-, and gamma-Proteobacteria, Cyanobacteria, Actinobacteria	Taylor *et al.,* 2004
Stylinos sp.	alpha-, beta-, and gamma-Proteobacteria, Cyanobacteria, Actinobacteria	Taylor *et al*., 2004

Table 1. Recent studies on sponges and their diverse microbial associates.

Probe Name	Sequence (5'-3')	Reference
EUB338	5'-GCTGCCTCCCGTAGGAGT-3'	(Loy *et al*. 2003)
α-CC01	5'-CGACCTCGCGATCTCGCT-3'	this study
act-CC08	5'-CGCTTGACCTCGCGGTGT-3'	this study
SpC1	5'-CTACACATTCCACCGCTA-3'	this study
α-CC01 NON	5'-CGACTTCGCGATCTCGCT-3'	this study
act-CC08 NON	5'-CGCTTGACCTCGCAGTGT-3'	this study
SpC1 NON	5'-CTACTCATTCCACCGCTA-3'	this study

Table 2. List of oligonucleotide probes used in this study. The general eubacterial probe, EUB338, was used to detect general bacterial populations. Oligonucleotides were designed to target specific groups of sequences from the *C. candelabrum* embryo clone library. The probes target an α-proteobacterial species (α-CC01), an actinobacterial species (actino-CC08), and a deeply-branching clade of bacteria in a sponge clade (SpC1). Single base mismatch probes for each of the specific probes were designed to confirm the specificity of the probes.

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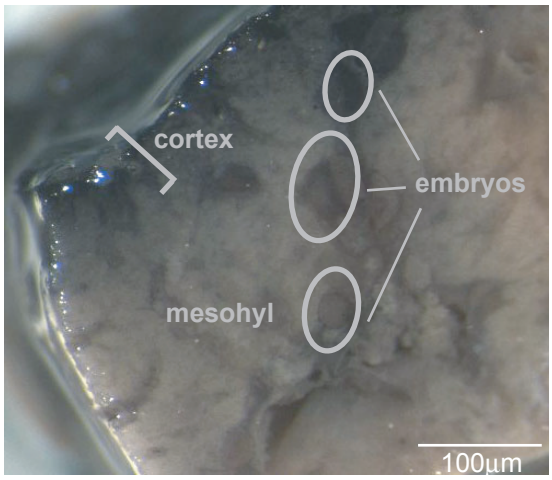


Figure 1. *Corticium candelabrum*. A cross-section of *C. candelabrum*, showing the heavily pigmented cortex and the mesohyl. Circled are embryos at various stages brooding within the sponge.

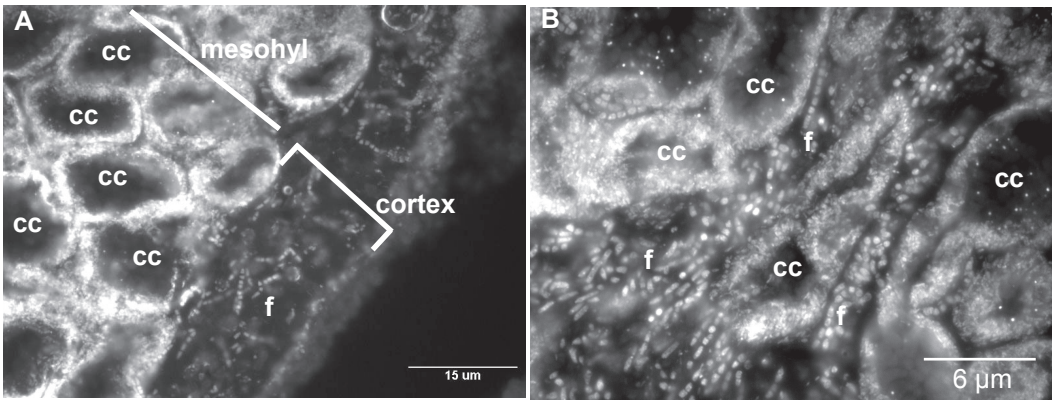


Figure 2. FISH on a *C. candelabrum* cross-section. (A) CY5-EUB338 hybridizes to large, filamentous cells present in the cortex, and unicellular bacteria surrounding the choanocyte chambers in the mesohyl. (B) The large, filamentous morphotype is also present in between the choanocyte chambers. cc: choanocyte chambers; f: filaments.

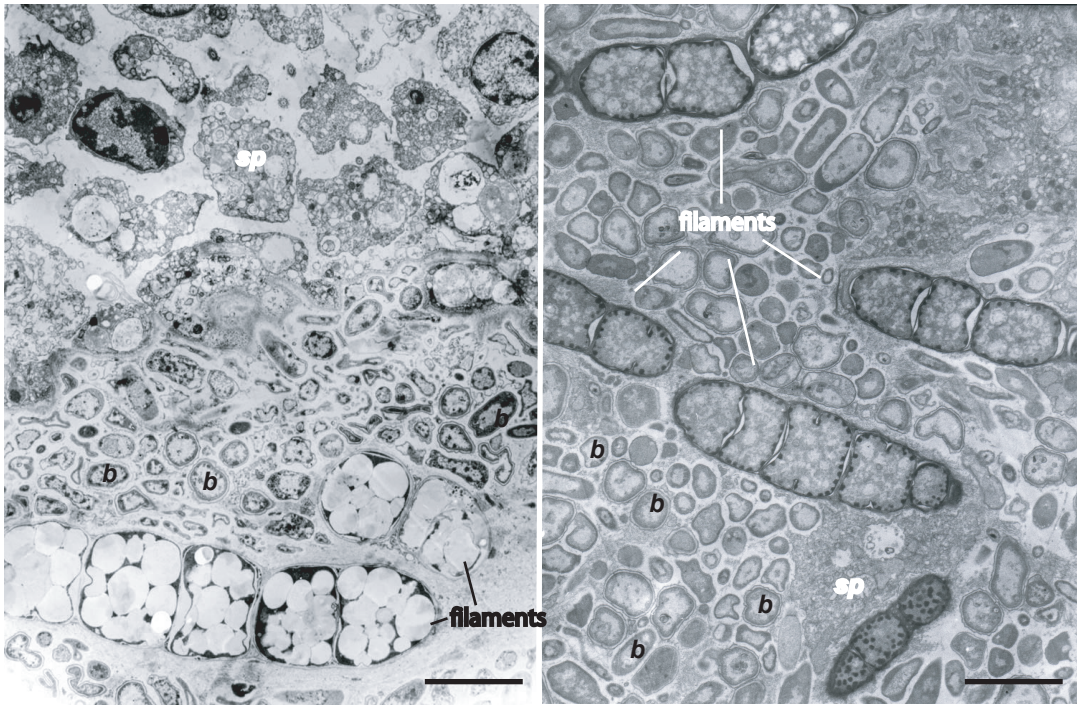


Figure 3. Large filamentous microbes in *C. candelabrum*. TEM shows unicellular bacteria and large filamentous microorganisms that contain large vacuoles of electron-transparent material (A), and electron-dense inclusions (B). b: bacteria; sp: sponge cells.

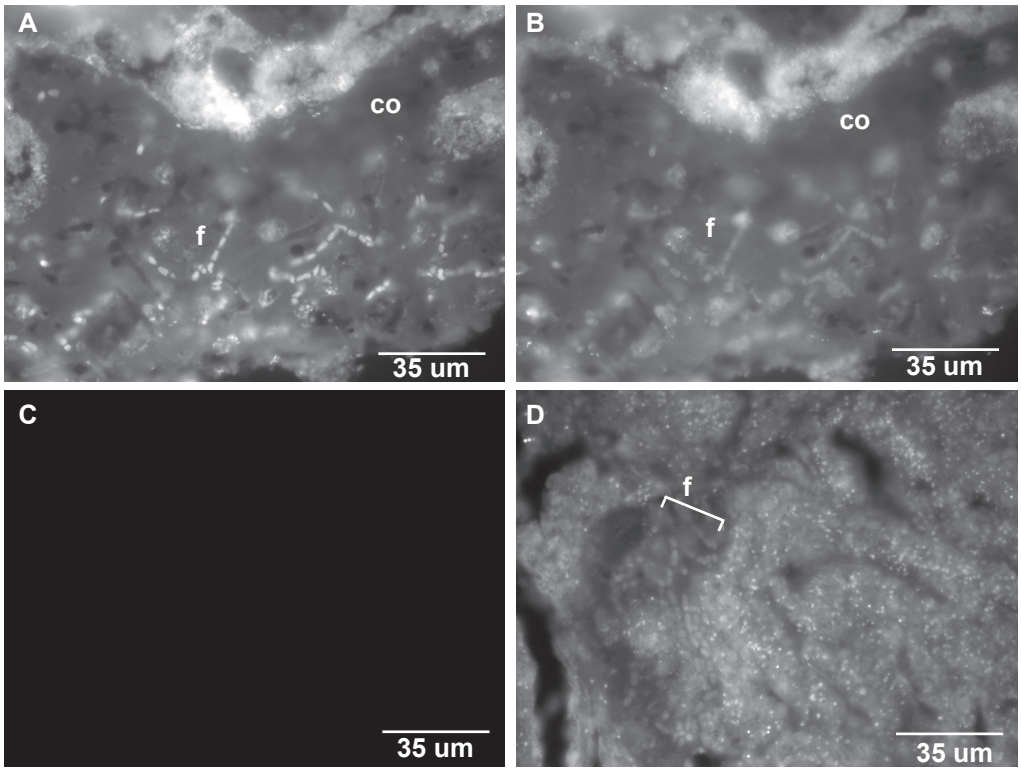


Figure 4. FISH probes for identification of the large filamentous cells.
(A) General eubacterial probe CY5-EUB338 hybridizes to unicellular bacteria in the mesohyl, and to filamentous microbes. (B) Archaeal probe CY3-ARK915 hybridizes to many unicellular microbes in the sponge, and the filaments also fluoresce. (C) Negative control without the addition of hybridization buffer or probe shows that the filamentous cells do not exhibit autofluorescence, but (D) with the addition of hybridization buffer and no probe, the filaments have light levels of signal. co: cortex; f: filaments.

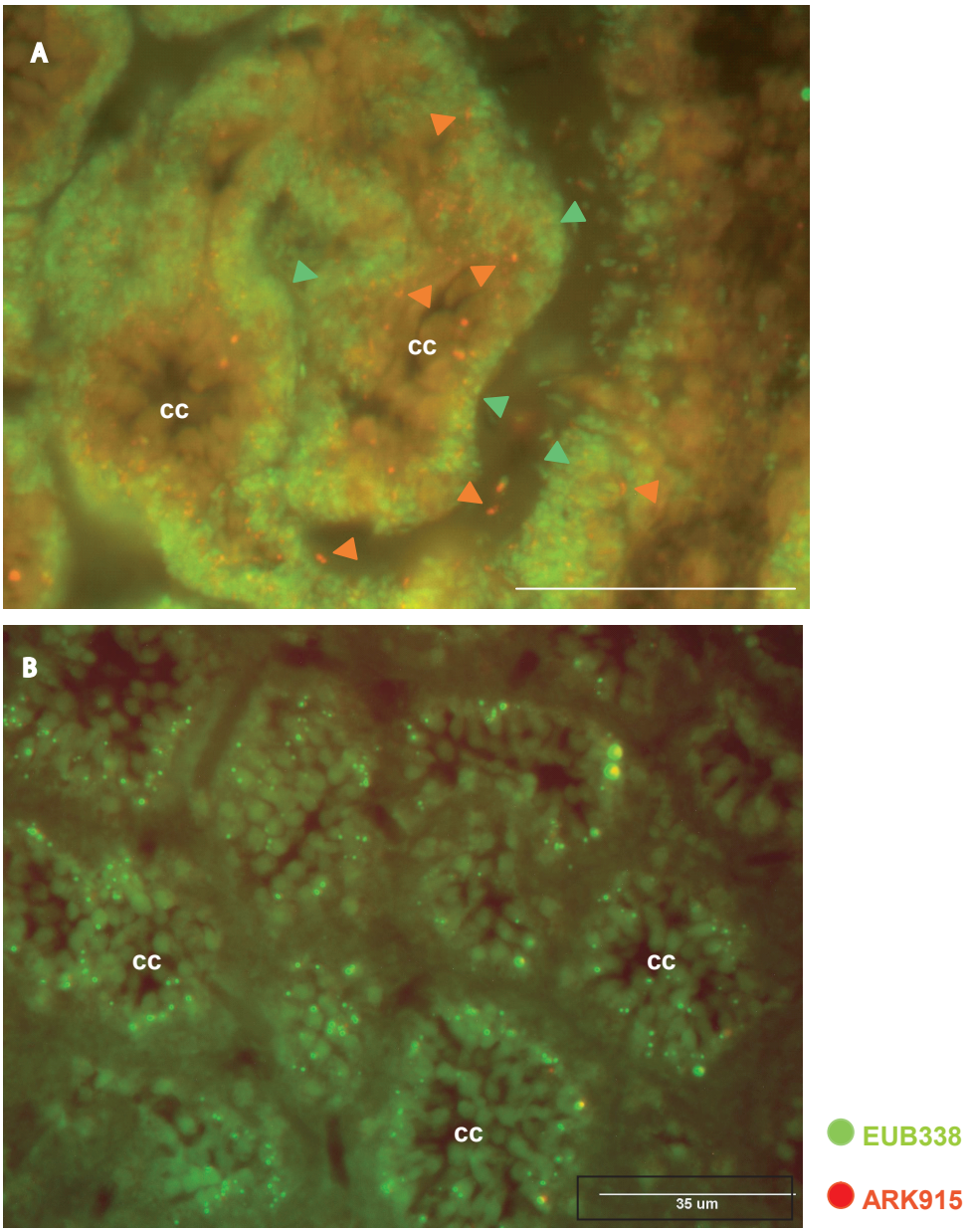


Figure 5. Eubacteria and Archaea in *C. candelabrum*.
(A) General eubacterial and archaeal probes (CY5-EUB338 and CY3-ARK915, respectively) localize microbes lining the choanocyte chambers and in the interior of the sponge choanosome. CY5-EUB338 (green) hybridizes to high numbers of cells, and the CY3-ARK915 reveals archaea lining the choanocyte chambers. (B) Negative control probes for each show no signal. Bar = 35um.
cc: choanocyte chambers.

