Title

Biology of the Bryostatins in the Marine Bryozoan Bugula neritina: Symbiosis, cryptic speciation and chemical defense

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Biology of the Bryostatins in the marine bryozoan *Bugula neritina*: Symbiosis, cryptic speciation and chemical defense

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

Doctor of Philosophy in Marine Biology

by

Seana Kelyn Davidson

Committee in charge:

Professor Margo G. Haygood, Chair
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1999

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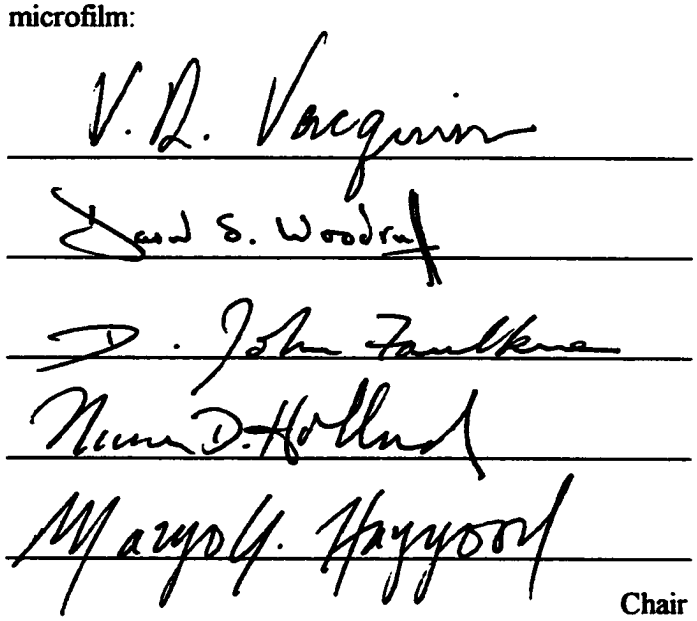

Chair

University of California, San Diego

1999

Dedicated to my brother David whose open mind amazed us all

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LIST OF ABBREVIATIONS

BB	Bodega Bay, Spud Point Marina, Northern California.
CI	Catalina Island, California
CO I	mitochondrial, cytochrome *c* oxidase subunit I gene
DGGE	Denaturing Gradient Gel Electrophoresis
dNTPs	phosporylated dideoxy nucleotides
ESIMS	Electrospray Injection Mass Spectrometry
^{3}H	tritium
HB	Humboldt Bay, Woodley Marina, Northern California.
HPLC	high-pressure liquid chromatography
NC	North Carolina
NCI	National Cancer Institute
NP	Newport Bay
PCR	Polymerase Chain Reaction
PDBu	phorbol dibutyrate
PKC	Protein Kinase C
PKS	Polyketide Synthase
PP3m	Patrick's Point, Northern California, 3 meters depth
PP10m	Patrick's Point, Northern California, 10 meters depth
PV	Palos Verdes, Los Angeles, California
SSU rRNA	gene encoding the small subunit of the ribosomal ribonucleic acid
SP	Scripps Pier, La Jolla, California.
TAE	Tris, Acetate, EDTA
TP	Torrey Pines artificial reef 2
UV	ultra violet

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Thank you members of the Haygood and Tebo lab, especially Deeanne Edwards who took time to show me my first molecular techniques and has continued to be very helpful. Some day I hope to drive a red sports car and go diving in the tropics every year like she does. Grace E. Lim started as a graduate student the year that I was preparing to complete my dissertation. She has provided a new infusion of enthusiasm for the chemistry and biology of these bryozoans and their symbionts. Grace conducted the denaturing gradient gel electrophoresis required for the microbial population SSU rRNA gene profile data presented in the Chapter 3 supplemental pages and in Chapter 5. Grace will appear as a co-author for the paper resulting from the studies conducted in Chapter 5.

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Paul Dayton showed me the desert.

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My mom deserves a very big thanks. She has always been very supportive. She raised me to believe that I can be whoever I want and do whatever I want. I don't know if I'll ever forgive her (grin). She also showed me what a hard life a career in the arts provides. You must work very hard, long hours, create from nothing, sometimes feel inadequate and insecure of your direction but pull through by opening night. There is a lot of trial and error, doing things over again till they're just right and then doing it again. Thinking this seemed too difficult, I decided not to go down that path, and chose scientific endeavors instead. They are not very different. The tools and the outcomes vary, but the process is the same. My mother also let me leave cups on my window sill containing a little orange juice in the bottom so that I could see what kind of molds grew, let me rescue an entire pond full of tadpoles one summer, capture

spiders in jars and provided me with all the art supplies, stage make-up, and vinegar, baking soda and food coloring a kid could want.

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Schmidt, E. W., A. Y. Obraztsova, S. K. Davidson, D. J. Faulkner, and M. G. Haygood. (1999) Identification of the antifungal peptide-containing symbiont of the marine sponge *Theonella swinhoei* as a novel δ-proteobacterium, "Candidatus Entotheonella palauensis." Submitted.

Haygood, M.G., E.W. Schmidt and S.K. Davidson. (1999) Microbial symbionts of marine invertebrates: opportunities for microbial biotechnology.

Davidson, S. K. and M.G. Haygood. (1999) Identification of sibling species of the bryozoan *Bugula neritina* that produce different anticancer bryostatins and harbor distinct strains of the bacterial symbiont "*Candidatus* Endobugula sertula." *Bio. Bull.* **196:** 273-280

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ABSTRACT OF THE DISSERTATION

Biology of the Bryostatins in the marine bryozoan *Bugula neritina*:

Symbiosis, cryptic speciation and chemical defense

by

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Doctor of Philosophy in Marine Biology

University of California, San Diego, 1999

Margo G. Haygood, Chair

Abstract

This dissertation investigates the identity and function of a bacterial symbiont described in the marine bryozoan *Bugula neritina* by R.M. Woollacott in 1981. *B. neritina* is the source of bryostatins, unique cytotoxins suspected to have a bacterial source, and is considered a single species throughout its cosmopolitan temperate range. Bryostatins found from different collections of *B. neritina* vary, and only certain populations produce bryostatins that possess an octa-2,4-dienoate substituent. In this dissertation the bacterial symbionts of the larvae are identified by small subunit ribosomal rRNA (SSU) gene sequences and named "*Candidatus* Endobugula sertula." The variable regions of these genes were used to design oligonucleotides specific to the symbiont. These specific oligonucleotides were used for *in situ* hybridization to the bacteria in the pallial sinus to confirm the origin of the sequence, and for specific amplification of symbiont SSU rRNA genes by PCR. Then the mitochondrial

cytochrome *c* oxidase subunit I gene was used to identify two distinct species of *B. neritina* each harboring a different symbiont as determined by SSU rRNA sequence. Variation in the bryostatin profiles is associated with this genetic difference. Only one *B. neritina* / "*E. sertula*" association can produce bryostatins with an octa-2,4-dienoate substituent (bryostatins 1-3, 12 and 15). In order to elucidate the possible involvement of the symbiont in production of bryostatins, experiments were conducted to eliminate "*E. sertula*" from *B. neritina* to determine whether *B. neritina* can continue to grow normally without the symbiont, and/or continue to produce equivalent levels of bryostatins. Symbiont levels were estimated using a symbiont-specific PCR assay, then bryostatin activity levels were compared between control and treated *B. neritina* colonies. When symbiont levels were greatly reduced, bryostatin activity declined by approximately 50%. Genetic evidence was discovered that indicates "*E. sertula*" has the potential to synthesize complex polyketides like bryostatin. Finally evidence was gathered to address the hypothesis that bryostatins serve as defensive compounds for the bryozoan host. The distribution of bryostatins in the colonies is suggestive of a defense, and it was found that predatory nudibranchs of *B. neritina* sequester bryostatins and concentrate them in their egg ribbons. In summary, the symbiont's most likely function is to provide a chemical defense, bryostatins, for the host.

Chapter 1

INTRODUCTION

Symbiosis:

Harnessing the metabolic and synthetic potential of microorganisms

Symbiotic associations between microorganisms and metazoans are known from almost every phylum. These associations may form and persist as a result of improved evolutionary potential, two genomes working in concert to adapt to environmental conditions. In most associations the host obtains an additional capability from their symbiont and thus a competitive advantage. If the host can prevent a damaging invasion by a microorganism, and this microbe can elude the host's immune defenses, then the infection may persist eventually developing into a tightly regulated association. Prokaryotes have incredibly diverse metabolic and synthetic capabilities, utilizing a variety of substrates as energy sources including those to drive autotrophic fixation of carbon. Microorganisms, including bacteria, fungi and dinoflagellates, also synthesize a wide range of secondary metabolites, toxins that have evolved as ammunition against competing organisms. A symbiotic association allows an animal or plant to take advantage of a microorganism's metabolic and biosynthetic capabilities. As well, people have been using these biosynthetic capabilities in the development of therapies against disease, including antibiotics, anticancer compounds and anti-inflammatory agents.

In marine habitats, symbionts include diverse microorganisms forming associations with a variety of animals. At the hydrothermal vents and methane seeps distantly related animals have evolved symbiotic associations with bacteria in order to survive in a potentially toxic and inhospitable habitat with scarce food. The hydrothermal venting of water enriched in reduced metal compounds, including sulfides, provides chemical energy for autotrophic bacteria, but is potentially toxic to

metazoans. Chemoautotrophic symbionts provide the primary production for entire vent ecosystems. A variety of invertebrates harbor symbiotic bacteria which fix carbon using the energy derived from oxidation of reduced compounds. Other organisms then feed upon these invertebrates. Bivalve mollusks contain chemoautotrophic symbionts which provide the host with a food source (Brooks et al., 1987; Distel et al., 1988; Distel and Wood, 1992; Eisen et al. 1992). Vestimentiferan tube worms, related to polychaetes, derive their carbon food source from chemoautotrophic bacteria living off the sulfide (for review see Nelson and Fisher, 1995). Influenced by the symbiosis, the tube worms of this association have evolved to such a modified form that the digestive system has been lost entirely and replaced with the massive trophosome which contains the bacterial symbionts. The polychaetes *Alvinella pompejana* and *A. caudata* have external bacterial communities associated with the worms' integuments (Cary et al., 1997; Gaill et al., 1987; Haddad et al., 1995). The dominant filamentous bacterial species are ε-Proteobacteria and they are suspected to have a role in detoxifying sulfide (Cary et al., 1997). There are even crustaceans (shrimp) in these environments that have bacterial symbionts to enable their hosts to live in such an inhospitable environment (Polz and Cavanaugh, 1995).

In other habitats as well, symbionts allow their hosts to exploit resources otherwise unavailable, improve resource acquisition or provide a means of defense. For example the wood-boring Teredinid mollusks (shipworms) would not survive on their wood diet without the symbiotic proteobacteria that degrade cellulose and lignin, and fix nitrogen (Dean, 1978; Distel et al., 1991; Waterbury et al., 1983). Symbioses

exist between photosynthetic dinoflagellates and cniderians (anemones, soft-corals and hard corals) in which the symbionts provide a fixed carbon source to their hosts. In the coral reefs, as at the hydrothermal vents, the entire ecosystem is supported by a symbiosis.

A number of organisms harbor luminescent bacteria for a variety of functions, some of which have not yet been proven. Among cephalopods, species from two families of squid, Sepiolidae and Loliginidae, harbor luminescent proteobacteria in highly developed light organs (Nealson and Hastings, 1991; McFall-Ngai 1994; Ruby 1996) believed to provide counter illumination for predator avoidance. Most of the identified luminous symbionts are closely related suggesting that these species have a feature in common which allows them to form stable associations with metazoan hosts. *Vibrio fischeri* has been identified as the symbiont in at least five species of animal hosts including bobtail squids and Monocentrid fishes. *Vibrio logei* has recently been identified from the light organ of squid *Sepiola* spp. (Fidopiastis et al., 1998). Among the vertebrates, seven families of fish contain species that harbor luminescent γ-proteobacteria for counter illumination in some species and hunting prey in other species (Haygood, 1993; Nealson and Hastings, 1991). The described light organ symbionts from fishes include *V. fischeri* (Monocentrid fishes) and very closely related bacteria, *Photobacterium phosphoreum* and *P. leiognathi*.

With the field of marine natural products exploration expanding, so has our awareness of symbionts that provide their host with bioactive compounds, presumably to act as chemical deterrents to predators, competitors and settling larvae. There are

diverse invertebrates, including sponges (bacteria, fungi, dinoflagellates), flat worms (dinoflagellates), tunicates (dinoflagellate), crustacea (bacteria defending eggs), a bivalve (dinoflagellate in gill cells) and soft coral (dinoflagellate), from which symbionts have been isolated that produce bioactive compounds (reviewed in Kobayashi and Ishibashi, 1993). Sponges produce a variety of bioactive compounds many of which are thought to be for chemical defense against predators and competitors. Symbionts may be the true source of the bioactive compounds for many of these sponges including unique peptides and cytotoxic macrolides which resemble bacterial metabolites (Faulkner, et al., 1994; Kobayashi and Ishibashi, 1993; Fusetani and Matsunaga, 1993). These primitive multicellular organisms are made up of cell aggregates, lacking true tissues and immune systems, and may harbor entire microbial communities. Many of these microbes may not be specific symbionts, but reside there as a result of the filtering activity of the sponges. In certain species of sponges it is clear that there are specifically associated microorganisms including examples from the Bacteria (cyanobacteria, actinomycetes, γ- and δ-proteobacteria; Bewley et al. 1996; Unson et al., 1994; Schmidt et al, 1999 submitted) and Archea (Preston et al., 1996). For the lithistid sponge *Theonella swinhoei* and the dictyoceratid sponge *Dysidea herbacea* the hypothesis that certain symbionts provide unique bioactive compounds has been confirmed (Unson and Faulkner, 1993; Unson et al., 1994; Bewley, et al., 1996; Schmidt et al., 1998). E.W. Schmidt et al. (1999 submitted) successfully cultured and identified the large filamentous bacterial symbionts of *Theonella swinhoei* as novel δ-proteobacteria. These filaments were shown earlier to contain unique

peptide, theopalauamide, and this and related peptides are proposed as markers for the symbiosis and the presence of related symbionts in other *Theonella* species.

In symbioses for which the symbiont is suspected of providing a chemical defense, the role of the symbiont has often remained speculation with little proof. These are hard to demonstrate because the noxious or toxic compound must be linked to the symbiont as the source, and the defensive function of the compound must be demonstrated. Symbionts are notoriously difficult to isolate and culture. Once in culture there is no guarantee that the microbial symbionts will produce the same compounds that are produced when in association with their host. Often other methods to show production by microbes in an association must be employed. For some sponges, cell separations and fractionations have been successfully accomplished (Bewley et al., 1994; Unson and Faulkner, 1993). The compounds of interest were then detected in particular cell fractions. Other methods could involve using a marker, such as an antibody, to label the compound in the whole animal. If the compound contains certain elements (i.e. chlorine or bromine) they can be detected in particular cells using Scanning Electron Microscopy with an energy dispersion X-ray detector. This method has been used with some success to localize bromide containing compounds to bacterial cells on the surface of a bryozoan *Amathia convoluta* (Walls et. al., 1995) and to cyanobacterial cells in the sponge *Dysidea herbacea* (Unson et al, 1994). Both the cell separation and the localization approaches are problematic if the compound is excreted and not found in the symbiont cells exclusively.

Another approach is to eliminate the symbionts and measure levels of compounds in aposymbiotic animals. If successfully eliminated, the relative level of protection from predation, fouling, pathogens and competition could be assessed. In the case of the shrimp *Palaemon macrodactylus* and the lobster *Homarus americanus* Gil-Turnes and Fenical successfully identified and demonstrated the protective role of epibiotic bacteria, *Alteromonas* sp., in protecting embryos from fungal infection by producing anti-fungal agents (Gil-Turnes et al., 1989; Gil-Turnes and Fenical, 1992). The bacteria were consistently associated with healthy eggs, successfully cultured and produced the compound. Eggs lacking the bacteria did not contain the compound and were susceptible to the fungal pathogen. In this association the embryos were protected by their epibiotic bacteria from a pathogenic fungus.

In an analogous association, several squid species (*Loligo pealei*, *L. forbesi*, *L. opalescens* and *Sepia officinalis*) harbor bacterial symbionts in the accessory nidamental gland (AN) which is involved in excretion of a gelatinous layer around the eggs within the egg capsules (Kaufman et al., 1998 and refs. therein). The egg capsules of *Loligo opalescens* are deposited on the sea floor, remain there for a month, and seem resistant to fungal, bacterial and predatory attack. Bacteria have been found associated with the capsules of *L. opalescens* (Biggs and Epel, 1991) and it is suspected that the bacteria in the AN gland and in the capsules produce toxins that protect the embryos. However, the defensive role of these symbionts and the toxin they may produce remains to be shown. It is interesting to note that the AN gland may be the evolutionary precursor to the light organ in the squid species harboring

luminescent bacteria (Buchner, 1965; Bloodgood, 1977; Montgomery and McFall-Ngai, 1993).

For a number of symbioses, the speculated function of the symbionts has not been demonstrated or is unknown. A great number of Echinoderms have been shown to harbor bacteria for unknown purposes (Kelly et al., 1995; Kelly and McKenzie, 1995). Brittle stars (Burnett and McKenzie, 1997) host subcuticular α-proteobacteria. The spatangoid echinoderm *Echinocardium cordatum* harbors *Thiothrix* spp. in their digestive tube possibly as a means of detoxifying sulfide in the ingested sediment (Brigmon and De Ridder, 1998). Ascidians, both solitary and colonial, are known to harbor photosynthetic symbionts (Prochloron and cyanobacteria; Lambert et al. 1996; Lewin and Cheng, 1989) but their role in providing a fixed carbon source has not been shown. The role of symbionts in many associations remains elusive. Many of the bacteria associated with sponges have not been identified nor has their benefit to the sponge, if any, been described. In some situations it is possible that the microbial symbionts are merely residing in their host with no benefit gained by the host. Among the bryozoans, internal bacteria, possibly symbionts, have been described primarily in microscopical studies (Lutaud, '64, '65, '69, '86; Woollacott, 1981; Woollacott and Zimmer, 1975) and in a culturing study (Zimmer and Woollacott, 1983). In several species of bryozoans, secondary metabolites are suspected to be of microbial origin. Brominated metabolites of *Amathia wilsoni* (Walls et al., 1995) have been localized to surface bacteria, however additional studies are required to confirm the microbial origin. Four species, *Phidolopora pacifica, Diaperoecia californica, Heteropora*

alaskensis, Tricellaria ternata, and *Hippodiplosia insculpta,* from British Columbia contain similar nitrophenols which may have a dietary origin (Tischler et al., 1986). *Bugula dentata* (Matsunaga et al., 1986) and *Sessibugula translucens* (Carté and Faulkner, 1986) both contain Tambjamines and a blue tetrapyrrole pigment that are also found in colonial ascidians *Atapozoa* spp. (Paul et al., 1990). These compounds may have a microbial source. The blue tetrapyrrole is known from a marine bacterium *Serratia marcescens* (Matsunaga, et al. 1986). The defensive properties of these compounds have been demonstrated in studies examining the predatory nudibranchs feeding on these bryozoans and the ascidians *Atapozoa* spp. However the microbial source, if there is one, has not been demonstrated for any of these species.

The research presented in this dissertation investigates the identity and function of a bacterial symbiont described in electron micrographs of the marine bryozoan *Bugula neritina* by R.M. Woollacott in 1981. *B. neritina* is the source of novel cytotoxic macrolides, the bryostatins, which are suspected to have a bacterial source. Bryostatin 1 has been investigated for therapeutic potential in the treatment of various cancers. In early studies the bacteria observed in the larvae were identified and specific molecular detection methods were developed. The findings of this work led to the discovery of two distinct strains of the symbiont and bryozoan host. This genetic difference is associated with distinct bryostatin profiles indicating a genetic cause for the differences in chemistry observed in field collections. In order to elucidate the possible involvement of the symbiont in the production of bryostatins, experiments were conducted to eliminate the symbiont from *B. neritina* to address the question of

whether *B. neritina* can continue to grow normally without the symbiont, and/or continue to produce equivalent levels of bryostatins. Finally, evidence was gathered to define the possible role bryostatins may play in the chemical defense of the host.

The two fundamental questions linking the chapters together are; 1) are the bacterial symbionts necessary for the production of bryostatins and 2) what benefit might the symbionts provide their host? Bryostatins are likely used as a chemical deterrent to predators and competitors. If the symbiont is providing these potent cytotoxins, this association between the bryozoan and its bacterial symbiont probably arose to exploit the benefits of a chemical defense. The bryozoan obtained and domesticated the γ-proteobacterium, harnessing the chemical potential of the bacterium to improve the bryozoans ability to compete for space in the fouling community and avoid predation.

Bugula neritina

Bugula neritina is a cheilostome bryozoan (Class Gymnolomata; Order Cheilostomata; Suborder Anasca; Division Cellularina; Family Bugulidae; Woollacott and Zimmer, eds., Biology of Bryozoans 1977). Bryozoa are colonial invertebrates made up of uniserially and multiserially budding individuals termed zooids (figure 1). The colonies, which may be encrusting or erect, have chitinous exoskeletons with varying degrees of calcification ranging from uncalcified to thick stony coraline colonies. Each individual may be described as a chitinous box (zooecium) within which resides the feeding animal (polypide). The autozooids are the feeding members of the colony bearing a lophophore which is a crown of ciliated tentacles forming a funnel.

The lophophore is everted for feeding and withdrawn into the zooecium when disturbed. The cilia create a current which brings particles into the funnel and through the tentacles. Heterozooids are non-feeding individuals with diverse morphologies that serve for protection (avicularia, vibracula), reproduction and brooding (gonozooids, ovicells), or as anchors for the colony (rhizozooids) (Silén, 1977). Avicularia are essentially pincers that have a reduced non-feeding polypide that serves as a sensory organ. Vibracula are long movable spines that sweep across the colony surface. Ovicells brood larvae, and rhizozooids grow out from the base of the colony to anchor it to the substratum. These non-feeding portions are supplied with nutrients from the autozooids via a network of epithelial tissue forming tubules (the funicular system) that run throughout the colony.

There are no known specialized excretory organs. Instead the polypides degenerate and regenerate periodically. The gut tract condenses down into a small brown body and may remain in the zooecium. These can easily be seen in zooids of bryozoan colonies and repeated cycling of the polypide results in multiple brown bodies deposited in the zooecium (Gordon, 1977 and refs therein).

Bugula neritina forms upright flexible branching colonies that are only lightly calcified and lack specialized defensive heterozooids. *B. neritina* has been very well studied both in the laboratory and in the field. The adults are easily found in the field and larvae are readily obtained from adults in the lab. The larvae settle well in the laboratory after a brief swimming period. These features have allowed detailed study of larval ecology and settlement (Jaeckle, 1994; Maki, et al., 1989; Mihm and Banta,

1981; Wendt, 1996; Wendt and Woollacott, 1995; Woollacott, 1984), metamorphosis (Reed and Woollacott, 1982, 1983; Woollacott and Zimmer, 1971, 1978), feeding (Okamura, 1990) and variations in growth (Keough, 1989, 1989; Walters, 1992). *B. neritina* broods its larvae individually in specialized brood chambers, ovicells, that form proximal to the parental zooid (Woollacott and Zimmer, 1972). Although each zooid is hermaphroditic, self-fertilization is believed to be avoided by various means. Only a few large macrolecithal eggs are produced in each reproductive zooid. The egg migrates from the parental zooid into the ovicell where the developing embryo is nourished by the colony through funicular cords that lead to a 'semiplacental' system in the ovicell (Woollacott and Zimmer, 1975). Upon release these relatively large (200-300 μm), lecithotrophic larvae settle within 12 hours, often sooner, and stores are used to develop the first zooid, termed the ancestrula (Keough, 1989; Wendt, 1996; figure 2). Feeding usually occurs in 48 hours post settlement. This short swimming period leads to a small dispersal area. The formation of ovicells is coordinated throughout a colony such that ovicells will form on each zooid along a branch beginning proximally and progressing distally. Along any given row older ovicells, and presumably larvae, will be towards the bottom with the youngest near the tips of the branch (Personal observation, and Carolyn Sheehan, CalBioMarine Tech. Inc. pers. comm.).

Despite the low dispersal potential of the larvae, and the apparent lack of good physical defensive structures *Bugula neritina* is a highly successful member of fouling communities world wide in temperate coastal waters. This bryozoan is considered a single species throughout its range and its occurrence on boat hulls and docks allows

for trans-oceanic transport which certainly makes dispersal over great distances a possibility, although the genetic structure of the populations has yet to be thoroughly investigated. This subject is partially addressed in Chapter 4.

Bryostatins

Bryostatins, cytotoxic macrocyclic lactones, were initially discovered from *B. neritina* in 1968 by G. Pettit while on a bioprospecting mission to detect anticancer compounds from marine invertebrates. Extracts from this bryozoan showed potent activity against certain cancer cell lines including murine P833 leukemia. Further investigations led to the structural elucidation of the active component bryostatin 1 (Pettit et al., 1982). In years that followed 18 bryostatins in all have been described, all of which share a central bryopyran ring and differ in their C7 and C20 substituents (Pettit, 1991; Pettit et al., 1991; Pettit et al., 1996; figure 3). Three polar groups (boxed in figure 3) on the bryopyran ring mimic diacylglycerol, the natural ligand which binds to the activator site of protein kinase C (PKC) in cell membranes. The binding of bryostatin 1 to PKC activates a secondary messenger cascade which alters cell fate. The variation in the affinity of bryostatin 1 for different members of the PKC family may be the source of tissue specific effects. Depending on the cell type, undifferentiated cells may differentiate or undergo apoptosis in response to bryostatin 1 (Mohammad et al., 1995; Rao et al., 1996; Steube and Drexler, 1993).

Bacterial symbionts

Certain facts indicate bryostatins are produced by bacterial symbionts, and not by the bryozoan host (Anthoni et al., 1990). First, bryostatins are complex polyketides,

which places them in a class of compounds made primarily by bacteria and fungi. Metazoans are not known to synthesize this class of compound, except for several sponges which also harbor bacteria. The two other natural products related to bryostatins in structure are miyakolide (Higa et al., 1992) and aplasmomycin (Nakamura, 1977; Anthoni, 1990; figure 3). Miyakolide was isolated from a sponge. Sponges are known to harbor many bacteria and in at least one case a complex polyketide from a sponge has been shown to originate from their symbiotic bacteria (Bewley et al., 1996). Aplasmomycins are synthesized by the marine actinomycete *Streptomyces griseus*. Furthermore, bryostatins are unlike any other bryozoan secondary metabolites described to date, most of which are alkaloids (reviewed in Chapter 2).

Bugula neritina harbors bacterial symbionts that are passed along to the larvae from the parent prior to release (figure 4). These rod-shaped gram negative bacteria were first described in the pallial sinus of the coronate larvae in electron micrographs by R.M. Woollacott (1981) and are prime candidates for the producers of bryostatins. We can infer from the evolution of vertical transfer (parent to larvae) of the symbionts that they are crucial to the success of the animal. The larvae hold the bacteria appressed between foldings of the pallial epithelium which seems specialized for this function. In the adults, bacteria of similar morphology were described in the funicular cords, including those leading to the ovicells (Woollacott and Zimmer, 1975). The funicular cords nourishing the developing larvae may be the route by which bacteria enter the larvae before they are released from the parent. There are bacteria and

microsporidia in the brown bodies as well (Dr. N.D. Holland, unpublished electron micrographs), but these are likely a mixture originating from the gut of the animal. Microsporidia recently have been found to be very closely related to fungi (Hirt et al., 1999).

Bacteria have been observed in several other bryozoans, including two other *Bugula* species, *B. pacifica* and *B. simplex* (Woollacott, 1981). In *Bugula* spp. with observed bacteria, the bacteria were found in the pallial sinus of the larvae upon release from the adults. However this is not a general feature of *Bugula* since *B. turrita* and *B. stolonifera* did not have these symbionts (Woollacott, 1981). In the other species, bacteria were observed in sacules associated with the funicular system, in cells of the esophagus and in avicularia (Lutaud, '64, '65, '69, '86; Zimmer and Woollacott, 1983). For most of these associations neither the bacteria nor their function have been described. In the case of *Watersipora arcuata*, the symbiont observed in the larvae and the adult have been cultivated and identified as a mycoplasma-like organism (Zimmer and Woollacott, 1983; Boyle et al., 1987). However these symbionts have not been confirmed by specific probes designed against the cultured organisms. Additional investigations into the associations of bacteria with bryozoans will undoubtedly reveal more symbioses. Anthoni et al. (1990) suggested that secondary metabolites of several bryozoans may be of microbial origin. In this dissertation the possible production of bryostatins by the bacterial symbiont originally observed in the larvae is investigated, testing the hypothesis that bryostatins are of microbial origin.

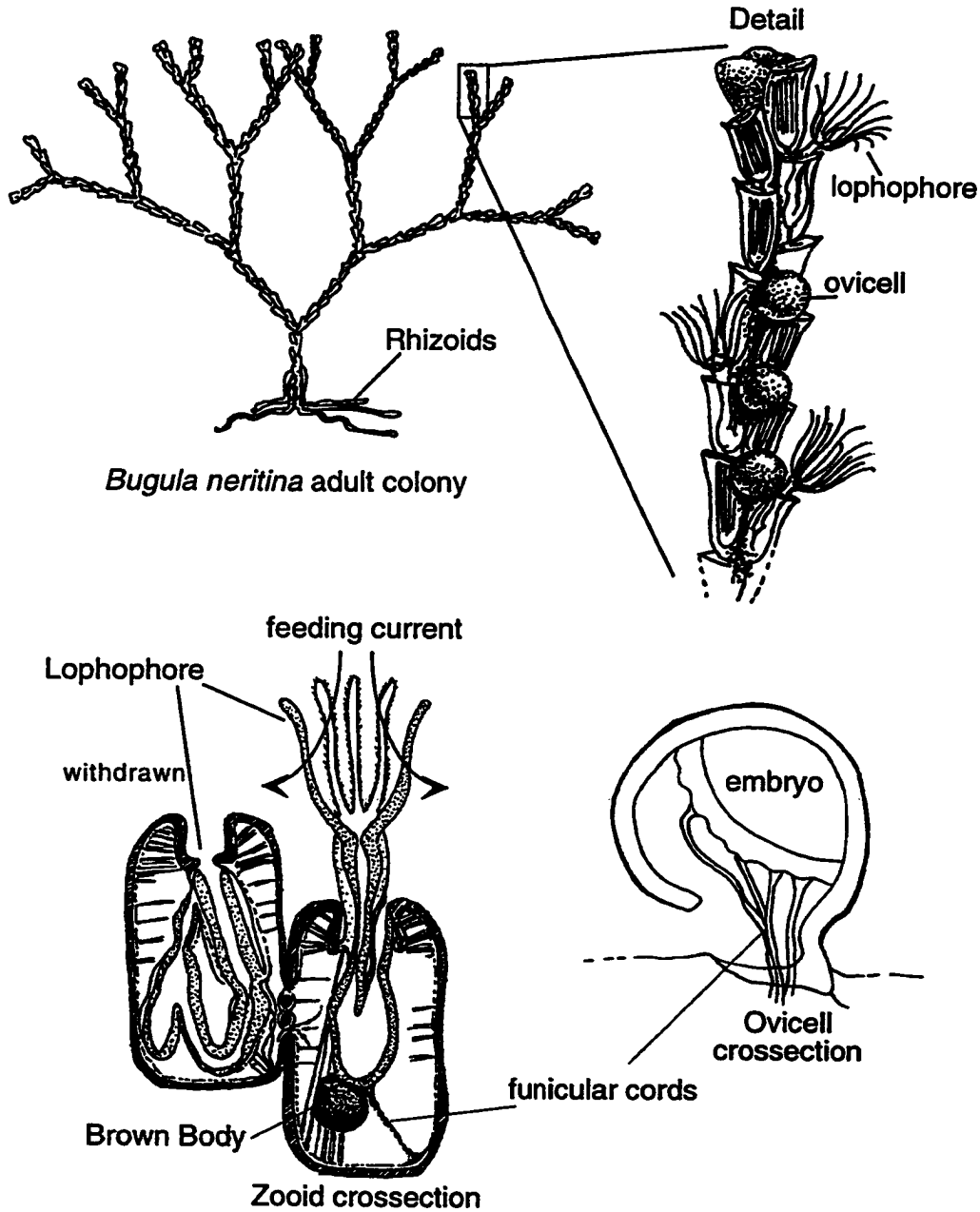

Figure 1. *B. neritina* adult colony form and details of zooid morphology.