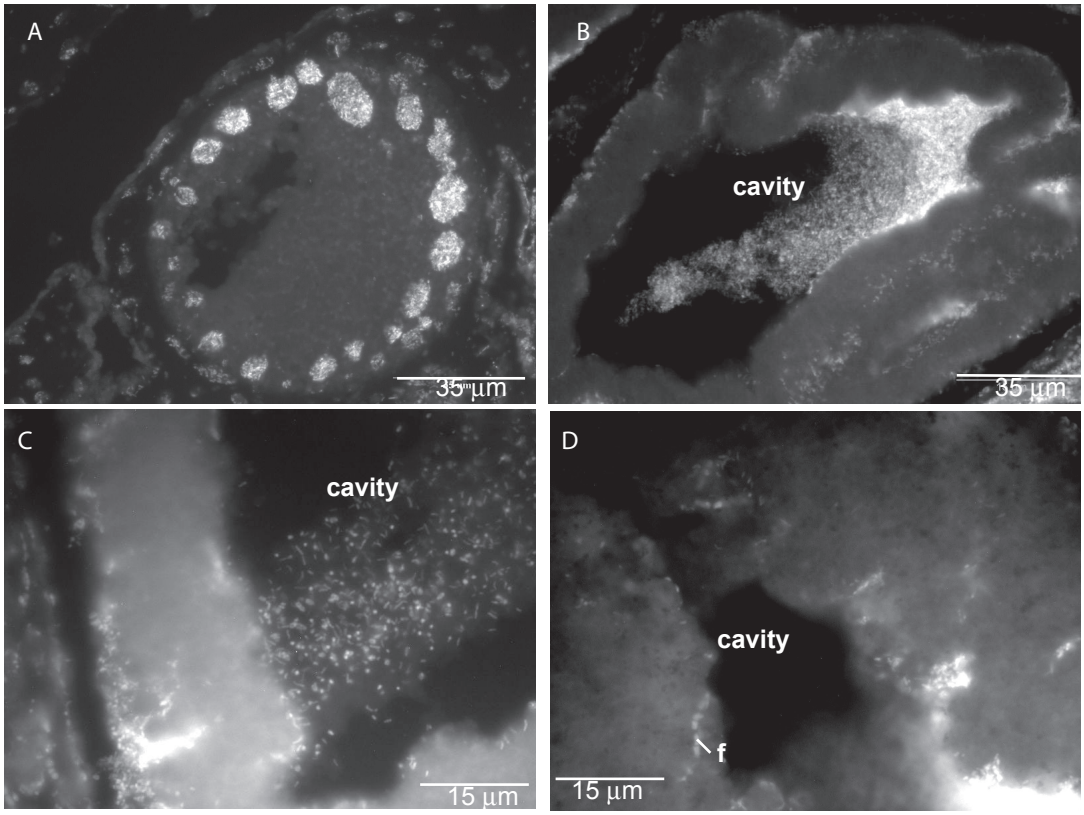


Figure 6. *C. candelabrum* embryos probed with CY5-EUB338. Bacterial aggregates line the embryo (A) and occur in a mass in the central cavity of a later stage brooded embryo (B). (C) Diverse bacterial morphotypes occur inside the cavity, including large filamentous cells (D). f: filament.

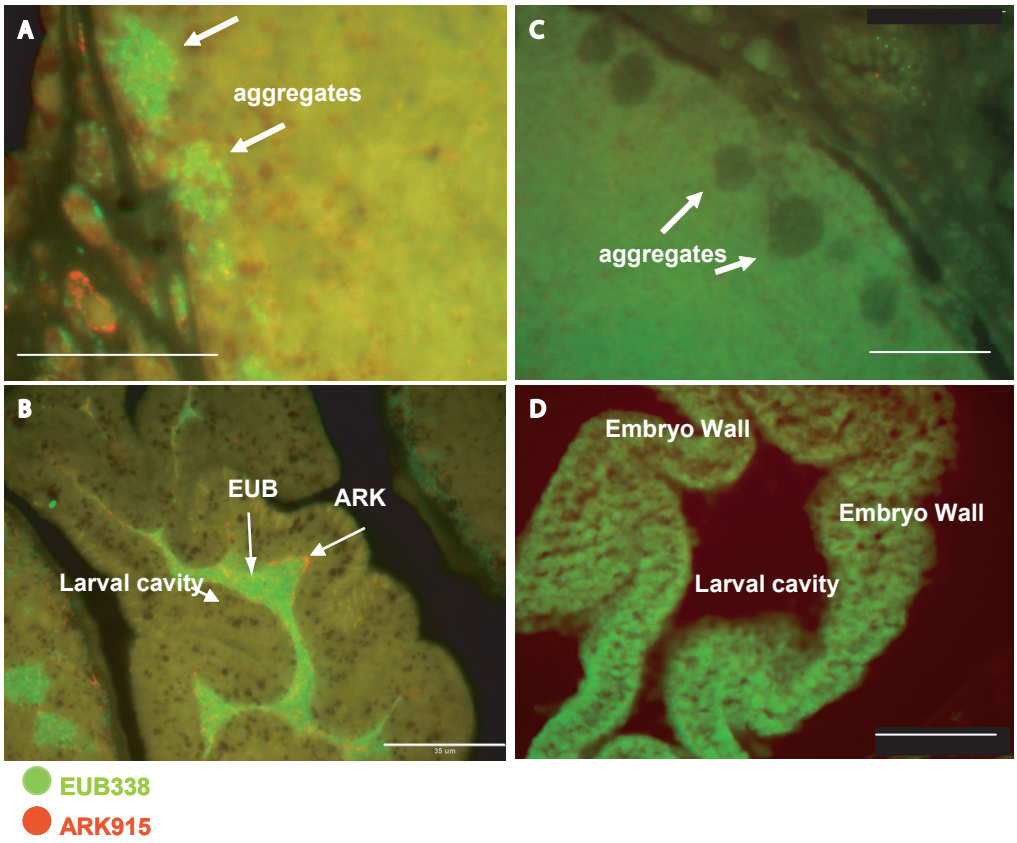


Figure 7. Eubacteria and archaea in *C. candelabrum* embryos. (A) Both ARK915 and EUB338 hybridize to cells in aggregates in early stage embryos. (B) Cavity in a developing embryo possesses high numbers of bacteria in addition to archaea. Negative controls show no signal in early stage embryos (C) or in later stage embryos with cavities (D). ARK: archaea (red); EUB: eubacteria (green). A,C: Bar = 10um. B,D: Bar = 35um.

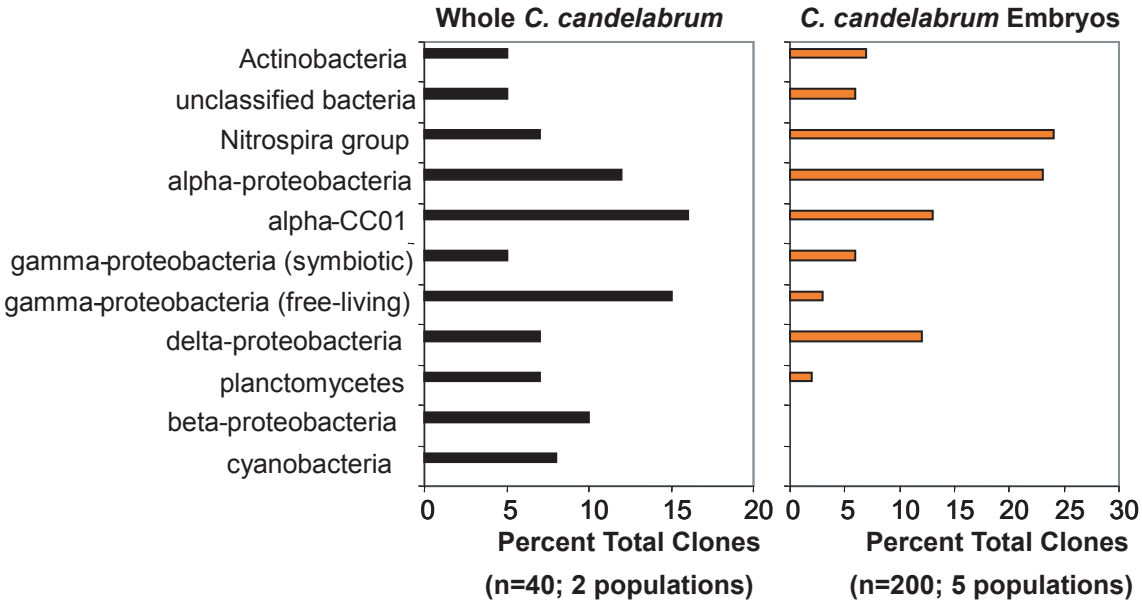


Figure 8. Effective enrichment of embryo-associated sequences in clone libraries. Histogram showing the distribution of sequences into groups. Based on 200 sequences, 16S rRNA gene sequences from *C. candelabrum* embryo clone libraries possess representatives from fewer phyogenetic groups than found in clone libraries from the whole sponge. Groups that were represented in the whole sponges but not detected in the embryos include free-living cyanobacteria.

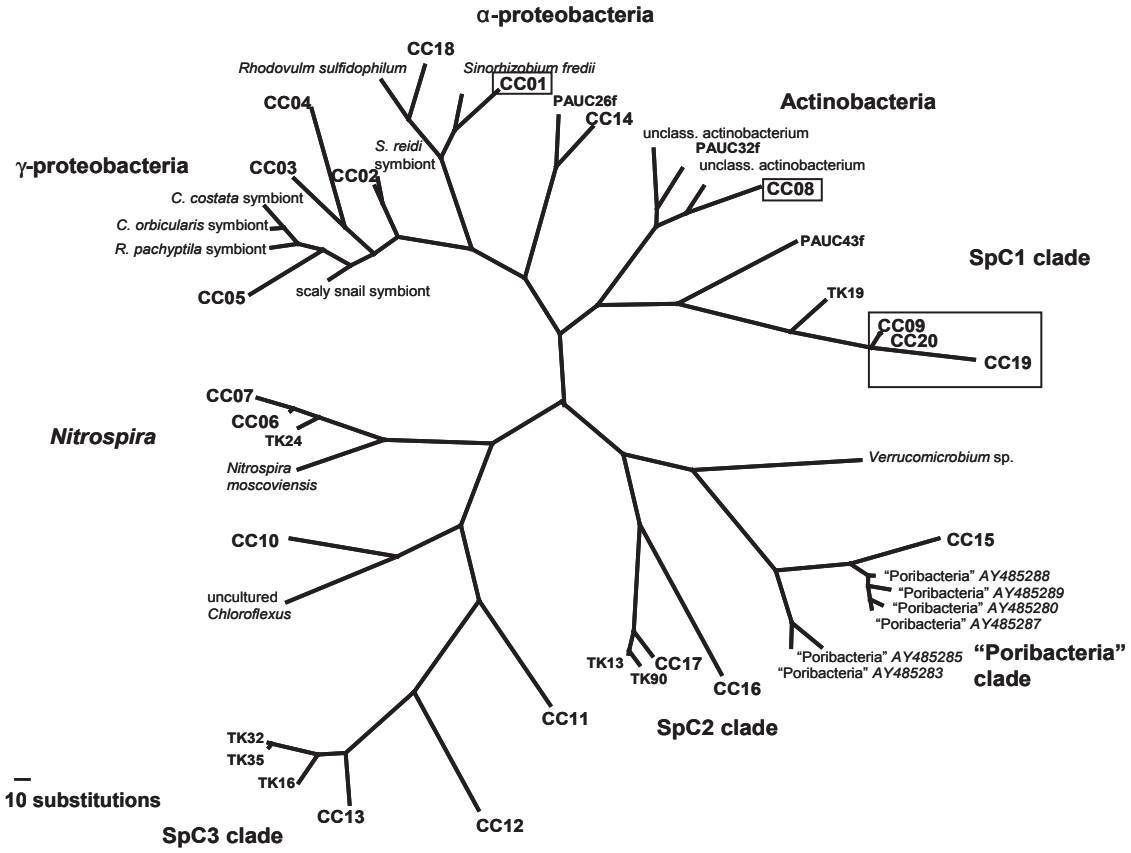


Figure 9. Phylogenetic affiliations of bacterial 16S sequences consistently found in embryo libraries. Libraries contained diverse sequences from the proteobacteria and actinobacteria, as well as several clades of sequences that are most closely related to unclassified bacteria that originated in other marine sponge 16S rDNA clone libraries. CC##: *C. candelabrum* sequences. Boxes indicate sequences targeted by specific probes (this study).

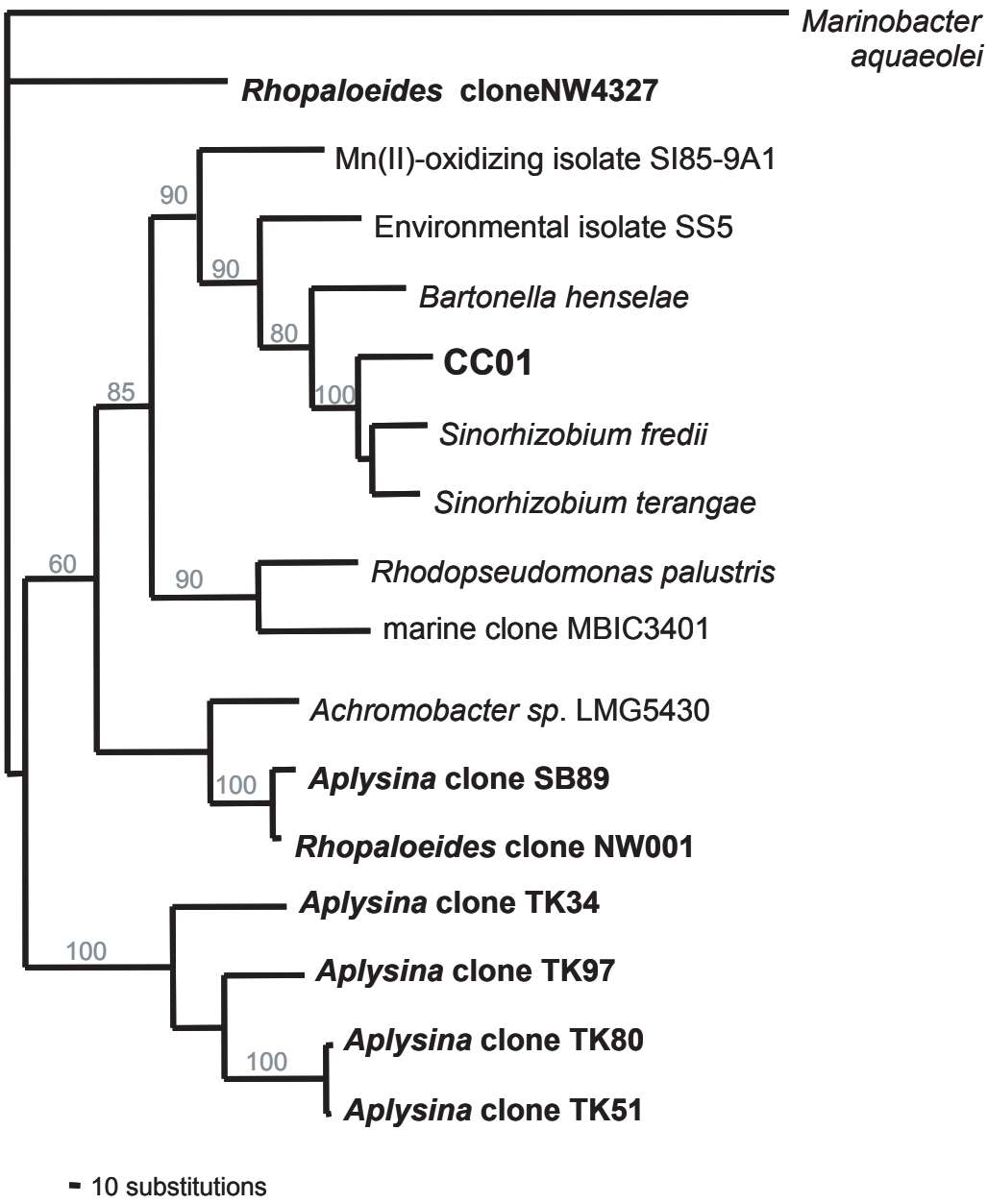


Figure 10. Parsimony tree showing clone CC01 is an alpha-proteobacterium that is closely related to pathogenic and symbiotic bacteria. Other bacterial 16S sequences found in marine sponges are shown in bold.