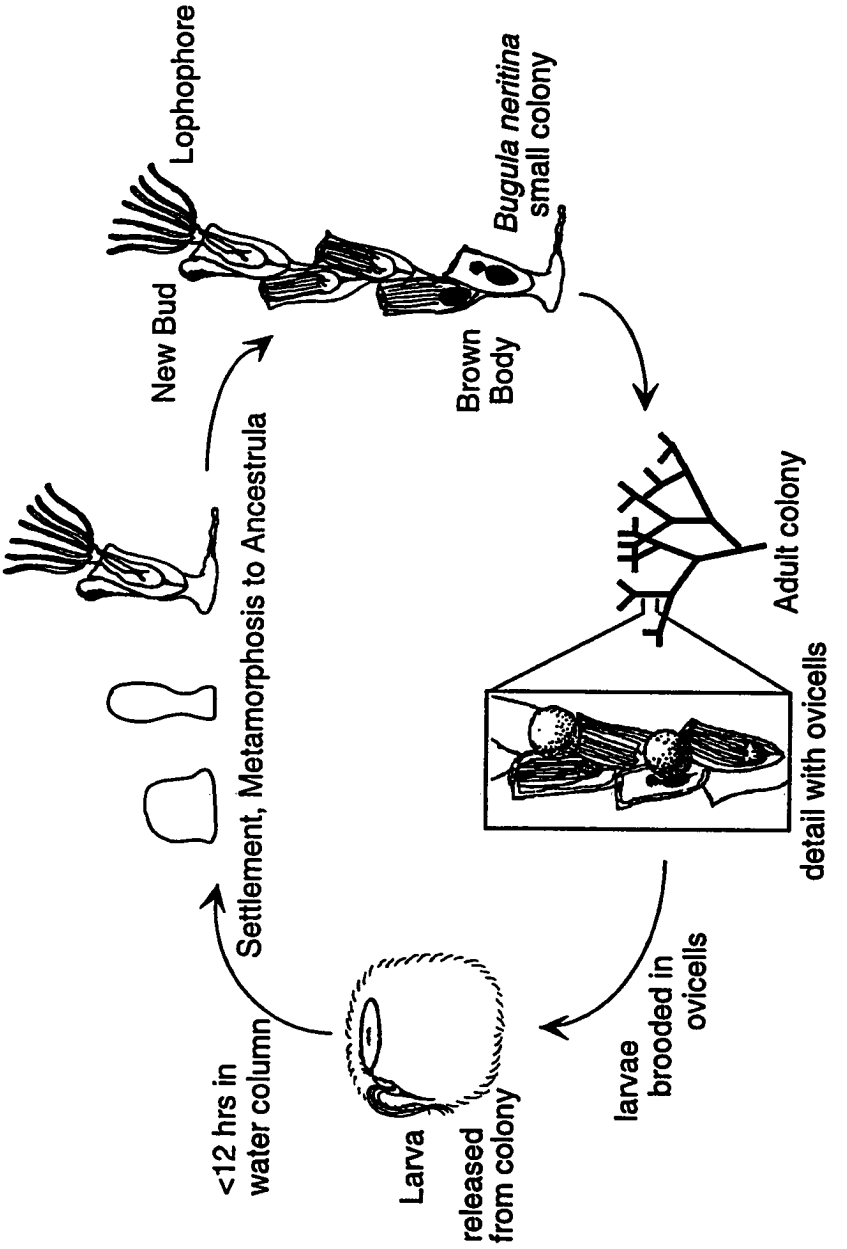

Figure 2: Life cycle of *Bugula neritina.*

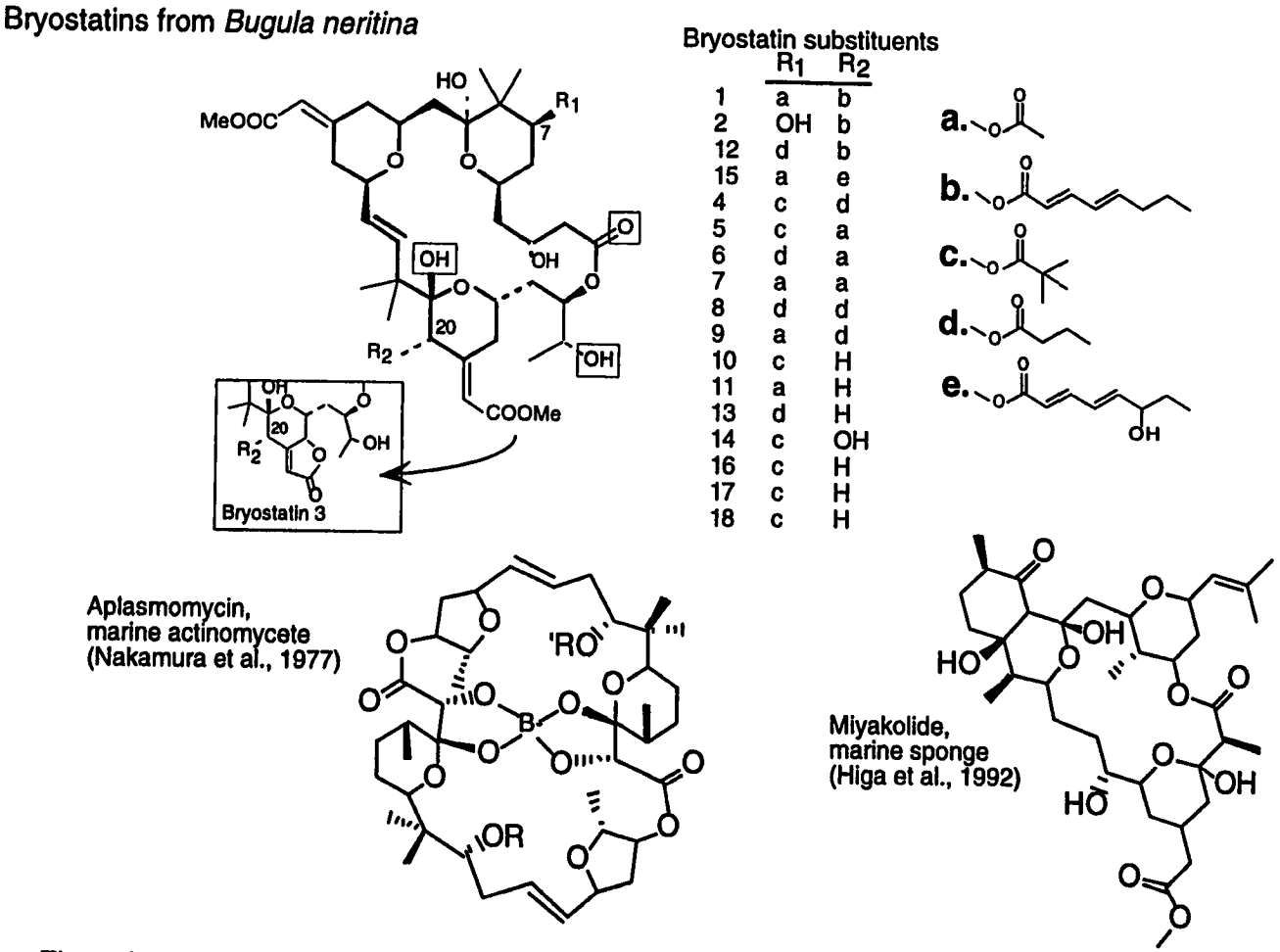

Figure 3: Bryostatins from *Bugula neritina* and other related compounds. The bryopyran ring is highlighted. Polar groups on the bryopyran ring that mimic diacylglycerol are boxed.

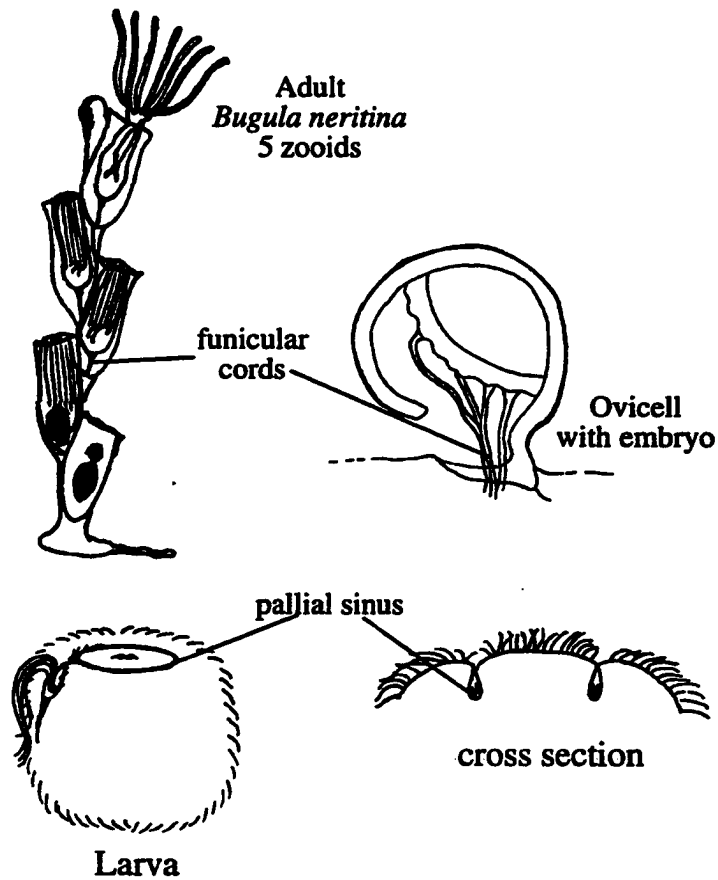

Figure 4. Locations of bacteria in the bryozoan *Bugula neritina*

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Chapter 2

Better living through chemistry: the possible role of bryozoan secondary metabolites in the evolution of defense.

Marine invertebrates use several strategies to escape predation, avoid overgrowth and defend space. These include physical reinforcement, chemical deterrents, camouflage, mimicry, and escape. The physical defenses used by bryozoans, which are sessile, colonial invertebrates, have been well described. However, the role of chemical defenses is not well studied. This paper will examine the relationship between the possession of noxious chemicals and elaborate physical defensive structures. I hypothesize that the bryozoans known to contain toxins or bioactive secondary metabolites have reduced physical defenses. If this is true throughout the bryozoa, which can not be shown definitively in this comparison alone, then possessing toxic or repugnant secondary metabolites may influence the evolution of defense structures of bryozoa. Other sessile organisms are well known for defensive secondary metabolites. Sponges and plants (terrestrial and marine) are the best known examples of organisms that have developed effective chemical defenses. Ascidians and gorgonians are also commonly documented with bioactive metabolites. Bryozoans face the similar challenge of a sessile habit in a competitive environment full of predators. Sessile invertebrates and plants cannot escape and must sit and fight with any means available. The least energetically expensive and most effective defense will be favored evolutionarily. In theory, production and maintenance of physical structures is more expensive than synthesizing or sequestering toxins. With this logic it seems likely that the secondary metabolites found in bryozoans have an important evolutionary and ecological role in the defense strategies of these organisms.

The reduction or loss of physical structures as effective chemical deterrents evolved was used to explain the loss of the shell in the opistobranch molluscs (Faulkner and Ghiselin, 1983). Manufacturing and carrying a shell around is considered more expensive than synthesizing a toxin or sequestering a toxin found in the diet. The synthesis of the shell is avoided, as is the energy expended in carrying and maintaining the shell. If sequestered from the diet, *de novo* synthesis of the toxins is not required. Although many nudibranchs sequester compounds from their diet, there are examples of those that synthesize their own toxins. Interestingly, many opistobranchs feed on and sequester compounds from sponges and marine algae. Sponges are the source of a variety of potent bioactive secondary metabolites. Although sponges express a variety of physical defenses including silicious spicules and heavy calcification, they are sessile and unable to escape predators able to tolerate their physical defenses. A sessile lifestyle is probably a key determinant in the evolution of chemical defenses. Incidentally, sponges also harbor a variety of microorganisms which may be the source of much of their chemical diversity.

The pattern of loading chemicals where there is a lack of physical fortification occurs in algae as well. In young growing parts of certain calcareous algae, the growing tips contain toxins until they calcify sufficiently to protect them (Hay and Fenical, 1988). The conditions leading to evolution of chemical defenses are likely a complex combination of a sessile lifestyle, lack of a good physical fortification, and the intensity of predation and competition. In the tropics, where competition is very intense, there is a greater abundance of highly toxic organisms. There are also plenty

of examples of organisms that use both chemistry and structure for defense (i.e. the rock-like toxic sponges and spiny toxic fish like the lion fish). In general, however, if organisms are not well equipped with protective structures, they are more likely to have repugnant compounds. Which mode of defense came first is not always clear. The ancestor may have had physical protection, then gradually developed a chemical defense that diminished the need for the protective structures. Or the ancestor may have been physically weak and developed toxins in response to predation. In this chapter I examine the physical descriptions of bryozoa known to contain bioactive secondary metabolites. Bryozoans, much like sponges, range in their physical form from soft to very calcified coraline structures, and from very spiny to smooth. In comparing the physical forms and chemistry from bryozoans I will attempt to show a pattern of chemically reinforced defenses where structures are lacking. The correlation may indicate an evolutionary loss of structures no longer needed for protection, or development of chemistry in lieu of structure. The weakness in this comparison is that it relies on published chemistry from bryozoans, which is fairly sparse and many genera are not represented.

The defenses typically seen in the bryozoa are calcification (from light to rock-like), avicularia, vibracula, spines and modified spines forming protective plates (see Fig 1). An individual of a bryozoan colony, the zooid, forms a box-like structure. The frontal plate faces outward and can be rigid, as in the Cheilostome Ascophora, or flexible as in the Cheilostome Anasca. In the Anasca the flexibility of this frontal plate must be maintained to allow the lophophore to be everted by contractions of muscles

that pull the plate inward, forcing the lophophore out. In this group there are a number of structures that have developed to protect this vulnerable frontal plate, including a variety of spines. Avicularia are specialized zooids that form a beak-like pincer. They range in size and morphology from species to species. Avicularia are thought to defend against small predators such as picnogonids, isopods, and amphipods that pierce individual zooids (in Biology of Bryozoans, 1977). Protective plates, denticles, form across the vulnerable frontal membrane of the zooid protecting it from small predators that feed by piercing or scraping this frontal plate away from the soft tissue inside. Vibracula are long hair-like spines that sweep the colony surface to remove debris and small predators. I have observed in cultures that the vibracula are effective in keeping *Scrupocellaria* sp. colonies very clean compared to a species lacking vibracula which become fouled under similar conditions (*B. neritina* in the same dish). Spines are projections of the exoskeleton. The actual effectiveness of all of these structures for defense has not been clearly shown. In *Membranipora membranacea* spines inhibited feeding by two different nudibranchs. The spines are induced only in the presence of the predator, and the number of zooids taken in a day was reduced from 44 to 3 in the presence of spines (Harvell, 1984). Larger predators such as fish and crabs may not be deterred by spines and avicularia. Calcification and toxins are likely to be more important when avoiding larger predators.

Most of the secondary metabolites isolated from bryozoans are alkaloids. It is interesting to note that terrestrial plants use a variety of alkaloids for defense. These are derived from aromatic amino acids. There are a few species that contain other

types of compounds, including the bryostatins from *Bugula neritina* and the methyl sulfoxonium ion from *Alcyonidium gelatinosum*. The three main orders of the marine bryozoans differ in their defensive morphologies. Members of the order Ctenostomata seemingly do not have any structural reinforcements. The colonies are not calcified, lack spines and avicularia, and have a leathery, gelatinous, or fuzzy appearance. The Cheilostomata, in contrast, have various spines, vibracula, avicularia, and some calcification of their chitinous exoskeletons (zooecium). The order Cyclostomata is characterized by tubular zooids in a very heavily calcified colony, lacking additional defense structures. There were no Cyclostomes mentioned in the natural products literature. The highly calcified members of the Cyclostomata are less likely to use chemistry for defense, but this idea remains to be tested. Their absence from the natural products literature could be a lack of sampling or the absence of interesting chemistry. In the discussion below, first ctenostome then cheilostome secondary metabolites are discussed.

Order Ctenostomata

The ctenostome *Acyonidium gelatinosum* is well known for the contact dermatitis, Dogger Bank itch. This species of the suborder Carnosa produces (2-hydroxyethyl)dimethyl sulfoxonium ion (Christophersen, 1985). It forms large gelatinous colonies, and poses a serious health hazard to fishermen in the North Sea area. By genetic analyses, *A. polyoum* is considered a junior synonym of *A. gelatinosum* (from Illustrated keys for the classification of Mediterranean bryozoa).

The secondary metabolites from *A. polyoum* should be compared to those of *A. gelatinosum.*

Zoobotryon verticillatum, although considered tropical, is widely distributed and found elsewhere. Metabolites of specimens from the southern coast of Spain and San Diego California are similar. *Z. verticillatum* (suborder Stolonifera) contains gramine-derived alkaloids and a brominated indole carbaldehyde: from San Diego; 2,5,6-tribromo-*N*-methyl gramine and the corresponding *N*-oxide side chain (Sato and Fenical, 1983); from San Diego and Spain, 2,5,6-tribromo-*N*-methyl indole-3-carbaldehyde (0.088-0.1% and 0.01% dry wt. respectively) and the related *N*-oxide side chains (0.058-0.02% dry wt.) were found (Ortega *et al.*, 1993). The related indole-3-carbaldehyde and 6-bromoindole-3-carbaldehyde have been described from an unidentified yellow marine bacterium, genus *Pseudomonas.*

In the suborder Stolonifera, several species of the genus *Amathia* produce similar alkaloids (figure 2). The amathamides, brominated proline-derived alkaloids, have been isolated from the Australian populations of *A. wilsoni* (A-F), *A. convoluta* (G, 0.04%), and *A. pinnota* (C, 0.06%) (Blackman *et al.*, 1993). These compounds differ in degree of bromination, methylation and by the stereochemistry about a double bond. The biosynthetic precursor 2-(2,4-dibromo-S-methoxyphenyl)-ethylamine was obtained from *A. wilsoni, A. convoluta,* and *A. pinnota* (Blackman *et al.*, 1993). *A. convoluta* found in Florida has yielded the convolutamides and convolutamines (Zhang, Shigemori *et al.*, 1994; Zhang, Kamano *et al.*, 1994). The convolutamides A-F have a unique *N*-acyl-γ- lactam moiety with a dibromophenol group (figure 2). These

chemicals show bioactive cytotoxic properties and may have a protective function. Only convolutamides A and B show cytotoxic activity in the National Cancer Institute (NCI) 60-cell line panel against L1210 murine leukemia and KB human epidermoid carcinoma cells (Zhang, Shigemori *et al.*, 1994). Convolutamines A-E are related to the amathamides and consist of cytotoxic brominated ß-phenylethylamine alkaloids which inhibited cell growth in P388 lymphocytic leukemia (Zhang, Kamano *et al.*, 1994). Evidence suggested that the biogenesis of the convolutamines and amathamides is related.

The ecological relevance of some of these compounds made in *Amathia* has been partially shown by studies on the antifeedant and antibacterial activity of the extracts from *A. convoluta* and *A. wilsoni* respectively. Volutamides (figure 2) from *A. convoluta* inhibited pinfish and urchin feeding (volutamides C and D) and were toxic toward larvae of a co-occurring hydroid (volutamides B and D; Montanari et al., 1996). These compounds are biosynthetically related to the amathamides from *A. wilsoni* and *A. pinnota* and to a lesser extent to the convolutamides and convolutamines.

In 1993, Walls, Ritz and Blackman examined the surface bacteria, fouling organisms and antibacterial activity of extracts from four different species of bryozoans. Two were "chemically defended" and these were from different orders (Cheilostomata, Ctenostomata). The other two were considered not "chemically defended" based on lack of antimicrobial activity (both Cheilostomes). The results correlated antimicrobial activity of extracts to the amount of fouling on the colonies.

Both *A. wilsoni,* and *Orthoscuticella ventricosa* showed the least amount of fouling and had the most active antibacterial extracts. The extracts from *Bugularia dissimilis* and *Cellaria pilosa*, two species with spines, avicularia and calcification, exhibited no antibacterial activity and had the greatest amount of fouling. Fouling increased from tip to base (3%-30% in *O. ventricosa* and *A. wilsoni*; 10-70% in *B. dissimilis* and *C. pilosa*). The bryozoan colonies with lower fouling also had invested less in physical defense.

Both the *A. convoluta* and *A. wilsoni* studies are significant in that they used bioassays relevant to the bryozoan. The antifeedant properties of the volutamides were tested on co-occurring potential predators and larvae that may settle on the colonies. The antibacterial properties *of A. wilsoni* extracts were tested on bacteria obtained from the waters where the bryozoans were collected. Therefore, these were marine bacteria that may actually come in contact with these bryozoa. Often studies screen extracts against bacteria that have no natural contact with the bryozoan. *Bacillis subtilis* and *Pseudomona aeruginosa* are often used, but these assays do not test activities for which the compound evolved. Defensive metabolites may have developed to protect against very specific targets found in their environment.

The extracts from *A. wilsoni* and *O. ventricosa* strongly inhibited both early and late bacterial films but showed some selectivity. One strain was not affected by either, and a second did not respond to the *A. wilsoni* extracts. In nature, *O. ventricosa* showed an evenly distributed low level of bacteria (1.22cells/100 μm^2), while *A. wilsoni* growing in the wild had the highest bacterial densities of all four species even

though the distribution was patchy. It was suggested that the bacterial films on the *A. wilsoni* were specifically recruited as an inhibitor of larval settlement. In a later study the amathamides were shown to be localized in the surface bacteria, although the data were not entirely convincing (Walls *et al.*, 1995). It is possible that these bacteria are symbiotic with *A. wilsoni*, recruited for the purpose of chemical protection. These bacteria have yet to be identified. However, the pattern of amathamide distribution does not match the bacterial distribution. *A. wilsoni* shows a concentration gradient of amathamides increasing from the base to the tips (Walls *et al.*, 1991). The oldest portions, the base, lacks amathamides whereas the tips (0.087g/g) have ten times the concentration of the middle portions of the colonies (0.0087g/g). The growing tips are devoid of bacteria. The distribution of the amathamides may explain the patchiness of the bacterial film and why this species had a higher bacterial count than *O. ventricosa*, and suggests a defensive role for the compounds.

Order Cheilostomata

To date the greatest number of bryozoans containing bioactive metabolites are in the order Cheilostomata. This may simply be a sampling bias. Even within the Cheilostomes, because of the difficulty in sampling, few encrusting types have been investigated. The suborder Ascophora will be discussed first followed by the suborder Anasca. Members of the Cheilostome suborder Ascophora typically have rigid frontal plates on the zooids. In contrast, the zooids of Anascan Cheilostome bryozoa are characterized as having flexible frontal plates.

Suborder Ascophora

Several Ascophora species identified from Australia make related ß-carboline alkaloids. However this suborder has not been well surveyed. For several species the discussion is limited to the chemistry due to a lack of good Australian Bryozoa taxonomies.

Cytotoxic ß-carbolines have been isolated from two families, Mucronellidae and Catenicellidae. *Cribricellina cribraria* (Beutler *et al.*, 1993) and *Catenicella cribraria* (Prinsep, 1991) both produce cytotoxic 1-vinyl-8-hydroxy-ß-carboline as their major secondary metabolite (tested using NCI 60-cell human tumor panel). This compound was especially effective against melanomas. The vinyl group was found to be important for the cytotoxicity. Replacement of the vinyl with an acyl group decreased activity significantly (Prinsep, 1991). Five different ß-carbolines have been isolated from *Catenicella cribraria*. They have varying activities against bacteria and fungi, with compound 2 being ineffective, and 5(Pavettine) being very effective (figure 3). Interestingly, the vinyl group was not important to the antimicrobial activity. Simple ß-carboline alkaloids have been isolated from *Costata hastata* as well (Prinsep, 1991). *Catenicella cribraria* lacks spines, is only lightly calcified, and has large avicularia, but they appear to lack the mandible.

Dakaria subovoidea, family Cheiloporinidae, forms deep red colonies found in harbors around Japan as a fouling organism. This bryozoan yielded two new compounds which contain a 6H anthra [1,9-bc] thiophene ring which has never been

seen in nature although it has been synthesized as a dye (Shindo *et al.*, 1993). The major metabolite isolated was 1,8-dihydroxyanthraquinone, and the two minor compounds were: 5,7-dihydroxy-1-hydroxymethyl-6-oxo-6H anthra [1,9-bc] thiophene, and 5, 7-dihydroxy-1-methoxy carbonyl-6-oxo-6H anthra [1,9-bc] thiophene (figure 4). Because the bioactivities of these compounds were not well tested, it is difficult to comment on their defensive significance. However, *D. subovoidea* does lack spines, avicularia and is the only encrusting species for which the secondary metabolite chemistry is described in the literature to date.

Suborder Anasca

The family Flustridae, suborder Anasca, is the best represented in the chemistry literature currently. The frondose colonies of *Flustra foliacea* have yielded a number of different, but related bromoalkaloids called flustramines. The flustramines are physotigmine, tryptamine and quinoline alkaloid types. These compounds exhibit a wide antibacterial and antifungal activity (Laycock *et al.*, 1986; Holst, 1994). Two major populations have been studied, one in Nova Scotia the other in Scandinavian waters. There is geographic variation in the flustramines suggesting an ability of *F. foliacea* to make a variety of bromoalkaloids. The Bay of Fundy population contains physotigmine bromoalkaloid dihydroflustramine C and its *N*-oxide; flustramine D and its *N*-oxide; and isoflustramine D (Laycock, *et al.* 1986). *F. foliacea* of the North Sea contains related but different flustramines which include flustramines A-C,

dihydroflustramine C (Wright, 1984), flustramine E and debromoflustramine B (Holst *et al.*, 1994; figure 5)

The antibacterial properties of *F. foliacea* extracts were described in 1977 in the context of inhibiting larval settlement on the colonies. A smaller bryozoan, *Scrupocellaria reptans*, readily colonizes only the growing tips of *F. foliacea*, which lack antibacterial activity. In contrast, the older parts of the colony exhibit antibacterial activity, and seem to inhibit larval settlement, although this latter assumption was not rigorously tested. Incidentally, *S. reptans*, and others in this genus, are calcified and display long vibracula, spines widened into protective plates and many avicularia typically of two sizes. This genus was investigated, but there was no mention of interesting chemistry (Al-Ogily, 1977). *F. foliacea*, on the other hand, is thin-walled, has only short thick spines, and few small avicularia. Members of the family Flustridae characteristically have few defensive structures and little calcification. This underscores the difference in defense strategies that may exist among the bryozoans. It is not known whether *S. reptans* prefers to settle on *F. foliacea*, but that is an interesting relationship to investigate as it may be an example of *F. foliacea* providing a refuge.

Several other members of the family Flustridae contain related alkaloids. *Membranipora perfragilis* (or *Biflustra*) from South Australia has yielded unique cytotoxic isoquinoline alkaloids, perfragilin A and B which are similar to mimosamysin from the bacterium *Streptomyces lavendulae* (Choi, 1993). Mimosamycin has also been found in the marine sponges *Reniera* sp. (Frincke and Faulkner, 1982) and

Xestospongia caycedoi (McKee and Ireland, 1987). Although the thiomethyl ether substituents render these unique substances, a microbial origin for these compounds has been suggested in the literature. There is a possibility that mimosamycin in these sponges is an artifact due to oxidation during analysis (Dr. D.J. Faulkner, pers. comm.).

Biflustra perfragilis from Bass Strait, Tasmania, has a noxious odor due to volatile fractions identified as mainly methanol and dimethyldisulfide. Other volatile substances listed in decreasing amounts included dichloromethane, dimethylsulfide, chloromethane and methanethiol (Blackman et al, 1992). Also found were two novel sulfur-containing isoquinolines, 2-methyl-6,7-di(methylthio) isoquinoline-3,5,8(2H)-trione (0.07%) and 2-methyl-6-methylthioisoquinoloine-3,5,8(2H)-trione (0.03% dry wt.; figure 6). The first shows activity against brine shrimp and marine bacteria but not against any human pathogens. This result emphasizes the need for relevant bioassays if the goal is to understand the chemical ecology. These isoquinolines are closely related to perfragilins A and B, also related to mimosamycins, found in *M. perfragilis* (S. Australia) (Blackman et al, 1993).

M. perfragilis and *B. perfragilis* are believed to be the same species. The slight differences in the compounds made by the two chemotype species is an example of geographic variation in the secondary metabolites (Blackman *et al* , 1993). *B. perfragilis* forms deep red to yellow orange large colonies that are highly calcified though brittle. Colonies as large as 0.5 m in diameter have been found free of epibionts and unbroken. The antimicrobial activity of extracts and the lack of fouling organisms indicates that the secondary metabolites of this species has a protective role.

A study conducted in Antarctica provides some evidence that *Carbesea curva*, also of the Flustridae, is a chemically defended species (Winston and Berheimer, 1986). The fouling community in the study area was characterized as highly competitive and dominated by a diverse group of cheilostome bryozoans typically having elaborate defensive structures. *Carbesea curva* was the only one of five species that tested positive for haemolytic activity and was the only species lacking defensive structures. *C. curva* is weakly calcified with a membranous frontal wall, and lacks spines and avicularia. Despite the absence of good structural defenses *C. curva* is one of the most abundant species in the community, suggesting a competitive advantage. The weakness in this study is the lack of a relevant bioassay for the chemical extracts. Heamolytic activity against mammalian cells may not be a good proxy for function in a marine invertebrate defense system. Other species may have contained secondary metabolites that were not detected by the assay but acted as effective toxins. Unfortunately, no other references to *Carbesea curva* were found, and its metabolite is unknown. This genus is characterized by simple zooids lacking avicularia, spines, and having only weak calcification.

Other species in the family Flustridae mentioned in the chemistry literature include *Hincksinoflustra denticulata*, *Securiflustra securifrons*, and *Chartella papyracea*, all of which make indole alkaloids. *S. securifrons* produced halogenated indole-imidazole alkaloids, securamines A-D and securines (Rahbaek et al., 1996; figure 7). *C. papyracea* makes a unique indole alkaloid, Chartelline A (0.07% dry wt.), derived from a tryptamine, histidine, and an isoprene unit (Christophersen, 1985; figure

7). *C. papyracea* has thin walls, only slight calcification, one short spine on the zooid, and lacks avicularia. Similarly *S. securifrons* lacks avicularia, and spines and has a membranous frontal wall with lightly calcified thin walls. These would appear to be easily pierced by many of the typical predators. *H. denticulata*, however, is armored by denticles that come across the frontal surface of the zooids, and small conical avicularia. There are geographic variations in the numbers of denticles, some having few to very small denticles. It would be interesting to compare the regional differences in chemistry to see if they correlate with the differences in denticle morphology.

In summary, the family Flustridae is characterized as having thin lightly calcified walls, few small or no avicularia, and short, dull spines. This family also contains many species producing bioactive metabolites. The ability to produce toxic metabolites may have influenced the evolution of protective structures in this family.

Several bryozoans have both the typical structural defenses complemented with bioactive chemicals. This pattern is also seen in the algae where grazing is especially heavy, such as in tropical regions (Hay and Fenical, 1988). Members of the family Bugulidae, are characterized as having the most highly modified avicularia, and often have sharp spines along the distal margins of the zooids. *Bugula dentata* (Australia) contains bipyrroles (tambjamines) and a tetrapyrrole (blue pigment; figure 8). The tambjamines were originally described from nudibranch predators of the bryozoan *Sessibugula translucens*, *Tambje abdere* and *T. eliora* (Carté and Faulkner, 1983). *B. dentata* contains tambjamines C, E, G-J (Blackman and Li, 1994). *B. dentata* has the typical suite of physical defense structures--avicularia and marginal spines. The ovicells

are blue in this species indicating a concentration of the blue tetrapyrrole in the reproductive structure which may be a defense of the developing embryos.

The defensive properties of the tambjamines have been established by examination of nudibranch predators and extracts from tambjamine-producing prey including the bryozoan *Sessibugula translucens* (Carté and Faulkner, 1983) and an ascidian *Atapozoa* sp. The nembrothid nudibranch *Tambje abdere* sequesters tambjamines from *Sessibugula translucens* (containing tambjamines A-D) as defense against predators such as the nudibranch *Roboastra tigris*. *T. eliora* also sequesters tambjamines, but at a lower concentration so as to be defended against generalist predators but not against *R. tigris* which seems to specialize on *T. eliora* (Carté and Faulkner, 1983). Nembrothid nudibranchs also sequester tambjamines from an ascidian, *Atapozoa* sp. (Paul et al., 1990). Tambjamines E, G and I have shown activity against brine shrimp, and the tetrapyrrole is active against several gram positive and negative bacteria (Matsunaga, 1986) and acts as a feeding deterrent against fish (Paul et al., 1990). The blue tetrapyrrole pigment was also found in a marine bacterium *Serratia marcescens* (Matsunaga et al., 1986). Since the tetrapyrrole is found in bacteria, bryozoa and ascidians it is possible that it is of microbial origin, or a result of convergent evolution in synthesis. The enamines, tambjamines A and B, inhibit cell division and exhibit antibacterial activity. The isobutylamines, tambjamines Cand D, also inhibit cell division and have antifungal and antibacterial activity (Carté and Faulkner, 1983).

Most of the species within the genus *Bugula* have large avicularia with the typical bird head profile with the exception of *Bugula neritina* and *B. longissima* (Antarctic). *B. neritina* is unusual in its genus in that it lacks spines and avicularia (figure 9), and also produces potent cytotoxins--the bryostatins (figure 10). The bryopyran ring structure is believed to be synthesized from acetate units by a type I polyketide synthase pathway (Kerr, 1996). Bryostatins are only found in *B. neritina* and related compounds have not been identified from other bryozoans (with the exception of bryostatins found in a sample of *Amathia convoluta* which was contaminated with *B. neritina*; Pettit, 1990). Although the role of bryostatins as a predator deterrent is not established, the toxicity of bryostatins and the reduction of protective structures in *B. neritina* typical of its congeners is suggestive of a protective role for bryostatins. *B. neritina* is widely distributed in temperate and subtropical regions, and has been referred to as "a true weed" because of its success as a fouling organism (Bryozoan Evolution, p.100). The success of *B. neritina* could be explained by having rapid growth, however, the larvae of *B. neritina* have been shown to be chemically defended (Lindquist, 1996; Lindquist and Hay, 1996). *B. neritina* larvae and their extracts in squid pellets were rejected by fish, corals and sea anemones. Interestingly, in this same study, the extracts from the adults had little effect on the palatability of food pellets to fish, indicating the larvae have different chemical deterrents or levels than are found in the adults. The larvae have been shown to contain bryostatins. It is possible that the larvae contain additional compounds besides bryostatins. Bryostatins have not been tested in feeding assays. Determining the

palatability of bryostatins is crucial to understanding what ecological role these cytotoxic compounds may have, although there are other functions they may serve.

The pattern of bryostatin activity throughout the colony suggests a defensive function. Using a bryostatin activity assay described in Chapter 5, we found that in cultured colonies and in wild colonies bryostatin activity levels were highest in the tips and the rhizoids of colonies (Appendix). This suggests a function in competition and defense. The rhizoids anchor the colony and establish space on the surface in the fouling community. These may be susceptible to grazing predators and overgrowth by other fouling organisms including settling larvae. The loading of the rhizoids with bryostatins may ensure the colony's place on the surface and protect it from being snipped off at the base. In fouling communities, competition for space is fierce and having toxins in the portion that grows along the substrate likely ensures space for the next generation and protects the existing colony from detachment and overgrowth. *B. neritina* larvae tend to settle on other *B. neritina* colonies, including the rhizoids. Extracts from *B. neritina* have been shown to deter larval settlement (Martín and Uriz, 1993) and compounds in the rhizoids may defend space against the settlement of other species. Furthermore, rhizoids have been documented to overwinter after the larger part of the coloy has died off (Silén, 1977, and refs therein). These rhizoids then bud new feeding zooids and eventually colonies. If the rhizoids must survive without the rest of the colony, having a higher dose of bryostatins may assist in preventing overgrowth and grazing during this time.

The tips, also higher in bryostatin than the middle portions of the colony, are a highly exposed area of the colony. The colonies tend to form rounded bushes, with growing tips highly exposed at the tops. The tips are the first portions of the colony encountered by swimming predators such as fish. In addition, the ovicells that harbor larvae tend to form on the younger portions of the colonies near the tips. Protection of this region of the colony is logical and similar in pattern to the loading of toxins in the vulnerable tips of algae (Hay and Fenical, 1988). *A. wilsoni,* mentioned earlier, also concentrates their defensive alkaloids in the tips of the colonies (Walls *et al.*, 1991).

Other evidence that bryostatins have a defensive role for *B. neritina* comes from the nudibranch predators of *B. neritina*. *Polycera atra* and *P. hedgpethi* both are commonly found feeding on *B. neritina*. *P. atra* is far more abundant than *P. hedgpethi*. Very small (5-8 mm) *P. atra* and *P. hedgpethi* both appeared to scrape away the frontal plate with the radula and ingest the exposed zooid inside (personal observations). Larger adults may feed in a different way and take colony tips into the mouth. Extracts of these nudibranchs were found to contain levels of bryostatin activity similar to and higher than the prey *B. neritina* itself (Chapter 6). Furthermore, the eggs of *P. atra* contained $\geq$ 17 x higher concentrations than the prey showing that the nudibranchs sequester the bryostatins and concentrate it in their eggs. Although there is no direct evidence that the nudibranchs use bryostatins for defense, it is highly likely that the conspicuous eggs are loaded with these cytotoxins as a means of protection. The white egg cases are highly visible against the dark brown to purple *B.*

neritina colonies. If the nudibranchs use bryostatins from their diet as a chemical defense, then the bryozoan probably benefits from them as well.

The Cheilostomata characteristically have specialized defensive structures. Species from this order containing bioactive metabolites tend to have reduced, or lack the specialized defensive structures. In contrast, members of the order Ctenostomata lack calcification, avicularia, spines or other defensive appendages. The colonies are gelatinous, leathery or furry and sometimes covered with detritus. It seems probable that ctenostomes rely on camouflage and chemical defenses to a greater extent than cheilostomes to escape predation and compete for space. However, there is not enough information on the secondary metabolites of either of these groups to draw a strong conclusion regarding the evolution of defensive structures in relation to defensive metabolites. The investigation of bryozoan secondary metabolites is relatively recent (most described in the last fifteen years or so), and there are very few families represented in the natural products and chemical ecology literature. In addition, negative results are not often published so that it is very difficult to identify species that are clearly not chemically defended. There is a need for screening assays of extracts that are relevant to the probable activity of the compounds. The term "chemically defended" is often used when the chemical has not been tested for its effectiveness as a deterrent. Even if the chemistry has been characterized, and its activity tested on a few bacteria and NCI cell lines, there is often no evidence that the chemicals actually serve as a predator deterrent or antifoulant. Often there is an assumption that if an unusual bioactive chemical is there, it must be for defense.