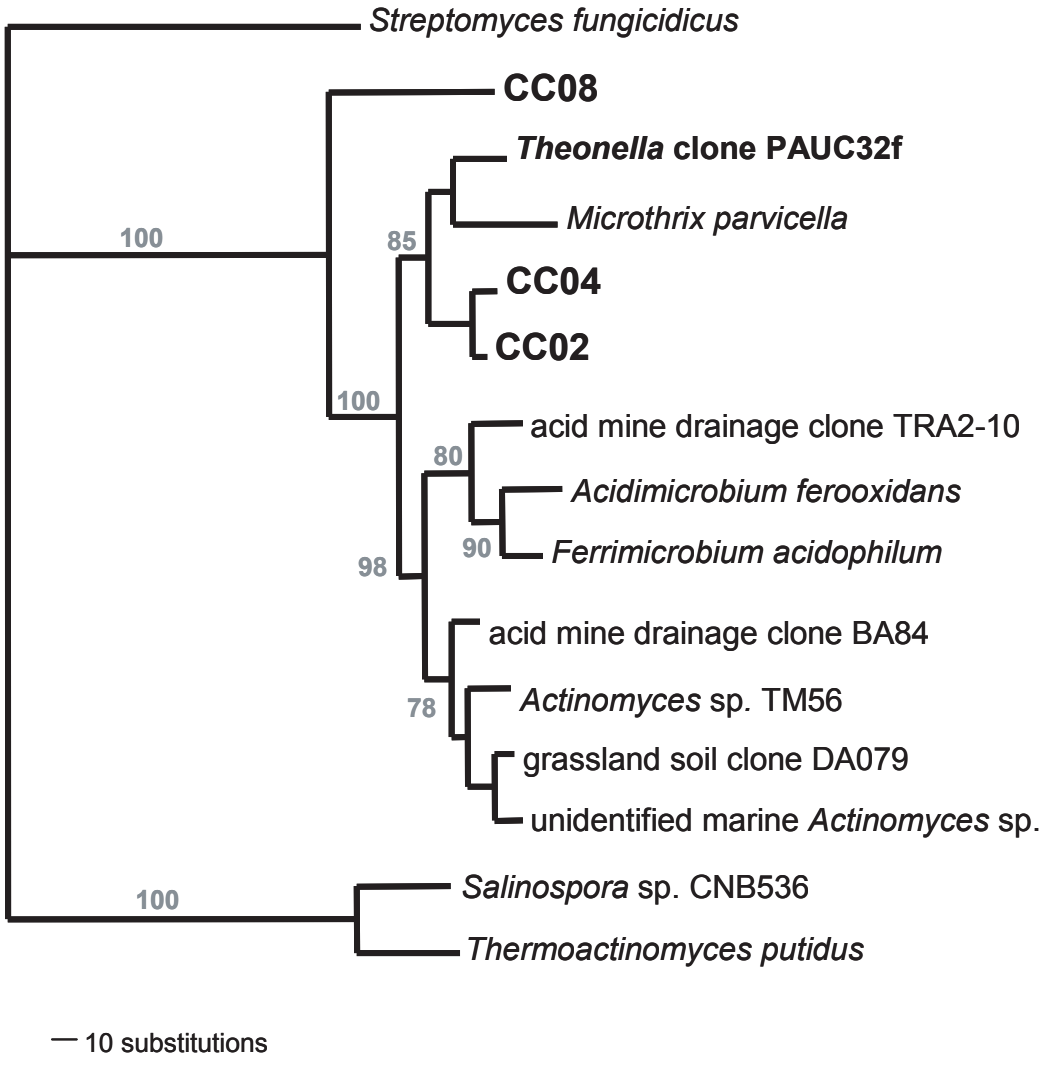


Figure 11. Parsimony tree showing clone CC08 falls into the actinobacteria. The cloned sequence is not very closely related to many known sequences. In addition to two other sequences from this study, it is also related to other bacterial 16S sequences found in sponges (shown in bold). Sequences from this study: CC##.

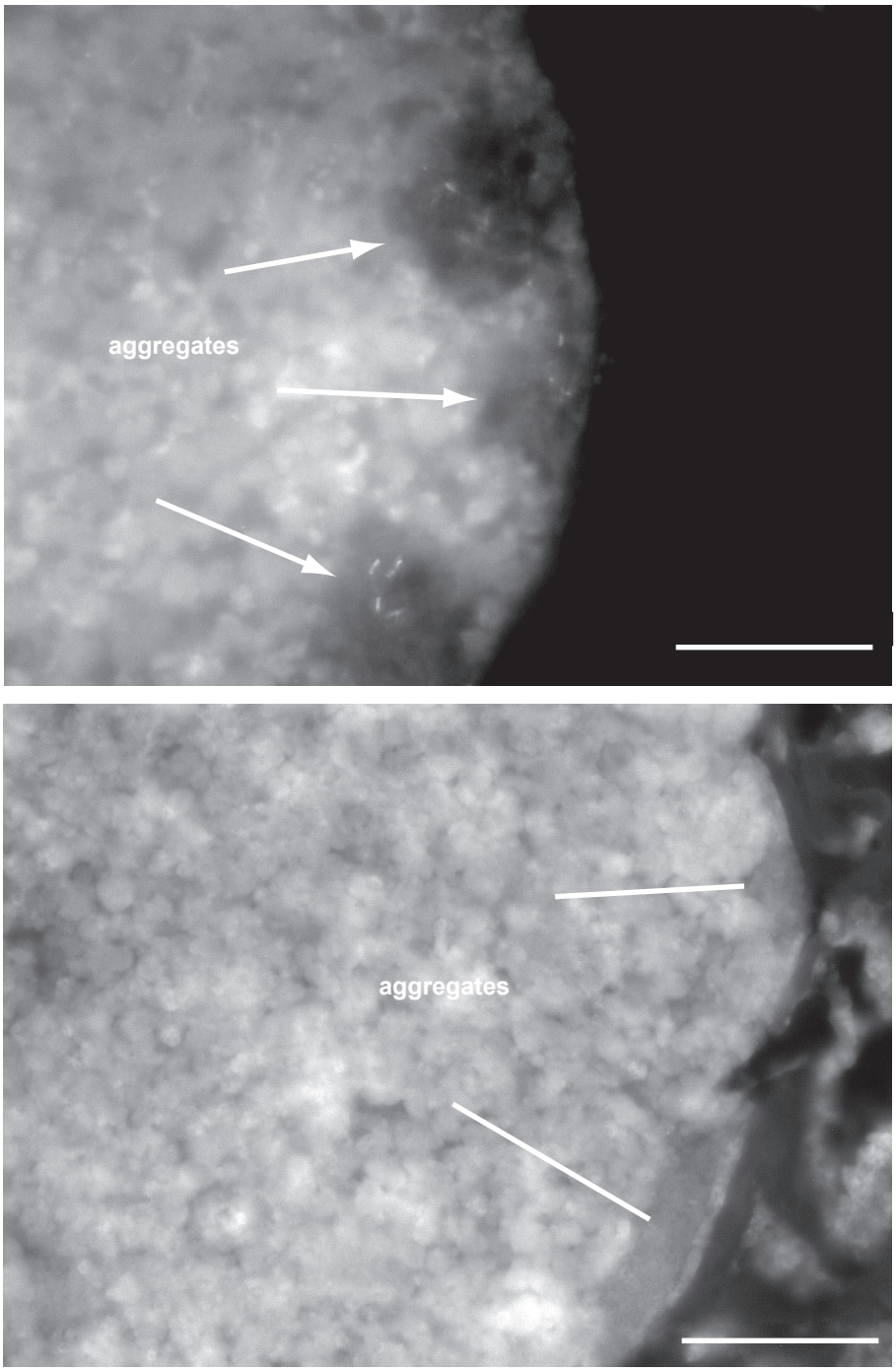


Figure 12. Sequence-specific probe (α-CCO1) hybridizes to cells in bacterial aggregates in embryos. (A) Specific probe for the alpha-proteobacterium α-CCO1 hybridizes to a small number of cells within the aggregates around the early stage embryo. (B) Single base mismatch negative control probe shows no signal in the aggregates. arrows: bacteria in aggregates. Bar = 20um.

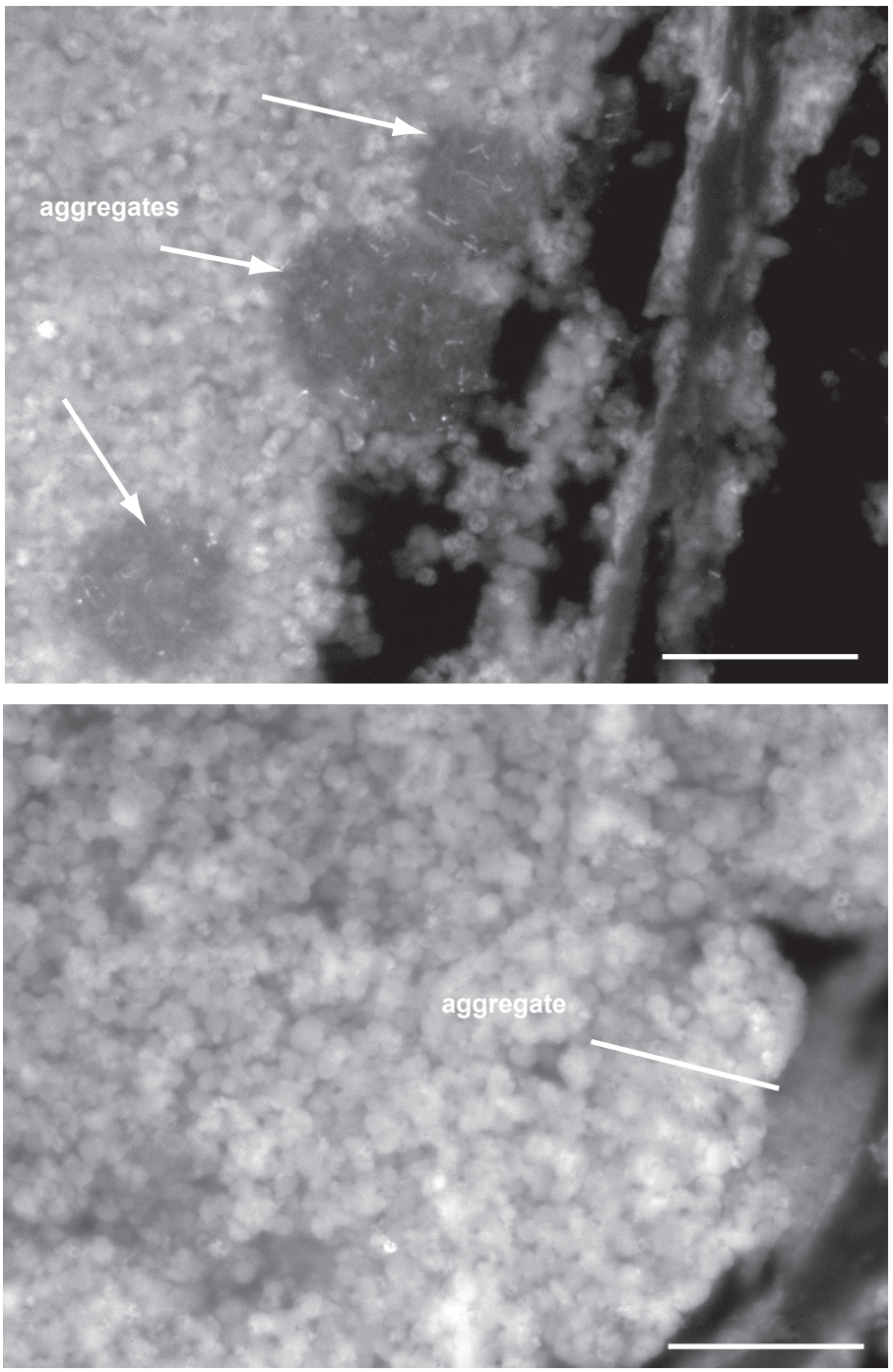


Figure 13. Sequence-specific probe (actino-CCO8) hybridizes to cells in bacterial aggregates in embryos. (A) Specific probe for the actinobacterium CC08 hybridizes to a small number of cells within the aggregates around the early stage embryo. (B) Single base mismatch negative control probe shows no signal in the aggregates. arrows: bacteria in aggregates. Bar = 20um.

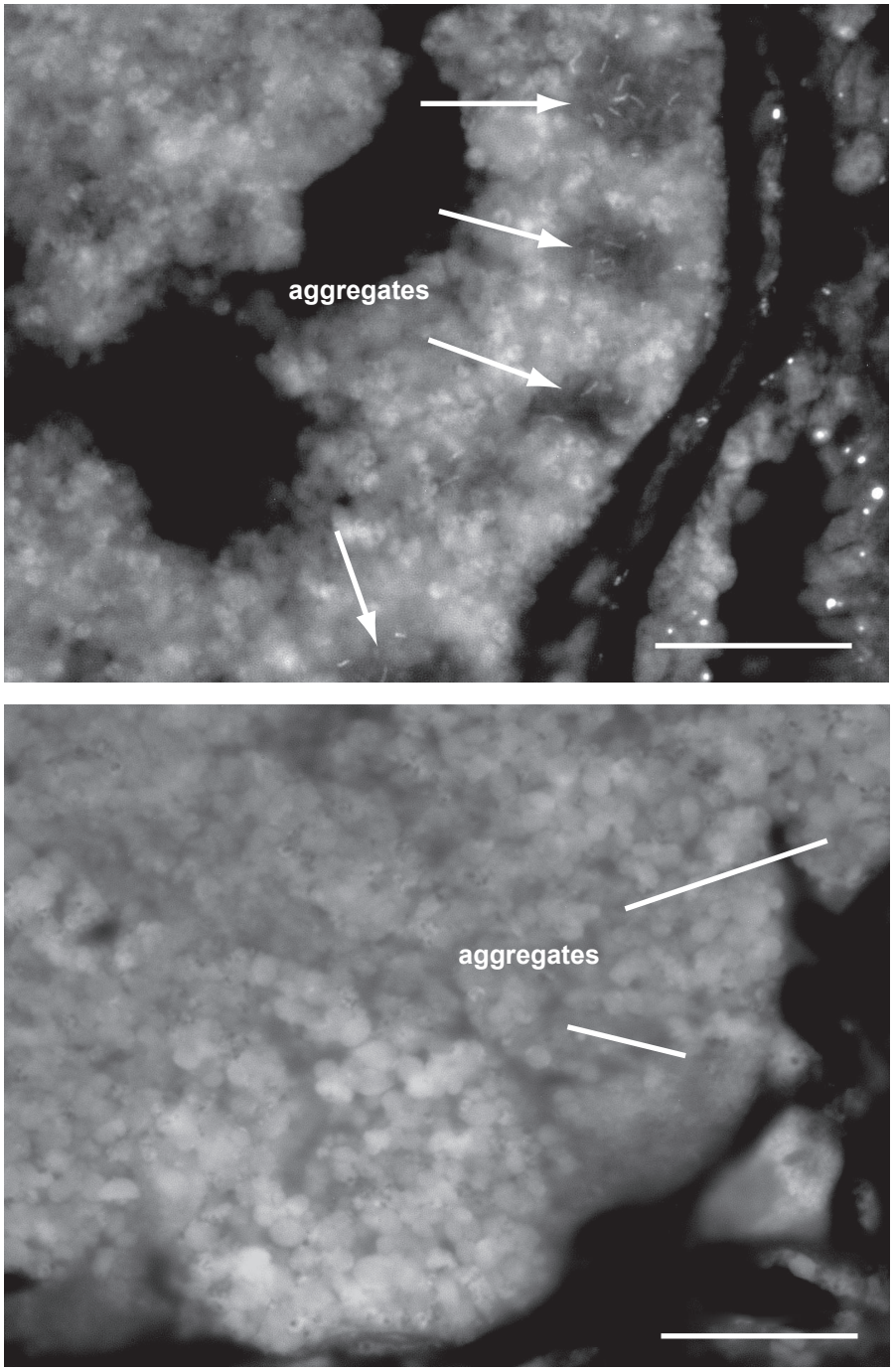
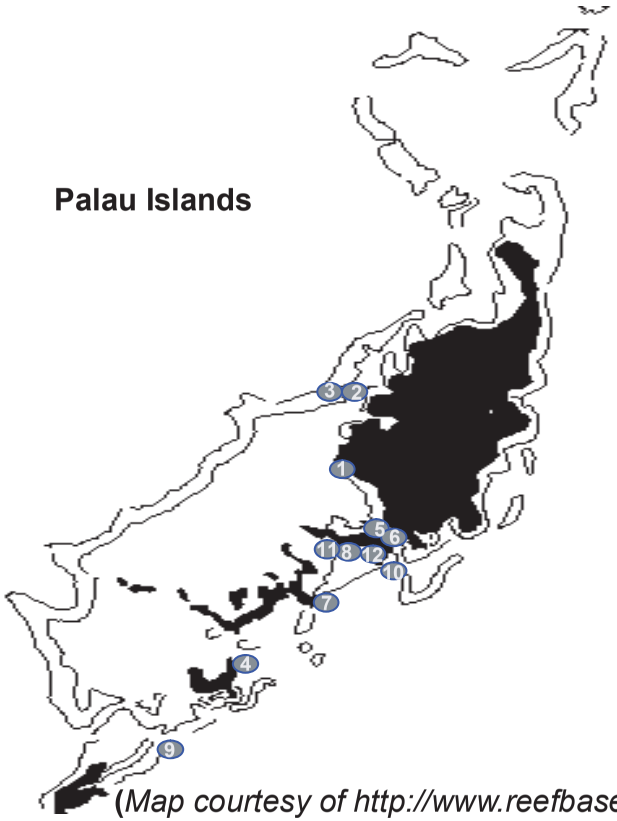
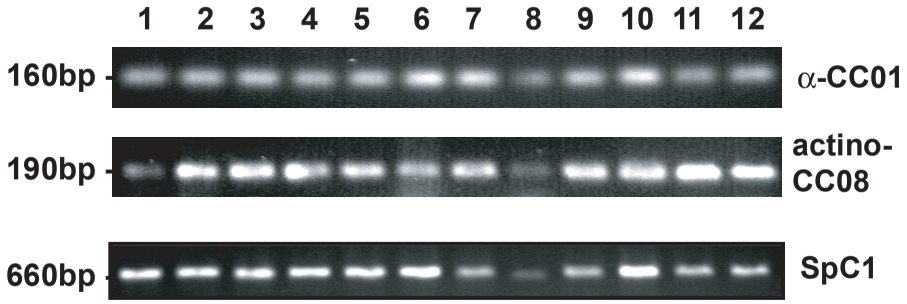


Figure 14. Sequence-specific probe (SpC1) hybridizes to cells in bacterial aggregates in embryos. (A) Specific probe for a clade of sponge associates (SpC1) hybridizes to a small number of cells within the aggregates around the early stage embryo. (B) Single base mismatch negative control probe shows no signal in the aggregates. arrows: bacteria in aggregates. Bar = 20um.





Collection		Year
1.	Ibobong	2002
2.	West Channel	2002
3.	West Reef	2002
4.	Wonder Channel	2002
5.	Sunken Bridge	2002
6.	Sunken Bridge	2001
7.	Chenoi Channel	2002
8.	Antipathes Cave	2002
9.	Ngerchong Channel	2004
10.	Lighthouse Channel	2004
11.	Venture Lake Opening	2004
12.	Antipathes Cave	2004

Figure 15. Sequence-specific primers amplify 16S products from *C. candelabrum* across spatial and temporal boundaries. *C. candelabrum* from twelve different locations in Palau, across three different years. Products from twelve sponges using three specific primer sets are shown here.

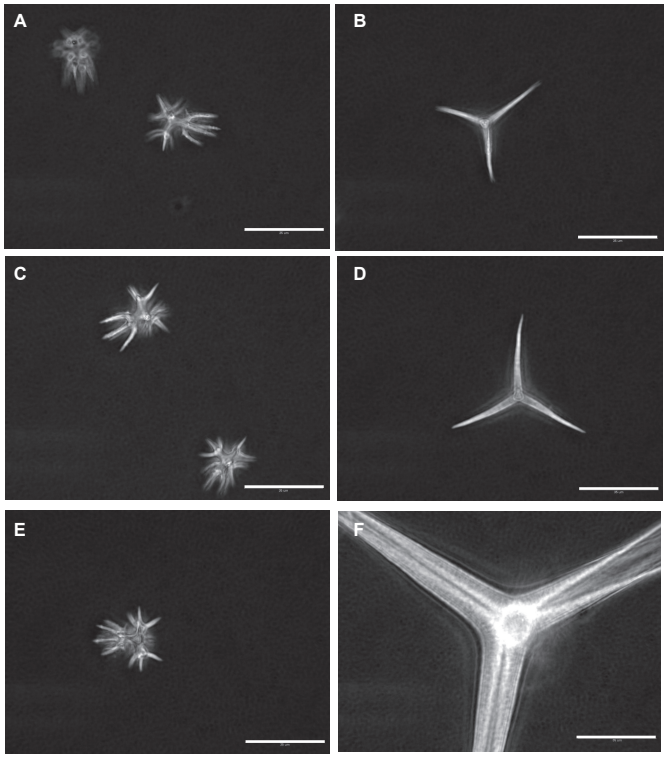


Figure 16. Spicules from *Corticium* collected in the Pacific. In three different chemotypes of sponges appearing to be *Corticium sp*., spicule morphology is similar. All three have small heterolophose calthrops, and though all three have triaene calthrops, the triaenes from the Guam sponge are much larger than those in the Palauan sponges.(A, B) Spicules from the black tedanolide-containing *C. candelabrum* (C, D) Spicules from the gray *Corticium* (non tedanolide-containing) collected from an adjacent rock in Palau. (E,F) *Corticium* sp., shown to contain steroidal alkaloids but no tedanolides, collected in Guam. Bar=35um in all micrographs.

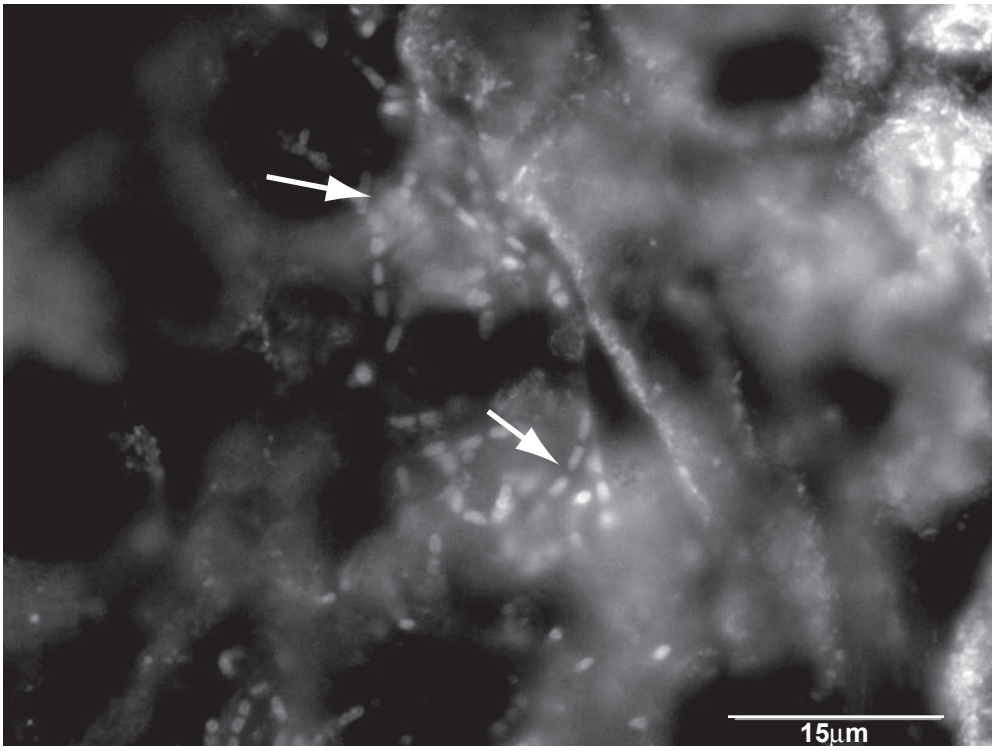


Figure 17. Bacteria in gray *C. candelabrum.* CY5-EUB338 hybridizes with large filamentous microbes in addition to small unicellular bacteria in *C. candelabrum.* Arrows: filamentous cells.

REFERENCES

Altschul, S. F., W. Gish, W. Miller, E. W. Wyers and D. J. Lipman (1990). Basic Local Alignment Search Tool. *J. of Mol. Biol.* 215(3): 403-410.

Behrens, S., C. Ruhland, J. Inacio, H. Huber, A. Fonseca, I. Spencer-Martins, B. M. Fuchs and R. Amann (2003). In Situ Accessibility Domains of Small-Subunit rRNA of Members of the Domains Bacteria, Archaea, and Eucarya to Cy3-Labeled Oligonucleotide Probes. *Appl. Environ. Microbiol.* 69(3): 1748-1753.

Bewley, C. A., N. A. Holland and D. J. Faulkner (1996). Two classes of metabolites from *Theonella swinhoei* are localized in distinct populations of bacterial symbionts. *Experientia* 52: 716-722.

Blazejak, A., C. Erseus, R. Amann and N. Dubilier (2005). Coexistence of Bacterial Sulfide Oxidizers, Sulfate Reducers, and Spirochetes in a Gutless Worm (Oligochaeta) from the Peru Margin. *Appl. Environ. Microbiol.* 71(3): 1553-1561.

Boury-Esnault, N., A. Ereskovsky, C. Bezac and D. Tokina (2003). Larval development in the Homoscleromorpha (Porifera, Demospongiae). *Invertebrate Biology* 122(3): 187-202.

Brusca, R. C. and G. J. Brusca (1990). Invertebrates. Sunderland, MA, Sinauer Press.

Buss, L. W. and J. B. C. Jackson (1981). Planktonic Food Availability and Suspension-Feeder Abundance - Evidence of Insitu Depletion. *Journal of Experimental Marine Biology and Ecology* 49(2-3): 151-161.

Cannone, J. J., S. Subramanian, M. N. Schnare, J. R. Collett, L. M. D'Souza, Y. Du, B. Feng, N. Lin, L. V. Madabusi, K. M. Muller, N. Pande, Z. Shang, N. Yu and R. R. Gutell (2002). The comparative RNA web (CRW) site: an online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. *BMC Bioinformatics* 3(2).

Cary, S. C. (1994). Transovarial inheritance of endosymbiotic bacteria in the protobranch bivalve, *Solemya reidi*. *Eos* 75(3): 60.

Cole, J. R., B. Chai, T. L. Marsh, R. J. Farris, Q. Wang, S. A. Kulam, S. Chandra, D. M. McGarrell, T. M. Schmidt, G. M. Garrity and J. M. Tiedje (2003). The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res.* 31: 442-443.

Davidson, S., S. W. Allen, G. E. Lim, C. M. Anderson and M. G. Haygood (2001). Evidence for the biosynthesis of bryostatins by the bacterial symbiont "*Candidatus* Endobugula sertula" of the bryozoan *Bugula neritina*. *Appl. Env. Microbiol.* 67: 4531-4537.

Distel, D. L., D. J. Beaudoin and W. Morrill (2002). Coexistence of multiple proteobacterial endosymbionts in the gills of the wood-boring bivalve Lyrodus pedicellatus (Bivalvia: Teredinidae). *Applied & Environmental Microbiology* 68(12): 6292-6299.

Donadio, S., M. J. Staver, J. B. McAlpine, S. J. Swanson and L. Katz (1991). Modular organization of genes required for complex polyketide biosynthesis. *Science 759*: 675-679.

Ereskovksy, A. V. and N. Boury-Esnault (2002). Cleavage pattern in Oscarella species (Porifera, Demopspongiae, Homoscleromorpha): transmission of maternal cells and symbiotic bacteria. *Journal of Natural History* 36: 1761-1775.

Erpenbeck, D., J. A. J. Breeuwer, d. V. H. C. van and S. R. W. M. van (2002). Unravelling host and symbiont phylogenies of halichondrid sponges (Demospongiae, Porifera) using a mitochondrial marker. *Marine Biology (Berlin)* 141(2): 377-386.

Faulkner, D. J. (2000). Marine pharmacology. *Antonie van Leeuwenhoek.* 77(2): 135-145.

Faulkner, D. J. (2001). Marine Natural Products. *Natural Products Reports* 18: 1-49.

Fiala-Medioni, A., Z. P. McKiness, P. Dando, J. Boulegue, A. Mariotti, A. M. Alayse-Danet, J. J. Robinson and C. M. Cavanaugh (2002). Ultrastructural, biochemical, and immunological characterization of two populations of the mytilid mussel Bathymodiolus azoricus from the Mid-Atlantic Ridge: Evidence for a dual symbiosis. *Marine Biology (Berlin)* 141(6): 1035-1043.

Fieseler, L., M. Horn, M. Wagner and U. Hentschel (2004). Discovery of the Novel Candidate Phylum "Poribacteria" in Marine Sponges. *Appl. Environ. Microbiol.* 70(6): 3724-3732.

Flatt, P., J. Gautschi, R. Thacker, M. Musafija-Girt, P. Crews and W. Gerwick (2005). Identification of the cellular site of polychlorinated peptide biosynthesis in the marine sponge Dysidea (Lamellodysidea) herbacea and symbiotic cyanobacterium Oscillatoria spongeliae by CARD-FISH analysis. *Marine Biology* 147(3): 761-774.

Fusetani, N., T. Sugawara, S. Mastunaga and H. Hirota (1991). Cytotoxic metabolites of the marine sponge *Mycale adhaerens* Lambe. *Journal of Organic Chemistry* 56: 4971-4974.

Gros, O., L. Frenkiel and H. Felbeck (2000). Sulfur-oxidizing endosymbiosis in Divaricella quadrisulcata (Bivalvia: Lucinidae): Morphological, ultrastructural, and phylogenetic analysis. *Symbiosis* 29(4): 293-317.

Haygood, M. G. and S. K. Davidson (1997). Small subunit ribosomal RNA genes and *in situ* hybridization of the bacterial symbionts in the larvae of the bryozoan *Bugula neritina* and proposal of "*Candidatus* Endobugula sertula". *Appl. Env. Microbiol.* 63: 4612-4616.

Hentschel, U., L. Fieseler, M. Wehrl, C. Gernert, M. Steinert and J. Hacker (2003). Microbial Diversity of Marine Sponges. Marine Molecular Biotechnology. W. E. G. Muller. Berlin Heidelberg, Springer-Verlag. Sponges (Porifera): 59-88.

Hentschel, U., J. Hopke, M. Horn, A. B. Friedrich, M. Wagner, J. Hacker and B. S. Moore (2002). Molecular evidence for a uniform microbial community in sponges from different oceans. *Applied and Environmental Microbiology* 68(9): 4431-4440.

Hentschel, U., M. Schmid, M. Wagner, L. Fieseler, C. Gernert and J. Hacker (2001). Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges Aplysina aerophoba and Aplysina cavernicola. *FEMS Microbiology Ecology* 35(3): 305-312.