Nonetheless, there is a pattern among the bryozoan species that have been described having bioactive compounds. They typically have reduced structural defenses relative to those known to be without chemical defense. The few studies that have attempted to place identified bioactive compounds in an ecological context have demonstrated a tendency to lack specialized protective structures when a toxin was available. This evidence is preliminary, and further research will be needed before any strong conclusions can be made. Additional bryozoans must be surveyed for bioactive metabolites and those that show no interesting activity need to be recorded as well as those that do contain interesting noxious compounds. The chemistry of species that are clearly well protected structurally should be examined and compared to other species from the same area that lack these types of specialized defenses. With additional data from a variety of families and morphologies a pattern can be expected to emerge.

Table 1: Bryozoan secondary metabolites and defensive structures.

Order, Suborder, family, genus	Types of secondary metabolites	activity	Spines	Avicularia	Calcification
Ctenostomata					
Suborder Carnosa					
Alcyonidiidae					
Alcyonidium gelatinosum	dimethyl sulfoxonium ion	cantact dermititis	no	no	no
Suborder Stolonifera					
Vesiculariidae					
Amathia wilsoni (Tasmania)	amathamide alkaloids A-F	antibacterial,antifungal	no	no	no
Amathia convoluta (Florida, Australia)	amathamide G, convolutamines A-E	variable cytotoxicity	no	no	no
Amathia pinnata (Australia)	amathamide C		no	no	no
Zoobotryon verticillatum (S.CA., Spain)	brominated indole carbaldehydes		no	no	no

Note: Ctenostomes do not have spines, avicularia, or calcification

Order, Suborder, family, genus	Types of secondary metabolites	activity	Spines	Avicularia	Calcification
Cheilostomata					
Suborder Anasca					
Flustridae					
Flustra foliacea (Scandanavia, Nova Scotia)	indole alkaloids, quinoline	antibacterial	short	small	weak, thin
Carbasea curva (Antarctica)	?	haemolytic activity	no	no	weak, thin
Hincksinoflustra denticulata	indole alkaloids		short	small	denticles
Securiflustra securifrons	indole alkaloids		no	small	weak, thin
Chartella papyracea	indole alkaloids, chartelline A		no	one small	weak, thin
Biflustra perfragilis (Tasmania)	S-cantaining isoquinoline alkaloids	antibacterial,cytotoxic	?	?	yes, brittle
Bugulidae					
Bugula dentata (Australia, Japan)	tetrapyrrole, tambjamines G-J, C,E	kills brine shrimp,antibact.	yes	yes	weak(?)
B. neritina (temperate)	bryostatins	cytotoxic	no	no	weak
Sessibugula translucens	bipyrroles=tambjamines A-D	antibacterial	?	?	?
Suborder Ascophora					
Mucronellidae					
Cribricellina cribraria (Australia)	beta-carboline alkaloids	cytotoxic			
Catenicellidae					
Catenicella cribraria (Australia)	beta-carboline alkaloids	cytotoxic	no	yes,no mandible	
Costaticella hastata	beta-carboline alkaloids	cytotoxic			
Cheiloporinidae					
Dakaria subvoidea (Japan)	6H-anthra[1,9-bc]thiophene	antioxidant	no	no	

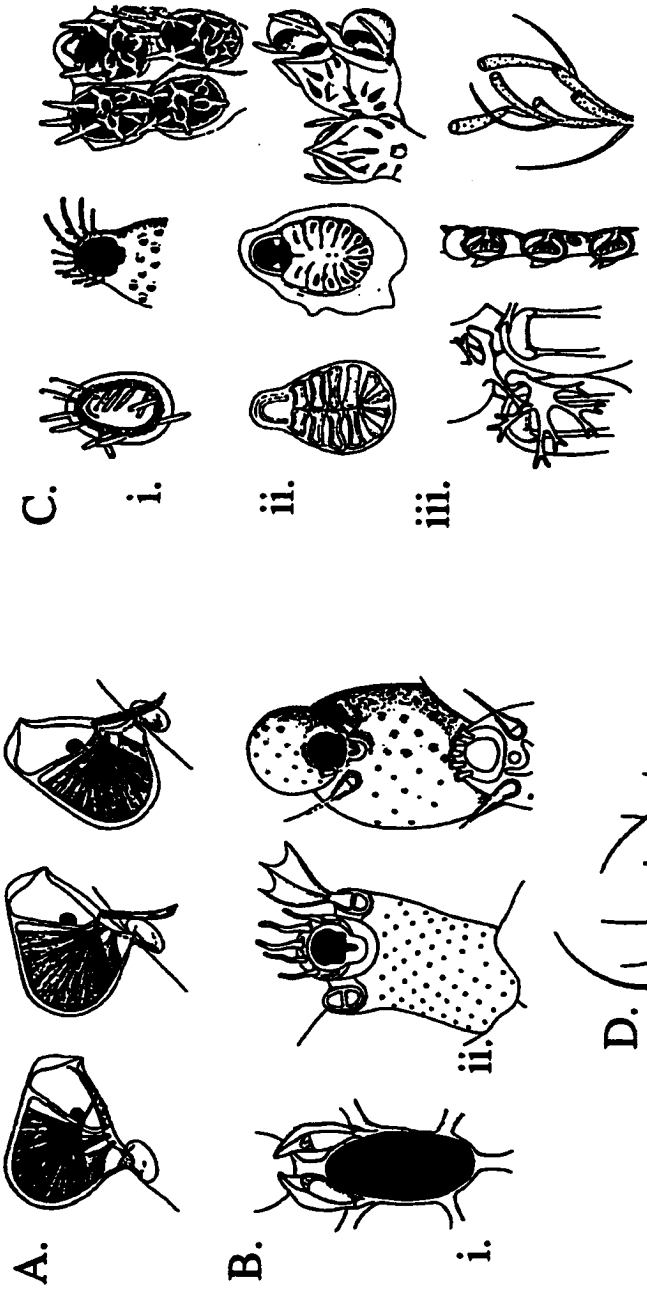
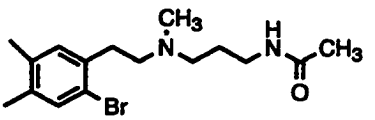
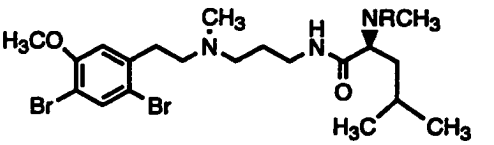


Figure 1. Examples of bryozoan defensive structures. A, avicularium from *Bugula* sp. illustrating movements. B, i. paired avicularia, ii. avicularia with mandible protruding beyond rostrum. C, i. spines, ii. spines fused into frontal shield, iii. variations of spine morphologies. D, vibracula.

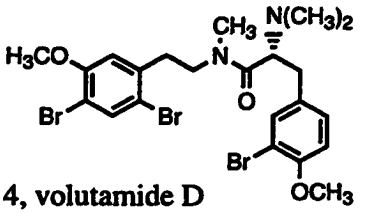
Volutamides A-E



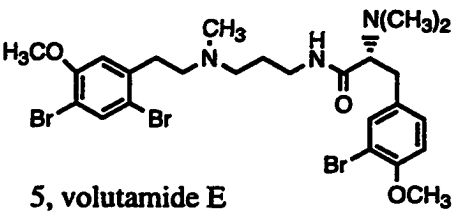
1, volutamide A



2, volutamide B, R=H
3, volutamide C, R=CH_3

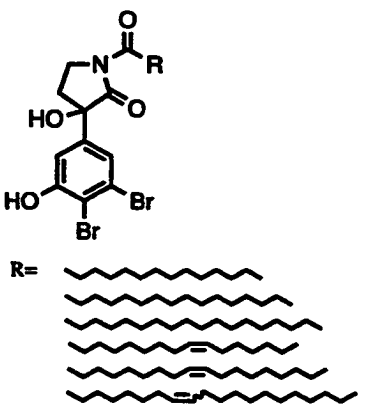


4, volutamide D

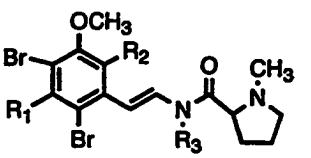


5, volutamide E

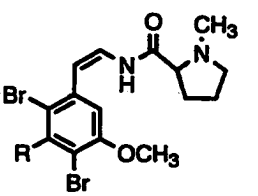
Convolutamides



Amathamides

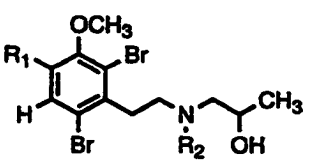


	R1	R2	R3
A	H	H	H
C	Br	H	CH3
D	Br	H	H
E	Br	H	H
G	Br	OCH3	CH3

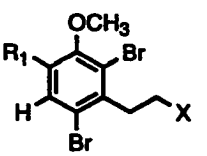


B R=H
F R=Br

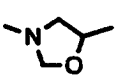
Convolutamines



A R1=Br, R2=CH3
B R1=H, R2=CH3
C R1=Br, R2=H



D X=



E X=

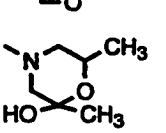
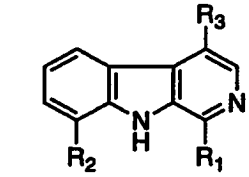
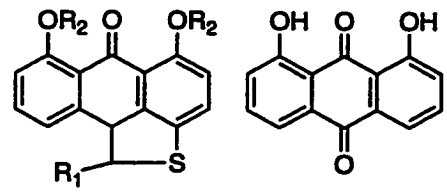


Figure 2. Compounds from *Amathia*, volutamines, convolutamides and convolutamines.



	R_1	R_2	R_3
1	$CH=CH_2$	OH	H
2	Et	H	$S(O_2)Me$
3	Me	H	H
4	Et	H	H
5	CH=CH2	H	H

Figure 3. Beta carbolin alkaloids from *Cribricellina cribraria*



R_1	R_2
CH_2OH,	H
CO_2CH_3,	H

Figure 4. *Dakaira subovoidea* metabolites

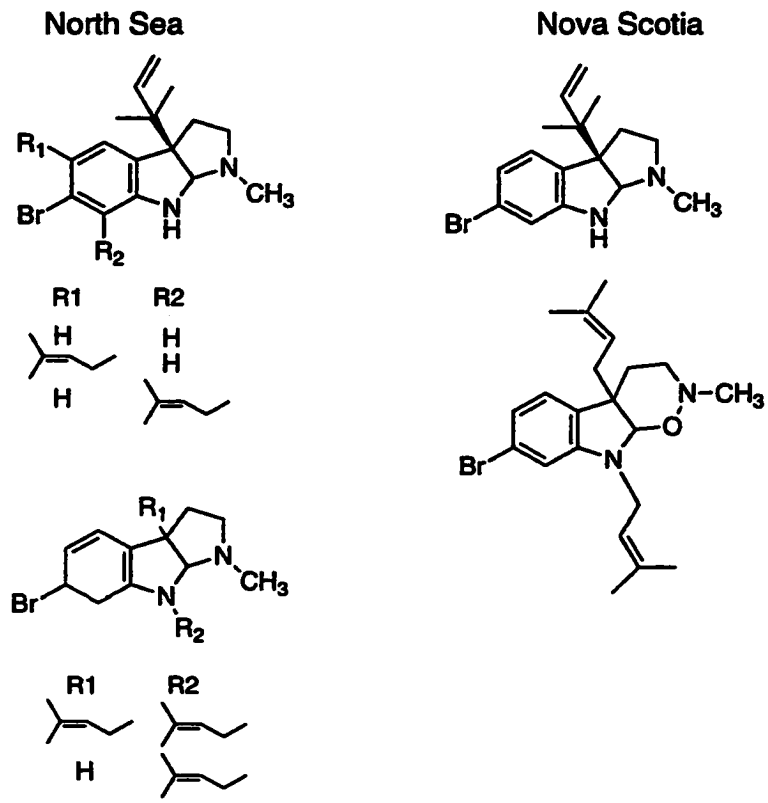


Figure 5. Flustramines from *Flustra foliacea*

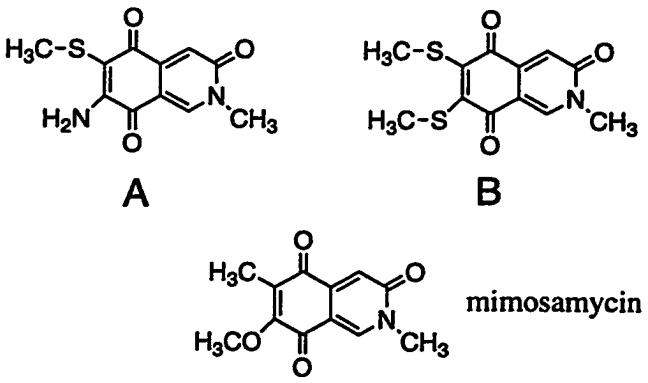
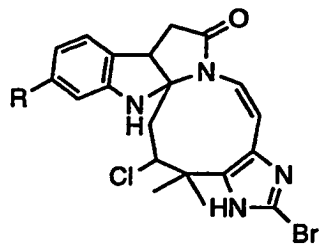
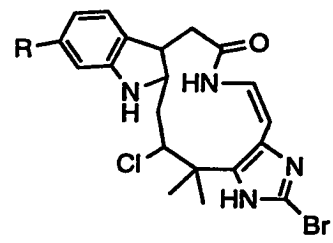


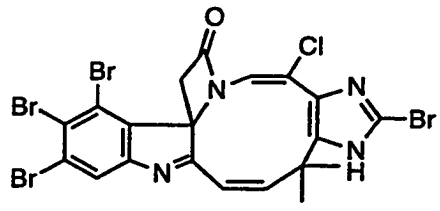
Figure 6. Perfragilins from *Biflustra perfragilis*



R=H: Securamine A
R=Br: Securamine B



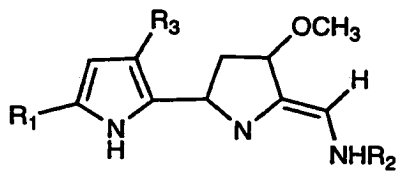
R=H: Securine A
R=Br:Securine B

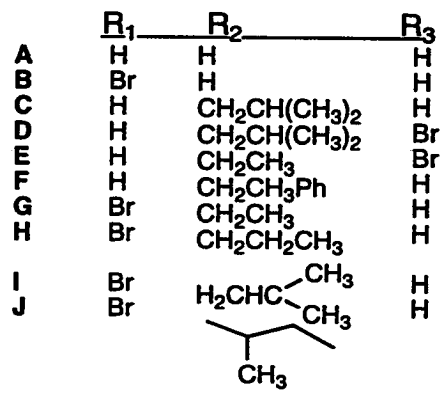


Chartelline A

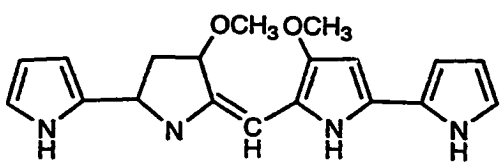
Figure 7. Indole alkaloids from *Securiflustra securifrons* and *Chartella papyracea*

Tambjamines

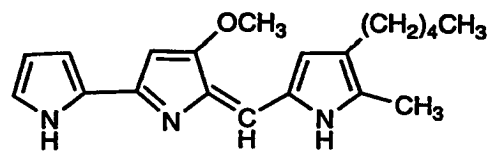




	R_1	R_2	R_3
A	H	H	H
B	Br	H	H
C	H	$CH_2CH(CH_3)_2$	H
D	H	$CH_2CH(CH_3)_2$	Br
E	H	CH_2CH_3	Br
F	H	CH_2CH_3Ph	H
G	Br	CH_2CH_3	H
H	Br	$CH_2CH_2CH_3$	H
I	Br	$H_2CHC(CH_3)CH_3$	H
J	Br		H



Blue tetrapyrrole pigment



prodigiosin from the marine bacteria genus *Benechea* (Giovannoni and Margulis, 1981)

Figure 8. The tambjamines and a possible microbial source

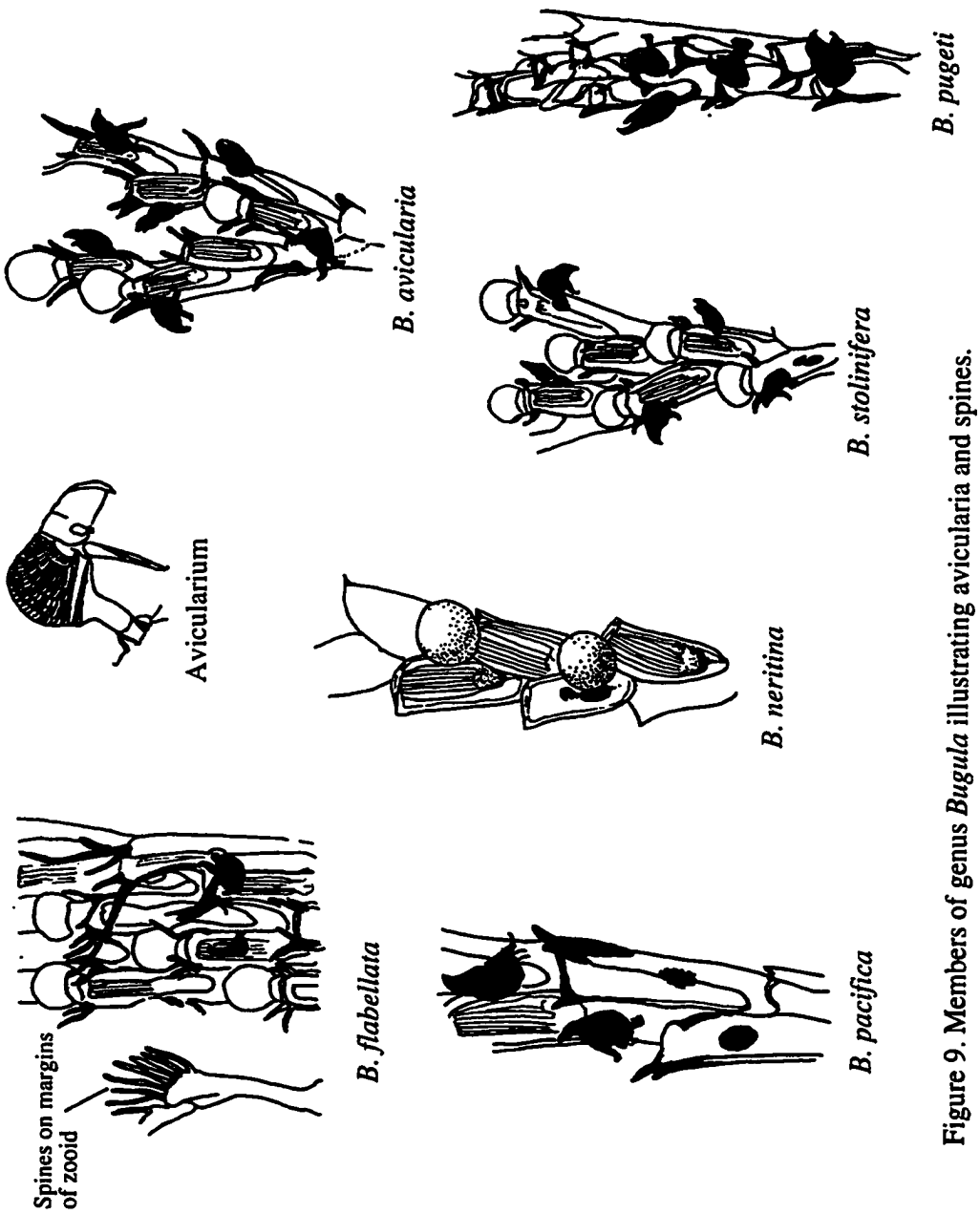


Figure 9. Members of genus *Bugula* illustrating avicularia and spines.

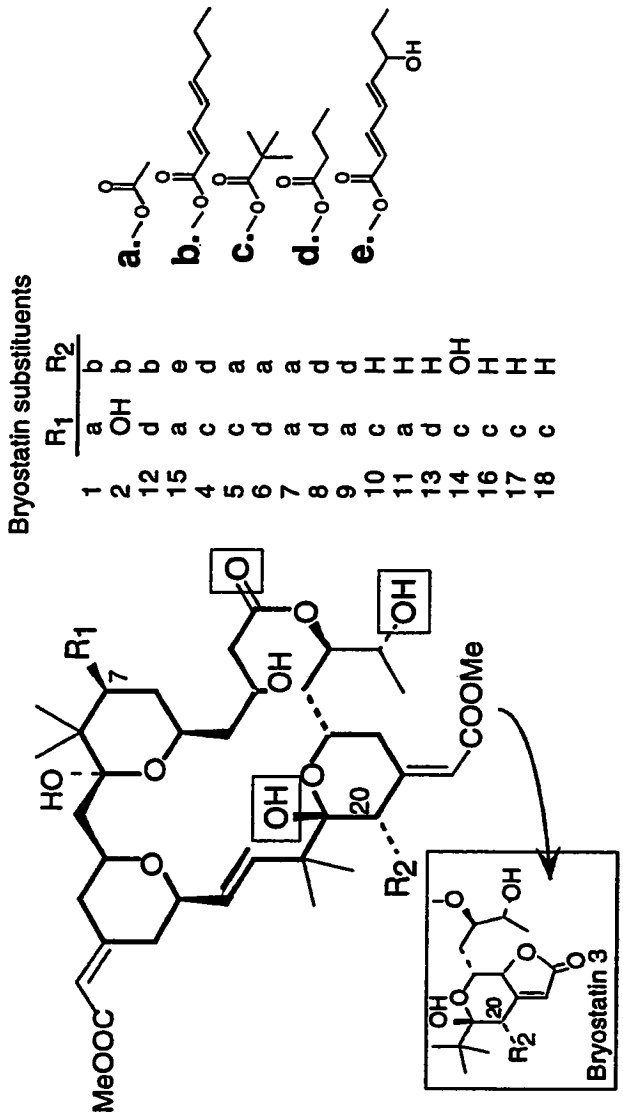


Figure 10: Bryostatins from *Bugula neritina*. The bryopyran ring is highlighted. Polar groups on the bryopyran ring that mimic diacylglycerol are boxed.

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Chapter 3

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"

Abstract

Larvae of the bryozoan *Bugula neritina* harbor bacterial symbionts. These symbionts were identified as a novel species of γ-proteobacterium, on the basis of ribosomal small-subunit rRNA gene sequences. *In situ* hybridization with oligonucleotides specific for the symbiont confirmed origin of the sequence. The taxonomic status "*Candidatus* Endobugula sertula" is proposed for the larval symbiont.

Bugula neritina (Cheilostomata, Cellularoidea) is the source of bryostatins (13), a family of cytotoxic macrocyclic lactones, one of which, bryostatin 1, is currently being tested in clinical trials for treatment of cancer (9, 14, 15). R.M. Woollacott has described extracellular rod-shaped bacterial symbionts located in the pallial sinus of the larvae of *B. neritina* (18). It has been suggested that bacterial symbionts may play a role in bryostatin production (3).

This study aimed to obtain a small-subunit (SSU) rRNA gene sequence from the larval symbionts of *B. neritina*, verify the sequence using *in situ* hybridization with specific oligonucleotide probes based on the SSU sequence, analyze the phylogenetic affiliations of the symbiont, and determine whether any of the bacteria readily cultured from the larvae were symbionts (4-7).

Collection of larvae, DNA extraction and SSU rRNA gene PCR amplification.

Free-swimming larvae were collected in the lab from adult *B. neritina* colonies obtained in different seasons and at indicated depths from five California locations: Palos Verdes (PV; 16m, provided by CalBioMarine Technologies, Inc.); Torrey Pines Artificial reef #2, La Jolla (TP, 13 m); Scripps Institution of Oceanography pier pilings, La Jolla (SP, 6 m); Bodega Bay marina (BB, 0 m); and Catalina Island (CI, 11-16 m). Larvae were concentrated in 1.5 ml microcentrifuge tubes (approximately 25 mg of larvae per tube) by gentle centrifugation, then rinsed four times in filtered seawater (0.2 μm-pore-size filter) to minimize the number of contaminating seawater bacteria. Excess water was removed and the larval pellets were placed in lysis buffer or frozen at -80°C for later use. A QIAamp tissue kit (QIAgen Corp.) was used to purify DNA. PCR was conducted with 200 ng of larval DNA with *Taq* polymerase and buffer (Boehringer Mannheim) as described previously (7), with 30 cycles of 94°C (1 min), 50°C (1 min), and 72°C (1 min) and a final elongation step of 72°C for 7 min. The amplification primer pairs were 27f-1492r, 27f-1101r, and 926f-1392r (10).

SSU rRNA gene sequencing and analysis.

The PCR products were sequenced with standard SSU rRNA primers (10) with fluorescently labeled dideoxynucleotide terminators in a standard cycle sequencing protocol (Prism ready reaction kit FS [Applied Biosystems, Inc.] and run on an Applied Biosystems 373A automated sequencer. Both strands were fully sequenced. Sequence runs were assembled and checked for errors with MacVector version 4.5 and

AssemblyLIGN™ software (Kodak) and then aligned on the *Escherichia coli* SSU rRNA secondary structure (4) to check for base pairing in helical regions. The CHECK CHIMERA program was run on the assembled sequences (11).

Amplification of larval DNA with the eubacterium-specific primer 27f and universal primer 1492r produced almost the entire SSU gene fragment, but a much smaller product also appeared. In order to obtain clean sequence most sequence was obtained from two additional PCR products using primer pairs 27f-1101r and 926f-1392r that resulted in single overlapping (165 bp) fragments. The sequences from all three products were in agreement. Double stranded and error-checked sequences were obtained for three Southern California (PV, TP, SP) populations corresponding to *E. coli* positions 38 to 1265 (1,221 bp); for two additional populations (BB, CI) a smaller region (1030 bp) was later determined with the symbiont-specific primers 240f and 1253r (Table 1).

Phylogenetic analysis was performed with sequence alignments from the Ribosomal Database Project (RDP)(11), supplemented with sequences from Genbank that were identified by BLAST. Most analysis trees were evaluated by maximum parsimony with PAUP (17) with a transversion cost of 2. Tree evaluation was done by using the branch and bound algorithm or, with large data sets, by using heuristic searches with multiple rounds of random addition of taxa. Bootstrapping analysis was used to test the significance of phylogenetic affiliations. For analysis of the relationships within the γ-proteobacteria, PHYLIP version 3.572 was used (6). The tree was constructed by neighbor-joining, with distances calculated by the Kimura

two-parameter estimation in PHYLIP, and tested with 100 bootstrap replications with random addition of sequences.

The 1,221-bp fragment was used for phylogenetic analysis and showed that the larval symbionts are γ-proteobacteria. Affiliation with the γ-proteobacteria was well supported by bootstrapping 1000x (89%; Fig 1). The symbionts from the five populations were very closely related (Table 2), but the symbiont group was not closely affiliated with any group of γ-proteobacteria in the RDP database (Fig. 2).

Oligonucleotide design.

Symbiont-specific oligonucleotides (Table 1) for use in *in situ* hybridization and specific PCR were designed from variable regions of the SSU rRNA gene. Target sites were selected based on previous studies (2, 5) and analyzed for sequence matches in CHECK PROBE (RDP) and BLAST (Genbank). The minimum numbers of mismatches found for each oligonucleotide are listed in Table 1. A negative control probe (Bn control) was designed by scrambling the sequence of one of the specific oligonucleotides (Bn1253r), thus maintaining the base composition. This scrambled probe sequence showed no significant matches to sequences in the databases. The positive control was the EUB338 eubacterial probe (1). Oligonucleotide probes were 5' end labeled with biotin or fluorescein.

Testing of oligonucleotide specificity.

Probes were hybridized to whole cells from the bacterial strains found to match the probe sequence, but with two mismatches (Table 3): *Pseudomonas aeruginosa* (ATCC 25330) and *Oceanospirillum kriegii* (ATCC 27133). The sequences of these test strains were checked by sequencing the portions of the SSU rRNA gene that the probe should have matched. An additional mismatch was found in the *P. aeruginosa* strain. These strains were grown overnight in the appropriate medium and then fixed in 4% paraformaldehyde-0.1 M MOPS buffered saline pH 7.4. The hybridization protocol was adapted from that used by Holland et al. for whole-larva hybridizations (8). For consistency hybridization conditions for bacterial cells were the same as those used in the whole-larva hybridizations. Bacterial cells in suspension were treated with proteinase K, refixed, treated with acetic anhydride, washed with buffer, then resuspended in prehybridization buffer (1 mg/ml total RNA, 100 µg/ml heparin, 10% formamide, 5 x SSC, 0.1 % Tween 20, 5 mM EDTA, 1 X Denhardt's solution) and incubated at the hybridization temperature for 30 minutes to one hour.

Fluoroscein-labeled probe was added to a concentration of 2 ng/µl and samples were heated to 55°C and then incubated for 2to 4 hours at the hybridization temperature. Hybridization temperatures tested ranged from 42 to 55°C in increments of approximately 3°C. Each wash was conducted twice for 15 minutes as follows: high stringency wash solution 1 was 10% formamide, 5 X SSC, and 1 % sodium dodecyl sulfate (SDS); wash solution 2, used at 37°C, was 10% formamide, 2 X SSC, 1 % SDS; wash solution 3, used at room temperature, was 10% formamide, 2 X SSC, and

0.1 % Tween 20. The high stringency wash temperatures varied depending on hybridization temperature. The cells were suspended in Vectashield (Vector) mounting media and viewed under epifluorescence. The eubacterial probe bound to the test cells at all hybridization temperatures. The Bn1253r probe did not bind.

***In situ* hybridization of larvae.**

Larvae for *in situ* hybridization were fixed in 4% paraformaldehyde-0.1 M MOPS (pH 7.4)- 0.5 M NaCl. Fixed larvae were either used immediately or stored in 70% ethanol at -20°C. The fixed larvae were treated with proteinase K (Boehringer Mannheim), refixed in 4% paraformaldehyde, treated with acetic anhydride in 0.1 M triethanolamine, washed with buffer and incubated in hybridization buffer (described in previous section) at 55°C for one hour. Biotinylated probes were added in fresh hybridization buffer at a concentration of 2 ng/µl. The samples were heated to 55°C for 5 to 10 minutes and then incubated at 43°C for four to six hours. Washes were as above, with solution 1 at 47°C, solution 2 at 37°C, and solution 3 at room temperature. Probes were detected by visualization of dark brown precipitate with horseradish peroxidase conjugated to avidin (Pierce ABC detection kit) and metal-enhanced diaminobenzidine (Pierce) as the substrate. Reactions were allowed to incubate until signal was sufficient; then reactions were stopped by dilution with buffer, rinsed, refixed and mounted for light microscopy.

The eubacterial probe bound to the bacteria in the pallial sinus (Fig. 3 D); bacteria outside the pallial sinus were rarely observed. The specific probe Bn1252r

bound to the bacteria in the pallial sinus under hybridization conditions that did not permit binding to the test strains (Fig. 3C). The negative control probe Bncontrol did not bind under the same hybridization conditions used for the specific probe, nor was there any endogenous peroxidase signal detected in the pallial sinus (Fig. 3B).

Cultivation of associated bacteria.

Larvae were washed with autoclaved seawater three to five times and used to culture associated bacteria by either homogenization and dilution plating or smearing of individual larvae on plates. The medium contained 1% peptone, 0.1% yeast extract, 3.7 mM succinate, 4 mM MgSO4, and 1.5% agar in 75% seawater at pH 7.2 (16). Plates were incubated aerobically at 18°C, and single colonies were isolated and maintained. There were relatively few bacteria associated with the larvae that could be isolated on the medium used. Typically, a variety of colony types was observed at densities of approximately 1 to 5 colony forming units per larva.

Specific PCR assay of associated bacterial isolates.

Symbiont-specific oligonucleotides 240f and 1253r were used with a temperature profile for specific PCR of 94, 53, and 72°C for 1 minute each. DNA (10 ng) from cultures of associated bacteria was amplified with the universal eubacterial primers 27f and 1492r as a positive control, and the symbiont-specific pair to test for identity as the symbiont. *B. neritina* larval DNA (200 ng) was used as a positive control for amplification with the symbiont-specific primer pair.

DNA was extracted and amplified from 15 representative strains; all tested negative for symbiont identity (Fig. 4).

Conclusions

We have obtained SSU rRNA sequences from the bacterial symbionts in the pallial sinus of *B. neritina* larvae, and phylogenetic analysis reveals this symbiont to be a novel γ-proteobacterium. The positive results of *in situ* hybridization with specific probes derived from the sequence confirm that the sequence is from bacteria in the pallial sinus. The results strongly suggest that a single organism is present in the pallial sinus. For three different *B. neritina* populations we obtained a clean (uncontaminated) sequence from directly sequenced PCR products amplified with universal bacterial primers, which would give unreadable sequence if a significant number of additional bacteria were present. In addition, *in situ* hybridization with the eubacterial probe, which would bind to all bacteria present, gave results indistinguishable from those given by the symbiont-specific probe. This organism was not readily cultivated suggesting that it may require specialized conditions for isolation. Closely related strains of this organism were found in all five populations of *B. neritina* examined, indicating that this is a specific association.

We propose the taxonomic status of *Candidatus* (12) for the larval symbiont of *B. neritina* with the following description: "*Candidatus* Endobugula sertula" [(γ-*Proteobacteria*) NC; gram negative; R; NAS (GenBank no. AF006607),

oligonucleotide sequence complementary to unique region of 16S rRNA 5'-CATCGCTGCTTCGCAACCC-3'; S (*Bugula neritina*, pallial sinus of larvae); M].

Nucleotide sequence accession numbers.

Sequences reported herein have been assigned GenBank accession numbers: strain BnSP, AF006606; strain BnPV, AF006607; strain BnTP, AF006608.

Acknowledgements

This work was supported by California Sea Grant College grant #RMP-61. We are grateful to CalBioMarine Technologies, Inc. for advice, technical assistance and the donation of *B. neritina* samples. We thank Linda Z. Holland for advice on *in situ* hybridization methods, Edward F. DeLong for the gift of oligonucleotides, and Ronald McConnaughey for expert assistance with collecting. Meredith Leong assisted with bacterial isolations and PCR evaluation of isolates.

Table 1: Oligonucleotides used in this study

Oligonucleotide	Sequence 5'-3'	Positions[a]	minimum mismatches[b]	Reference
Symbiont specific				
Bn1253r	CATCGCTGCTTCGCAACCC	1271-1253	1	This study
Bn240f (only for PCR)	TGCTATTTGATGAGCCCGCGTT	240-219	2	This study
Controls				
Bncontrol	ACGTCACCGTCCAGCCTCT	NA[c]	NA	
Eubacterial	GCTGCCTCCCGTAGGAGT	338-356	NA	Amann, et al, 1990

[a] Number denote corresponding positions in the *E. coli* sequence.
[b] Minimum number of mismatches with sequences from RDP and Genbank databases.
[c] NA, not applicable.

Table 2: Percent identities among SSU rRNA genes of *B. neritina* symbionts from California populations

	% Identity with symbionts from location[a]				
Location	BB	PV	CI	TP	SP
BB	–	99.61	99.61	99.61	99.81
PV	97.87	–	99.81	99.81	99.42
CI	99.22	97.48	–	100.00	99.42
TP	98.16	97.38	98.93	–	99.52
SP	97.77	96.90	97.67	97.28	–

[a] Above the diagonal are maximum percent identity values, counting ambiguous positions as identities. Below the diagonal are minimum percent identity values, counting ambiguous positions as different.

Table 3: Sequence of strains closely matching symbiont probe Bn1253r

Bugula neritina symbiont	G G G U U G C G A A G C A G C G A U G
Oceanospirillum kriegii	G G G U U G C G A A G C G G C G A C G
Pseudomonas aeruginosa	G G G U U G C C A A G C C G C G A G G

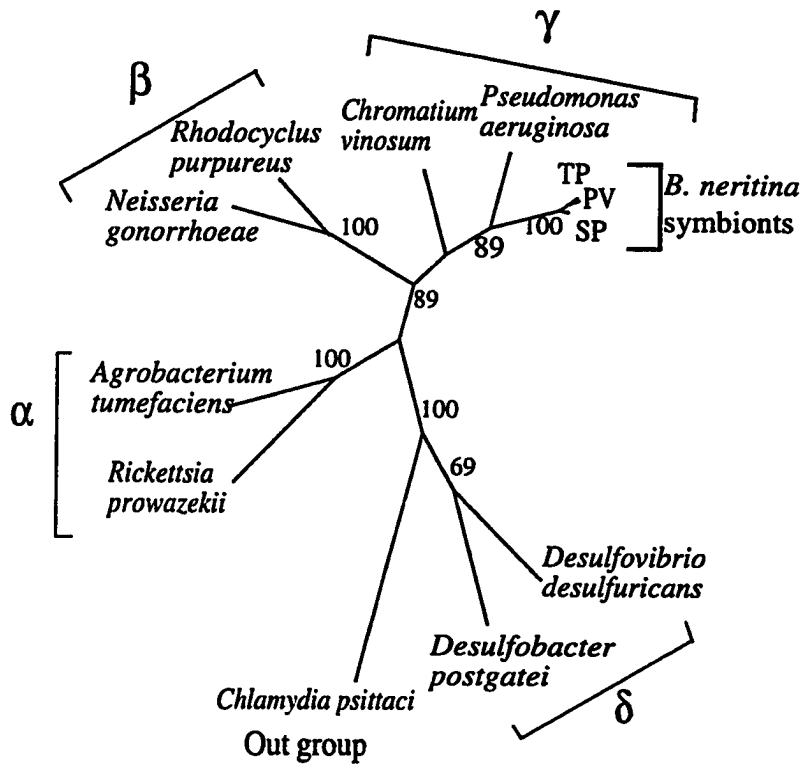

Figure 1. Phylogenetic relationships among proteobacteria. The tree was constructed with approximately 1100 base pairs of alignable sequence from positions 38 to 1265 (*E. coli* numbering), with *Chlamydia psittaci* as the outgroup. A branch-and-bound search was conducted with PAUP. Significantly supported nodes (> 80%) are labeled with the percentage of bootstrap replications (out of 1000) in which the node was found. PV, Palos Verdes; TP, Torrey Pines Artificial reef #2, La Jolla; SP, Scripps Institution of Oceanography pier pilings, La Jolla.

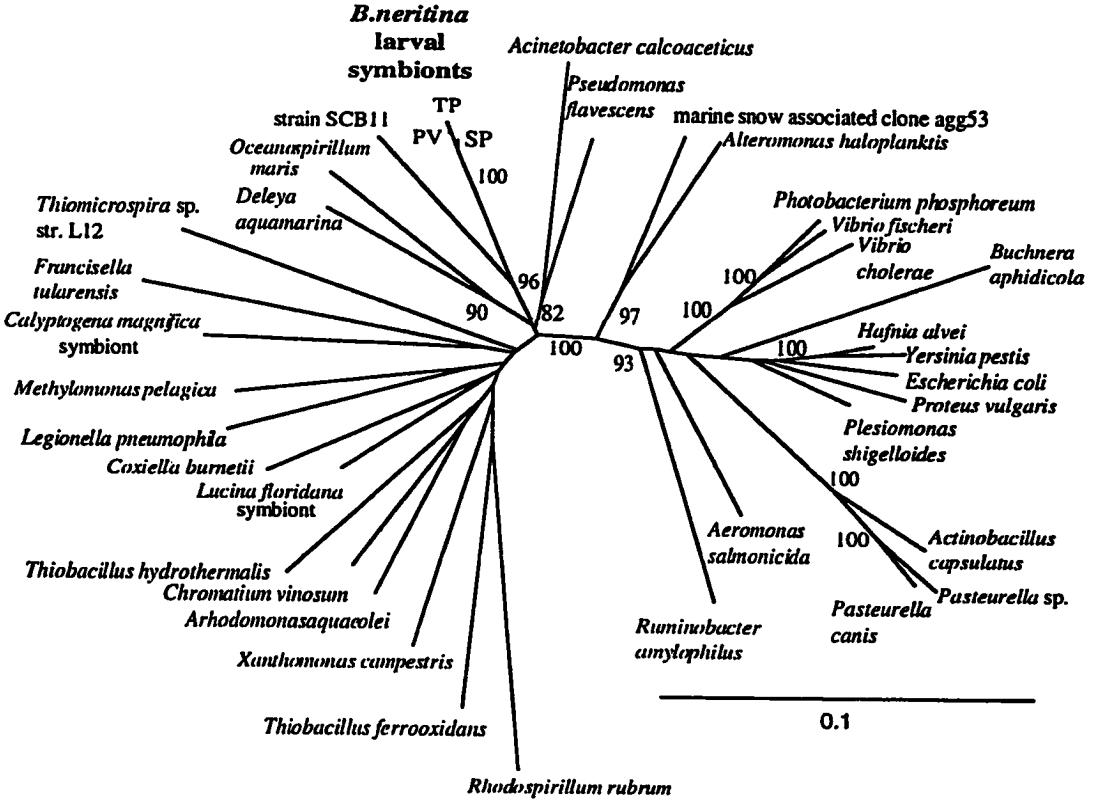

Figure 2. Neighbor-joining tree of phylogenetic relationships among γ-proteobacteria. The tree was constructed using 1204 base pairs of alignable sequence from positions 38-1265 (*E. coli* numbering), with the α-proteobacterium *Rhodospirillum rubrum* as the outgroup. Significantly supported nodes (> 80%) are labeled with the percentage of bootstrap replications (out of 100) in which the node was found. The bar shows distance. PV, Palos Verdes; TP, Torrey Pines Artificial reef #2, La Jolla; SP, Scripps Institution of Oceanography pier pilings, La Jolla.

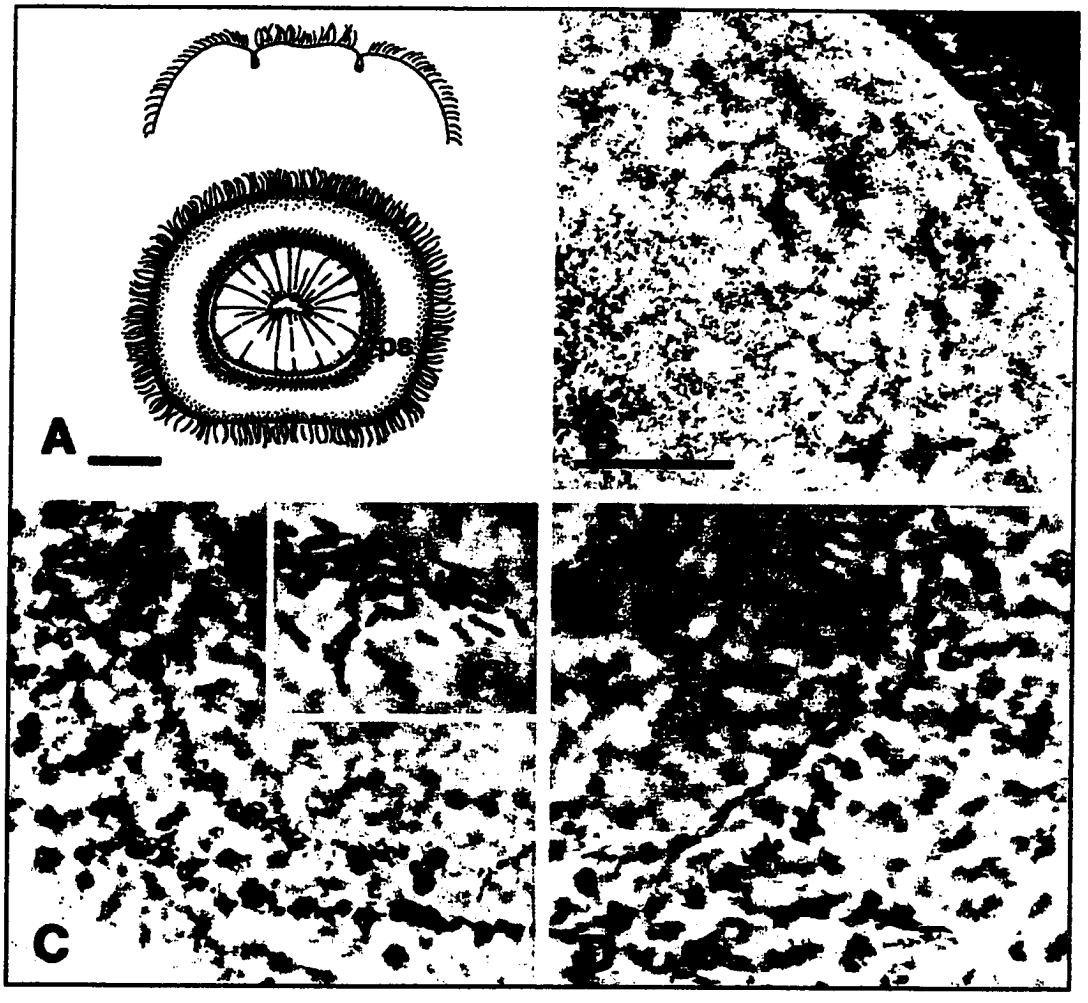

Figure 3. A, drawing of *Bugula neritina* larva, top drawing represents a crossection through the pallial sinus, bottom illustrates the aboral surface, PS pallial sinus, scale bar 100 μm; B-D phase micrograph of aboral surface of *B. neritina* larva after *in situ* hybridization. B, hybridization of larva with negative control probe, no staining was observed in the pallial sinus, arrows show approximate width and location of pallial sinus, scale bar 50 μm; C, hybridization of larva with symbiont specific probe Bn1253r; D, hybridization of larva with eubacterial probe.

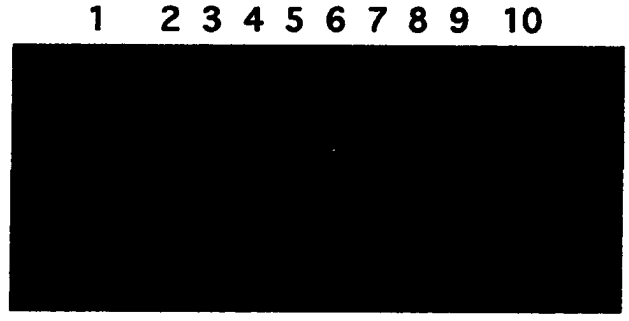

Figure 4. Agarose gel image showing specificity of symbiont specific primer pair Bn1253r and Bn240f. Lane 1,10 molecular weight standards (λ HindIII, EcoR1), lane 2 *Bugula neritina* larval DNA amplified with universal eubacterial primers 27f and 1492r, lane 3 *B. neritina* larval DNA amplified with symbiont specific primers Bn240f and Bn1253r. Lanes 4, 6, 8 bacterial isolates DNA amplified using universal eubacterial primers 27f and 1492r. Lanes 5, 7, 9 bacterial isolates DNA amplified using symbiont specific primers Bn240f and Bn1253r.

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Chapter 3 Supplement

This section is a supplement to Chapter 3 which has been previously published. Additional data was gathered since the studies presented in Chapter 3. The data presented in this update indicate that the identified larval symbiont "*E. sertula*" is the only bacterial symbiont specific to *B. neritina* and that this bacterium has the genetic potential to make complex polyketides such as bryostatins. The work in this supplement was done in collaboration with Grace E. Lim and Dr. Scott W. Allen (CalBioMarine Technologies, Inc.).

1) Analysis of the microbial community associated with *B. neritina* by denaturing gradient gel electrophoresis (DGGE). Seana K. Davidson and Grace E. Lim.

Introduction

In recent years denaturing gradient gel electrophoresis (DGGE) has been successfully used to identify bacteria in mixed populations based on small subunit ribosomal RNA gene (SSU rRNA) sequence(Muyzer et al., 1993; Ferris et al., 1996; Muyzer and Smalla, 1998). A variable region of the SSU rRNA gene is amplified using universal bacterial primers in PCR resulting in a mixed population of SSU rRNA genes. These diverse SSU rRNA genes are then separated on a polyacrylamide gel containing a gradient of denaturant (urea and formamide) such that the gene fragments melt and halt migration through the gel depending on the DNA GC content and sequence. Gene fragments of different sequences halt at different denaturant concentrations resulting in a separation of distinct species' genes. The individual bands are then cut directly from the gel, reamplified using the same primers in PCR and sequenced. The pattern of the

bands is indicative of the complexity of the community and identifies which bacteria are common between samples. The genes from the same species migrate the same distance on the gel. However there are cases where different species will migrate the same distance on the gel due to similar melting properties which are dependant on the sequence as well as the base composition.

We used the DGGE technique to identify bacteria associated with *B. neritina* . Our goal was to see if there were other bacteria in addition to "*E. sertula*" consistently associated with *B. neritina.* To analyze the microbial community associated with *B. neritina* SSU rRNA genes were amplified, using universal eubacterial primers, from *B. neritina* DNA samples collected at various locations and depths (surface to 24 m) along the California coast and one from North Carolina. The amplification products were separated using DGGE and compared. If there was an additional bacterial species consistently found in all samples this could be a source of bryostatins. Three other bryozoan species, *B. pacifica*, *Scrupocellaria sp.* and an unidentified white bryozoan, were also analyzed as negative controls for "*E. sertula*" and positive controls for ubiquitous environmental bacteria.

Methods

Gene amplification:

A 330 base pair portion of the SSU rRNA gene was amplified using a conserved bacterial primer 1055f, 5'-ATggCTgTCgTCAgCT-3', and a universal primer with a GC clamp 1392r-GC clamp, 5'-CgCCCgCCgCgCCCCgCgCCCggCCC gCCgCCCCCgCCCC-ACgggCggTgTgTAC-3' (Ferris et al., 1996). For amplification from *B. neritina* DNA 100 ng of DNA was used in 50 µl reactions using *Taq* polymerase and buffer (Boehringer Mannheim) as described in Haygood et al. 1992,

using 30 cycles of 94°C (1 min): 50°C (1min): 72°C (1min), and a 7 min 72 °C final primer extension. PCR products were cleaned using the QIAGEN QIAquick PCR clean up kit, and total eluted volume of PCR products was 30 ul to allow most of the reaction to be loaded onto the DGGE gel (20 µl).

Gel preparation and electrophoresis:

Acrylamide gels (6.5 %, 1:37 Bis-Acrylamide ratio) were prepared and run using 0.5X TAE buffer (1X TAE buffer is 0.04 M Tris base, 0.02 M sodium acetate, and 1 mM EDTA, pH adjusted to 7.4). Acrylamide solutions (6.5%, 0.5X TAE buffer pH 7.4) containing 0% and 100% denaturant (urea and formamide, UF) are mixed to make 35% and 65% UF solutions. 100 % UF solution is defined as 40 % vol/vol formamide and 7.0 M urea. TEMED (N',N',N',N'-tetramethylethylenediamine) and 10 % APS (ammonium persulphate) solutions are then added to initiate polymerization of the acrylamide. These two solutions are mixed with a gradient mixer as the gel is poured to create a gradient from 35% UF (top) to 65% UF (bottom) with denaturant increasing in the direction of electrophoresis. The optimal gradient is determined empirically by initially running a gel with a 0 to 100 % UF gradient for about eight hours, and examining the migration points of most of the bands. The midway point on the gel is taken to be 50 % UF. Subsequent gels are run with a gradient that should achieve the maximum spread of gene fragments for the particular sample run.

Samples are loaded into wells with Bromophenol Blue running solution (10x: 0.25% Bromophenol blue, 40 % w/v sucrose). The gel is run in 0.5X TAE at 60°C using the CBS Scientific Inc. DGGE 2000 system, with 200 V running voltage for five hours. The gel is then stained with 1X SYBR Gold (Molecular Probes, Eugene, OR) in 1X TAE for at least 20 minutes. Bands are visualized on a UV transilluminator, cut

from the gel and placed into 1.5 ml epitubes, then 400 ul of 1X SSC buffer is added, the gel is homogenized and incubated at 37°C overnight. Gel fragments are spun down, supernatant is transferred to a clean tube and the DNA is precipitated with 0.5 M LiCl in 95% ethanol. DNA is pelleted, resuspended in 20 µl sterile dH2O and used in reamplification of the band for sequencing. For PCR reamplification and sequencing the original PCR primers listed above were used. Cycle sequencing was accomplished using the ABI (Applied Biosystems, Inc.) BigDye reaction kit following manufacturers instructions. Sequencing reactions are separated and analyzed on an ABI 373 automated sequencer.

Sequences corresponding to the suspected "*E. sertula*" band were compared with the "*E. sertula*" sequence to confirm identity. A few of the other distinct bands were reamplified and sequenced to confirm that they contained only a single sequence not a mix.

Results

From the wild *B. neritina*, no additional bacteria consistently occurred that were unique to *B. neritina*. The triplet of strong bands near the top of the band ladder occur in all samples including non-*Bugula* negative controls (unidentified white bryozoan), *Bugula pacifica* and larvae (figure 1). The uppermost band was demonstrated to be a heteroduplex by reamplifying, running on another gel and showing separation into component products. This was repeated several times and occasionally there was full separation with the dominant band disappearing. The others are mixed products but may contain unique bacterial signals. These are likely environmental bacteria colonizing surfaces. Several other distinct bacterial bands

appear, but not in all samples. A few of the dominant bands that occurred in several samples were sequenced. However since the bacteria did not occur consistently with *B. neritina*, further characterization was not pursued. "*E. sertula*" remains the only known consistently associated bacterium found in all *B. neritina* samples tested so far.

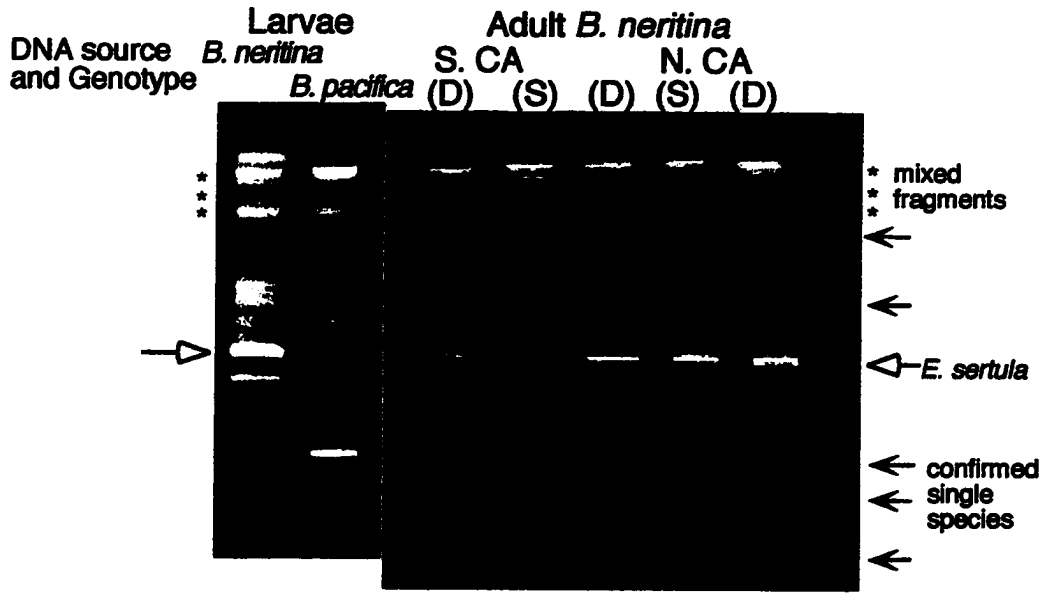


Figure 1: Representative DGGE gel of wild *B. neritina* bacterial SSU rRNA gene fragments.

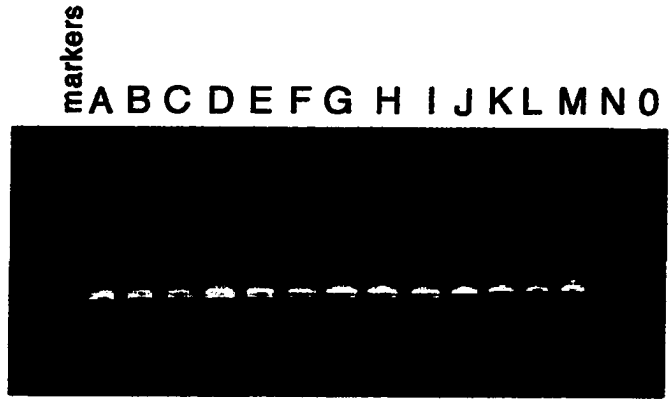
2) Type I complex polyketide synthase gene clones from *B. neritina*.

Degenerate primers designed from conserved regions of beta-keto acyl synthase domains of bacterial type I polyketide synthase (PKS-I) genes were successfully used to clone a 316 base pair portion of a bacterial PKS-I ß-keto acyl synthase domain from both larvae and adult *B. neritina* DNA (KSa). Since the larvae contain only one type of bacteria, "*E. sertula*", in great excess of others the KSa fragment most likely came from either the "*E. sertula*" or the host DNA. From this cloned fragment, specific primers were designed and tested on DNA from *B. neritina* collected from various locations and depths along the California coast. Two other bryozoans found with the *B. neritina* were also included in the screening as negative controls.

Results:

All PCR amplifications from *B. neritina*, including larval DNA, using the KSa-specific primers were positive. The two other bryozoan species tested, *Scrupocellaria* and an unidentified white bryozoan, were negative for the presence of the gene (Figure 2). Since the other two bryozoans were growing with *B. neritina*, we would expect them to have a similar consortium of non-specific environmental bacteria which, if containing this particular PKS gene, might amplify with these KSa primers. They showed no signal. This evidence together with the DGGE data finding no other consistently associated bacteria indicate that the PKS KSa clone originated from the "*E. sertula*" in both the larvae and the adult *B. neritina*. The presence of a bacterial type I polyketide synthase pathway in "*E. sertula*" shows a potential for synthesis of complex polyketides.

This work was conducted in collaboration with Dr. Scott Allen, CalBioMarine Technologies, Inc.



A. Torrey Pines, 9-12 m, 6/97
B. Scripps Pier, 0-7.6 m, 6/97
C. Palos Verdes, 16.5 m, early '97
D. Palos Verdes, Larvae, early '97
E. Catalina Island, Blue caverns Pt., 9-12, 8/97
F. Catalina Island, Harbor Dock, surface, 8/97
G. Catalina Island, Larvae, 8/97
H. Newport Boat hull, 0-2 m, 8/97
I. Humboldt Bay Dock, surface, 9/97
J. Tomales Bay, 0-1.5 m, 9/97
K. Patrick's Point (outside Humboldt Bay), 3 m, 9/97
L. Bodega Bay Dock, surface, 9/97
M. North Carolina (Atlantic), 2 m, fall '97
N. Scrupocellaria sp., Scripps Pier, '97
O. Unidentified white bryozoan,Scripps Pier, 3/98

Figure 2: Amplification of a PKS gene fragment KSa using specific primers designed from a beta-keto acyl synthase gene clone from *B. neritina* larvae and adult DNA. The gel image shows amplification of the gene fragment in a variety of *B. neritina* samples collected throughout the year from California, N. Carolina, deep and shallow genotypes. Included are two other species of bryozoa collected with *B. neritina* from the Scripps Pier.

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Chapter 4

Identification of sibling species of the bryozoan *Bugula neritina* that produce different anticancer bryostatins and harbor distinct strains of the bacterial symbiont "*Candidatus* Endobugula sertula."

Abstract

Although the cosmopolitan marine bryozoan *Bugula neritina* is recognized as a single species, natural products from this bryozoan vary among populations. *B. neritina* is the source of the anticancer drug candidate bryostatin 1, but it also produces other bryostatins and different populations contain different bryostatins. We defined two chemotypes on the basis of previous studies: chemotype O contains bryostatins with an octa-2,4-dienoate substituent (including bryostatin 1), as well as other bryostatins; chemotype M lacks bryostatins with the octa- 2,4 -dienoate substituent. *B. neritina* contains a symbiotic γ-proteobacterium "*Candidatus* Endobugula sertula," and it has been proposed that bryostatins may be synthesized by bacterial symbionts. In this study, *B. neritina* populations along the California coast were sampled for genetic variation and bryostatin content. Colonies that differ in chemotype also differ genetically by 8% in the mitochondrial cytochrome *c* oxidase subunit 1 (CO I) gene; this difference is sufficient to suggest that the two chemotypes represent different species. Each species contains a distinct strain of "*E. sertula*" that differs at four nucleotide sites in the small subunit ribosomal RNA (SSU rRNA) gene. These results indicate that the chemotypes have a genetic basis rather than an environmental cause. Gene sequences from an Atlantic sample matched sequences from the California chemotype M colonies, suggesting that this type may be cosmopolitan due to transport on boat hulls.

Introduction

The recognition of sibling species throughout the world oceans has gained attention in recent years (Knowlton, 1993; Knowlton and Jackson, 1994; Muricy et al.,1996; Palumbi, 1996). The identification of species that are difficult to distinguish by morphology alone is clearly important to understanding the ecology of a system. This has been demonstrated in studies of the coral *Montastraea annularis* and symbiotic dinoflagellates which until recently were treated as a single species of coral and a single symbiont (Knowlton et al.,1997; Knowlton et al.,1992). Distinguishing these species is essential to understanding patterns of bleaching and interpreting data regarding environmental degradation and global climate changes (Knowlton et al.,1992; Rowan et al.,1997). In designing research programs, distinguishing sibling species is critical to understanding the biology of a system since these species can differ in their physiology and behavior in ways that affect their relationships and geographic distributions. If multiple species are treated as one species, we are likely to misinterpret the cause of observed patterns. Secondary metabolite chemistry is another character that probably varies among sibling species. The ability to distinguish closely related but apparently identical marine invertebrate species is critical to bioprospecting for pharmaceuticals from the sea and understanding the chemical ecology of the community. One of the obstacles in efforts to find potential drugs is the inconsistent recovery of compounds from different collections of the same organism. This has proven especially troublesome in work with sponges, and for certain species of sponges

there is genetic evidence of extensive cryptic speciation (Muricy, et al.,1996). Christophersen attributed geographic variation in bioactive compounds from some bryozoans to environmental factors (Christophersen,1991). However, in this study we show that a variation in chemistry from the common marine bryozoan *Bugula neritina* is most likely due to genetic differences.

B. neritina is a common member of fouling communities on boat docks, boat hulls and submerged rocks. This species has been treated as a single species throughout its cosmopolitan temperate range, although there are consistent chemistry differences among populations (Pettit,1991). Some features of the biology of *B. neritina* could promote divergence between populations separated by long distances. The dispersal of the larvae is limited, which hinders mixing between populations by larval dispersal alone (Keough, 1989; Wendt, 1996). Larvae are brooded on the colony until they are ready to be released, are nonfeeding, and spend only a few hours in the water column before settling. On the other hand, settlement of these organisms on boat hulls and floating debris allows wide dispersal and mixing between populations separated by long distances.

B. neritina is commonly studied in the laboratory and in field studies because of its abundance and the ease with which larvae can be collected and induced to settle in the laboratory. In recent years, attention has focused on the chemistry of this invertebrate. *B. neritina* produces a series of cyclic macrolides, the bryostatins, which have cytotoxic properties including action against certain cancer cell lines. There are currently 18 different described bryostatins; all have in common the bryopyran ring but

vary at two substituent sites (Fig. 1). The structural similarity between the bryostatins and compounds produced by bacteria has led to the proposal that the bryostatins are produced by bacterial symbionts due to (Anthoni et al.,1990). Bacterial symbionts are borne inside the larvae (Woollacott, 1981); these are γ-proteobacteria named "*Candidatus* Endobugula sertula" (Haygood and Davidson, 1997). These bacteria have not been cultivated; *Candidatus* is a taxonomic status used for bacteria when it is not possible to deposit a type strain (Murray and Stackebrandt, 1995).

Clinical research efforts have concentrated on bryostatin 1 which is in Phase II clinical trials for the treatment of leukemias, lymphomas, melanomas and solid tumors (Pluda et al.,1996). Pettit and colleagues observed that bryostatin 1 was found in *B. neritina* samples from California, but not from the Gulf of Mexico (Pettit, 1991). In more extensive work in Southern California, Mendola observed that bryostatin 1 is typically found in deeper samples, but is absent from shallow populations (D. Mendola, pers. comm.). We have defined two bryostatin chemotypes, O and M, on the basis of observations of Pettit and colleagues in California and Gulf of Mexico populations (Pettit,1991) (Fig. 1). Chemotype O (octa-2,4-dienoate) contains bryostatins with an octa-2,4-dienoate ester at C-20 (bryostatins 1-3, 12 and 15) and small amounts of the minor bryostatins 4-9. The presence of bryostatins 1, 2 or 3 is diagnostic of chemotype O. Chemotype M (minor) contains the minor bryostatins 4-9 only, and lacks the octa-2,4-dienoate ester at C-20. The presence of minor bryostatins and the absence of bryostatins 1, 2 or 3 is diagnostic of chemotype M. Because the occurrence of

bryostatins 10-18 is not well characterized, they are not included in the chemotype descriptions.

To investigate the relationship between chemotype and genetic variation in *B. neritina*, three characters were analyzed in samples from 13 locations at various depths along the California coast (Fig. 2), and from one Atlantic location (North Carolina): (1) bacterial symbiont small subunit ribosomal RNA gene sequence (SSU rRNA), (2) host bryozoan mitochondrial cytochrome *c* oxidase subunit I gene sequence (CO I), and (3) bryostatin content.

Materials and Methods

Sampling Regime:

Colonies of *B. neritina* were collected by hand, off docks or subtidally using scuba, along the California coast from Humboldt bay to San Diego (Fig. 2). *B. neritina* was collected from different depths (ranging from surface to 24 m) at the same location when possible, and at nearby locations in most cases. In addition, one sample was collected in the Atlantic Ocean, from a settling panel array in Beaufort, North Carolina. Samples for chemical analysis were frozen live or dried in the field, then stored frozen at -20° or -80°C. At each site, individual colonies were selected for DNA analysis and placed immediately in 95% ethanol in the field and kept cool until stored at -20°C.

DNA Extraction, polymerase chain reaction and sequencing:

DNA was extracted from individual colonies by using the QIAGEN QIAamp tissue kit and following the instructions for tissue. To avoid contaminating parent-colony DNA with progeny DNA, only the portions of a colony lacking ovicells, which contain larvae, were used. If necessary ovicells were removed with forceps before DNA was extracted.

Polymerase chain reaction (PCR), using symbiont-specific primers and conditions described in Haygood and Davidson (1997), was used to amplify about 1060 bp of the "*E. sertula* " SSU rRNA gene. Approximately 710 bp of the bryozoan mitochondrial cytochrome *c* oxidase subunit 1 gene (CO I) was amplified using primers and conditions described in Folmer *et al.*. 1994. Taq polymerase (Boehringer Mannheim) and included buffer were used for all reactions.

Sequencing was performed using the Applied Biosystems, Inc. (ABI) Prism ready reaction kit FS using guidelines provided by ABI, and reaction products were analyzed on an ABI 373A automated sequencer. The SSU rRNA gene was sequenced using symbiont-specific and universal eubacterial SSU rRNA primers (Haygood and Davidson,1997; Lane,1990). The CO I gene was sequenced using the amplification primers. Sequences were aligned in AssemblyLign (Kodak), and differences were counted and compared against the total base pair count to calculate percentage differences. PAUP was used for maximum parsimony phylogenetic analysis

(Swofford,1990), with a transversion cost of 2 relative to transition. Position 365 was excluded from the analysis due to a possible sequencing artifact. A heuristic search was conducted with 10 starting trees created by random addition and one best tree was found; bootstrap resampling was done 100 times.

*Nhe*I restriction digest

A sequence difference in the SSU rRNA gene between two detected strains of "*E. sertula*" allows rapid identification of strain type by digestion with *Nhe*I (see Fig. 3). The *Nhe*I restriction enzyme cuts only the amplified SSU rRNA gene from one strain (type D) into two fragments. The SSU rRNA gene amplification products were cleaned using the QIAGEN QIAquick PCR purification kit, then digested with *Nhe*I (Boehringer Mannheim) and analyzed by agarose gel electrophoresis.

Bryostatin content analysis

Bryostatin content analyses were performed by Dr. M. Koleck of SAIC-Frederick, under contract to the National Cancer Institute (NCI). *B. neritina* samples, 50-90 grams wet weight from each sample site, were sent frozen to SAIC-Frederick, where they were lyophilized, extracted and analyzed for bryostatin using high-pressure liquid chromotography (HPLC) (Schaufelberger et al., 1990). A portion of each dried sample was removed for extraction and HPLC analysis. Subsample size was 5 grams except for samples HB1 (4.44 g) and HB2 (3.47g). A Rainin-Microsorb column (3 μm

C-18, 100 x 4.6 mm I.D.) and corresponding precolumn (15 x 4.6 mm I.D.) were used for HPLC separations. The system includes a Waters 600 E System Controller, a Waters 715 Ultra Wisp Sample processor, and a Waters 996 Photodiode Array Detector. The mobile phase was aqueous 78% MeCN, UV, at 1 mL/min. Samples were dissolved in MeOH (2mL). All sample solutions were filtered through Anotop 0.2 μm filters prior to injection (10 μL).

A bryostatin 1 standard was used to establish a bryostatin 1 standard curve to determine the amount of bryostatin 1 in each sample (data not shown). The NCI standard method for bryostatin analysis was established on the basis of HPLC retention times, absorbance spectra, and phorbol dibutyrate displacement assays of peaks from many *B. neritina* samples. Co-injections of bryostatin 1, 2 and 3 standards confirmed the validity of the method. The results of extensive analysis of *B. neritina* samples has shown that retention times and absorbance spectra are sufficient to identify bryostatins (David Newman, NCI, pers. comm.). The presence of bryostatins 1, 2 and 3 was determined by the appearance of peaks at established HPLC retention times (~11.3, ~5.2, and ~8.1 min respectively) and confirmed by absorption spectra for these peaks scanned from 200 to 600 nm. The diagnostic UV absorbance peaks for the bryostatins that contain the octa-2,4-dienoate ester are at 230 nm and 265 nm.

The presence of the minor bryostatins was determined, but individual minor bryostatins were not identified. The minor bryostatins (4-11) each have different HPLC retention times and similar absorbance peaks at 230 nm but not at 265. Most of

the common minor bryostatin HPLC peaks are observed at ~9.8 min and ~6.5 min retention. If HPLC peaks cannot be confirmed by the absorbance spectra, the bryostatin in question is scored as not detected.

Chemotypes were assigned as follows: if the minor bryostatins were detected in the absence of bryostatins 1-3, chemotype M was assigned. If bryostatins 1-3 were detected, chemotype O was assigned. In chemotype O *B. neritina*, bryostatins 1-3 are found at much higher levels than the minor bryostatins 4-9 (Pettit, 1991). Thus if minor bryostatins are detectable and bryostatins 1-3 are absent, the sample can be assigned to chemotype M with confidence.