Hirose, E. (2000). Plant rake and algal pouch of the larvae in the tropical ascidian Diplosoma similis: An adaptation for vertical transmission of photosynthetic symbionts Prochloron sp. *Zoological Science (Tokyo)* 17: 233-240.

Hoffmann, F., O. Larsen, V. Thiel, H. T. Rapp, T. Pape, W. Michaelis and J. Reitner (2005). An anaerobic world in sponges. *Geomicrobiology Journal* 22(1-2): 1-10.

Hopwood, D. A. and D. H. Sherman (1990). Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. *Annu. Rev. Genet. 24*: 37-66.

Koropatnick, T. A., J. T. Engle, M. A. Apicella, E. V. Stabb, W. E. Goldman and M. J. McFall-Ngai (2004). Microbial factor-mediated development in a host-bacterial mutalism. *Science* 306(5699): 1186-1188.

Krueger, D. M., R. G. Gustafson and C. M. Cavanaugh (1996). Vertical transmission of chemoautotrophic symbionts in the bivalve Solemya velum (Bivalvia: Protobranchia). *Biological Bulletin* 190: 195-202.

Lopanik, N., N. Lindquist and N. Targett (2004). Potent cytotoxins produced by a microbial symbiont protect host larvae from predation. *Oecologia* 139(1): 131-139.

Mullins, T. D., T. B. Britschgi, R. L. Krest and S. J. Giovannoni (1995). Genetic Comparisons Reveal the Same Unknown Bacterial Lineages in Atlantic and Pacific Bacterioplankton Communities. *Limnology and Oceanography* 40(1): 148-158.

Muricy, G., C. Bezac, M. F. Gallissian and N. Boury-Esnault (2002). Anatomy, cytology and symbiotic bacteria of four Mediterranean species of Plakina Schulze, 1880 (Demospongiae, Homosclerophorida). *Journal of Natural History* 33(2): 159-176.

Nyholm, S. V. and M. J. McFall-Ngai (2004). The winnowing: Establishing the squid-Vibrio symbiosis. *Nature Reviews Microbiology* 2(8): 632-642.

Peek, A. S., R. A. Feldman, R. A. Lutz and R. C. Vrijenhoek (1998). Cospeciation of chemoautotrophic bacteria and deep sea clams. *Proceedings of the National Academy of Sciences of the United States of America* 95(17): 9962-9966.Pile, A. J. P., Mark R.; Savarese, Michael; Chernykh, Valeri I.; Fialkov, Vladimir A. (1997). Trophic Effects of Sponge Feeding Within Lake Baikal's Littoral Zone. 2. Sponge Abundance, Diet, Feeding Efficiency, and Carbon Flux. *Limnol. Oceanogr.* 42(1): 178-184.

Preston, C. M., K. Y. Wu, T. F. Molinski and E. F. Delong (1996). A psychrophilic crenarchaeon inhabits a marine sponge: Cenarchaeum symbiosum gen. nov., sp. nov. *Proceedings of the National Academy of Sciences of the United States of America* 93(13): 6241-6246.

Regoli, F., C. Cerrano, E. Chierici, S. Bompadre and G. Bavestrello (2000). Susceptibility to oxidative stress of the Mediterranean demosponge Petrosia ficiformis: Role of endosymbionts and solar irradiance. *Marine Biology (Berlin)* 137(3): 453-461.

Richter, C. and M. Wunsch (1999). Cavity-dwelling suspension feeders in coral reefs - a new link in reef trophodynamics. *Marine Ecology-Progress Series* 188: 105-116.

Ridley, C. P., P. R. Bergquist, M. K. Harper, D. J. Faulkner, J. N. A. Hooper and M. G. Haygood (2005a). Speciation and Biosynthetic Variation in Four Dictyoceratid Sponges and Their Cyanobacterial Symbiont, Oscillatoria spongeliae. *Chemistry & Biology* 12(3): 397-406.

Ridley, C. P. and D. J. Faulkner (2003). New cytotoxic steroidal alkaloids from the Philippine sponge Corticium niger. *Journal of Natural Products* 66(12): 1536-1539.

Ridley, C. P., D. J. Faulkner and M. G. Haygood (2005b). Investigation of *Oscillatoria spongeliae*-Dominated Bacterial Communities in Four Dictyoceratid Sponges. *Appl. Environ. Microbiol. in press.*

Rinehart, K. L., J. Cheng and J. Lee (1996). US Patent. S.A. USA, Pharma Mar.

Schloss, P. D. and J. Handelsman (2005). Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Applied and Environmental Microbiology* 71(3): 1501-1506.

Schmidt, E. W., A. Y. Obraztsova, S. K. Davidson, D. J. Faulkner and M. G. Haygood (2000). Identification of the antifungal peptide-containing symbiont of the marine sponge *Theonella swinhoei* as a novel d-proteobacterium , "*Candidatus* Entotheonella palauensis". *Marine Biology* 136: 969-977.

Schmidtz, F. J., S. P. Gunasekera, G. Yalamanchili, M. B. Hossain and D. Van Der Helm (1984). Tedanolide: a potent cytotoxic macrolide from the Caribbean sponge *Tedania ignis*. *Journal of the American Chemical Society* 106: 7251-7252.

Schonberg, C. H. L. and D. Barthel (1997). Inorganic skeleton of the demosponge Halichondria panicea. Seasonality in spicule production in the Baltic Sea. *Marine Biology* 130(2): 133-140.

Sciscioli, M., E. Lepore, M. Gherardi and L. S. Liaci (1994). Transfer of symbiotic bacteria in the mature oocyte of Geodia cydonium: (Porifera, Demospongiae): an ultrastructural study. *Cah. Biol. Mar.* 35: 471-478.

Sipe, A. R., A. E. Wilbur and S. C. Cary (2000). Bacterial symbiont transmission in the wood-boring shipworm B*ankia setacea* (Bivalvia: Teredinidae). *Applied and Environmental Microbiology* 66: 1685-1691.

Steindler, L., S. Beer, A. Peretzman-Shemer, C. Nyberg and M. Ilan (2001). Photoadaptation of zooxanthellae in the sponge Cliona vastifica from the Red Sea, as measured in situ. *Marine Biology (Berlin)* 138(3): 511-515.

Swofford, D. L. (2003). PAUP*: phylogenetic analysis using parsimony (* and other methods), version 4.0b 10. Sunderland, MA, Sinauer Associates.

Taylor, M. W., P. J. Schupp, I. Dahllof, S. Kjelleberg and P. D. Steinberg (2004). Host specificity in marine sponge-associated bacteria, and potential implications for marine microbial diversity. *Environmental Microbiology* 6(2): 121-130.

Thacker, R. W. and S. Starnes (2003). Host specificity of the symbiotic cyanobacterium Oscillatoria spongeliae in marine sponges, Dysidea spp. *Marine Biology* 142(4): 643-648.

Unson, M. D. and D. J. Faulkner (1993). Cyanobacterial symbiont biosynthesis of chlorinated metabolites from *Dysidea herbacea* (Porifera). *Experientia* 49(4): 349-353.

Unson, M. D., N. D. Holland and D. J. Faulkner (1994). A brominated secondary metabolite synthesized by the cyanobacterial symbiont of a marine sponge and accumulation of the crystalline metabolite in the sponge tissue. *Mar. Biol.* 119(1): 1-11.

Usher, K. M., J. Kuo, J. Fromont and D. C. Sutton (2001). Vertical transmission of cyanobacterial symbionts in the marine sponge Chondrilla australiensis (Demospongiae). *Hydrobiologia* 461: 15-23.

Usher, K. M., D. C. Sutton, S. Toze, J. Kuo and J. Fromont (2005). Inter-generational transmission of microbial symbionts in the marine sponge Chondrilla australiensis (Demospongiae). *Marine and Freshwater Research* 56(2): 125-131.

Vacelet, J. and C. Donadey (1977). Electron microscope study of the association between some sponges and bacteria. *Journal of Experimental Marine Biology and Ecology* 30(3): 301-314.

Vacelet, J., A. Fiala-Medioni, C. R. Fisher and N. Boury-Esnault (1996). Symbiosis between methane-oxidizing bacteria and a deep-sea carnivorous cladorhizid sponge. *Marine Ecology Progress Series* 145(1-3): 77-85.

Webster, N. S. and R. T. Hill (2001). The culturable microbial community of the Great Barrier Reef sponge Rhopaloeides odorabile is dominated by an alpha-Proteobacterium. *Marine Biology (Berlin)* 138(4): 843-851.

Webster, N. S., K. J. Wilson, L. L. Blackall and R. T. Hill (2001). Phylogenetic diversity of bacteria associated with the marine sponge Rhopaloeides odorabile. *Applied and Environmental Microbiology* 67(1): 434-444.

Woollacott, R. M. (1981). Association of bacteria with bryozoans larvae. *Mar. Biol.* 65: 155-158.

Yahel, G., J. H. Sharp, D. Marie, C. Hase and A. Genin (2003). In situ feeding and element removal in the symbiont-bearing sponge Theonella swinhoei: Bulk DOC is the major source for carbon. *Limnology and Oceanography* 48(1): 141-149.

CHAPTER 4

Morphological Studies of Vertical Transmission of Specific Bacteria in the Tropical Sponge *Corticium candelabrum*

ABSTRACT

Vertical transmission of microbes was investigated in the Palauan sponge *Corticium candelabrum*. Though oocytes in this sponge do not appear to contain bacteria, bacteria are present in the brooded embryos and remain associated with developing embryos and larvae after release into the seawater. The embryos are associated with numerous bacterial morphotypes, indicating a diverse vertically transmitted assemblage. The bacteria are extracellular in the embryos and larvae, and there is no indication of microbe digestion in any stage of embryonic development. The presence of three bacterial taxa previously identified to be consistently associated with *C. candelabrum* over three years of sampling and 100km distance are present throughout embryonic development. One of those three taxa, α-CC01, an alpha-proteobacterium most closely related to other known symbionts, is also present in the central cavity of swimming *C. candelabrum* larvae after release from the adult.

INTRODUCTION

Many sponges harbor microbes, not just in conjunction with filtering seawater or feeding, but in their internal structures. Enumeration of microbes in sponges has demonstrated that microbes may account for over half of the biomass in some sponges (Wilkinson 1987; Fuerst *et al.* 1998). Though highly specific associations with one microbial species have been demonstrated in several sponge species (Unson 1993; Schmidt *et al.* 2000; Usher *et al.* 2004; Ridley *et al.* 2005), suggesting great host-symbiont selectivity, other sponges harbor diverse microbial assemblages. The diversity of microbial communities in some sponges has previously been interpreted as a result of promiscuity in sponge-microbe associations, but recent research indicates that many sponges harbor microbes belonging to diverse but consistent, specific lineages (Erpenbeck *et al.* 2002; Hentschel *et al.* 2002; Taylor *et al.* 2004).

Although it has been difficult to identify microbes inside of sponges and demonstrate that they are symbiotic (stably associated) as opposed to randomly acquired from the environment, there are few examples in which functions of specific microbe-sponge associations have been clearly demonstrated. Sponges have been reported to contain heterotrophic and phototrophic symbionts (Unson *et al.* 1994; Bewley *et al.* 1996), as well as psychrophilic and methane-oxidizing archaeal species (Preston *et al.* 1996; Vacelet *et al.* 1996). Biochemical experiments have demonstrated translocation of carbon, nutrients, and antioxidants from photosynthetic symbionts to sponges (Regoli *et al.* 2000; Steindler *et al.* 2001). In addition to cycling inorganic nutrients and producing fixed carbon, some microbial symbionts produce bioactive compounds in their sponge hosts (Faulkner 2000; Faulkner 2001).

The origin of specific associations between microbes and sponges is not well understood, but it has been proposed that most sponges maintain symbioses with microbes via vertical transmission, rather than capturing them from surrounding seawater. In addition, the unique ribosomal RNA sequences of most microbes in sponges indicate that the bacteria and archaea within sponges are highly evolved and specialized. Morphological characterization of sponge reproduction and development have localized microbial cells within embryos and larvae of many sponges (Vacelet and Donadey 1977; Vacelet *et al.* 1996; Usher *et al.* 2001; Ereskovksy and Boury-Esnault 2002; Boury-Esnault *et al.* 2003; Ereskovksy *et al.* 2005), though few embryo-associated bacteria or archaea have been identified with molecular methods. Passage of microbes indicates a role in the survival of the host sponge.

C. candelabrum inhabits reefs in the equatorial Pacific, in addition to the Mediterranean Sea. Our lab's interest in this sponge began in 1999, when it showed high levels of cytotoxicity against human colon tumor cells. The cytotoxic compounds, later identified as tedanolides, are suspected to be of microbial origin because of their structural similarity to other microbial compounds, and because tedanolides and tedanolide derivatives have been isolated from other diverse marine invertebrates. An investigation of the bacteria and archaea associated with *C. candelabrum* revealed that the adult sponges are packed with microbes, and that a highly diverse, specific group of bacteria and archaea are present in developing embryos.

This study addresses basic questions about the morphology of microbial transmission in sponges. Are microbes in embryos to ensure transmission to future

generations, or simply as a food source? Previous investigations on the role of bacterial cells in the Antarctic sponge *Halisarca dujardini* demonstrated that some bacterial cells are digested in oocytes but there is no evidence of bacteria playing a nutritional role in embryonic or postembryonic development (Ereskovsky *et al.* 2005). I hypothesize that the microbes in *C. candelabrum* are not merely a source of nutrients. The embryos are lecithotrophic (Ereskovksy and Boury-Esnault 2002), and my previous study suggests that very specialized aggregates that consistently include specific bacterial taxa form during embryogenesis.

This chapter investigates the fate of microbes in *C. candelabrum* during embryonic development and larval release, using scanning electron microscopy (SEM), transmission electron microscopy (TEM), and fluorescence in situ hybridization (FISH). The objective of this work is to test the hypothesis that microbes that I previously identified in *C. candelabrum* embryos (see previous chapter) are present not only in embryos, but throughout embryogenesis and in the swimming larvae after released from the adult sponge.

MATERIALS AND METHODS

Sponge and Larval Collection. Reproducing specimens of *C. candelabrum* were collected via SCUBA in several locations in the Palau archipelago from a depth of 3-20m in Sept-Oct 2001, 2002, and March 2004. Sponges were collected together in plastic bags at depth and brought to the surface. Small numbers of sponges were kept in separate aquaria, larvae were collected via pipet in the mornings from the surface of

the seawater in the tanks, and identified microscopically and rinsed in sterile filtered seawater before fixation.

FISH. Samples were frozen, preserved in 100% ethanol, 2.5% glutaraldehyde, or frozen solid at -20°C until use. For FISH, whole sponges were fixed in 4% paraformaldehyde (4% in buffer: 20mM K_2HPO_4, 0.5M NaCl, pH 7.4) for 2 hours at room temperature and transferred to 70% ethanol for long-term storage at -20°C. For FISH and microscopy, individual sponges were embedded in paraffin wax and sectioned to 10μm. Sections were deparaffinized (2x5min in xylene; 2x5min in 100% ethanol, one rinse in Milli-Q water) and air-dried. FISH was performed in a humidity chamber in hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.4], 0.01% sodium dodecyl sulfate) with 35% percent formamide for 2 h. All probes, including the general eubacterial probe (EUB338: 5'-GCTGCCTCCCGTAGGAGT-3') and the sequence-specific probes designed in this study, were used at a concentration of 5ng/μl final concentration in hybridization buffer. After hybridization, the slides were incubated at 48°C in wash buffer (0.7 M NaCl, 20 mM Tris-HCl [pH 7.4], 50 mM EDTA, 0.01% sodium dodecyl sulfate) for 20 minutes. The wash buffer was rinsed off with Milli-Q water, and slides were air-dried and mounted in VectaShield (*Vector Labs*, Burlingame, CA). Slides were visualized on an Axioskop epifluroescence microscope (Zeiss).