Results

Symbiont strain analysis

The "*E. sertula*" SSU rRNA sequences from populations in Southern California, Northern California, and North Carolina (Atlantic) were identical except for consistent differences at four nucleotide sites within the 1024 bp sequenced from seven locations (Fig. 3). These differences are fixed, not base-paired with each other; they divide the samples into two strains, D (deep) and S (shallow). One of these polymorphisms fell within a restriction enzyme recognition site such that digestion of the SSU rRNA gene amplification product with *Nhe*I could be used to screen individual *B. neritina* colonies for "*E. sertula*" strain (Fig. 3). In Southern California strain, sites less than a mile apart were found to have different strains: strain S at sites shallower

than 9m and strain D at sites deeper than 9m (figs. 2,4). In Northern California, colonies containing strain D symbiont were found on the same docks (HB2, Humboldt bay, Woodley Marina) and shallow rocks (PP3m2, Patrick's Point, 3 m) as colonies containing the strain S symbiont (HB1 and PP3m1). In Bodega Bay (BB) and Tomales Bay (TB; not shown in Fig. 4 or 5, sequence data similar to BB) only the strain S symbiont was detected. To date, both strains of "*E. sertula*" have not been found within the same colony of *B. neritina*.

Host bryozoan CO I gene sequence analysis

At most locations sampled a single "*E. sertula*" strain was detected within the *B. neritina* population. For sequence analysis of the CO I genes, a region of 480 nucleotides was examined. For locations shown to contain a single "*E. sertula*" strain, one or two samples were sequenced, on the assumption that hosts with similar genotypes would contain similar "*E. sertula*" strains. In the case of Northern California, where both symbiont types were detected at the same location, the CO I gene was sequenced from two colonies of each representative type to ensure detection of differences between the hosts. To confirm the accuracy of using the single-stranded CO I sequence, five samples (each from a different site) were sequenced for 638 bp on both strands; all showed similar results. Two distinct lineages (D and S) of *B. neritina* were found. The difference between the two was 8.1% for the 480-bp region (Figs. 4, 5) and 9.1% over 638 bp data not shown). Additional sequence differences were

detected within the type S *B. neritina*, distinguishing two groups that are much more closely related. Both S types were found in Southern California. At Catalina Island, both S_1 and S_2 were present, showing that these two genotypes are found in the same area and at similar depths.

Colonies of CO I genotype D contained "*E. sertula*" strain D, and colonies of CO I genotype S contained "*E. sertula*" strain S.

Bryostatin content analysis

Bryostatin analysis by HPLC showed that bryostatins of chemotype O (bryostatin 1-3) were detected consistently in *B. neritina* collections from deeper than 9 m in Southern California, and the minor bryostatins of chemotype M (bryostatins 4-9) were detected in *B. neritina* from shallower than 9 m (Figs. 2, 4). Several of these populations have been sampled repeatedly for bryostatin analysis for other studies as well as for this paper and have given consistent results.

At sites farthest north, separation of chemotypes by depth was not apparent, but this result must be interpreted with caution because the chemistry data from *B. neritina* taken in the Humboldt area are ambiguous. HPLC peaks indicative of minor bryostatins, but no peaks for bryostatin 1, 2, or 3, were detected in a *B. neritina* sample from deeper than 9 m (PP10m, Patrick's Point, submerged rocks outside of Humboldt Bay). These findings are indicative of chemotype M. Absorbance spectra were weak, but consistent with minor bryostatins. This sample had CO I type S and "*E. sertula*"

strain S. One of the dock samples from Humboldt Bay (HB2) yielded HPLC peaks at the correct retention times for bryostatins 1, 2, and 3, and the minor bryostatins, indicating chemotype O. However, the absorbance spectra were too weak (bryostatin 3), or were possibly obscured by compounds in overlapping peaks, to positively confirm the bryostatin identification. For these two Northern California samples, chemotype designations are tentative due to lack of clear confirmation by absorbance spectra.

Consistently, the collections of *B. neritina* with type D CO I genotype and strain D "*E. sertula* " contained the bryostatins of chemotype O. Those samples with genotype S host and symbiont contained bryostatins of chemotype M.

Discussion

In this study we identified two types (D and S), probably species, of the marine bryozoan nominal species *Bugula neritina* that differ in their mitochondrial CO I sequences by approximately 8 %. Each type harbors a distinct strain of the bacterial symbiont "*E. sertula*" and has a characteristic chemistry profile. Both of these *B. neritina*/ "*E. sertula* " associations produce bryostatins, but only type D contains the additional bryostatins, 1-3, characterized by the octa-2,4-dienoate ester at C-20 of the bryopyran ring (Fig. 1). We found that, without exception, the type D *B. neritina* lineage contains strain D "*E. sertula*" and chemotype O bryostatins. The type S *B. neritina* lineage contains strain S "*E. sertula*" and bryostatins of chemotype M (Fig. 4).

In Southern California, the two types of *B. neritina* were found in the same areas but at different depths (transition at about 9 m). In Northern California, colonies containing type D host and symbiont were found on the same docks (Humboldt Bay) and shallow 3-m rocks (Patrick's Point) as colonies containing the type S host and symbiont. This pattern suggests that the two types are separated by an environmental parameter such as temperature, as has been found in some other marine invertebrates (Hellberg, 1998; Knowlton, 1993). In Southern California, where the two types are separated, surface waters become several degrees warmer in the spring through early fall. In Northern California, however, the surface remains much cooler. Additional research must be conducted before any conclusions can be drawn regarding the cause of the separation. The North Carolina *B. neritina* matches the type S *B. neritina* in California. The predominance of type S on boat docks and hulls and its appearance in both the Atlantic and Pacific indicates that this genotype of *B. neritina* is transported on boat hulls and may have a worldwide distribution mediated by human transport. The type D genotype, however, has a more restricted distribution that relies on larval dispersal and promotes divergence between widely separated populations. Additional samples from deep locations in the Atlantic and other areas will be valuable in confirming this hypothesis. The type S may have a broader temperature tolerance or other characters, such as better pigment protection from UV, that allow it to survive conditions it may experience on boat hulls. Taken together, these data suggest that two sympatric species of *B. neritina* exist in California.

A serendipitous experiment provided evidence that the type D *B. neritina* does not colonize shallow habitats in Southern California even if type D larvae are introduced there. *B. neritina* is a normal inhabitant of the Scripps Pier, La Jolla, California. Over a period of 3 years, larvae from Palos Verdes parent stock (consistently chemotype O and type D genotype) collected at about 16.5 m have been poured into the water around the pier as a consequence of aquaculture research. Cumulatively, these releases, each lasting 2 weeks and taking place three or four times yearly from spring through fall, put hundreds of thousands of larvae into the water. In November 1997, approximately a month after the end of the summer season of larval release, samples were taken from the Scripps Pier pilings in an attempt to detect any type D *B. neritina* that may have become established over the 3-year period. At the time, the only *B. neritina* colonies present were located at the bottoms of the pilings (4.5-7.5 m). The colonies were small (5 cm tall), which matched expected colony size for the settlement dates of the recently released larvae. Collections were taken from each northern piling along a transect from just below the location of release out to the end of the pier. Five colonies from each of the 6 pilings sampled were tested for symbiont genotype. Strain D symbionts were not detected in any of the samples. All the sequences and *Nhe*I digests of the SSU rRNA "*E. sertula*" gene taken from previous pier samples over 3 years have been strain S. The host bryozoan CO I gene was sequenced from two individuals, and these sequences matched type S. Bryostatin analysis of each of the pier samples and of previous collections revealed only the

bryostatins of chemotype M, consistent with the genotype found. These results suggest that the type D *B. neritina* is not able to establish a major population on the pier.

The finding that chemotypes O and M occur in distinct lineages of *B. neritina* indicates that there is a genetic basis for patterns of bryostatin synthesis. Previous experimental work has shown that bryostatins do not accumulate from the diet (Thompson and Mendola,1993), but did not rule out the influence of other environmental factors in determining the types of constituents used in bryostatin synthesis. A type D shallow sample (HB2) probably contains chemotype O bryostatins (by HPLC retention times), whereas a type S sample at 10 m (PP10m) does not, and is probably chemotype M. If so, these samples strengthen the argument that bryostatin substituents are determined by genetic capabilities and not by environmental factors associated with depth. It is possible that the chemotype differences are due to the different strains of "*E. sertula*" in the two types, to a difference in the host bryozoan, or to an additional unknown bacterium. In any case, one *B. neritina* / "*E. sertula*" mutualism is capable of making the octa-2,4 dienoate substituents of bryostatins 1-3, 12, and 15, and the other is not. It is also possible that there are additional genotypes of *B. neritina* /"*E. sertula*," and new bryostatins may be discovered in areas that remain to be sampled.

In some cases the consistent re-isolation of natural products from marine invertebrates has been problematic, possibly because of the difficulty in distinguishing closely related species or subspecies. On the other hand, geographic variation in bioactive compounds from a few bryozoans has been attributed to environmental

factors (Christophersen,1991). The present study provides evidence that invertebrate sibling species can contain different natural products, and it illustrates that distinguishing variants is important to ensure consistency in natural product chemistry. Variations in secondary metabolite chemistry should be included among the reasons, discussed by Knowlton and Jackson (1994), that proper taxonomy is critical to understanding the diversity, evolution, and ecology of a system. Molecular characterization provides a powerful tool for this endeavor. The chemistry, chemical ecology, and biology of variants may differ slightly but significantly, as in the case of *B. neritina,* where the genotypes are indistinguishable in the field, but one produces the commercially important compound and the other does not. Moreover, these chemical differences may determine the predators to which the colonies are susceptible. It is possible that these or other subtle chemical differences affect chemical defense and thus influence the ecology of the bryozoan.

Genbank Accession #s: PP3m1, AF061429; PP3m2, AF061430; PP10m1, AF061431; HB1, AF061427; HB2, AF061428; BB, AF061426; CI1, AF061417; CI2, AF061418; CI3, AF061419; CI4, AF061420; CI5, AF061421; PV, AF061422; NP, AF061423; TP AF061424; SP, AF061425; NC, AF061432; *B. pacifica*, AF061433.

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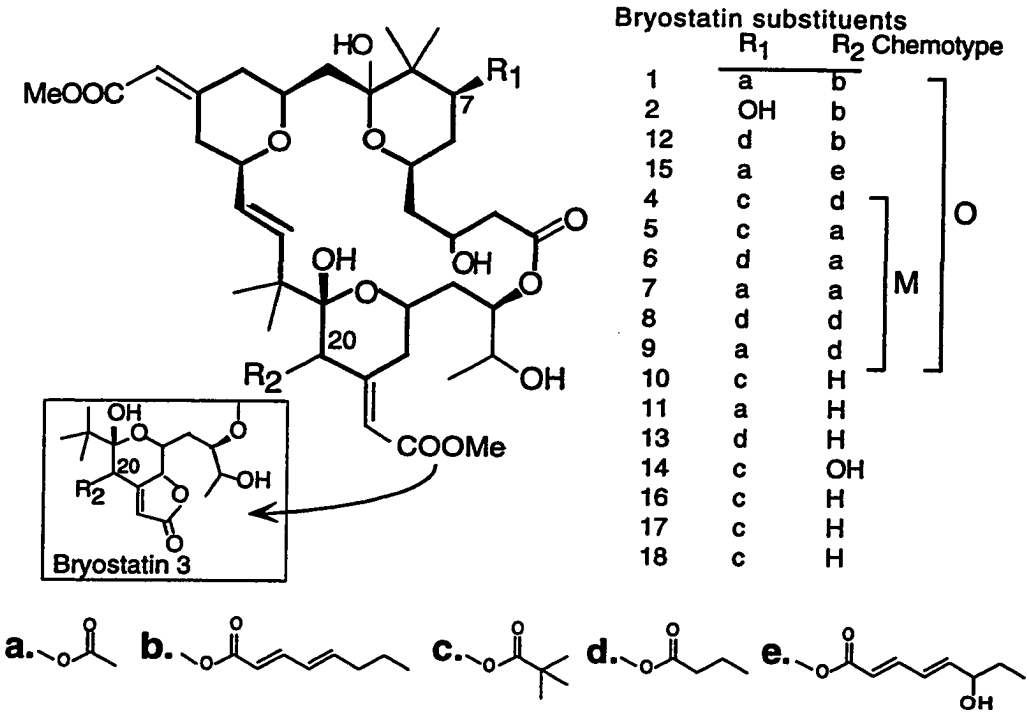

Figure 1. Structure of bryostatins. The bryopyran ring is in bold. The bryostatins of chemotypes M and O are bracketed.

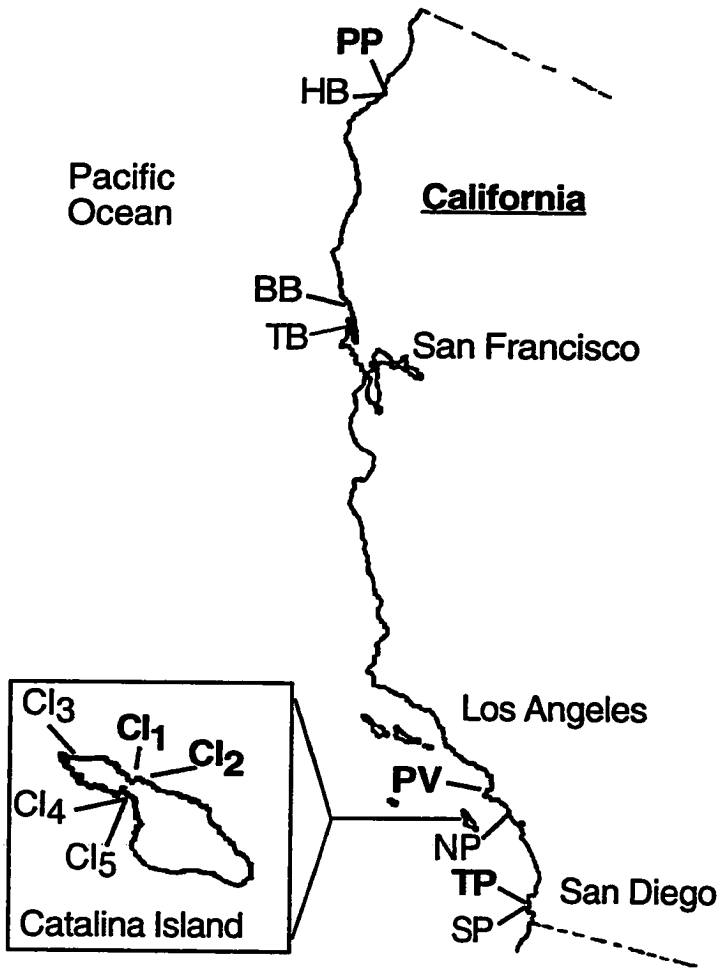

Figure 2. Sampling locations along the California coast. Locations labeled in bold are deeper than 9 m, locations in plain text are shallower than 9 m. Abbreviated site names are designated in Figure 4.

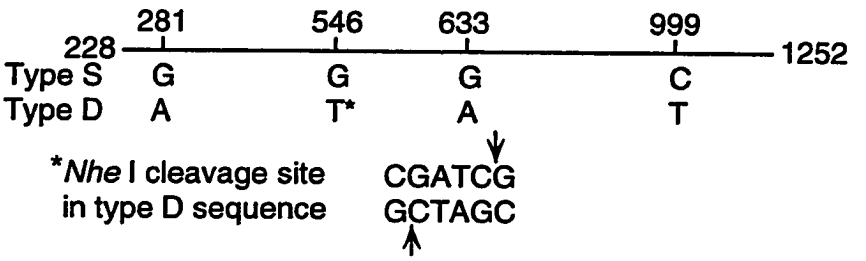

Figure 3. Polymorphic sites in the SSU rRNA gene of "*Endobugula sertula*" strains D and S. The fragment represented results from PCR amplification of the SSU rRNA gene using the symbiont specific primers from Haygood and Davidson, 1997. Nucleotide numbers are based on alignment with *E. coli* SSU rRNA gene.

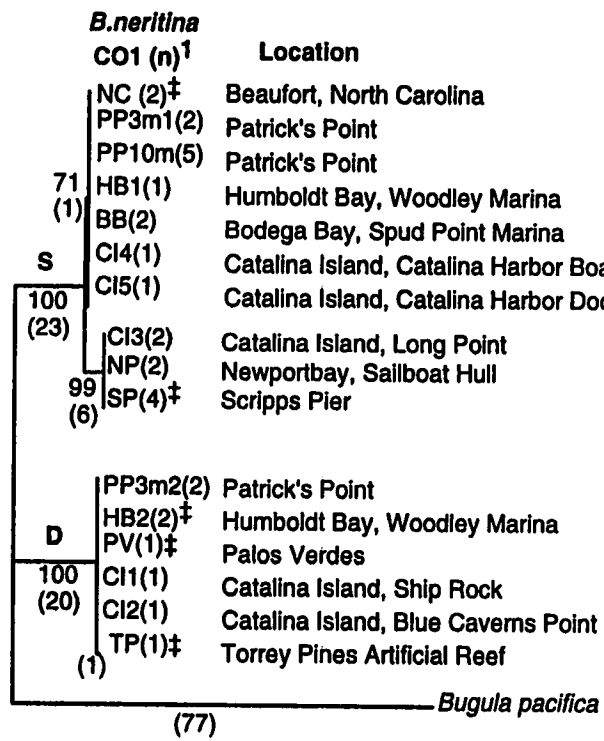

Figure 4. Summary of results. 1,Phylogenetic relationships among *B. neritina* CO I genes based on 480 nucleotides. *Bugula pacifica* is selected as the outgroup. Horizontal branch lengths are proportional to the number of steps separating groups. Numbers show the bootstrap support of the labeled nodes. Numbers in parentheses indicate the number of steps between nodes. ‡, 638 bp sequenced on both strands. 2,M, minor bryostatins detected; O, bryostatins 1-3 and minor bryostatins detected. *, tentative, based on HPLC retention times, and very weak absorbance spectra for bryostatin 3 and minors. ND, bryostatins not detected. 3,"*E.sertula*" SSU rRNA genotype determined by *Nhe*I digestion as shown in Fig. 2. †, genotypes confirmed by sequencing.

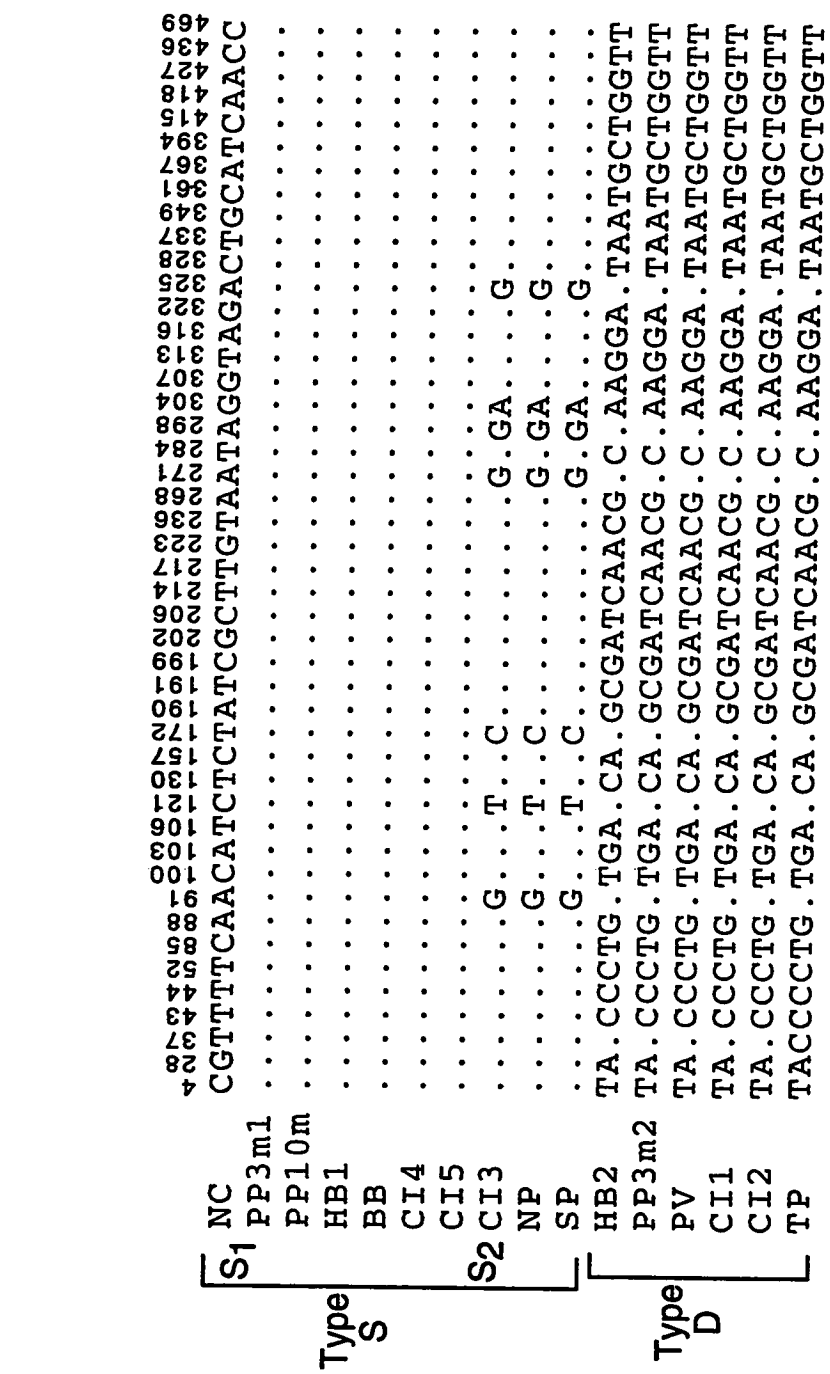

Figure 5: *Bugula neritina* cytochrome *c* oxidase subunit I gene alignment showing only the variable sites. Sequence corresponds to position 1521-1990 in the published *Drosophila yakuba* mitochondrial genome sequence (Clary and Wolstenholme, 1985) with nucleotide position number noted above. The brackets indicate sequences for *B. neritina* varieties type D and S, including the differences within type S as S1 and S2.

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Chapter 5

Effects of eliminating most of the symbiont "*Candidatus* Endobugula sertula" from *B. neritina* on growth and bryostatin production.

Introduction

Bugula neritina harbors the gamma proteobacterium "*Candidatus* Endobugula sertula" whose benefit to the host is unknown. This bacterial symbiont is passed to the larvae prior to their release from the adult. The larvae hold *"E. sertula"* cells in their pallial sinus which seems specialized for this function. The bacteria are located deep within the pallial sinus evenly distributed around the base, and held between folds of the epithelium, effectively closed off from the sea water (Woollacott, 1981). The evolution of this mechanism to ensure the inoculation of the next generation with *"E. sertula"* implies an important function for the symbiont in the survival of the host. Possible reasons for this mechanism may be that the symbiont is 1) difficult to obtain from the sea water, 2) an obligate symbiont or 3) only able to infect properly at a particular developmental stage in the animal, such as prior to or during metamorphosis of the larvae to the first zooid of the colony, the ancestrula. The larvae are released from the adults competent to settle and spend only a few hours in the water column which may make it difficult to acquire the proper bacteria from the water column prior to settlement. However, the larvae often settle near other *B. neritina* colonies. If the bacteria were free living or released from the adults and/or able to enter a developing colony, this highly developed mechanism for transfer of the symbiont would seem unnecessary.

A typical function for a symbiont is to provide an essential growth factor or nutrition of some type. An alternative is that the symbiont produces toxins that defend the host against predators and competitors in the fouling community. The *B. neritina* /

"E. sertula" association produces bryostatins, cytotoxic compounds currently under study for their anticancer therapeutic potential. Bryostatins are suspected of being bacterial products not synthesized by the host. The complex polyketide structure of bryostatins places them in a class of compounds made primarily by bacteria and fungi. Complex polyketides are known from a few sponges, Miyakalide, swinholide A, halichondrin and spongistatin (Uemura et al., 1985; Hirata and Uemura, 1986; Kobayashi et al., 1990; Higa, 1992; Bai et al., 1993; Litaudon et al., 1994) but the source of these is possibly bacterial. Sponges harbor numerous and diverse bacteria and at least in the case of swinholide A, the compound was shown to be of bacterial origin (Bewley et al., 1996). No other metazoans are known to synthesize complex polyketides. In addition, most other secondary metabolites known from bryozoans are alkaloids, and none are related to bryostatins (Chapter 2 for review). Other closely related compounds have been found in sponges (bryostatins A and B, miyakolide) and the marine actinomycete *Streptomyces griseus* SS-20 (aplasmomycins) (Nakamura et al., 1977; Pettit, 1991; Higa et al., 1992). Bryostatins A and B were found in a sponge growing around *B. neritina* colonies and may originate in the *B. neritina* (Pettit, 1991). Most well studied complex polyketides are known from actinomycetes, however they have been found from a diverse range of organisms including a gamma proteobacterium, *Pseudomonas fluorescens* (Nowak-Thompson et al., 1997).

Although the symbiont *"E. sertula"* is not an actinomycete, it is a prime candidate for the bryostatin producer. This bacterium is 1) found in all populations of *B. neritina* examined, 2) passed to the larvae in a highly evolved mechanism to ensure

transfer to the next generation, and 3) found throughout the colony. Moreover, a difference in symbiont strains is associated with a chemical difference in bryostatins (Davidson and Haygood, 1999; Chapter 4). Fungi have not been observed in any of the many histological studies of *B. neritina* and it is unlikely bryostatins originate from a fungus (Woollacott, 1971; Woollacott and Zimmer, 1975; 1977; 1978). Bacteria observed in the funicular cords by electron microscopy, including those that lead into the ovicells, are of similar morphology to the cells seen in the pallial sinus of the larvae (Woollacott, 1981; Zimmer and Woollacott, 1975).

This study addresses two main questions: 1) is the symbiont required for growth, providing a necessary nutritional factor, and 2) does the symbiont produce bryostatins? In an attempt to answer these in part, *B. neritina* colonies were treated with antibiotics to eliminate their symbionts and grown out over 2-3 months, then the symbiont levels, growth of the colonies and the bryostatin activities were compared between colonies with greatly reduced symbiont numbers and control colonies.

Hypotheses

1. If the bacterial symbionts are required for synthesis of the bryostatin then decreasing or eliminating the symbionts will lead to a decrease or lack of bryostatin in the *B. neritina* colonies.

2. If bacteria are of nutritional value, and necessary for normal development and growth, then elimination of most of the symbionts should have a detrimental effect on growth. If, on the other hand, bacteria are not providing necessary nutrients, elimination of symbionts should have no effect on growth.

Methods

Collection and settlement of *B. neritina* larvae in the lab:

Adult *B. neritina* was collected from Torrey Pines artificial reef (La Jolla), Palos Verdes (Long Beach) and Scripps Pier pilings (La Jolla), then maintained in aquaria with running seawater. To stimulate larval release, colonies were exposed to bright sunlight. It is important to keep colonies in the dark at night in order to stimulate release of larvae during the day. I found that if they are maintained under constant light, larvae may not be released in a daytime pulse as needed for collection. A series of screens were placed in the outflow to collect larvae and eliminate larger particles. Larvae released from the colonies were collected on the 100 μm mesh screens then washed into freshly filtered (0.2 –1.0 μm filter pore size) sea water and allowed to swim. A light source attracted larvae to one side of the tank or beaker and

larvae were transferred into fresh-filtered seawater. This process was repeated two or three times to obtain clean larvae, and eliminate as many other invertebrate larvae as possible. This method does not eliminate other positively phototactic larvae of similar size. Larvae were concentrated (approximately 8-10 per ml) and placed in appropriate containers for settlements.

Determining appropriate settlement substrate

For preliminary settlements to determine appropriate settlement substrate to use during antibiotic treatments and grow-out experiments, larvae were settled in aquaria with a variety of possible settlement surfaces. Larvae were placed in 5 liter aquaria with vigorous aeration on both ends and in each corner, to prevent settlement of larvae on the air-water interface and minimize settlement on the bottom of the tank. Plastic plates, ceramic tiles with a glazed side and un-glazed rough side, clean glass slides and seasoned glass slides (in sea water for 36-48 hrs) were placed in the aquarium and examined after 24 hrs allowed for larval settlement.

For antibiotic treatment experiments, larvae were settled onto polycarbonate petri dishes (Keough, 1989) mounted on plastic plates (13 cm by 13 cm, four dishes per plate) by 'hot-glue'. The plastic glue was allowed to cool and air overnight before settlements. Plates were placed in the dark for settling to avoid uneven settlement. If there is a light source the larvae will tend to settle to one side of the plate.

Determining appropriate antibiotic, dose and duration

Initially, small scale settlements were exposed to various antibiotics for varying lengths of time (5-14 days) and doses to determine effective treatment. Appropriate antibiotics would not affect growth of the host as compared to the controls, and would eliminate the symbiont as determined by a specific symbiont PCR assay (see below). Table 1 shows the antibiotics tested and the results. In general none of the cell wall synthesis inhibitors had any affect, which was expected given that *"E. sertula"* is a gram negative proteobacterium.

Settlement of *B. neritina* in the lab for Gentamicin sulfate treatments

Adult *B. neritina* was collected from Torrey Pines artificial reef (9/96, 6/98) and from Palos Verdes (3/97, 6/98), and maintained in aquaria with running seawater. Both of these populations have been previously shown to contain bryostatins 1-3. Larvae were collected as above, and settled in petri dishes mounted on plastic plates (approximately 200 larvae per dish). The larvae were allowed to settle overnight, then half of the plates containing the developing colonies were treated in the petri dishes with seawater containing 100 μg/ml Gentamicin sulfate from day 1 to day 8 post settlement (figure 1). The other half were left untreated as controls. Any free swimming larvae remaining were removed at this time. The sea water was changed daily including freshly prepared antibiotic solution. Once the ancestralae developed a feeding lophophore (48-72 hrs), then phytoplankton was added to the plates after each water change.

After antibiotic treatments were completed, the plates were suspended vertically in tanks (25-40 L) with aerated sea water. Water temperature was maintained at 18-20°C, with occasional fluctuations to 16°C and 21°C. Water was changed every 48 hours, and phytoplankton was added after the exchange. *B. neritina* cultures were maintained on a mixed phytoplankton diet in non-sterile filtered seawater. Fresh seawater passes through a series of sand filters before coming into the labs. In addition the water was filtered to remove large particles and marine invertebrate larvae that pass through the sand filters. Preliminary experiments had shown that it was not necessary to maintain the antibiotic-treated *B. neritina* in sterile seawater in order to prevent the symbiont, *"E. sertula"*, from repopulating the colonies. After antibiotic treatments, the level of *"E. sertula"* infection remains very low in the treated bryozoan colonies even after several months of growth in natural, non-sterile seawater. Other free living bacteria are allowed to colonize both the control and treated colonies such that the resident symbiont *"E. sertula"* (originally obtained from the parent colonies prior to settlement) should be the only bacterial species that varies significantly between the control and treated colonies.

Phytoplankton culture and preparation

Phytoplankton was grown in Guillard's F/2 medium (Guillard, 1975), except for *Rhodomonas* which was grown in F medium, at 20°C under constant light. *Rhodomonas* sp., *Ellipsoidea* sp., *Nannochloris* sp., *Isochrysis*, and *Monochrysis* were used. The diet was 50 % *Rhodomonas* with approximately equal proportions of the

others. The phytoplankton cultures were harvested 4-6 liters at a time by centrifugation at 4000 x g for 15-20 minutes. The pellets were resuspended in fresh sea water and equal amounts were poured into each tank. The exact final cell density was not determined. The colonies were fed sufficient amounts for good growth (empirically determined), and so that when observed under the dissection scope phytoplankton could be seen in the guts, and fecal pellets were produced regularly. In final experiments 4-6 liters of dense culture were harvested for four 25 liter aquaria per feeding.

Tetraselmas sp. was initially used, but I observed *Tetraselmas* cells swimming away from the fecal pellets undigested (confirmed with compound microscope), and after two days, the colonies failed to clear these cells from the water in these dishes. *Tetraselmas* passes through *B. neritina* undigested and apparently unharmed.

Observations and Sampling colonies for DNA and Bryostatin activity assays

The cultures were examined weekly by eye and less frequently with a dissection microscope to check health, level of fouling and growth. Several plates and at least 25-35 colonies were examined. Detailed notes on growth were taken approximately every 2-3 weeks. However, failure to record some of the growth data has left gaps in some of the growth records.

Periodically healthy *B. neritina* colonies were plucked off the dishes at the base using forceps for use in symbiont assays and bryostatin assays. Multiple colonies were pooled together for the assays. Healthy colonies contain live zooids and may have

brown bodies. Empty colonies, obviously dead, were avoided.

Symbiont Detection Assays

DNA was extracted using the QIAGEN tissue kit, and quantified on a spectrophotometer at 260 and 280 nm wavelengths. The quality of the DNA was checked by running on an agarose gel stained with ethidium bromide and examined on a UV transilluminator. The relative levels of symbiont remaining were determined using symbiont-specific primers in a PCR assay which specifically amplifies the *E. sertula* small subunit ribosomal RNA gene (SSU rRNA) as described in Haygood and Davidson, 1997 (see Chapter 2). This assay is very sensitive, detecting *E. sertula* DNA in only 1 ng of host DNA (10 pg/µl). The symbiont SSU rRNA gene was amplified from a dilution series of DNA from both the treated and the untreated control colonies in order to estimate the relative levels of symbiont remaining in the antibiotic-treated bryozoan colonies. In addition 25 ng of both control and treated colony DNA was used to amplify total bacterial SSU rRNA genes using universal bacterial primers 27F and 1492R (Lane, 1990) to compare total bacterial DNA signal between the two treatments.

Denaturing Gradient Gel Electrophoresis (DGGE) studies

After growing in non-sterile sea water for 2-3 months, the *B. neritina* colonies have been colonized by other bacteria from the sea water (growing on their surfaces and ingested) in addition to the resident symbiont "*E. sertula*" which is in the larvae

prior to settlement. Differences in this microbial community between the control and antibiotic-treated colonies may arise as a result of either the antibiotic treatments, or the reduction of the bacterial symbionts. Changes in the levels of bryostatins could theoretically be due to a difference in another bacterium besides "*E. sertula*." In order to compare the total bacterial community between the control and treated colonies, denaturing gradient gel electrophoresis was employed (Muyzer et al., 1993; Ferris Muyzer et al., 1996).

Gene amplification

A portion of the SSU rRNA gene was amplified using a conserved bacterial primer 1055f, 5'-ATggCTgTCgTCAgCT-3', and a universal primer with a GC clamp 1392r-GC clamp, 5'-CgCCCgCCgCgCCCCgCgCCCgg CCCgCCgCCCCCgCCCC-ACgggCggTgTgTAC-3' (Ferris et al., 1996). For amplification from *B. neritina* DNA 100 ng of DNA was used in 50 µl reactions with *Taq* polymerase and buffer (Boehringer Mannheim) as described in Haygood et al., 1992, with 30 cycles of 94°C (1 min): 50°C (1min): 72°C (1min), and a 7 min 72 °C final primer extension. PCR products were cleaned using the QIAGEN QIAquick PCR clean up kit, and total eluted volume of PCR products was 30 µl to allow loading most of the reaction onto the DGGE gel (20 µl).

Gel preparation and electrophoresis

Acrylamide gels (6.5 %, 1:37 Bis-Acrylamide ratio) were prepared and run using 0.5X TAE buffer (1X TAE buffer is 0.04 M Tris base, 0.02 M sodium acetate, and 1 mM EDTA, pH adjusted to 7.4). Acrylamide solutions (6.5%, 0.5X TAE buffer pH 7.4) containing 0% and 100% denaturant (urea and formamide, UF) were mixed to make 35% and 65% UF solutions. 100 % UF solution was defined as 40 % vol/vol formamide and 7.0 M urea. TEMED (N,N,N',N'-tetramethylethylenediamine) and 10 % APS (ammonium persulphate) solutions were then added to initiate polymerization of the acrylamide. These two solutions were mixed as the gel is poured to create a gradient from 65% UF (bottom) to 35% UF (top) with denaturant increasing in the direction of electrophoresis.