Design and application of species-specific primers and probes. Probes were designed targeting three groups of 16S rRNA gene sequences from clone libraries constructed from five *C. candelabrum* samples (see Chapter 3, Figure 9). The sequences, representing one α-proteobacterial 16S sequence from the library, one

actinobacterial sequence, and a deeply-branching clade of bacteria, were targeted with the oligonucleotide primers CC01-1216 (5'-CGACCTCGCGATCTCGCT-3'), CC08-1245 (5'-CGCTTGACCTCGCGGTGT-3'), and SpC1 (5'-CTACACATTCCACCGCTA-3'), respectively. PCR cycling conditions with the specific primers were: initial denaturation (3 min at 95°C); 35 cycles of denaturation (30 sec at 95°C), annealing (1 min at 65°C), and elongation (1 min at 72°C); and a final extension step (7 min at 72°C). The primers were designed to be specific by targeting a hypervariable region of the 16S molecule, and to be efficient probes for 16SrRNA extremely high accessibility to oligonucleotide probes (Behrens *et al.* 2003). ProbeMatch (RDP; http://rdp.cme.msu.edu/ probematch/search.jsp) suggests that the three probes do not match to 16S sequence of any microbes known in the database. Single base mismatch probes were designed as negative controls to confirm the specificity of each of the three probes (see Table 1 for all probe sequences).

Transmission electron microscopy. *C. candelabrum* was fixed in 2.5% glutaraldehyde-phosphate/sucrose (2.5% glutaraldehyde in phosphate buffer: 20mM KH2PO4; 60mM K2HPO4; 350mM sucrose, pH 7.3) and stored at -20°C. Samples were stained with 4% uranyl acetate in 50% ethanol, and subsequently dehydrated using a graded series of ethanol solutions followed by propylene oxide and infiltration with epoxy resin (Scipoxy 812, Energy Beam Sciences, Agawam, MA). After polymerization at 65C overnight, thin sections were cut and stained with uranyl acetate (4% uranyl acetate in 50% ethanol) followed by bismuth subnitrate. Sections were examined at an accelerating voltage of 60kV using a Zeiss EM10C electron microscope (P. Reid, VA Hospital Core Imaging Facility, La Jolla, CA).

Scanning electron microscopy (SEM). Paraformaldehyde-fixed *C. candelabrum* adults that had been stored in 70% ethanol were sliced open to various thicknesses and examined for the presence of embryos. Sections containing multiple embryos were dehydrated in an ethanol series (85% ethanol, 20 min; 95% ethanol, 20 min), critical-point dried with liquid CO_2, and coated with a gold-palladium mixture (Au/Pd sputter coater). Samples were examined at an accelerating voltage of 30 kV on an environmental SEM (ESEM, FEI Quanta 600).

RESULTS

The abundance of embryos in *C. candelabrum* appeared to be greater during the fall months of collection (2001, 2002) than in the March collection (2004). The oocytes and embryos, full of yolk granules, are scattered throughout the mesohyl. In SEM micrographs (Fig 1A), developing embryos are present throughout the mesohyl, and a closer look (Fig 1B) shows bacterial cells, including large filaments, on the exterior of the embryos. Bacteria are densely packed in the mesohyl of *C. candelabrum*, but bacteria do not appear to be associated with the oocytes until the onset of embryogenesis, during which they form distinct aggregates within the inside of the embryo (Figure 2A). The bacteria appear to be extracellular. Once the oocyte starts to divide and vitellogenesis begins (yolk granules form and get larger), bacteria are between the follicle wall and the developing embryos and starting to move toward the center of the dividing mass of embryonic cells (Figure 2B). During central cavity formation, bacteria are seen in between the cells of the embryonic wall and are in a mass within the cavity (Figure 2C). Figure 3A shows bacterial cells in a large mass

within the central cavity of a developing embryo. Around the outside of the developing embryos, bacteria occur along strands of mucus connecting the outer wall of the follicle to the developing embryo (Figure 3B). Bacteria also appear to be on mucus strands connecting one follicle to another (Figure 3C). After embryogenesis, bacteria remain in the central part of the embryo, close to the anterior end of the central cavity, consistent with previous ultrastructural findings that bacteria occur in a mass in the anterior end of the cavity of swimming *C. candelabrum* larvae (Boury-Esnault *et al.* 2003).

Transmission electron microscopy (TEM) on the embryos demonstrates that the embryo-associated bacteria are not intracellular, nor are there membranes surrounding the aggregates. In Figure 4A, an early stage embryo that does not appear to contain microbes is in the mesohyl surrounded by microbial cells. Figure 4B shows densely packed microbial cells associated with strands of mucus passing into the developing embryo.

Specific CY3-labeled oligonucleotide probes (α-CC01, actino-CC08, and SpC1) hybridize to bacterial cells within the peripheral clusters of microbes in early stage embryos (Figure 5A, B, C) and in central cavities of later embryonic developmental stages (Figure 5D,E,F), in addition to the mesohyl of the adult sponge (Figure 5G,H,I). Single-base mismatch probes show no hybridization, as previously described in Chapter 2. Negative control probe shows a similar image, which contains particulate autofluorescence, and this is easily distinguished from true hybridization signal.

Because of limited larval supply, it was possible to get conclusive results only for the presence of α-CC01 in the *C. candelabrum* larvae. Figure 6A shows a mass of

bacteria in the anterior end of the central cavity. Cells appearing yellow, hybridizing with both the α-CC01 probe (CY3-CC01, green) and the general eubacterial probe (CY5-EUB338, red) make up a small portion of this mass (Figure 6B). Cy5-EUB338 also hybridizes to many other bacterial cells that are on the surface of the larva. General eubacterial probe CY3-EUB338 reveals the presence of a large filamentous morphotype associated with the larva (Figure 6C), though because the larva was damaged, the exact localization is impossible to discern.

DISCUSSION

The presence of bacteria and archaea in adult sponges and sponge embryos and larvae has been well-documented. However, key questions about sponge-microbe associations remain unanswered. For example, it is not yet known if microbes in sponges serve specific symbiotic functions for sponges. With the growing number of examples of species-specific interactions between microbes and sponges, there is an indication that diverse sponges have evolved very old, obligate associations with bacteria or archaea that contribute to the nutrition or chemical defense of the sponge host.

Ereskovsky *et al* (2005) suggest that in addition to small populations of nonspecific bacteria found in sponges (populations similar to those observed in surrounding seawater), there are large populations of specific microflora in the mesohyl, and in some sponges, specific intracellular symbionts. In *C. candelabrum,* there is no evidence of intracellular bacteria, but here my observations the concept of this species as a "bacteriosponge" (de Caralt *et al.* 2003). Bacteria are packed densely

throughout the mesohyl and choanosomes of the adult sponge, and my results show that at least three specific bacterial taxa of the assemblage in the choanocytes are consistently inoculated into early stage embryos and remain within the embryos throughout development. FISH images show that none of the three phylotypes tested is a dominant phylotype in the bacterial biomass in the embryos, suggesting additional bacterial phylotypes are transmitted via the embryos. At least one of these species (α-CC01) remains associated with the larva after release from the adult. Though other bacteria are observed within the larval cavity, they have yet to be identified. A large, filamentous bacterial morphotype is in the *C. candelabrum* larva, which may be the same – or closely related to the – large filamentous microbes abundant in adult *C. candelabrum*. It is unknown whether the bacteria on the outside of the larvae are residual seawater microbes that stuck to the larval exterior during collection, but the larvae were rinsed in sterile seawater before fixation.

C. candelabrum produces large, yolk-rich cinctoblastula embryos, which develop asynchronously within the adult mesohyl, surrounded by follicles. Previous work suggests that different orders of sponges employ different morphological mechanisms of microbial inoculation into oocytes or embryos (Ereskovksy *et al.* 2005). Bacteria have been shown to penetrate the follicle during late oogenesis in *C. candelabrum* and other Homoscleromorph sponges (Ereskovksy and Boury-Esnault 2002). However, in my study, *C. candelabrum* also appears to utilize a mucus umbilicus, which has been described in embryonic development in at least four Dictyoceratid sponges (*Spongia barbera, S. cheisis, S. graminea,* and *Hippospongia lachne*) (Kaye 1991; Kaye and Reiswig 1991).

Transmission of microbes in sponges is just starting to be investigated at the molecular level, and there are a few examples of one species/one host associations. The work I present here indicates that *C. candelabrum* has evolved to inoculate its embryos with specific microbes, and these microbes remain in swimming larvae when they are expelled into the water column to settle and form a new adult.

Many researchers have excluded the possibility of microbes serving nutritional purpose by being ingested by embryos or larvae (Gallissian and Vacelet 1976; Sciscioli *et al.* 1994), but the role of larva-associated microbes is not understood. My results indicate that at least three specific groups of bacteria are consistently present in embryos throughout embryonic development, and at least one of those species occurs in larvae. Future work investigating the presence of these three bacterial taxa, in addition to other embryo-associated bacteria, should be explored. Identifying the species of microbes that remain in the host after larval metamorphosis and subsequent growth into the rhagon (primitive sponge) could reveal which – and how many species of – bacteria and archaea play a significant role in the survival of the sponge host.

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candelabrum in Palau. Nick Holland provide helpful protocols for sample fixation and embryo separation. Evelyn York at the SIO Analytical Facility made the SEM imaging possible, and Patricia Reid of the VA Medical Association, San Diego, provided her expertise and assistance with sample preparation and photography for transmission electron microscopy.

Probe Name	Sequence (5'-3')	Reference
EUB338	5'-GCTGCCTCCCGTAGGAGT-3'	(Loy *et al*. 2003)
α-CC01	5'-CGACCTCGCGATCTCGCT-3'	this study
act-CC08	5'-CGCTTGACCTCGCGGTGT-3'	this study
SpC1	5'-CTACACATTCCACCGCTA-3'	this study
α-CC01 NON	5'-CGACTTCGCGATCTCGCT-3'	this study
act-CC08 NON	5'-CGCTTGACCTCGCAGTGT-3'	this study
SpC1 NON	5'-CTACTCATTCCACCGCTA-3'	this study

Table 1. List of oligonucleotide probes used in this study. The general eubacterial probe, EUB338, was used to detect general bacterial populations. Oligonucleotides were designed to target specific groups of sequences from the *C. candelabrum* embryo clone library. The probes target an α-proteobacterial species (α-CC01), an actinobacterial species (actino-CC08), and a deeply-branching clade of bacteria in a sponge clade (SpC1). Single base mismatch probes for each of the specific probes were designed to confirm the specificity of the probes.

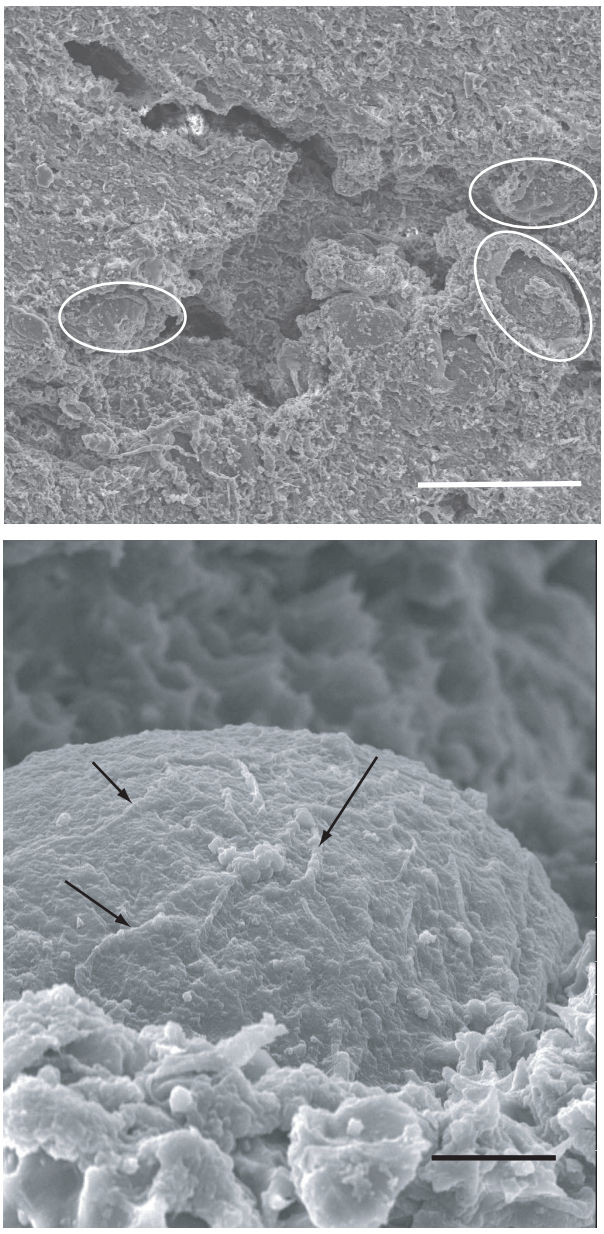


Figure 1. Embryos in *C. candelabrum.* (A) SEM shows embryos distributed throughout the mesohyl of a cross-section of *C. candelabrum*. Bar = 200um. (B) A closeup look at an embryo shows large filaments (arrows) on the surface of the developing embryo. Bar = 20um.

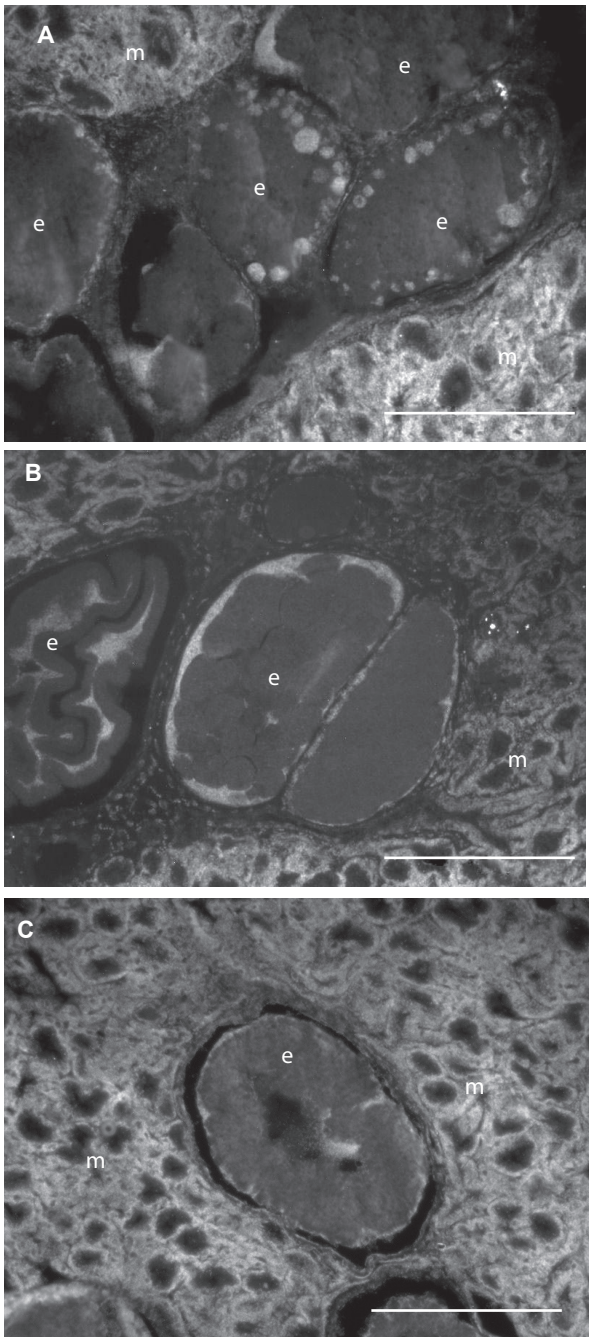


Figure 2. Bacteria in developing *C. candelabrum* embryos.
(A) Aggregates of bacteria inside early stage developing embryos, before the formation of a central cavity. (B) As the embryo develops, the bacteria spread out around the outer portion of the embryo and begin to migrate toward the center of the embryo. (C) Once the cavity is formed, bacteria occur in dense mass within the cavity. Other bacterial cells remain among the cells of the embryo. Bar = 100μm in all micrographs. m: mesohyl; e:embryo.