Samples were loaded into wells with Bromophenol Blue running solution (10x: 0.25% Bromophenol blue, 40 % w/v sucrose). The gels were run in 0.5X TAE at 60°C with the CBS Scientific Inc. DGGE 2000 system, at 200 V running voltage for five hours. The gel was then stained with 1X SYBR Gold (Molecular Probes, Eugene, OR) in 1X TAE for at least 20 minutes. Bands were visualized on a UV transilluminator, cut from the gel and placed into 1.5 ml epitubes, then 400 ul of 1X SSC buffer was added, the gel was homogenized and incubated at 37°C overnight. Gel fragments were spun down, supernatant transferred to a clean tube and the DNA was precipitated with 0.5 M LiCl in 95% ethanol. The DNA was pelleted, resuspended in 20 µl sterile dH_2O and used in reamplification of the band for sequencing. The original PCR primers listed above were used for PCR reamplification and sequencing. Cycle sequencing was

accomplished with the ABI (Applied Biosystems, Inc.) BigDye reaction kit following manufacturers instructions. Sequencing reactions were analyzed on an ABI 373 automated sequencer.

Sequences corresponding to the suspected "*E. sertula*" band were compared with the "*E. sertula*" sequence to confirm identity. Then an "*E. sertula*" standard was amplified with the primers described above from clean symbiont SSU rRNA gene amplification products. This standard is run on the gels along with the samples to locate the "*E. sertula*" bands. For the comparison between the bacterial signals from the control and treated colonies, the banding pattern was compared to see if there were differences between the two but these were not sequenced.

Polyketide Synthase gene detection

Degenerate primers derived from conserved regions of beta-keto acyl synthase domains of bacterial type I polyketide synthase (PKS-I) genes were used to clone a portion of a PKS-I ß-keto acyl synthase from both larvae and adult *B. neritina* DNA (KSa; see Chapter 3 supplemental pages). From this cloned fragment, specific KSa primers were designed and shown to specifically amplify the KSa gene fragment from *B. neritina* (Chapter 3 suppl. pgs.). These KSa primers were used to amplify genes from the control and treated *B. neritina* to assess the level of this gene signal in those colonies lacking most of their symbionts.

Bryostatin Activity Assays: Tritiated Phorbol dibutyrate Displacement from Protein Kinase C (PKC) on rat brain liposomes

Bryostatins bind to Protein Kinase C (PKC) at the same site as phorbol ester, but the bryostatins have a much higher affinity under assay conditions (below) and irreversibly displace the phorbol from the binding site on PKC (DeVries et al., 1988). Crude extracts containing bryostatins together with tritiated phorbol dibutyrate (^{3}H-PDBu) are added to a suspension of rat brain liposomes rich in PKC. The bryostatin displaces the tritiated phorbol on the receptor sites, then the liposomes are captured on a glass fiber filter, un-bound signal is rinsed off and the tritium signal is read on a scintillation counter. A decrease in the tritium signal relative to the negative control (total bound signal) indicates bryostatin activity. This method was adapted from the protocol described in Schaufelberger et al., 1990 with assistance from Mary Koleck of the NCI for detecting bryostatin activity in extracts (Schaufelberger et al., 1990; DeVries et al., 1988; DeVries et al., 1997). This assay is not specific to bryostatins but indicates the presence of PKC binding and correlates very well with the presence of bryostatins in *B. neritina* samples.

DeVries et al., 1997, describes using crude ethanol extracts directly in the phorbol dibutyrate displacement assay to screen marine organisms for PKC binding activity in a similar manner to our protocol. The NCI, however, purifies the extracts further by fractionating the extracts on a silica column using dichloromethane, ethyl acetate and methanol, and collecting the ethyl acetate fraction. All the bryostatins elute with the ethyl acetate. I chose not to use this additional clean up step because of the

small sample sizes and I wanted to be able to directly relate weight of the sample to activity in order to compare treatments. If I had run extracts through columns prior to the assay, an additional sample size variable is added, and I risk losing some of the sample. The weakness of the assay we conducted is that it identifies bryostatin-like activity, but does not confirm for certain that the activity is due to bryostatins. The NCI analyzed large numbers of samples of *B. neritina* over a period of years and did not find PDBu displacement activity associated with anything other than bryostatins in *B. neritina* (unpublished data). *Scrupocellaria* sp. occasionally settled and grew in the cultures of *B. neritina*, as did *Bowerbankia* sp. These *Scrupocellaria* colonies were used as controls for bryozoan extract PDBu displacemtent activity and showed none indicating that the activity in the *B. neritina* extracts is due to bryostatins (figure 2).

To prepare *B. neritina* extracts, fresh or frozen (-80°C) samples (4-8 mg) were lyophilized, weighed in pre-weighed 1.5 ml tubes then homogenized in 95% ethanol using an epitube pestle for a final concentration of 12 μg/ml dry weight to volume. Debris was spun down (full speed epifuge for 10 minutes), and the supernatants were used in assays. Samples were run in triplicate for each volume used to generate the displacement curves.

To prepare liposome suspension, one medium male Sprague-Dawley rat brain is homogenized in 30 ml of 10 mM Tris HCL buffer, pH 7.4, using a tissue tearor set at maximum (position 5) until blended fully (30 seconds). The suspension is spun at 16,000 X g for 10 minutes, then the supernatant is discarded and the process is repeated until the pellet is a buff color. Then 30 ml of 10mM Tris buffer are added,

homogenized for 20 sec on full speed with the tissue tearer, and aliquots (1-2ml) are stored at -80°C. This suspension is thawed, homogenized and spun down in a tabletop centrifuge on maximum speed for 10 minutes, then resuspended in the binding buffer (50 mM Tris, HCl pH 7.4, 2 mg/ml bovine serum albumin). If multiple aliquots are thawed for a single experiment, they are pooled, homogenized and this solution is used for binding reactions. For the current study, different batches of liposome suspension have consistently given similar total binding counts for the negative control, and similar displacement values for the positive control (PBBu, no tritium) indicating consistent concentrations of liposomes and PKC enzyme in suspension.

Data from DeVries et al., 1988, shows that the IC_{50} is highly dependent on tissue concentration (receptor concentration) in the assay. Standard amounts of tissue giving reasonable concentrations for IC_{50} values were determined from binding curves given in the De Vries 1988 paper and used in the Schaufelberger, 1990 paper. The 100 µl of suspension as prepared above is the standard amount used in the NCI protocol for screening extracts. This level gives reasonable curves for the sample sizes we test. Bryostatin 1 standard curves were generated to give an estimate of bryostatin levels in the samples. Incubation times, and washes were determined in De Vries et al., 1988.

To set up reaction tubes the appropriate amount of binding buffer, sample (between 2 and 15 ml) and 10 ml of ^{3}H-PDBu (10 nM, 10 µCi) is added to each tube for final volume of 0.9 ml. Then 100 µl of the rat brain liposome suspension is added to start the reaction. A positive control of 10 µl 20 µM cold phorbol dibutryrate, and a negative control with no sample added are also included. The negative control is run in

duplicate for each assay and used as the total signal bound.

Reactions are incubated for one hour at 30°C. This incubation time was empirically determined to allow complete binding, no additional binding, in De Vries et al., 1988. The 1 ml reactions are passed through glass fiber filters (Whatman GF/B) on a vacuum manifold, then rinsed with 2 ml 10 mM tris HCL buffer, pH 7.4. Filters are then placed in scintillation vials and 4 ml of ECOSCINT scintillation cocktail were added. Vials were placed on a shaker for 30 minutes prior to reading the tritium signal on a scintillation counter. To confirm that 30 minutes was sufficient for counts to be released from the filters, a series of samples were read at 30 minutes and again the following day. Although the counts did increase indicating further release of signal, the values for displacement did not change, nor did the variance, indicating that shaking for 30 minutes is sufficient.

The amount of tritium signal lost relative to the total tritium signal in the negative control was calculated and expressed as a percentage. The amount of crude extract required to displace 50 % of the bound tritiated phorbol is the IC_{50} and used for comparing samples. Levels higher than 50 % displacement begin to saturate the binding sites and the curve is no longer linear.

Results:

Determining settlement substrate

The larvae settled well on clean glass, clean plastic and rough parts of ceramic tiles and PVC plates. The best settlement was achieved on polycarbonate petri dishes.

For settlements to be treated with antibiotics, larvae were settled in the petri dishes (Keough, 1989) which allowed treatments in a small volume of water and easy observations under the dissection microscope. Approximately 200 larvae settled per petri dish, including the vertical sides of the dishes, with almost all larvae settling within 24 hours (usually larvae began settling immediately after being placed in the plates).

Determining antibiotic treatments

The results of the antibiotic treatments and symbiont analysis are summarized in Table 1. Antibiotic-treated colonies were initially screened for the presence of symbiont SSU rDNA by comparing equal amounts of DNA from control and treated colonies about 1 month old for symbiont signal. Initial gentamicin treatments as low as 10 μg/ml and 20 μg/ml resulted in a decrease of symbiont signal. Puromycin and polymixin (20 and 30 μg/ml) also reduced symbiont levels but seemed to affect the bryozoan host as well. Dual treatments combining gentamicin with puromycin and polymixin gave no additional symbiont elimination and the growth of the bryozoan was stunted. The cell wall synthesis inhibitors did not eliminate "*E. sertula*", as predicted by the fact that "*E. sertula*" is a gram negative proteobacterium. However, since cultures of many marine invertebrates are treated with streptomycin and penicillin, they were tested. Gentamicin up to 150 μg/ml did not affect the growth of the host bryozoan and was the most effective antibiotic against the symbiont in treatments lasting 7 to 10 days. A dose of 100 μg/ml for 7 days beginning immediately after larvae settle was chosen as the optimal treatment to reduce the levels of the symbiont.

Growth

The growth of both the treated and the control *B. neritina* colonies was similar, showing no detrimental effects of the gentamicin treatments (up to 150 µg/ml), or loss of symbionts. The zooids, lophophores, and growth form of the colonies appeared normal relative to the control colonies. In all cases the range of colony sizes increased as growth progressed. In all cultures there were colonies that did not thrive and colonies that did, probably due to variability among larvae (Keough 1989; CalBioMarine Technologies, Inc., pers. comm.). The range of sizes and average size were similar between the control and the gentamicin-treated colonies (figure 3). Data from several other gentamicin treatments and growth experiments showed similar results. Some trials failed when both the treated and the control colonies died at the same time for unknown reasons. These were not included in this analysis and final bryostatin activities and detailed symbiont levels were not assessed.

During the 1998 treatment and growth, puromycin was used to treat gentamicin-treated colonies a second time after a period of growth. The rationale behind this experiment was that if we allowed the remaining bacteria to actively grow, then possibly they would be more sensitive to a different protein synthesis inhibitor. For this experiment, plates of both treated and control colonies were removed from the aquaria and antibiotics were added to the petri dishes of the treated colonies. The colonies remained in the dishes outside of the tank for 7 days. Growth slowed during this time for both the control colonies and the puromycin-treated colonies (figure 3 C). For the Torrey Pines set the growth resumed after plates were placed back into the

large aquaria, but the Palos Verdes set remained stunted although they appeared healthy, continued feeding and polypides remained normal (figure 3, C and D).

Other factors besides the antibiotics that affected growth were: the movement of the water in the tanks, frequency and amount of phytoplankton, and temperature. Best growth was observed when water was very well aerated and mixed, and changed a few times a week, rather than when kept on constant flow through. With constant flow, phytoplankton are washed out which reduces the food supply. Phytoplankton added three or more times a week yielded best results. For four 25 liter tanks, 4-6 liters of dense phytoplankton culture were required per feeding. A diet including 50% Rhodomonas consistently yields the best growth. Suspending settled plates above the bottom in the tanks with flow transverse to the suspended plates minimized fouling and collection of detritus in the plates. Care must be taken not to place too many plates in a single aquarium and to suspend plates to allow flow around them.

Symbiont DNA levels in 100 µg/ml gentamicin-treated and control colonies

After an initial treatment with 100 µg/ml gentamicin sulfate for seven days, colonies were allowed to grow without further antibiotic treatments. Symbiont levels were analyzed at 1 month and 2-3 months. For comparison of symbiont DNA levels, control colony DNA was added in 50 µl PCR reactions as follows: 25, 10, 5, 2.5, and 1 ng and for treated colonies, 250 and 100 ng of DNA was required (figure 4). Total DNA concentration was brought to 250 ng in all reactions with salmon sperm DNA. Amplification products of the symbiont SSU rRNA genes for the untreated control

colonies were faint but visible on agarose gel when 10 µl of the PCR reaction using 1 ng of DNA template were loaded. The gentamicin treated colonies, however, showed very faint but visible product when using 100 ng of template. The intensity of the amplification products from 250 ng of treated colony DNA was comparable to the corresponding control products using between 2.5 and 5 ng of DNA indicating approximately 50-100 fold (99-98%) reduction in symbiont DNA signal from the treated colonies (figure 4). An artifactual second band of larger size appears occasionally in reactions with high levels of *B. neritina* DNA. This doublet appears in the treated colony lanes in figure 4 and the larger band could conceivably compete for primers, dNTPs and polymerase, resulting in less of the desired product. However, primers, dNTPs and polymerase are in excess and since the reaction was clearly not saturated this likely is not a concern. If we assume this second band suppressed the amount seen in the target band and take the next highest amount of control DNA to be the equivalent (10 ng) this is still a 25 fold reduction, or 96 %. The 10 ng amplification of control DNA band is much more intense than the 250 ng treated DNA product band and taking 10 ng to be the highest equivalent amount is conservative.

Microbial Community Analysis by Denaturing Gradient Gel Electrophoresis

The bacterial SSU rRNA fragments were amplified in triplicate for each sample then pooled and run on the denaturing gradient gel. Running triplicate reactions gives an average PCR amplification result. Clear banding patterns were achieved. The banding patterns were slightly different for the control colony DNA amplifications

compared to the treated colonies (figure 5 A). There is a strong "*E. sertula*" signal in the control colony DNA, but this signal is absent from the treated colony DNA lane. In addition, a few bands appear in the treated colony DNA lane that are absent from the controls, or are much more intense than they appear in the control. All of the bands that appear in the controls also appear in the treated colony lane, with the exception of the "*E. sertula*" band and one very faint band (figure 5, open arrow). This is a minor product and was not seen consistently in the wild sample DGGE analyses (Chapter 3 suppl. pgs.) indicating that this is not a candidate for the bryostatin producer. This pattern indicates that the only major bacterial species eliminated from the gentamicin treated colonies was the resident symbiont "*E. sertula*". Although there are a few additional bands in the treated colony lane, this would not explain reduction in bryostatin production if bryostatin is a bacterial product.

Detection of polyketide synthase gene KSa

The wild adult, larvae and control cultured *B. neritina* colony DNA all yielded strong signals for the polyketide synthase KSa gene. Amplification from the treated colony DNA resulted in only a small amount of product (figure 5 B), indicating a substantial reduction in the level of KSa present in these colonies.

Bryostatin activity assays

Extracts of *B. neritina* colonies from three settlements and grow-out experiments were tested for bryostatin activity using the binding assay. In addition,

colonies of another bryozoan, *Scrupocellaria* sp., that had fortuitously settled in the dishes along with the *B. neritina* larvae were assayed for activity. The *Scrupocellaria* sp. extracts showed no phorbol displacement activity even at sample extract sizes much higher than that tested for *B. neritina* (figure 2).

The percent of tritiated phorbol displacement plotted against the amount of sample added generated curves shown in figure 6. The IC_{50} values calculated from the binding curves were expressed as $1/IC_{50}$ (activity/μg of sample), compared and shown in figure 7. The IC_{50} values indicate that reduction of the symbiont "*E. sertula*" is consistently correlated with reduced bryostatin levels (22 % to 60 % less bryostatin activity by weight). The least differences were observed when the colonies had been growing quickly and the levels of bryostatins were low in the control colonies (1998 settlement). It is possible that the levels of bryostatin production could not keep up with the rate of growth. The greatest differences were observed when growth had slowed as in the TP 1996 end point measurements and the PV 1998 double treatments as if bryostatins were accumulating in the colonies, but at a higher rate in the controls. The highest bryostatin levels and the greatest difference between the control and treated colonies occurred with the PV 1998 double treatment in which the growth was stunted but the colonies appeared healthy and feeding.

The data were transformed into bryostatin 1 equivalents by comparing the IC_{50} values of the colony extracts to that of bryostatin 1 (calculated from a PDBu displacement assay conducted using bryostatin 1). Since PDBu assay measures binding to PKC by all bryostatins present in the sample, this conversion is referred to as

bryostatin 1 equivalents and does not represent the actual amount of bryostatin 1, which is presumably somewhat lower. The amounts by weight were converted into amount per zooid by estimating how much a dried zooid weighs. Zooids were counted on colonies that had been air dried from water and air dried from ethanol, then weighed and an average zooid weight of 12 μg was calculated. Values for bryostatin equivalents are shown in figure 8. The comparisons between treated and control colony amounts of bryostatins by weight and by zooid are of course almost equivalent to the $1/IC_{50}$ comparisons. But if we take into account the colony size and estimate total amount of bryostatin per colony, we find that the differences increase and that the colonies increase total bryostatin between 10 and 14 weeks (figure 8 C). The bryostatins per unit weight appear to decrease from 4 weeks to 14 weeks (figure 8 A), but the total bryostatin per colony may actually be increasing. This may explain why the fast growing colonies show smaller bryostatin level differences. If the colonies are growing fast, the activity by weight decreases even though the total may increase which makes the comparisons between the control and the treated colonies more difficult. However, the treated colonies were also growing quickly and should also show less activity by weight. The average of the three settlement and growth experiments shows 54 % bryostatin activity remaining in treated colonies when comparing total bryostatin 1 equivalents per colony. If we examine the values by $1/IC_{50}$ (activity/ μg), and by amount of bryostatin 1 equivalents by weight we get an average of 40 % decrease or 60 % remaining in the treated colonies. For the Torrey Pines 1998 settlement, growth was equivalent between the control and the treated colonies and the colonies were growing

well, but due to the very low levels of bryostatins in the controls compared to the other settlements at the end of the growth period, these data were not included in the analysis. All other controls tested had IC_{50} values between 40 and 80 μg. In the Torrey Pines 1998 sample bryostatin was undetectable.

Discussion

In this study, we eliminate most of the bacterial symbiont, "*Candidatus* Endobugula sertula", from newly settled *Bugula neritina* and examine the affect on growth and bryostatin production. The dose of bacteria passed to the next generation is approximately 3000 cells (counted in micrographs showing "*E. sertula*" labeled with a specific probe in larvae from wild *B. neritina*). The bacterial symbionts are suspected to be the true source of the bryostatins (complex polyketides). If the symbionts are not necessary for normal growth by providing some nutritional element, they may provide these toxins as a defense for the host. The current study addressed two main questions; first, can the colonies survive and grow normally with greatly reduced or no symbionts, and second, does *B. neritina* produce the same level of bryostatins with greatly reduced or no bacterial symbionts?

Studies to date show that gentamicin sulfate was the most effective antibiotic against the symbiont without harming the bryozoan host. Approximately 95 % of the symbionts were eliminated from colonies treated with gentamicin and remained at this low level during growth of the colonies for 2-3 months as determined by PCR assays. The estimated level of reduction by the serial dilution PCR method was 95-98 %

eliminated. The reactions were not saturated at the DNA concentrations tested as shown by the continued increase in signal up to 25 ng of control colony DNA. Additional control DNA did not give any greater signal indicating that 25 ng of control cultured *B. neritina* was saturating the reactions. This comparison is a rough estimate and the numbers of remaining symbionts could be slightly higher. However, the numbers are significantly reduced and remain so through the experiments. There is no evidence that the colonies are able to obtain "*E. sertula*" from the sea water. Treated *B. neritina* colonies mistakenly reared in the same tank as control colonies during the 1997 experiment showed no increase in symbiont levels relative to other treatments. In all grow out experiments, antibiotics were chronically present for only the first week of growth after which colonies were exposed to free-living bacteria in the sea water yet the colonies remained ~95 % free of "*E. sertula*."

The development and growth of colonies indicates no adverse effect of elimination of 95 % of the bacterial symbiont showing that the symbiont is not required for nutrition. Although there was a large variance of colony sizes, there were no significant differences observed between the control and treated colony growth. Both treatments had similar variance and average colony size. This variance has been documented in studies by Keough (1989). Two of the settlements shown in the figures show slightly smaller average colonies for the gentamicin treated colonies. This is likely by chance and not an effect of the antibiotic treatment and loss of symbionts. In several other growth experiments, growth rates and form were similar between treatments (data not shown). There is always some variability in the growth rates and

for the growth data from 1996, both the gentamicin 100 μg/ml and 50 μg/ml (not shown) treatments resulted in colonies similar in size or slightly larger (by a few zooids) than the controls. Treatments with 50 μg/ml of gentamicin gave similar levels of curing as the 100 μg/ml, so comparing these growth rates with that of the 100 μg/ml and controls is valid.

"*E. sertula*" does not seem to repopulate the colony after reduction, possibly due to the development and morphology of the colony. The treatments begin immediately after larvae have settled, during the metamorphosis to the ancestrula and the formation of the first bud. The treatment ceases after this period. Zooids are separated by communication plates that are perforated by pores (Bobin, 1977). The funicular cords run up to the pores, but the passage of material between the zooids is controlled by specialized cells that form rosettes in the pores. These cells transport solutes from one zooid directionally and specifically to the next. If the communication plate has formed, then the bacterial cells can not pass through. Likely the bacteria pass from one zooid to the next during the formation of the next bud. In *Watersipora cucullata* this has been observed for mycoplasma symbionts in special sacs associated with the funicular system (Zimmer and Woollacott, 1983). Only during the budding and growth of the next zooid are the symbionts incorporated into the next zooid. If the plate forms before the transfer, the cells do not migrate. If the "*E. sertula*" cells are transferred through the colony via the funicular system then once a new bud has formed and developed a closed communication plate, the path to the new zooid and its descendants is likely blocked for bacteria. This may be examined by cutting treated

colonies and looking for symbiont signal in the individual branches relative to the lower part of the colony. Absence of signal from colony tips will indicate a selective loss of symbionts from the upper portions of the colony.

An alternative explanation is that once the level of "*E. sertula*" is low then other bacteria colonize in its place. However this would seem potentially detrimental to the animal. The denaturing gradient gel electrophoresis profiles show similar communities colonizing both the control and the treated colonies. The banding patterns are similar with the only crucial difference being the lack of the "*E. sertula*" in the treated colonies. The "*E. sertula*" band is the dominant band present in the control colonies aside from the heteroduplexes and non-specific bacteria, indicating that "*E. sertula*" is the predominant bacterial species specifically colonizing *B. neritina*. Furthermore DGGE experiments with wild *B. neritina* have shown that only the "*E. sertula*" band is consistently found associated with *B. neritina* . Other bands appear, but not in all samples from various locations. The major bands appearing at the top of the gel image in figure 5 have appeared in all other *B. neritina* samples from the wild, and non-*Bugula* negative control bryozoans, that have been run on the denaturing gels indicating that these may be common environmental bacteria and not symbionts. The very top band has been demonstrated to be a mix of the other products, a heteroduplex complex. The other two may be mixes of unique environmental bacterial genes, but also contain some heteroduplexes. Even so, when comparing the treated with the controls these bands do not differ from each other. There are a few additional bands and bands that have a much stronger signal from the treated colonies which may

suggest that the lack of "*E. sertula*" allows colonization by other bacteria, but further work would need to be done to show this. There appears to be one minor bacterial signal eliminated from the treated colonies besides the "*E. sertula*," although this band is not consistently found in the wild *B. neritina* samples. If bacteria are producing bryostatins, then a reduction in bryostatin activity in the treated colonies would most likely be due to the reduction of "*E. sertula*", and not an unknown symbiont obtained from the seawater after settlement. The caveat here is that the DGGE study does not look at non-eubacterial sources, such as fungi or archea. However, the antibiotic used does not affect eukaryotic organisms, and any fungi, or other eukaryotes, should remain unaffected.

Phorbol dibutyrate displacement assays indicated an average reduction of 46 % in bryostatin activity from treated colonies relative to controls. This reduction, however, is not proportional to the loss of bacterial symbiont (46 % loss in activity compared to 95 % estimated loss of "*E. sertula*"). This finding may seem to contradict the hypothesis that the bacterial symbionts produce bryostatins, but there is no reason to expect a strict linear relationship between the two. If the symbionts produce the bryostatins, the production of bryostatins is most likely regulated in some way by the bacteria. Often production of bioactive secondary metabolites (i.e. antibiotics) is regulated by concentration. If the number of bacteria is greatly reduced, then the bacteria present may increase production of the compound to maintain the concentration expected with the full complement of bacteria. Conversely, in control colonies it is possible that not all of the bacteria actively produce bryostatins all of the

time. In addition, there is evidence that the bryostatins are outside of the bacterial cells and may move about the colony (Appendix). If this is the case, a colony may build up bryostatin levels outside of the bacterial cells allowing colonies with fewer bacteria to obtain levels much higher than expected from the number of bacteria present. Bryostatin assays coupled with symbiont assays have shown that the rhizoids of the wild colonies have the highest level of bryostatin activity, but a much smaller symbiont population (rough estimate of 25 fold less, see Appendix) compared to the other portions of the colony. In addition, the larvae have bryostatins throughout, but "*E. sertula*" are found only in the pallial sinus of the aboral side. This evidence indicates that the concentration of bryostatin is not directly proportional to the numbers of bacteria present in untreated, wild colonies, and provides an example of how the cured colonies with only 2-5% of their normal symbiont population can reach levels of bryostatin activity about half that of control colonies.

DNA from-field collected and cultured *B. neritina* colonies was screened by PCR for the presence of the polyketide synthase KSa gene. The only samples that did not give a strong signal were the two other bryozoan species (no signal) and the treated colonies (very weak signal; figure 5 B). All other *B. neritina* samples, including larval DNA, gave very good amplification products when using the specific KSa primers (see Chapter 3 suppl. pgs.). Of all the *B. neritina* DNA samples only the *B. neritina* with greatly reduced symbiont numbers did not give a strong signal for the presence of this gene indicating that the genes are present in "*E. sertula*".

Summary

- "*E. sertula*" is severely reduced in *B. neritina* colonies treated with gentamicin sulphate
- other members of the microbial community seem unaffected relative to controls
- Treatment with antibiotics and reduction of symbionts does not affect growth rates.
- The KSa (type I complex polyketide) signal is reduced in the treated colonies
- There is about half the amount of bryostatin activity in the treated colonies

In conclusion, the reduction of bacterial symbionts is correlated with a reduction in bryostatin activity and reduction of the KSa gene levels. Genetic evidence shows potential for production of complex polyketides by "*E. sertula*" and no other significant differences in the microbial community between treatments indicating that the difference in bryostatin levels is most likely due to the reduction of the "*E. sertula*" symbiont. If an additional bacterial type were present in high levels to give the strong PKS signal seen from *B. neritina*, it would have been detected in the DGGE studies. This evidence proves that "*E. sertula*" is required for maximum synthesis of bryostatins, but not necessarily that it is the source of bryostatins. "*E. sertula*" could be providing a factor required by the host to make bryostatins. The "*E. sertula*" symbionts are not providing a significant nutritional benefit, as shown by the unaffected growth of gentamicin-treated colonies, unless they are providing a nutrient only needed in small amounts. The symbionts must be essential for survival of the host, as

suggested by the evolution of symbiont transfer from parent to larvae. The information taken together strongly suggests that the symbiosis may have arisen through a competitive advantage of having noxious cytotoxins as a defense against predators and competitors. This defensive role remains to be shown.

Acknowledgements

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Table 1: Summary of antibiotic treatments of *Bugula neritina*.

Antibiotic	Mode of Action	Dose (µg/ml, 7days)	Effectiveness	Toxicity	Conclusion
Gentamicin	Protein synthesis inhibition	5,10,20,50,100,200, 250	Most effective at 50-100 µg/ml	normal growth up to 200 µg/ml	provides nearly complete curing, approximately 95% symbiont levels detected relative to controls
Streptomycin	Beta lactam, cell wall synthesis inhibition	50, 100	ineffective	none	not suitable
Tetracycline	protein synthesis inhibition	50, 100	ineffective	none	not suitable
Penicillin	Beta lactam, cell wall synthesis inhibition	50, 100	ineffective	none	not suitable
Chloramphenicol	protein synthesis inhibition	5, 10, 25, 50		TOXIC even at lowest dose	not suitable
Puromycin	Protein synthesis inhibition	10, 20, 30	As effective as Gentamicin at 20-30 µg/ml	slow growth at 20-30 µg/ml, but recover after a week	not as good as Gentamicin due to mild toxicity
Polymixin	electron transfer inhibition	10, 20, 30	similar results to puromycin	toxic at 20-30 µg/ml wich is most effective dose	not as good as Gentamicin due to toxicity
Furolizidone				TOXIC	not suitable
Immipenum	cell wall synthesis inhibition	50, 100, 150	ineffective	none	not suitable
Gentamicin, Puromycin		Gentamicin 50, Puromycin 10, 20	most effective at Gent 50, Puro 20	slow growth, but recover after a week	equivalent to gentamicin
Gentamicin, Polymixin		Gentamicin 50, Polymixin 10, 20	most effective at Gent 50, Poly 20	somewhat toxic slow growth, some deformation	equivalent to gentamicin effect on bryozoan problematic

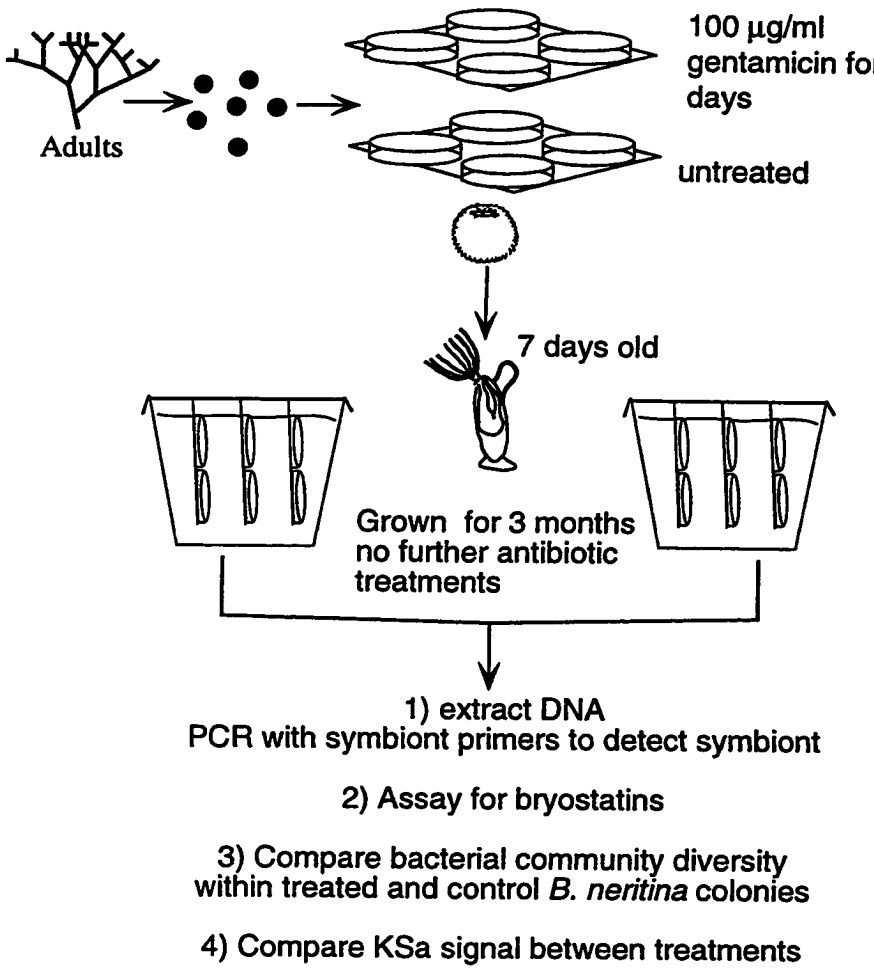

Figure 1. Schematic showing gentamicin treatment of *Bugula neritina*. Larvae are released from adults then settled into plates mounted on plastic panels. The newly settled larvae are treated in the plates with 100 µg/ml of gentamicin sulfate daily for 7 days. Then the panels are hung in aquaria and the *B. neritina* are reared for 2-3 months.

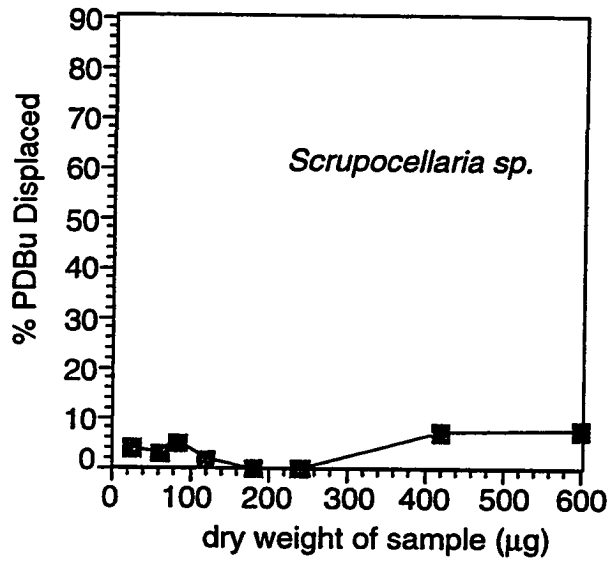

Figure 2. PDBu Displacement activity in *Scrupocellaria* sp., a bryozoan occuring with *B. neritina* but with no known bryostatins. This serves as a negative control for PDBu displacement activity in bryozoan extracts.

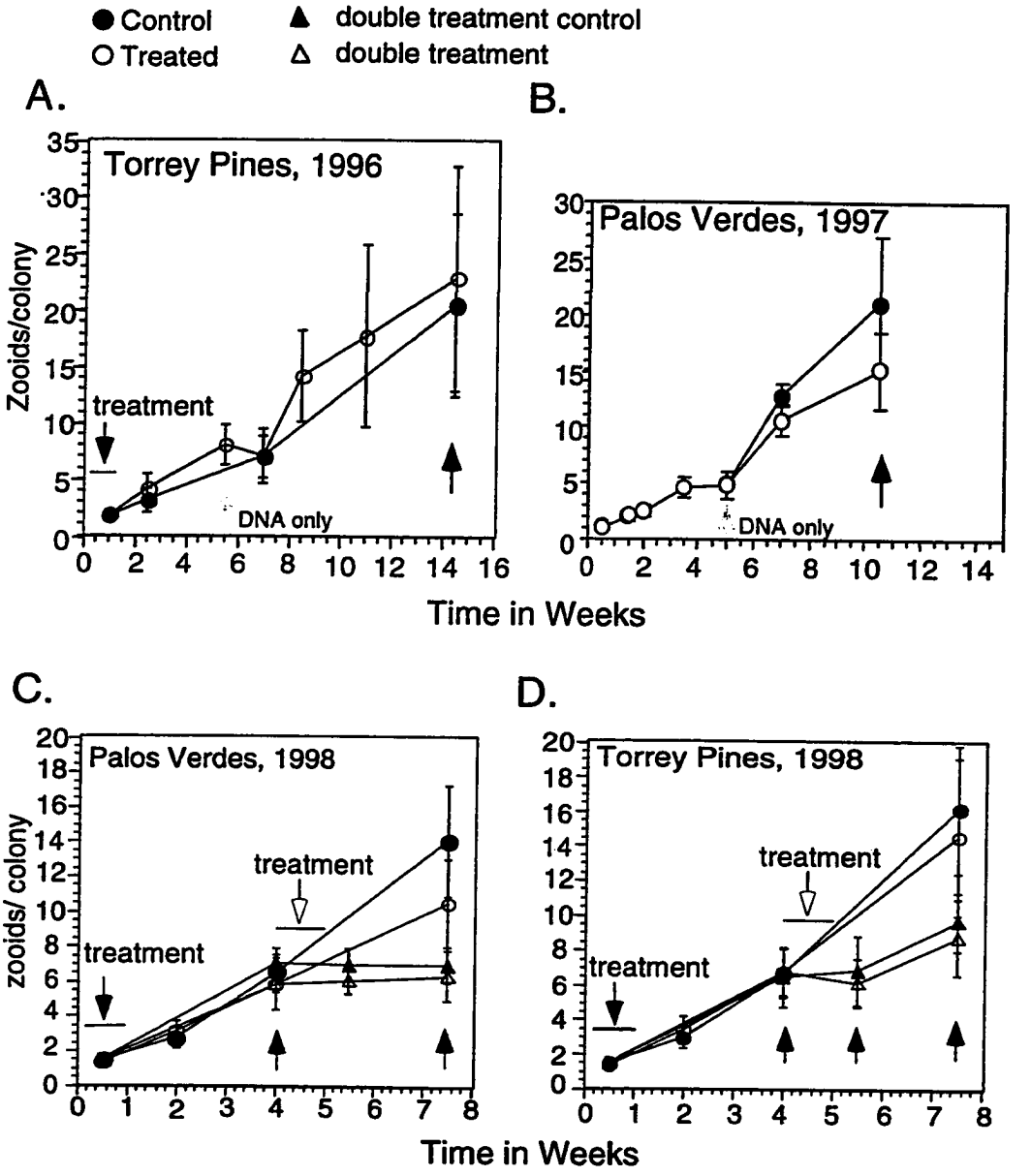

Figure 3. Growth of cultured *B. neritina* colonies. Treatment periods are indicated. Unlabeled arrows show sampling times. Second treatments with puromycin are indicated with the open arrows.

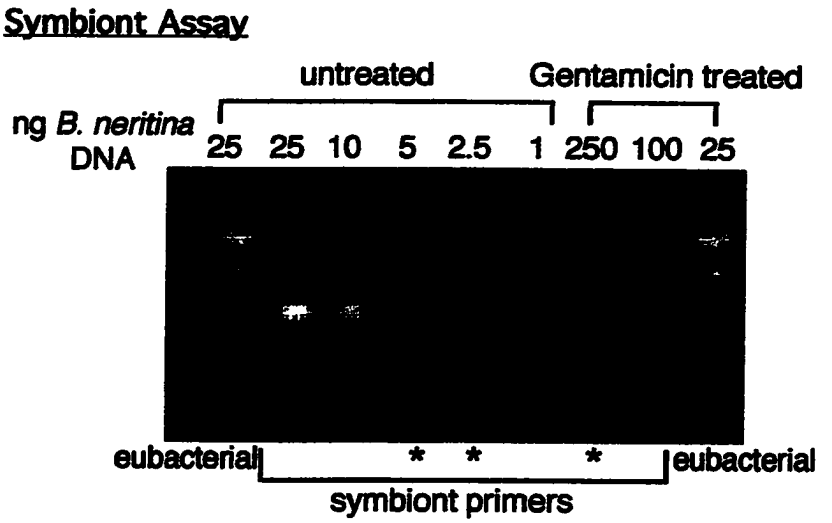

Figure 4. Gel image showing the amplification signal from a series of DNA concentrations from treated and control colonies. 10 µl was loaded in each lane. Eubacterial, universal eubacterial primers amplify total eubacterial SSU rRNA genes. Symbiont primers amplify the "*Endobugula sertula*" SSU rRNA genes specifically. Asterisks show similar levels of amplification indicating approximately equal amounts of symbiont signal.

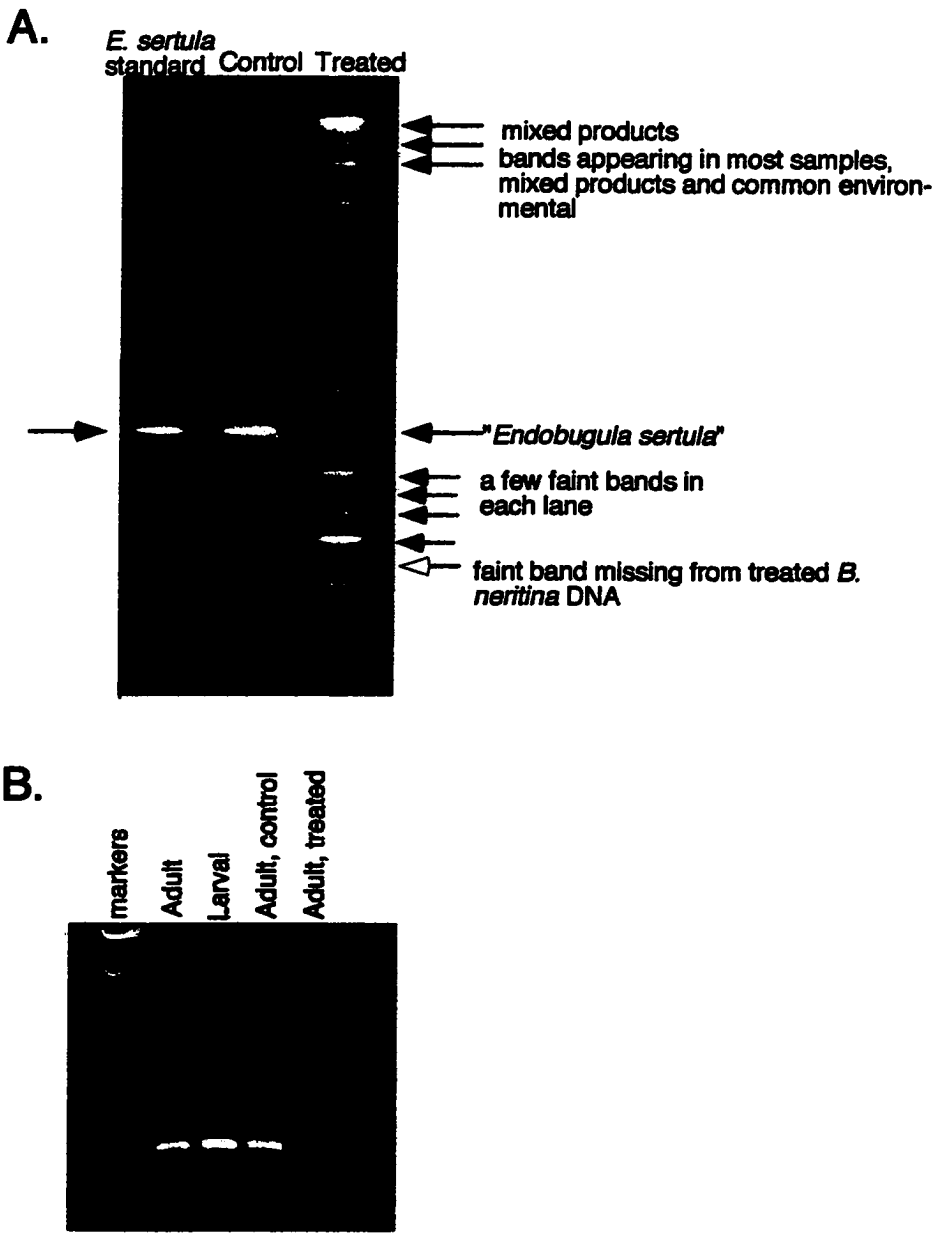

Figure 5: A, Denaturing gradient gel showing bacterial SSU rRNA fragments from treated and control colonies. B, polyketide synthase KSa gene fragments amplified from DNA of *B. neritina* larvae, adults, control and treated colonies.

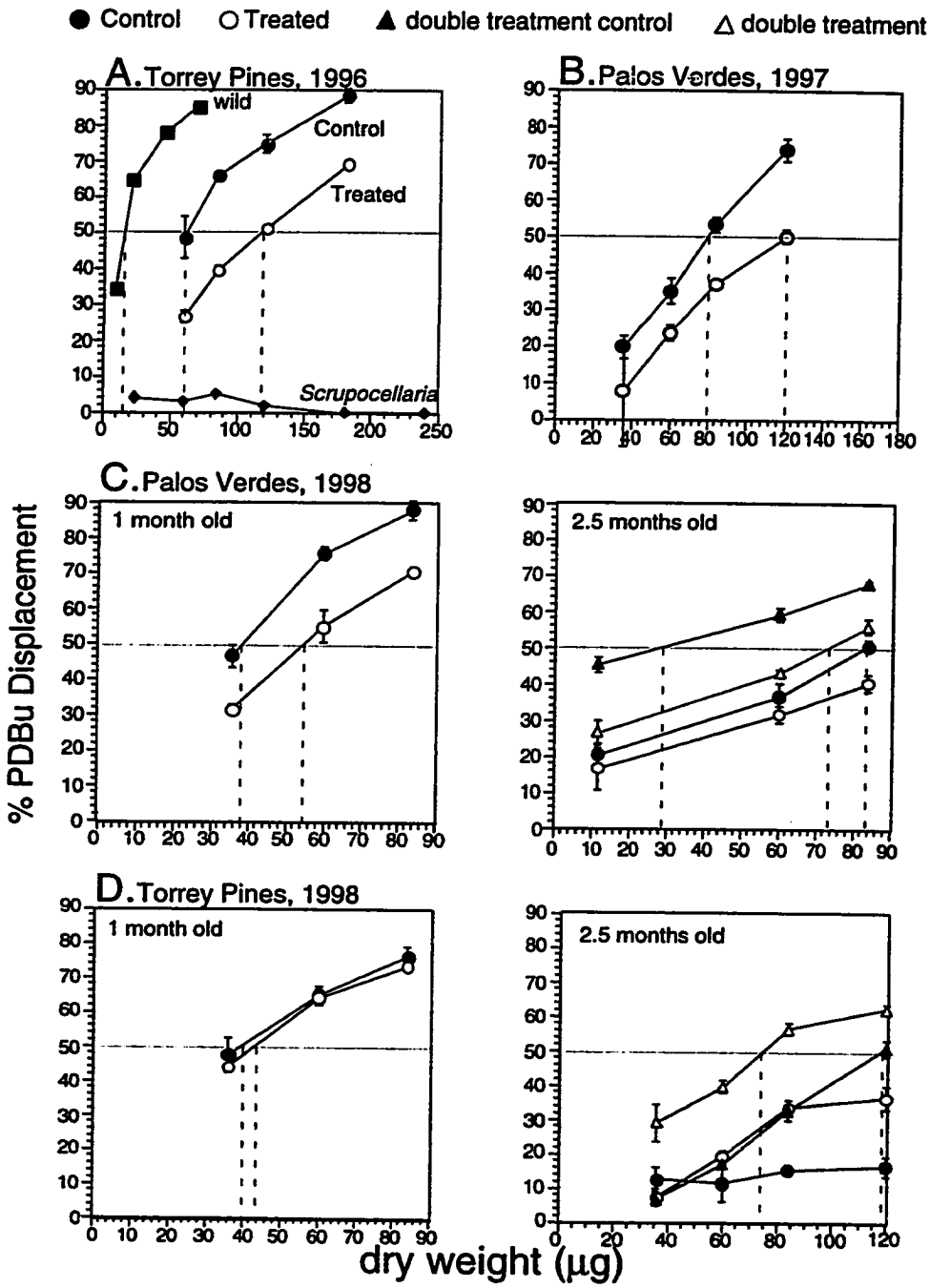

Figure 6. ^{3}H-Phorbol Dibutyrate displacement plotted against amount of *B. neritina* extracts. Lines dropped to axis indicate IC_{50} values (dose giving 50 % displacement).

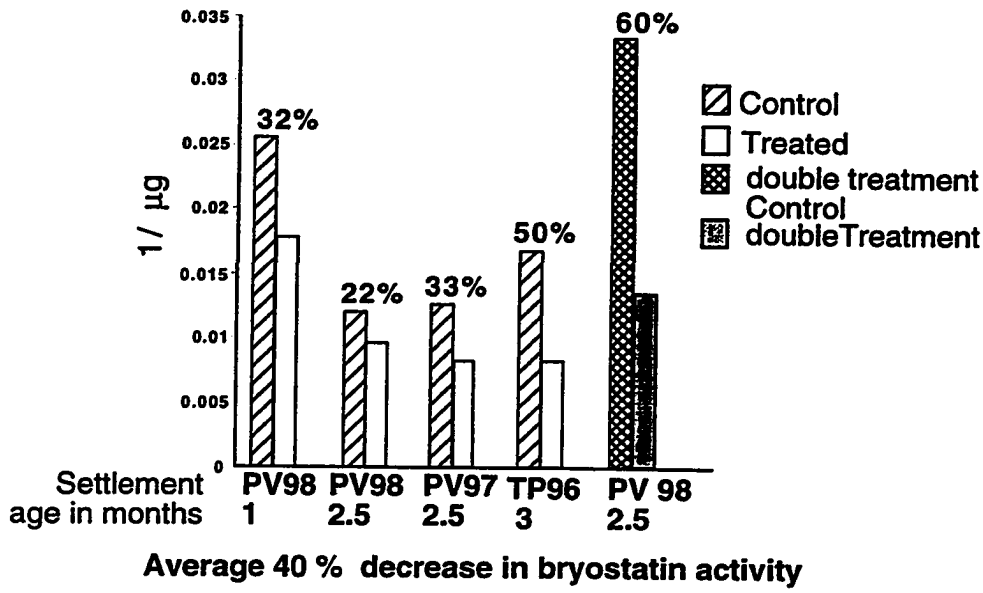

Figure 7. Relative Bryostatin activity in $1/IC_{50}$ (displacement activity per microgram) with amount of decrease in activity relative to controls shown above.

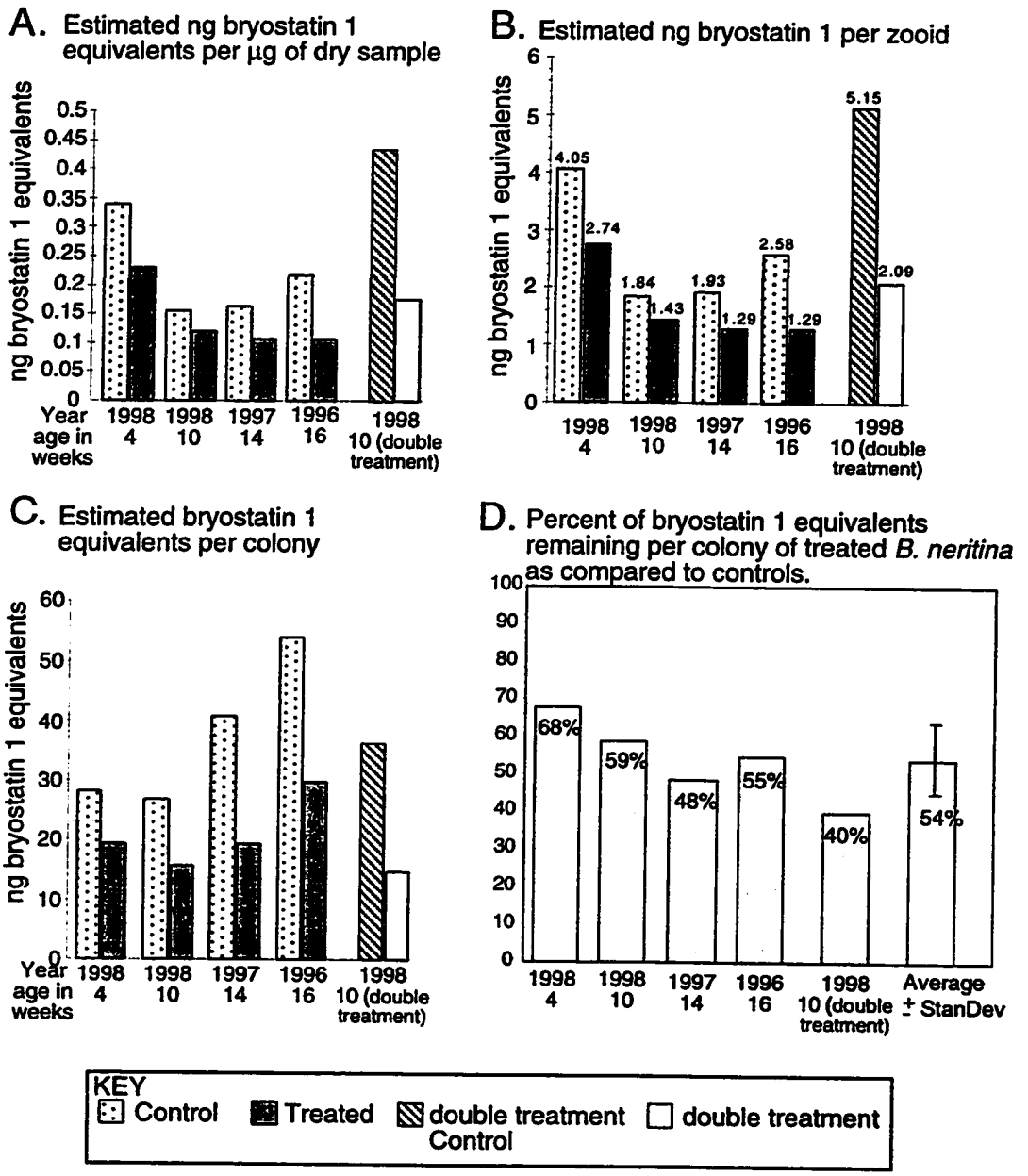

Figure 8. Bryostatin 1 equivalents in treated and control cultured colonies from 1996-1998 settlements viewed various ways. Bryostatin 1 equivalents were estimated from a bryostatin 1 PDBu displacement curve which gave an IC_{50} value of 13 ng.

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Chapter 6

The sorcerer's nudibranch and their eggs of death: evidence that *Polycera atra* sequesters bryostatins from their bryozoan prey, *Bugula neritina*.

The goal of this study was to determine if the nudibranch predators of *B. neritina* sequester bryostatins from their prey.

The main approach was to 1) test extracts of two nudibranch predators, *Polycera atra* and *P. hedgpethi* for bryostatin activity using a phorbol dibutyrate displacement (PDBu) assay and 2) use mass spectrometry to confirm the presence of compounds with mass similar to that of bryostatins.

In this chapter I show that the nudibranchs sequester bryostatins and concentrate them in their eggs. The loading of egg ribbons with bryostatins suggests that bryostatins may be used to defend the offspring. We can infer that the source is dietary, coming from their bryozoan prey. If the bacterial symbionts, "*Candidatus* Endobugula sertula", harbored by the bryozoan are responsible for production of bryostatins, as accumulating evidence indicates, then the symbionts provide chemical protection for both host and predators.

Introduction

It is well documented that dorid nudibranchs sequester toxins from their diet for their own chemical defense, mainly from sponges, soft corals, ascidians and bryozoans (reviewed in Karuso, 1987; Faulkner, 1992; Avila 1995). In their 1983 review Faulkner and Ghiselin developed the idea that opistobranch molluscs lost their shell after acquiring chemical defenses. Manufacturing and carrying a shell around is considered more expensive than sequestering a toxin found in the diet. The synthesis

of the shell is avoided, as is the energy expended in carrying and maintaining the shell. *De novo* synthesis of the toxins is also avoided, as they are produced by the ingested organism. The nudibranch ancestor must have evolved the ability to sequester the toxins and avoid being poisoned itself. How this occurs is of course a problematic evolutionary question. The organism must develop at least partial chemical defense before physical defenses will begin to diminish in importance and the shell can decrease in size. Therefore the ability to withstand the toxin, then sequester it safely, must evolve before the shell can diminish. This likely developed not as a defense but as a way of detoxifying an abundant food source to avoid competition. Feeding on a food source that is not widely preyed upon by others ensures a good supply, but in order exploit this the organism must develop a way to deal with the toxins. A side benefit that ultimately proved a to be a major selective advantage was that predators attempting to feed on the ancestral nudibranch found the toxins distasteful.

In many species these diet-derived compounds are selectively concentrated in the dorsal mantle, the exposed portion of the body first touched or tasted by a predator (Cimino et al., 1982; Faulkner and Ghiselin, 1983; Carté and Faulkner, 1986; Paul et al., 1990; Avila and Paul, 1997; Pawlik et al., 1988). Specialized mantle dermal formations that secrete mucous containing toxins have been documented and histologically examined (Avila and Paul, 1997; Avila and Durfort, 1996). Compounds are selectively sequestered, and in certain cases the dominant compound in the nudibranch is not the most abundant compound in the prey (Pawlik et al., 1988; Paul et al., 1990; Rogers and Paul, 1991). There are species whose egg masses also contain

the diet-derived compounds and have been shown to act as feeding deterrents to potential predators (McClintock and Baker, 1997; Pawlik et al., 1988). A particularly dramatic example is that of the brilliantly colored Spanish dancer, *Hexabranchus sanguineus*, and its conspicuous pink egg ribbons. The Spanish dancer selectively sequesters oxazole-containing macrolides from the sponge *Halichondria* sp., and concentrates them in the dorsal mantle (Pawlik et al., 1988). The egg ribbons are highly conspicuous, bright pink in color and laid in exposed places. These also contain the macrolides, but in concentrations about 10 fold or more than in the nudibranch itself. The macrolides from the nudibranch, egg ribbons and the sponge were shown to be highly deterrent to fish feeding. The eggs are not always assayed in the many studies conducted on the deterrent properties of dorid extracts so it is difficult to estimate how widespread is the habit of loading eggs with diet-derived metabolites.

In this paper I present evidence that two nudibranch predators of *Bugula neritina*, *Polycera atra* and *P. hedgpethi*, sequester bryostatins, cytotoxic macrolides found in the bryozoan. Although most nudibranch predators examined for secondary metabolites sequester compounds from sponges, there are good examples of nudibranchs feeding on bryozoa and sequestering their toxins. *Tambje abdere* and *T. eliora* feed on the cheilostome bryozoan *Sessibugula transluscens* and sequester tambjamines from the bryozoan as predator deterrents (Carté and Faulkner, 1986). In response to threat *T. abdere* excretes mucous containing the tambjamines, and the defensive role of the tambjamines has been well demonstrated (Carté and Faulkner, 1983, 1986).

Two nudibranch predators have been observed feeding on *B. neritina*, *Polycera atra* (the sorcerer's nudibranch) and *P. hedgpethi*. Both are cryptic on *B. neritina*. *P. atra* is more conspicuous, with yellow and black stripes, than *P. hedgpethi* which nearly matches the color of *B. neritina* being a dark brown. *P. atra* is more abundant and commonly found than *P. hedgpethi* along the California coast. *P. atra* tends to feed on the upper portions and tips of the colonies (personal obs.) and lays conspicuous white egg cases on *B. neritina*. Two other species, *Polycera negra* (Gulf of California) and *Dirona picta*, also have been found feeding on *B. neritina* but were not examined in this study. We hypothesized that the nudibranch predators sequester bryostatins and use them for defense. To investigate this possibility *B. neritina* was collected from Torrey Pines artificial reef in La Jolla, California, *P. atra* and egg masses and *P. hedgpethi* were collected and extracted in acetone. These extracts were tested for bryostatin activity and compounds of the proper mass using electrospray mass spectrometry. Evidence that the nudibranchs sequester the bryostatins would support the hypothesis that bryostatins provide chemical protection for the bryozoan.

Materials and Methods

Sample Collection

Bugula neritina was collected from Torrey Pines artificial reef in June of 1998. Colonies were maintained in large tanks with flowing seawater on the Scripps Pier. *Polycera atra* were collected from the colonies and brought to the lab where they were maintained on *B. neritina*. The following samples were collected: 15-20 individuals of

the actively feeding *P. atra* , 20-30 individuals left without *B. neritina* for 36 hrs (starved), and egg ribbons laid during this starvation period. A number of *P. atra* were left feeding in the aquarium for a week and eggs of variable ages were sampled at the end of the week. In addition, two actively feeding individuals of *Polycera hedgepethi* were collected. All samples were immediately placed in acetone and stored at –20°C prior to analysis. The prey colonies of *B. neritina* were stored frozen at –80°C until extraction with ethanol. See table 1 for sample summary.

Table 1: Sample summary

Species	Sample type	number	dried mass (mg)
Polycera atra	feeding	15-20	139.6
	starved ~36hrs	20-30	228.1
	eggs	15-20	42
Polycera hedgpethi	feeding	2	6.8
Bugula neritina	prey		

Extraction and Fractionation of *B. neritina, P. atra* and *P . hedgpethi*

The samples were homogenized in acetone. The homogenization step may be unnecessary if the adult nudibranchs excrete their toxins into the acetone. Metabolites of purer composition have been obtained in this manner (Avila and Paul, 1997; John Faulkner, pers.com.). Debris was pelleted in a table top centrifuge at 14 x g for 10 minutes then the acetone was removed. The pellets were dried and weighed as an estimate of nudibranch dry weight. Acetone extracts were dried down and resuspended in ethanol for bryostatin assays at 6 mg/ml and 3 mg/ml (eggs and feeding *P. atra*) dried animal weight to solvent volume. Only a small portion of the extracts were required for PDBu displacement assays (2, 4, 6 and 8 µl).

The remaining extracts were dried down and resuspended in a small volume of ethyl acetate. The ethyl acetate extract was added to hexane and loaded onto an ethyl acetate equilibrated silica column packed in a pasteur pipette. The column was eluted with approximately 3 mls of dichloromethane, followed by ethyl acetate, then methanol. Each fraction was collected for initial examinations to detect bryostatin masses by electrospray injection mass spectrometry (ESIMS). All of the bryostatins should elute with the ethyl acetate. This was confirmed by ESIMS of each of the extract fractions from *B. neritina* and the feeding *P. atra*. For the remaining samples, only the ethyl acetate eluates were tested. All eluates to be injected were dried down and resuspended in 100 µl of methanol prior to injection into the ESIMS.

Bryostatin activity assays

The phorbol dibutyrate displacement activity assay was used to detect bryostatin-like activity in the nudibranch extracts. This assay is described elsewhere (Chapter 4). In brief, bryostatins bind to protein kinase C (PKC) at the same site as phorbol esters. However, bryostatins bind with much higher affinity such that bryostatins will displace phorbol on the protein kinase C receptor site under assay buffer conditions (De Vries, 1988). A suspension of rat brain liposomes rich in PKC is prepared as described in Chapter 4. Extracts are added to a solution containing tritiated phorbol dibutyrate and incubated for one hour. The liposomes are then captured on glass fiber filters and rinsed with buffer to remove unbound excess tritiated phorbol. The remaining tritium signal is read on a scintillation counter and compared

to the total bound signal (negative control). A positive control of unlabelled phorbol is added to ensure consistency between assays, and that the PKC is intact in the liposome suspension. A reduction in the tritium signal indicates bryostatin activity by displacement of the phorbol. The displacement is expressed as a percentage and plotted against amount of extracts. The amount of extracts required to give 50% displacement (IC_{50}) is compared between samples. IC_{50} values are expressed as dry weight of sample (μg).

Electrospray Mass Spectrometry

To confirm that the phorbol displacement activity in the extracts was due to bryostatins, mass spectrometry was performed to identify compounds with the same masses as bryostatins. The mass spectra for several of the major bryostatins have been reported (Pettit et al., 1982; Pettit et al., 1991). Below is a list of masses commonly observed for bryostatin 1. The sample was delivered into the chamber at a rate of 5 μl/min, and the strength of the ion beam was adjusted to minimize fragmentation of the compounds. A few large peaks of smaller masses (400's) were suspected fragments of bryostatin. This was confirmed by isolating the bryostatin mass peak and further fragmenting it (i.e. 850 fragmented to 407). Masses obtained from the nudibranch extracts were compared to those obtained from the prey, Torrey Pines *Bugula neritina*.

Table 2: Bryostatin Mass Spec summary.

Bryostatin 1 [M]	M+Na	M-18 (H_2O)	M-36 (2 H_2O)	M-54 (3 H_2O)	M-acetate
904	926	886	868	850	844

Results

Bryostatin assays

Actively feeding *P. atra* showed activity levels 4.25 times higher than that of their prey (figures 1 and 2). However when starved for about 36 hours the activity level dropped to approximately 1.5 times that of the prey (figure 1 and 2) and equal to the feeding *P. hedgpethi* displacement activity level. The eggs that were laid during this starvation period contained much higher concentrations of bryostatin-like activity than either the prey or the parent nudibranchs tested. The smallest sample size tested yield greater than 80 % displacement. At this level the reaction is saturated and it is not possible to determine an IC_{50}. However a minimum IC_{50} value was estimated based on the amount of feeding *P. atra* required to displace 80 % of the ^{3}H-phorbol signal. *P. atra* displaced 80 % of the tritium signal at 24 µg of sample, which is minimally equivalent to the 6 µg of egg extracts required to displace 84% (see figure 1). Using this estimation, the eggs are at least 4 times higher in activity than the feeding *P. atra* and have at least 17 times more displacement activity than the original prey. An additional assay on a dilution series of egg extracts will show how much more concentrated the activity really is.

Actively feeding *P. hedgpethi* (IC_{50} 22 μg) yielded slightly higher levels of PDBu displacement activity as their prey *B. neritina* (IC_{50} 32μg) (figures 1 and 2) However only two individuals were tested, and additional assays are needed.

Mass spectrometry

Table 3 shows major mass peaks present in the extracts that matched bryostatin 1 masses. A peak at 851 (bryostatin 1) was consistently found in both the *B. neritina* prey and the *P. atra* adults and eggs. In addition, a peak consistent with bryostatin 3 mass at 888 was identified in the *P. atra* adults feeding and starved, and the eggs (figure 3). There were many other mass peaks present in each sample. Reported here are the mass peaks consistent with bryostatin 1. Other major bryostatin peaks were not consistently clear. Bryostatin 1 masses were the major peaks in the *B. neritina* being grazed by the *P. atra*. Peaks of similar masses were identified in the *P. atra* extracts as well as from the eggs. The eggs yielded peak patterns much cleaner and more concentrated (fewer small peaks of various masses) than the nudibranch extracts which is consistent with the PDBu displacement data.

Table 3: Mass peaks found by Electrospray Mass Spectrometry:

Bryostatin 1	904 (M)	886 (M-H2O)	868 (M-2 H20)	850 (M-3H2O)	other
B. neritina	+	+	+	+	926
P. atra feeding	+	+		+	926, 888, 875
P. atra starved	+			+	888, 875
Fresh eggs	+	+		+	888, 875, 830
eggs (mixed ages)	+	+		+	888, 875

926 amu is reported as bryostatin 1 (M+Na) and is an artifact created by the machine. 875 amu was a dominant peak in most of the samples and is the mass reported for bryostatins 6 and 9 (M+Na) (Pettit, 1991). The mass for bryostatin 3, 888 amu, also clearly appeared in the nudibranchs and eggs but was weak in *B. neritina* extracts.

Discussion

In this study I found that extracts from two nudibranch predators of *B. neritina* contained levels of bryostatin activity equal to or higher than that in the prey itself. Actively feeding *P. atra* contained PDBu displacement activity consistent with bryostatins that was much higher (4 x) than that of *B. neritina*. If starved for approximately 36 hours, the level of bryostatin-like activity dropped to an amount close to that in the prey. Eggs laid during this starvation period contained highly concentrated levels of bryostatin-like activity relative to either the prey or the feeding parent nudibranch. The levels of bryostatin activity per individual nudibranch may vary since the extracts were from many individuals pooled together and represents an average. The bryostatin content of an individual likely depends on whether or not it has recently laid eggs, the number of eggs and its feeding history. The feeding

nudibranchs were also leaving egg masses on the *B. neritina* as well as the sides of the tanks. Two individuals of *P. hedgpethi* showed bryostatin activity levels only slightly higher than that of the prey, however no additional analyses were run on this sample. Although the phorbol displacement assay is not specific to bryostatins, bryostatins are the only compounds in *B. neritina* shown to have PDBu displacement activity. In a survey of marine invertebrate extracts, De Vries used a similar PDBu displacement assay to find PKC binding compounds. In the De Vries et al., 1997 paper, 24 molluscs were tested and only one showed any activity indicating that the tissue itself does not contain PKC binding compounds with higher affinities than phorbol. However, the irritants aplysiatoxin and lyngbyatoxin of two other opisthobranch molluscs will bind the phorbol site on PKC (Fujike et al., 1989). These compounds are also sequestered from the diet and not intrinsic to the tissue.

To confirm the phorbol displacement activity was due to the presence of bryostatins, samples were analyzed by mass spectrometry and showed that bryostatins are indeed abundant in the *P. atra* extracts. Key peaks consistently included the bryostatin 1 masses at 904, 868 [M-18], 850[M-36], and 407(confirmed fragment from 850). Bryostatin 1 was the dominant bryostatin in the *B. neritina* from Torrey Pines, and this was consistent with previous analyses of the bryostatin content in *B. neritina* at this site. In addition a peak regularly appeared from *P. atra* and eggs with mass of 875, which is the published mass of bryostatin 6 and 9 with an added sodium ion.

From these data it is clear that *P. atra* and *P. hedgpethi* contain bryostatins, are sequestering them from their prey *B. neritina* and are most likely using these

compounds for defense. The levels found are much too high to be explained by bryozoan remaining in the gut. If bryostatin activity was due to *B. neritina* in the nudibranch gut, then the levels would be much lower in the *Polycera* than found in the prey since the nudibranch mass is much higher than that of the bryozoan in the gut. Furthermore, the concentrated levels of bryostatins in the egg masses confirms the ability to sequester bryostatins and manipulate their destination. Although bryostatin toxicity and ability to deter predators has not been tested on marine invertebrates or fish, the concentration of bryostatins in the egg masses strongly suggests a defensive role for the bryostatins which are toxic to cells in culture and to mice. The eggs are white and conspicuous on the dark brown to purple *B. neritina* where they are laid and would be highly visible to predators. Being on the bryozoan colonies may afford some protection in itself, but there are a number of invertebrates that colonize the *B. neritina* suggesting that egg cases would be fair game to small crawling predators and to fish able to pluck them off without ingesting the bryozoan. It is possible that placing bryostatins in the eggs is simply a means of excreting the toxins, however this seems unlikely and there is precedence in the opistobranchs, especially dorid nudibranchs, for sequestering toxins from the diet to defend the parent and the eggs. This is reminiscent of the Spanish dancer *Hexabranchus sanguineus* whose eggs have greater than 10 times the amount of the diet-derived compounds than the nudibranch or its prey (Pawlik et al, 1988). In this case the protective function of the compound in the eggs has been demonstrated. The deterrent potential of bryostatins is an important avenue to investigate in order to show the role these compounds play in the ecology of the

animal, and explain in part how the symbiosis between the host and bacteria may have arisen.

The ability of the *P. atra* and *P. hedgpethi* to take up the bryostatins, safely sequester them and place them into their egg ribbons indicates a highly evolved and long-established association between the nudibranch and its prey. Further analysis may reveal whether *P. atra* selectively sequesters particular bryostatins or alters them. There were certain mass peaks appearing in the nudibranchs and eggs that were not dominant peaks in the bryozoan. The bryostatins are believed to be produced by bacterial symbionts within the bryozoan. If this proves to be true, as growing evidence supports, then the symbionts are protecting not only the host bryozoan, and their progeny, but also the nudibranch predators and their progeny. The production of bryostatins by the bacteria likely evolved as a weapon against other microorganisms, and has become a valuable substance to obtain for protection against predation for metazoans.