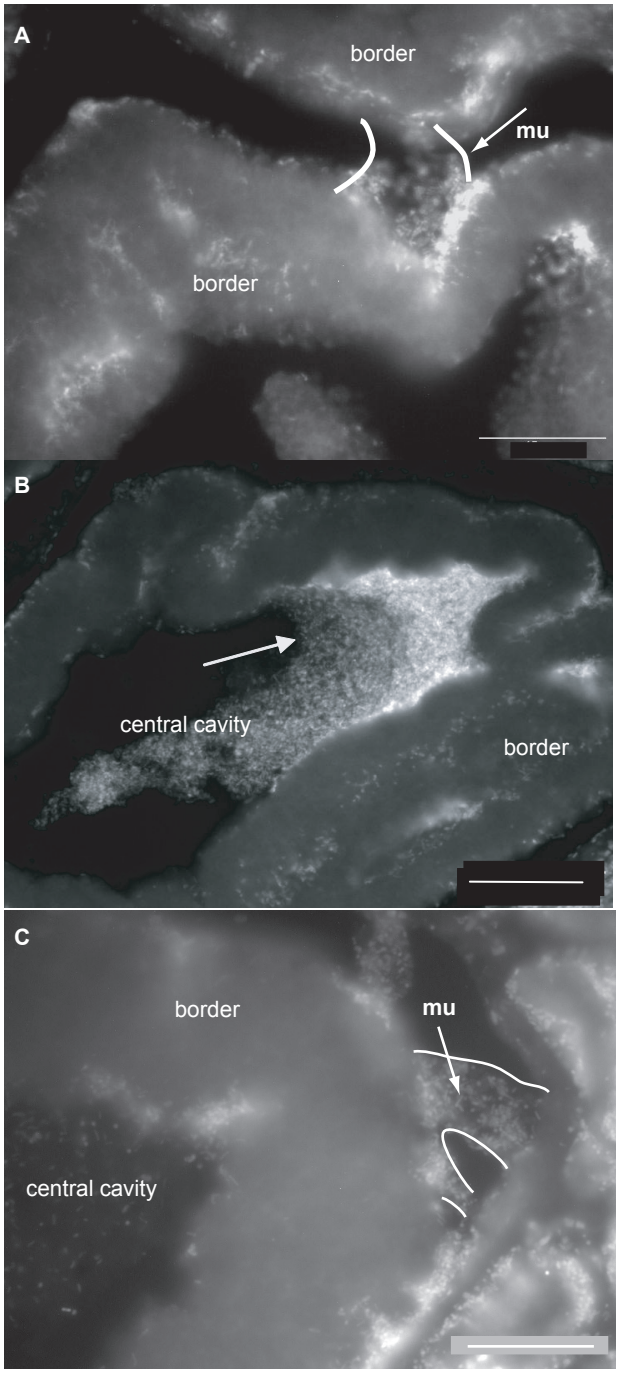


Figure 3. Bacteria in later stages of embryonic development.
(A) Bacteria occupy a portion of the central cavity in the embryo, in addition to the blastomeres. (B) Bacteria along a mucus umbilicus between the follicle wall and the embryo. (C) Bacteria colonize mucus that connects one follicle to another. Bar = 15μm in all micrographs. mucus strands (mu) outlined in white.

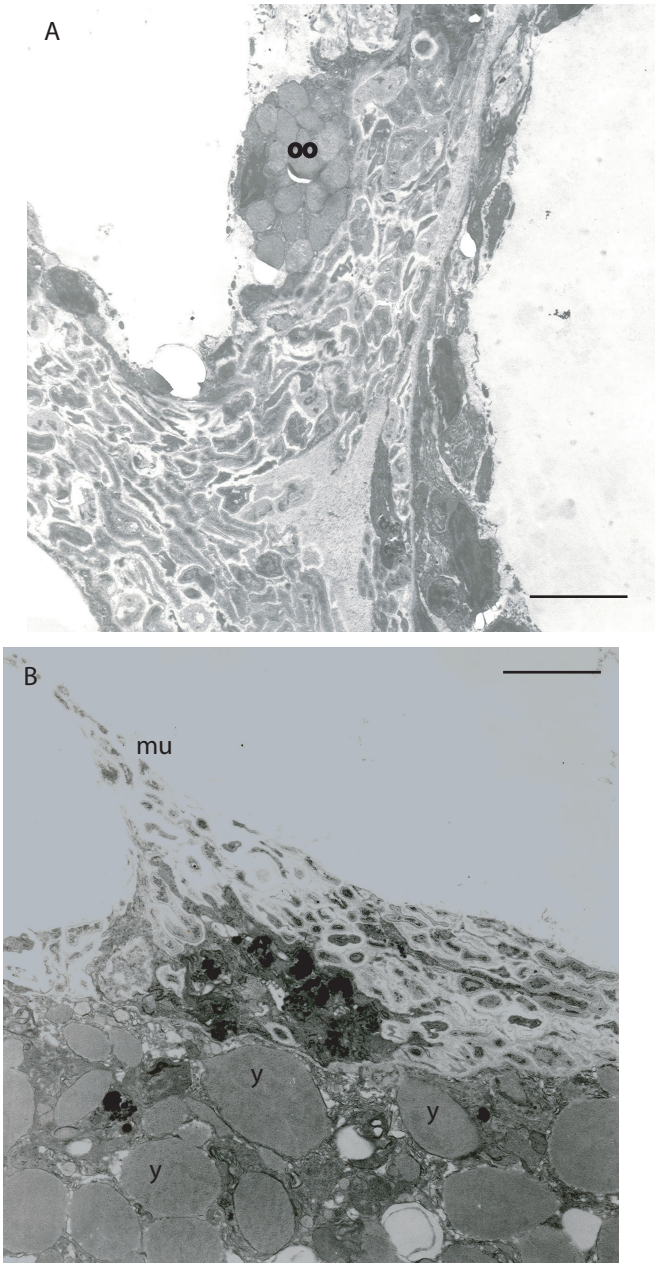
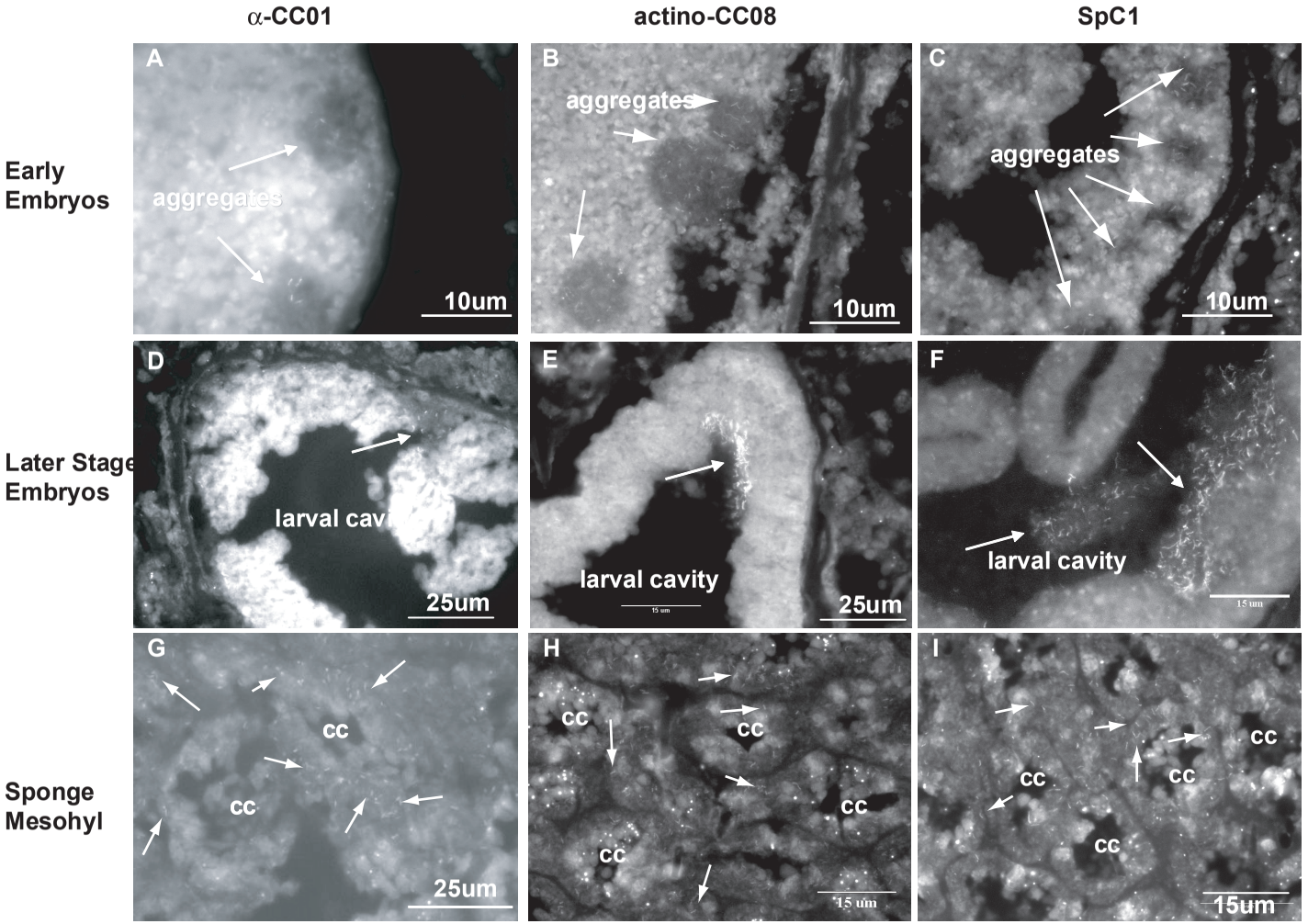


Figure 4. Transmission electron microscopy (TEM) shows bacteria on and around *C. candelabrum* oocytes and embryos. (A) An oocyte after vitellogenesis. Bacterial cells are not evident inside the oocyte but surround the oocyte. (B) Bacterial cells on mucus strands attached to developing embryos. Bar = 3um. oo: oocyte; mu: mucus; e: embryo; y: yolk granule.

Figure 5. Sequence-specific FISH probes in *C. candelabrum* embryos and adults. All three taxa are present in the aggregates of bacteria in the early stage embryos (A,B,C), in masses inside the embryonic cavity (D,E,F), and in the mesohyl of adults (G,H,I). arrows: bacteria; cc: choanocyte chamber.


α-CC01
actino-CC08
SpC1
Early Embryos
Later Stage Embryos
Sponge Mesohyl
A
B
C
D
E
F
G
H
I
aggregates
aggregates
aggregates
10um
10um
10um
larval cavity
larval cavity
25um
25um
15 um
cc
cc
cc
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cc
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15 um
15um

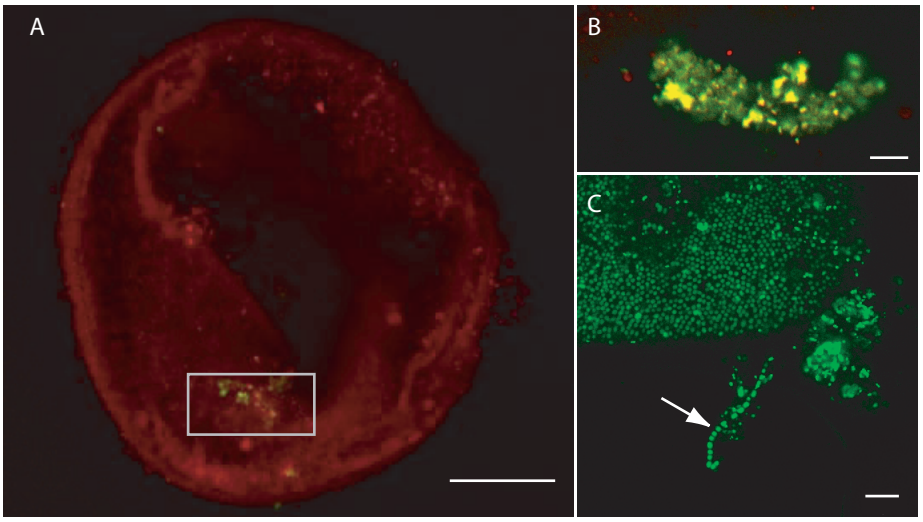


Figure 6. α-proteobacterium α-CC01 and other bacteria in *C. candelabrum* larvae. (A) *C. candelabrum* larva probed with CY5-EUB338 (red) and CY3-αCC01 (green) contains a mass of bacteria in the anterior pole of the internal cavity. Bar = 20μm. (B) Close-up image of αCC01 (yellow) make up a small portion of the bacterial population in the cavity. Bar = 5μm. (C) General eubacterial FISH probe (CY3-EUB338; green) reveals the presence of a large, filamentous bacterial morphotype associated with the larva, though the larva is damaged and it is unclear if the filament was inside or outside of the larva. Bar = 20μm.

REFERENCES

Behrens, S., C. Ruhland, J. Inacio, H. Huber, A. Fonseca, I. Spencer-Martins, B. M. Fuchs and R. Amann (2003). In Situ Accessibility Domains of Small-Subunit rRNA of Members of the Domains Bacteria, Archaea, and Eucarya to Cy3-Labeled Oligonucleotide Probes. *Appl. Environ. Microbiol.* **69**(3): 1748-1753.

Bewley, C. A., N. A. Holland and D. J. Faulkner (1996). Two classes of metabolites from *Theonella swinhoei* are localized in distinct populations of bacterial symbionts. *Experientia* **52**: 716-722.

Boury-Esnault, N., A. Ereskovsky, C. Bezac and D. Tokina (2003). Larval development in the Homoscleromorpha (Porifera, Demospongiae). *Invertebrate Biology* **122**(3): 187-202.

de Caralt, S., G. Agell and M.-J. Uriz (2003). Long-term culture of sponge explants: conditions enhancing survival and growth, and assessment of bioactivity. *Biomolecular Engineering* **20**(4-6): 339-347.

Ereskovksy, A. V. and N. Boury-Esnault (2002). Cleavage pattern in Oscarella species (Porifera, Demopspongiae, Homoscleromorpha): transmission of maternal cells and symbiotic bacteria. *Journal of Natural History* **36**: 1761-1775.

Ereskovksy, A. V., E. Gonobobleva and A. Vishnyakov (2005). Morphological evidence for vertical transmission of symbiotic bacteria in the viviparous sponge *Halisarca dujardini* Johnston (Porifera, Demospongiae, Halisarcida). *Marine Biology* **146**: 869-875.

Erpenbeck, D., J. A. J. Breeuwer, d. V. H. C. van and S. R. W. M. van (2002). Unravelling host and symbiont phylogenies of halichondrid sponges (Demospongiae, Porifera) using a mitochondrial marker. *Marine Biology (Berlin)* **141**(2): 377-386.

Faulkner, D. J. (2000). Marine pharmacology. *Antonie van Leeuwenhoek.* **77**(2): 135-145.

Faulkner, D. J. (2001). Marine Natural Products. *Natural Products Reports* **18**: 1-49.

Fuerst, J. A., R. I. Webb, M. J. Garson, L. Hardy and H. M. Reiswig (1998). Membrane-bounded nucleoids in microbial symbionts of marine sponges. *Fems Microbiology Letters* **166**(1): 29-34.

Gallissian, M. F. and J. Vacelet (1976). Ultrastructure of Some Stages of Oogenesis of Sponges of Genus Verongia (Dictyoceratida). *Annales Des Sciences Naturelles-Zoologie Et Biologie Animale* **18**(4): 381-404.

Hentschel, U., J. Hopke, M. Horn, A. B. Friedrich, M. Wagner, J. Hacker and B. S. Moore (2002). Molecular evidence for a uniform microbial community in sponges from different oceans. *Applied and Environmental Microbiology* **68**(9): 4431-4440.

Kaye, H. R. (1991). Sexual Reproduction in 4 Caribbean Commercial Sponges .2. Oogenesis and Transfer of Bacterial Symbionts. *Invertebrate Reproduction & Development* **19**(1): 13-24.

Kaye, H. R. and H. M. Reiswig (1991). Sexual Reproduction in 4 Caribbean Commercial Sponges .3. Larval Behavior, Settlement and Metamorphosis. *Invertebrate Reproduction & Development* **19**(1): 25-35.

Preston, C. M., K. Y. Wu, T. F. Molinski and E. F. Delong (1996). A psychrophilic crenarchaeon inhabits a marine sponge: Cenarchaeum symbiosum gen. nov., sp. nov. *Proceedings of the National Academy of Sciences of the United States of America* **93**(13): 6241-6246.

Regoli, F., C. Cerrano, E. Chierici, S. Bompadre and G. Bavestrello (2000). Susceptibility to oxidative stress of the Mediterranean demosponge Petrosia ficiformis: Role of endosymbionts and solar irradiance. *Marine Biology (Berlin)* **137**(3): 453-461.

Ridley, C. P., P. R. Bergquist, M. K. Harper, D. J. Faulkner, J. N. A. Hooper and M. G. Haygood (2005). Speciation and Biosynthetic Variation in Four Dictyoceratid Sponges and Their Cyanobacterial Symbiont, Oscillatoria spongeliae. *Chemistry & Biology* **12**(3): 397-406.

Schmidt, E. W., A. Y. Obraztsova, S. K. Davidson, D. J. Faulkner and M. G. Haygood (2000). Identification of the antifungal peptide-containing symbiont of the marine sponge Theonella swinhoei as a novel delta-proteobacterium, "Candidatus Entotheonella palauensis". *Marine Biology (Berlin)* **136**(6): 969-977.

Sciscioli, M., E. Lepore, M. Gherardi and L. S. Liaci (1994). Transfer of symbiotic bacteria in the mature oocyte of Geodia cydonium: (Porifera, Demospongiae): an ultrastructural study. *Cah. Biol. Mar.* **35**: 471-478.

Steindler, L., S. Beer, A. Peretzman-Shemer, C. Nyberg and M. Ilan (2001). Photoadaptation of zooxanthellae in the sponge Cliona vastifica from the Red Sea, as measured in situ. *Marine Biology (Berlin)* **138**(3): 511-515.

Taylor, M. W., P. J. Schupp, I. Dahllof, S. Kjelleberg and P. D. Steinberg (2004). Host specificity in marine sponge-associated bacteria, and potential implications for marine microbial diversity. *Environmental Microbiology* **6**(2): 121-130.

Unson, M. D., N. D. Holland and D. J. Faulkner (1994). A brominated secondary metabolite synthesized by the cyanobacterial symbiont of a marine sponge and accumulation of the crystalline metabolite in the sponge tissue. *Mar. Biol.* **119**(1): 1-11.

Unson, M. M. D. (1993). Chemical and biological studies of the symbiosis between a marine sponge and a cyanobacterium, University of California San Diego.

Usher, K. M., J. Fromont, D. C. Sutton and S. Toze (2004). The biogeography and phylogeny of unicellular cyanobacterial symbionts in sponges from Australia and the Mediterranean. *Microbial Ecology* **48**(2): 167-177.

Usher, K. M., J. Kuo, J. Fromont and D. C. Sutton (2001). Vertical transmission of cyanobacterial symbionts in the marine sponge Chondrilla australiensis (Demospongiae). *Hydrobiologia* **461**: 15-23.

Vacelet, J. and C. Donadey (1977). Electron microscope study of the association between some sponges and bacteria. *Journal of Experimental Marine Biology and Ecology* **30**(3): 301-314.

Vacelet, J., A. Fiala-Medioni, C. R. Fisher and N. Boury-Esnault (1996). Symbiosis between methane-oxidizing bacteria and a deep-sea carnivorous cladorhizid sponge. *Marine Ecology Progress Series* **145**(1-3): 77-85.

Wilkinson, C. R. (1987). Interocean Differences in Size and Nutrition of Coral-Reef Sponge Populations. *Science* **236**(4809): 1654-1657.

CHAPTER 5

Summary

In this dissertation, a bryozoan and a sponge were investigated to explore basic aspects of associations between invertebrates and bacteria and archaea. Animals in diverse habitats across the world have evolved to maintain persistent and specific relationships with microbes, harnessing the powerful metabolic and biosynthetic capabilities of their prokaryotic partners. Together, the partners of a symbiotic association form a new ecological entity, occupying a niche that would otherwise be unavailable to one or both members of the association. This thesis focuses on bioactive metabolite symbioses, in which the animal host harbors specific microbes that make compounds that control ecological interactions, by providing chemical defense against predation, settlement, or fouling, or by conferring competitive advantage in claiming substrate.

B. neritina studies: localization of symbionts and biaoctive compounds

The first research project presented here explores the symbiosis between the bryozoan *Bugula neritina* and its specific bacterial symbiont *Endobugula sertula*, which synthesizes bryostatins that protect the host larvae from predation (Davidson *et al.* 2001; Lopanik *et al.* 2004). Previous research demonstrated conclusively that bryostatins are synthesized by symbionts for the purpose of host protection, but the host has a complex life cycle, consisting both of soft-bodied lecithotrophic larvae, large colonies encased in chitinous exoskeletons, and intermediate soft-bodied metamorphic stages. Although earlier work demonstrated different levels of chemical defense in larvae and in adults, the shifting localization and levels of bryostatins on the host during metamorphosis and development into adult colonies had not been

explored. In addition, Davidson (1999) indicated that bryostatins are localized in rhizoids and growing tips of adult colonies. However, previous quantitative investigations of *E. sertula* in tissues have not shown significantly higher levels of symbiont in bryostatin-rich host tissues (Lopanik, unpublished). These results led to the hypotheses that 1) the *B. neritina- E. sertula* symbiosis regulates the symbiotic production of bryostatin, and *E. sertula* only produces bryostatins in particular tissues or during specific portions of the host life cycle; and 2) bryostatins are transported in the host tissue and do not co-localize with their symbiotic source.

Transmission of microbial symbionts is intensively studied across many symbioses. The mode of symbiont acquisition reflects the tightness of the association between the two partners, and whether the symbiont exists temporarily as a free-living microbe or solely as a symbiont within host tissue has a significant impact on host and symbiont evolution (Peek *et al.* 1998). *B. neritina* can reproduce both sexually – via internal fertilization and brooded embryos – and asexually through budding from autozooids or rhizoids. Presence in the swimming larvae suggests that the symbiont inoculates the offpsring during embryonic development in the ovicell chamber, but the mechanism for symbiont transmission during asexual reproduction remained unclear. My research investigated how *E. sertula* populations persist throughout *B. neritina* life history.

I demonstrate that *E. sertula* cells travel into the ovicell chambers via the funicular cords, inoculating the embryo before its release as a swimming larva. Moreover, during embryonic development, the embryo is covered with a protective layer of bryostatin that persists throughout metamorphosis until a chitinous cuticle forms

around the settled juvenile. The pallial epithelium, the larval tissue with which *E. sertula* is associated in swimming larvae, moves around the larva and eventually is internalized and destroyed after metamorphosis. *E. sertula* cells migrate with the pallial epithelium during metamorphosis, are internalized with the epithelium when it is pulled into the center of developing *B. neritina*, and migrate to the outer epidermal layer, the cystid. As *B. neritina* juvenile continues to develop into an ancestrula, the first functional unit of the colony, *E. sertula* localizes to the bud of tissue that eventually gives rise to the second zooid of the colony. I do not detect bryostatins in *B. neritina* preancestrulae, and in ancestrulae, *E.sertula* synthesizes bryostatins at apparently low levels. *E. sertula* also is present in the funicular system in the rhizoids, the root-like structures that anchor the colonies to substrate. In *B. neritina* sexual reproduction, developing embryos receive an inoculum of *E. sertula* that they retain upon release from adults. In asexual colony growth, *E. sertula* is present in budding tissue. The symbiont also appears to exist in the funicular system as a store for asexual production of new colonies from rhizoids. While *B. neritina* has varying levels of bryostatins throughout the life cycle, and bryostatins are concentrated in different tissues, *E. sertula* are constantly present in the life cycle. These results support our hypothesis that bryostatin biosynthesis and symbiont proliferation may be regulated, reserved for specific events in the life history, such as the protection of larvae.

The present study represents the first example of simultaneous localization of a bioactive compound and its symbiotic bacterial source in a marine invertebrate. Bryostatin localization can be useful for future studies. Previous results (Davidson

1999) indicate that *Polycera atra*, a specialized nudibranch predator of *B. neritina*, is not only resistant to bryostatins but also sequesters it and uses it for protection of its egg cases. The bryostatin localization technique described in this study should be used to confirm that *P. atra* eggs contain bryostatins. In addition, it would be interesting to explore how the bryostatins, acquired dietarily by the nudibranch, ultimately localize to the reproductive tract and become involved in egg case production.

C. candelabrum and diverse symbiotic associations

For simplicity's sake, symbioses have been historically explored in the context of one-host/one-symbiont relationships. There is sufficient evidence that many organisms harbor only one symbiotic species, and the symbiont supplies a clear benefit to the host. However, in recent years, with the advent of molecular biology tools and an increased understanding of cryptic speciation and biodiversity, there are new investigations suggesting that some animals harbor diverse microbes that may fulfill distinct roles in the host survival. Associations that include more than one animal host and one microbial symbiont are more challenging to research.

The second invertebrate studied in this dissertation is a marine sponge, an organism that harbors extremely high microbial diversity. Bioactive metabolite symbiosis has been suspected in many sponge species and has been demonstrated in a handful of examples. While this project was initiated as an attempt to identify a microbial source of the tedanolides in the tropical sponge *Corticium candelabrum*, our early results demonstrated the presence of diverse microbes in the embryos of *C.*

candelabrum. Discovered in ultrastructural studies of sponge reproduction, transmission of microbes via sponge oocytes and embryos has been an accepted phenomenon for decades. However, very few vertically transmitted microbes have been identified in sponges with current molecular techniques. One of the chapters in this dissertation identifies a specific but diverse vertically transmitted microbial assemblage in *C. candelabrum*.

For two reasons, identification of embryo-associated microbes is an important approach for understanding microbial ecology of sponges. First, vertically transmitted microbes are likely to play an important role in the survival of the sponge. To overcome the challenge of staggering diversity of microorganisms within the whole sponge, analyzing isolated embryos reduces the complexity, figuratively pulling the needle from the haystack. The experiments in this study demonstrate that specific microbes persistently associated with the sponge could be identified using the embryo-based approach. However, it is important to note that though diversity appears to be lower in embryos than in the whole sponge, there are still multiple species passed on to the larvae. The second motivation for studying vertically transmitted microbes in sponges is to undertand the specificity of sponge-microbe associations and how they are maintained intergenerationally in the environment. Before identifying how microbes function in sponges, it is crucial to understand how specific, persistent, and predictable microbial communities are in sponges.

The results of my studies indicate that many types of bacteria and even archaea are vertically transmitted in *C. candelabrum*. In the two chapters pertaining to C. candelabrum, I show that *C. candelabrum* maintains specific, obligate associations

with a diverse community of microorganisms via vertical transmission. In chapter 3, I describe the diversity of species found in the embryos, and the persistence of specific bacteria in *C. candelabrum* across time and space. Chapter 4 focuses on the morphological transmission of microbes in *C. candelabrum*, comparing it with previous ultrastuctural studies of microbes in sponge reproduction, and following specific microbes throughout embryonic development.

Recent research characterizes specific associations between sponges and one or two specific microbes, but my research is the first to describe transmission of multiple specific bacterial species in sponges. Though the studies here certainly do not show that vertical transmission of a specific community is a phenomenon common to all sponges, the composition of the *C. candelabrum* microbial community bears similarity to communities previously found in other sponges, and microbes have been shown to be present in embryos and larve of diverse sponge species.

Sponges are primitive organisms, but they consist of intricate water canals lined by highly specialized cells that may function to ingest bacteria or particles selectively – perhaps microbes that create particular microenvironments were selected over time, and eventually, like many other vertically transmitted microbes, they evolved to survive exclusively within the host. In turn, the sponge evolved physiological or behavioral mechanisms to inoculate embryos with symbionts. These microbes are in specific, persistent association – symbiosis – with the sponge, in contrast to other microbes in the sponge, which are ingested by filtering seawater.

The next step for understanding sponge-microbe associations is to characterize embryo-associated microbes in other sponge species. This should help to assemble a

larger picture, perhaps even patterns, of specificity of associations between bacteria and archaea and sponges.

A main theme that links all of the studies in this dissertation is vertical transmission of microbes in marine invertebrates. Isolating embryos or larvae is a valuable approach for identification of specific microbial associates in marine invertebrates. The embryo/larval approach has the power to decrease the microbial diversity in a working sample. In addition, understanding of microbial assemblages in marine invertebrate embryos and larvae provides valuable fundamental information for understanding the maintenance of species-specific associations in the environment.

REFERENCES

Davidson, S., S. W. Allen, G. E. Lim, C. M. Anderson and M. G. Haygood (2001). Evidence for the biosynthesis of bryostatins by the bacterial symbiont "*Candidatus* Endobugula sertula" of the bryozoan *Bugula neritina*. *Appl. Env. Microbiol.* **67**: 4531-4537.

Davidson, S. K. (1999). Biology of the Bryostatins in the marine bryozoan *Bugula neritina*: Symbiosis, cryptic speciation and chemical defense. Scripps Institution of Oceanography. La Jolla, University of California San Diego**:** 210.

Lopanik, N., N. Lindquist and N. Targett (2004). Potent cytotoxins produced by a microbial symbiont protect host larvae from predation. *Oecologia* **139**(1): 131-139.

Peek, A. S., R. A. Feldman, R. A. Lutz and R. C. Vrijenhoek (1998). Cospeciation of chemoautotrophic bacteria and deep sea clams. *Proceedings of the National Academy of Sciences of the United States of America* **95**(17): 9962-9966.