Acknowledgments

Dr. D. John Faulkner and Dr. Eric W. Schmidt provided assistance with the chemical analysis of the nudibranch extracts. Carolyn Sheehan (CalBioMarine Technologies, Inc.) helped with nudibranch collections.

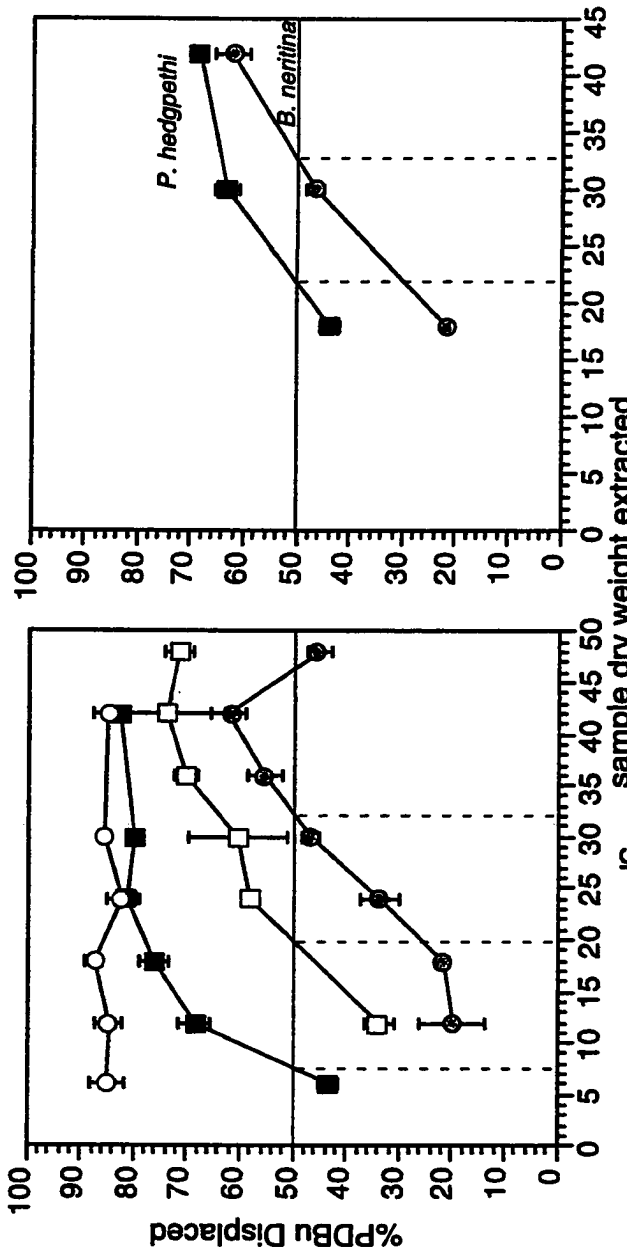


Figure 1: Phorbol dibutyrate displacement curves generated from extracts of *Bugula neritina* (prey), *Polycera atra*, their eggs and *P. hedgpethi*. The amount of sample required to displace 50 % of the tritiated phorbol signal is the IC_{50}. The IC_{50} for the eggs was estimated based on the amount of extract required to reach 80 % for the feeding *P. atra* .

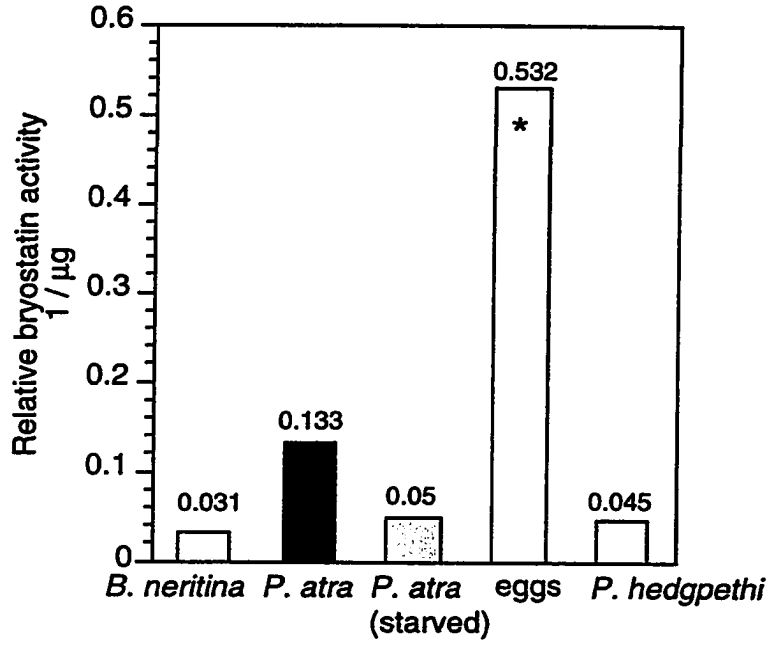
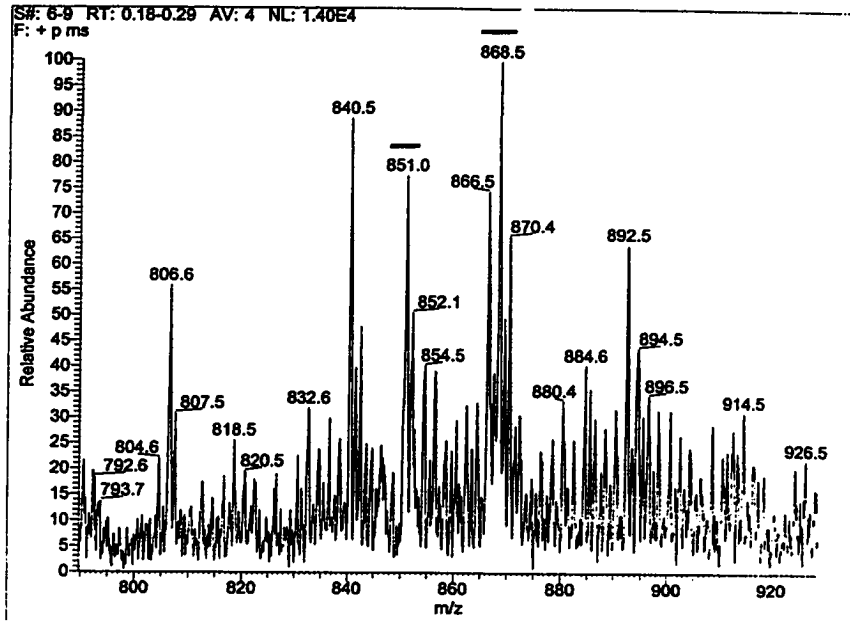


Figure 2. PDBu displacement activity in extracts from *Polycera atra* and *P. hedgpethi*, nudibranch predators of *Bugula neritina*. The 1 / IC_{50} values indicate an estimate of activity per unit dry weight of tissue extracted (per µg) based on the amount of extract required for 50 % displacement of the tritiated phorbol signal. *, estimated to be a minimum of 17 X more activity in the eggs than in the prey *B. neritina*.

Figure 3: Mass spectrometry traces from ethyl acetate fractions of extracts *from B. neritina, P. atra* and *P. atra* egg ribbons. Key peaks corresponding to bryostatin masses are indicated with a line above the peak.

A. *B. neritina* (prey)



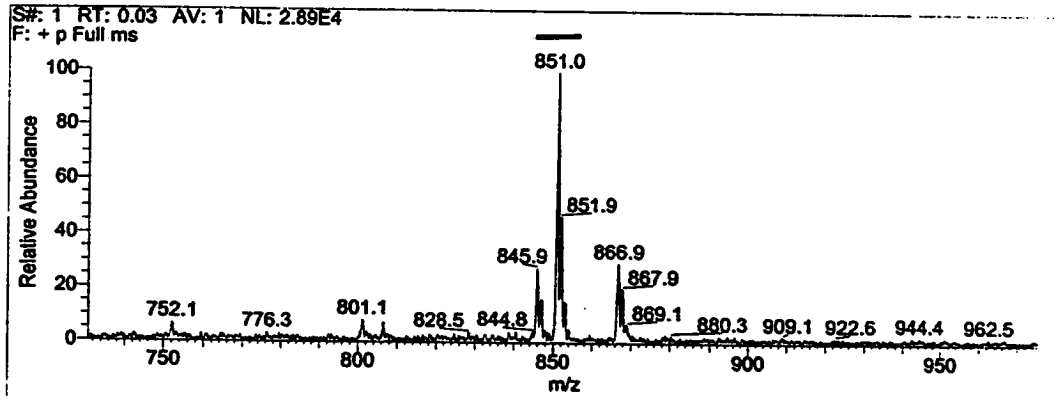
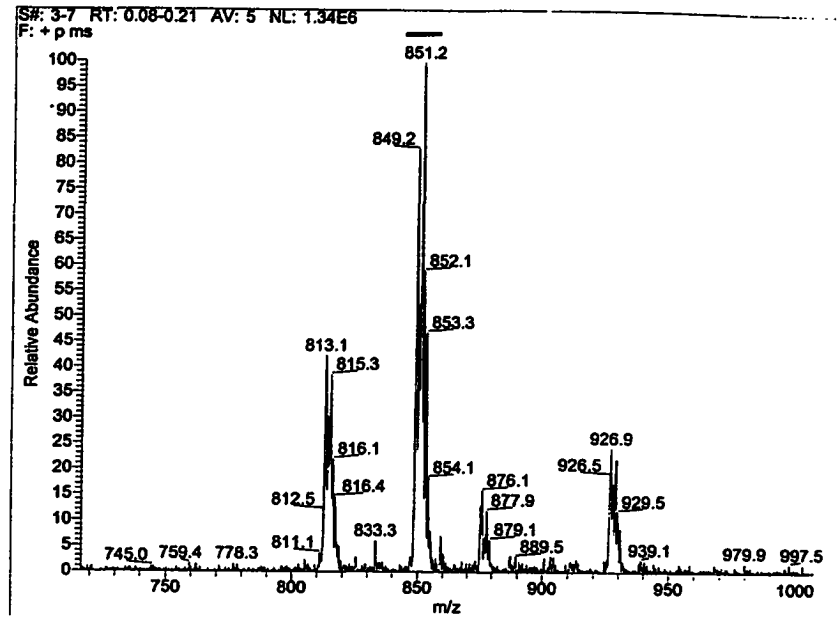
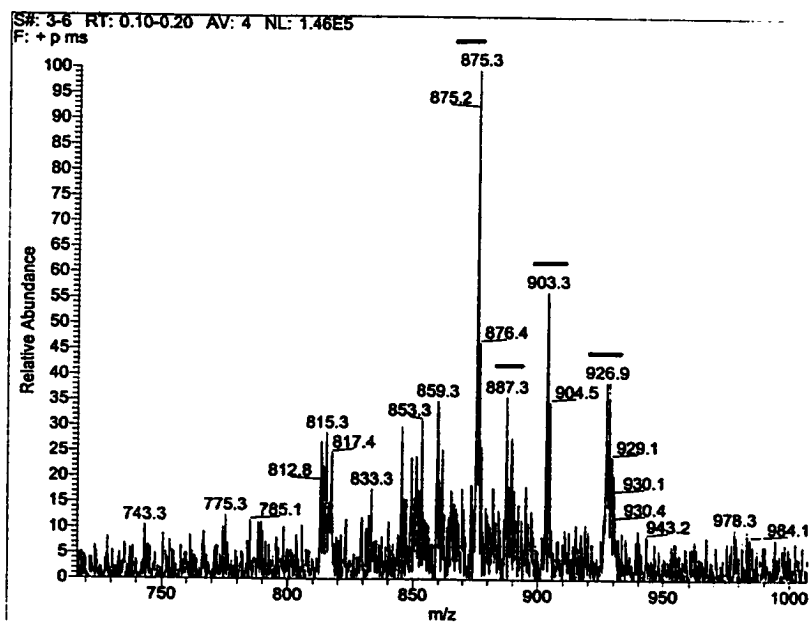


Figure 3:

B. *P. atra* feeding





B. *P. atra* feeding

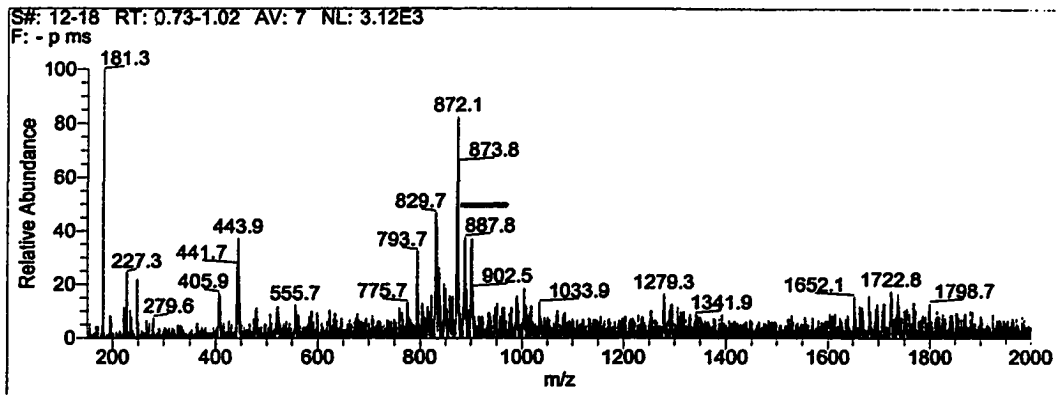


Figure 3.

C. *P. atra* starved for 36 hours

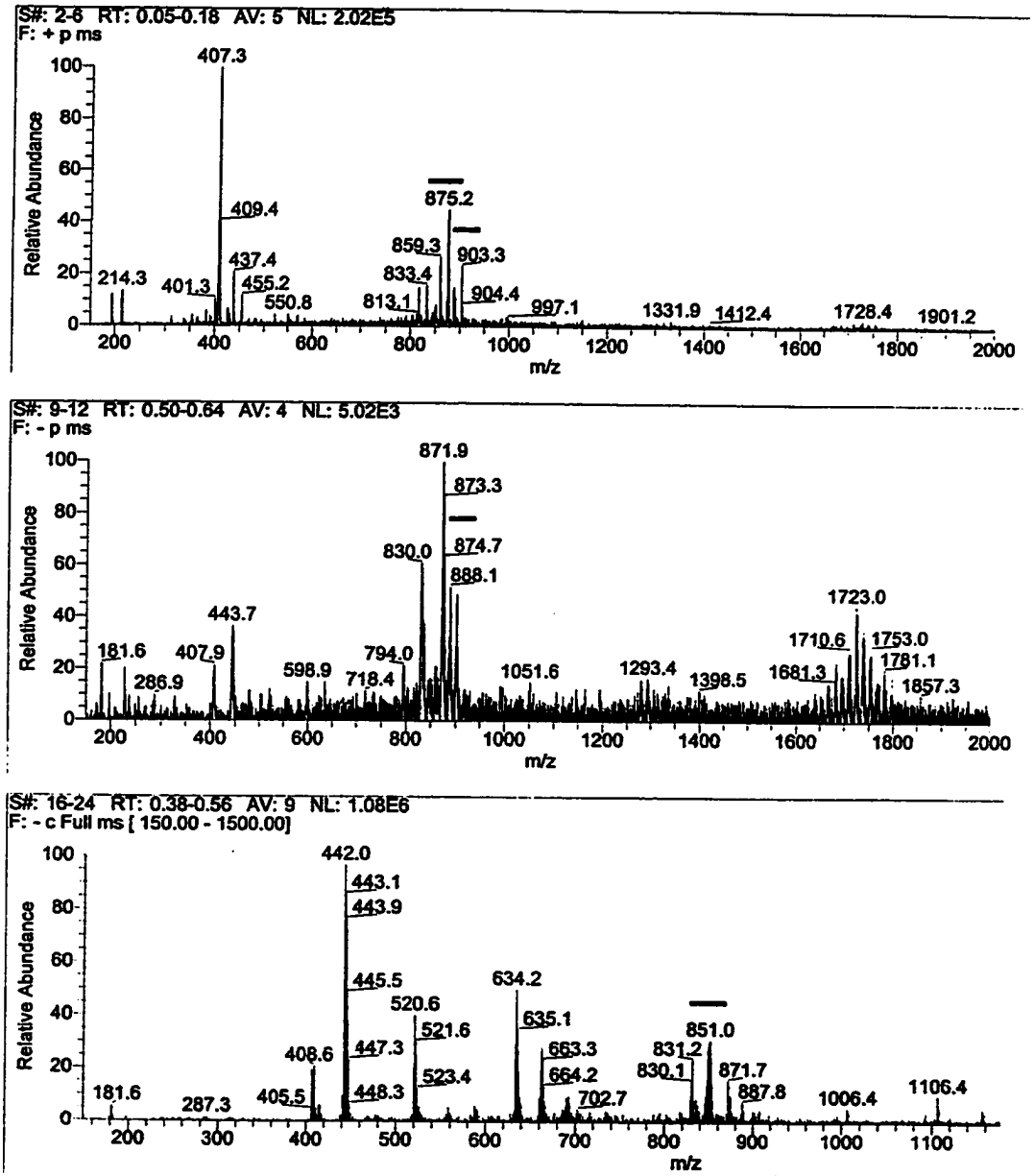
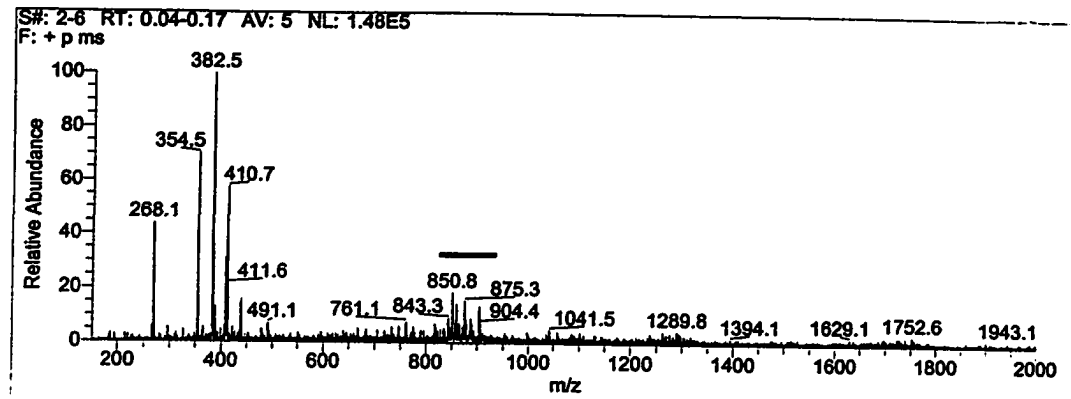


Figure 3.

D. *P. atra* egg ribbons of mixed ages



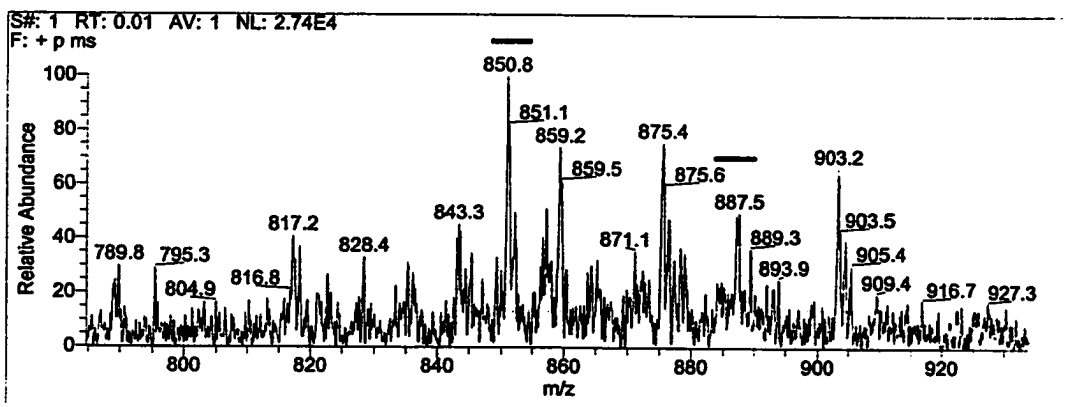
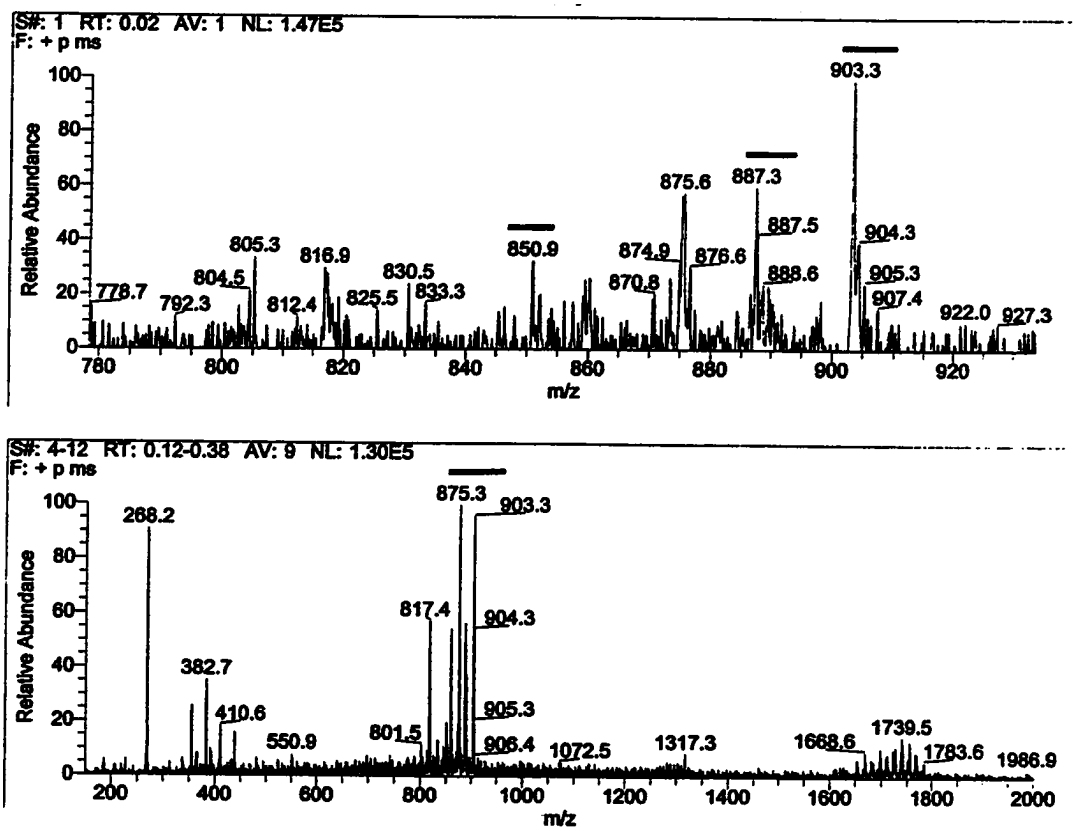


Figure 3.

E. *P. atra* fresh egg ribbons



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Appendix

The data presented here are in the form of an appendix because they are referred to in several chapters and do not constitute a full fledged manuscript for publication, but preliminary studies needing additional experiments. This work was conducted in collaboration with Meredith Protas.

Introduction

In Chapter 2 I discuss possible chemical defenses of bryozoa and suggest that *B. neritina* uses bryostatins as a means of protection against predation and competitors for space. In this study we asked two questions; 1) Is bryostatin concentrated in vulnerable areas of the colony, or distributed evenly? 2) Does the location of the bryostatin activity correlate with the location of the symbionts "*Endobugula sertula*"? In order to address these questions we investigated the distribution of bryostatin activity along the length of *B. neritina* colonies, and measured the relative symbiont DNA signal. Bryostatin activity was measured from different portions of *B. neritina* colonies ranging in age from one month to several months. The spatial pattern of bryostatin concentration may suggest where the colony needs it the most and reveal whether or not bryostatins are concentrated in vulnerable areas of the colony, or areas that would give *B. neritina* a competitive advantage in the fouling community. If bryostatins are involved in the defense of the bryozoan colonies, we would expect bryostatin activity to be concentrated in the tips relative to the rest of the colony, because the outermost tips are the first portions encountered by swimming predators when approaching *B. neritina*.

In order to correlate bryostatin activity with the presence of symbiont cells, the relative amounts of "*Endobugula sertula*" DNA were measured throughout the colony and this distribution was compared with that of the bryostatin activity. A strong correlation could support the hypothesis that "*E. sertula*" is involved with the synthesis of bryostatins. However co-localization at this macroscopic level is hard to interpret. There are a number of plausible explanations for the bryostatins to be associated with areas that also contain symbionts. If symbionts are distributed throughout the colony as are bryostatins, this pattern does not tell us much. If bryostatins are absent from areas devoid of symbionts, that suggests the symbionts may themselves contain the bryostatins. However, bryostatins may be excreted from the cells that produce them, and be transported around. In this case, detection of bryostatins at the site of synthesis will be very difficult. In fact, even if the symbionts are producing bryostatins, it is possible to have higher concentrations in areas devoid of symbionts if the compound is excreted, transported and concentrated in areas away from the site of synthesis.

Within the larvae the bacterial symbionts are segregated to the pallial sinus. If bryostatins are produced by the symbionts, and are not excreted and moved about, then bryostatins should be in the area of the pallial sinus and not elsewhere. This pattern would also be observed if the symbionts excreted bryostatins or were involved in the synthesis but they were not transported away. Examining bryostatin activity in the different portions of the larvae may answer whether we find bryostatins where there are no symbiont cells. For this examination we divided both larvae and adults to assay different portions for bryostatin activity and symbiont levels.

Methods

Cultured *B. neritina* adult colonies

Cultured *B. neritina* colonies of known ages were provided by CalBioMarine Technologies, Inc. thanks to the efforts of Carolyn Sheehan. These were from three different settlements of larvae from two different broodstock locations, Palos Verdes (deep genotype, Chemotype O) and Scripps Pier pilings (shallow genotype, Chemotype M). (see Chapter 4 for explanation of chemotypes and genotypes).

Larvae were settled onto PVC perforated plates and suspended in large flow-through aquaria on the Scripps Pier where they were maintained and fed a diet of mixed phytoplankton (50% *Rhodomonas*, 50% mix of *Ellipsoidea*, *Isochrysis*, and *Nanochloris*).

One month old Palos Verdes, three month old Scripps Pier, and six month old Palos verdes cultured colonies and Palos Verdes wild adult colonies (age unknown, estimated three to five months) were selected and examined under a dissection scope for division into parts to be assayed for bryostatin activity and symbiont levels. Colonies were divided into tips (upper most growing portions), middles, bottoms (lower portions including first branch point) and when available, rhizoids (anchoring structures, rhizozoids) (figure 1 A). Upper portions of large colonies (wild) were divided additionally into tips and lower tips.

Adult tissue preparation for bryostatin and symbiont assays

To prepare tissue for analysis portions of the colony were kept on ice before drying, then excess seawater was blotted away and the tissue was dried in a preweighed 1.5 ml epitube. After larval and colony tissue was dried and weighed, bryostatins were extracted by adding the appropriate amount of 95% ethanol to give 12 mg/ml sample to volume concentration then homogenized using an epitube pestel (Konte). Initial trials showed that the *B. neritina* colonies must be ground up in the ethanol to release the bryostatins. Tissue that was not homogenized gave very little or no signal compared to tissue from the same colony that had been homogenized. Debris was centrifuged into a pellet using a table top centrifuge at full speed for 10 minutes (14 K x g). The ethanol was removed for use in the bryostatin activity assays and the DNA was extracted from the pellets using QIAGEN tissue kit. Initial experiments showed that if the samples were blotted dry then dried under vacuum (10 min) DNA could be successfully extracted from the pellet after ethanol extraction. This allows for the same tissue to be assayed for both bryostatin activity and symbiont signal by PCR. If the sample is preserved in ethanol prior to drying, weighing and DNA extraction, then the DNA appears degraded. For this reason samples must be kept on ice and dried immediately.

Preparation of larvae for bryostatin activity assays

Larvae were fixed in buffered 4% paraformaldehyde (0.1 M MOPS pH 7.4, 0.5 M NaCl), rinsed in buffer three times and divided in half with a needle along the median line created by the contraction of the equatorial neuromuscular ring (pg 104, Biology of

Bryozoans; figure 1). The aboral portion containing the pallial sinus and bacterial symbionts were separated from the portion containing the metasomal sac. For each portion 120 larvae were used (tops, bottoms, tops and bottoms, and whole larvae). The collection of cut larvae placed together (tops and bottoms) was to control for any effect cutting the larvae in half may have on loss of bryostatins. Each portion was assayed for bryostatin activity. Symbiont assays were not performed on larval fractions because the pallial sinus has been well established as the location of the bacteria by electron microscopy (Woollacott, 1981) and by in situ hybridizations (Haygood and Davidson, 1997; Chapter 3). To control for possible effects of residual fixative in the assay, live colonies of the bryozoan *Scrupocellaria sp.* were fixed in buffered 4 % paraformaldehyde, rinsed in buffer three times, dried and assayed for PDBu displacement activity. In a previous experiment, *Scrupocellaria* showed no activity in this assay (Chapter 5, figure 2).

Bryostatin activity detection

The tritiated phorbol dibutyrate displacement assay (% PDBu displacement as an indicator of a protein kinase C binding) to detect relative bryostatin activity was performed as described in Chapter 5, with each sample weight run in triplicate.

Symbiont detection

The "*E. sertula*" SSU rDNA-specific amplification was performed to detect symbiont as described in Chapter 3 and 5. In this case a dilution series of DNA was

used for amplification of the symbiont SSU rDNA gene using polymerase chain reaction (PCR) to give a rough estimate of the relative levels of symbiont signal in each portion of the colony. The amount of DNA required to give adequate signal in PCR detected on an agarose gel (10 µl of a 50 µl reaction) were as follows 5, 2, 1, 0.5 and 0.1 ng. For the rhizoids 30, 20, and 10 ng were required. Rhizoids were not tested for all samples in part due to the small amount of tissue, but mainly because we decided to test them midway through the study and some samples were no longer available.

Polyketide synthase gene detection

Detection of beta-keto-acyl synthase gene amplified using specific primers designed from the KSa sequence found in *B. neritina* (see Chapter 3, supplemental pages) was performed using a DNA dilution series in PCR as above. Only the wild colonies, 3 month old Pier Piling cultured colonies, and the 1 month old colonies were assayed. 50, 5 and 1 ng of DNA were tested from the tips and the rhizoids of the older colonies, and 5 and 1 ng of DNA was tested from the 1 month old colonies.

Results

PDBu displacement activity, symbiont detection and PKS signal

Results are summarized in Table 1 and Figure 1. PDBu displacement plots are shown in Figure 2 and 3.

Scrupocellaria sp.

The fixed *Scrupocellaria* controls gave no PDBu displacement up to 400 μg sample weight. This is consistent with an earlier assay in which *Scrupocellaria* preserved in ethanol showed no activity (figure 2).

Larvae

The tritiated PDBu displacement curves for all larval fractions were similar, yielding equal IC 50 values close to 75 μg/ml dry sample weight.(Table 1; figure 1, 2) The level of bryostatin activity detected in each half was equal to each other and equal to that found in whole larvae and larvae that had been cut in half and placed back together.

1 Month Old Colonies

Bryostatin activity

In the one month old colonies, phorbol displacement activity was about 1.5 times higher in the lower portion than the mid and upper portions of the colonies (Table 1; figure 1, 3A). These colonies have only one branch point. When assayed one week later, with an additional branching point, all portions of the colony showed similar levels of PDBu displacement activity (Table 1) indicating a shift of bryostatin into the upper portions during early growth, either by new synthesis or transport.

Symbiont detection:

In one month old colonies, symbiont signal was strongest in the bottom portion of the colony and weakest in the tips. 1 ng of base DNA yielded symbiont signal that was comparable to signal from 5 ng of tip DNA.

PKS gene detection

The KSa primers yielded amplification products from the bottom (1 ng of DNA) of these colonies only. 5 ng of tip DNA did not give products.

3 Month Old Colonies

Bryostatin activity

These *B. neritina* colonies were substantially larger than 1 month old colonies. PDBu displacement activity was highest in the tips and in the rhizoids. Since only one rhizoid sample size was tested due to limited amount of tissue, the IC_{50} was estimated from the IC_{50} of the tips which gave similar displacement at the same sample size (~80 % at 120 µg). The activity for the rhizoids is a minimum estimate and could be higher since the reaction was saturating at the sample size tested (80 % displacement) and beyond the linear portion of the curve (Table 1; Figure 3B). The tips and rhizoids had approximately 2.4 and 3.2 times more than the mid and bottom portions of the colonies respectively (Figure 1B).

Symbiont detection:

From the three month old colonies, the strongest symbiont signal amplified from DNA of the tips, and approximately equal signal from the rest of the colony including the rhizoids. The least symbiont DNA was detected from the middle portions of the colony and the rhizoids. 5 ng of middle and rhizoid DNA was required to give a weak signal whereas only 1 ng of tip DNA yielded a strong symbiont gene signal. It is not

possible to determine exactly how much more symbiont DNA is present in the tips from this assay, only that there is more than in the rest of the colony.

PKS gene detection

A greater signal was detected in the tips relative to the rhizoids but the amount difference was not determined.

6 Month Old Colonies

Bryostatin activity

The six month old cultured colonies also had the highest levels of PDBu displacement activity in their tips with the middle and bottom portions of the colonies about equal. Rhizoids from these colonies were not examined. Tips gave activity 2.3 and 2 times higher than the middles and bottoms respectively (Table 1; Figure 1 B; Figure 3 C).

Symbiont detection

Within the 6 month old colonies, the tips clearly have the strongest signal and the middles the weakest. This pattern is very similar to that seen in the 3 month old colonies in which the mid portions of the colonies showed reduced signal relative to the bottom and upper portions of the colony. 1 ng of tip DNA yielded a symbiont signal much stronger than 5 ng of DNA from either the bottom or middle portions of the colonies.

Wild Palos Verdes adult colonies of unkonwn age

Data for the distribution of bryostatin activity and symbiont DNA in wild colonies is from one collection. An additional collection was tested for symbiont distribution but not PDBu displacement activity. This additional examination of wild colonies aimed to confirm results from the symbiont assays of the first sample. We were concerned that the low signal in the rhizoids may have been due to degradation of DNA during the processing for PDBu displacement assays. From the second collection DNA was extracted from freshly divided samples without drying, and yielded similar results for the distribution of symbiont gene signal.

Bryostatin activity:

These colonies were probably between 3 and 5 months old. The lower tips and mid portions of the colonies gave similar PDBu displacement activity, with the upper most growing portion only slightly less (14 %). The rhizoids gave the highest activities and the bottoms the least activity. Displacement activity in the rhizooids was 1.7 and 2.7 times higher than the mid (lower tips, middle) and bottom portions of the colonies respectively (Table 1; Figure 1 B; Figure 3 D).

Symbiont detection:

In the wild colonies, from two separate collections separated by a few months, the results were similar. The bacterial symbiont signal is concentrated in the tips of the colony (upper tips and lower tips). The amount of DNA required to give symbiont signal from the tips was less than that used for the three and six month old lab-cultured colonies (5, 3, and 1 ng of DNA vs. 5, 1, and 0.1 ng DNA). The rhizoids yielded the

least signal for symbiont DNA requiring at least 30 ng of DNA from both wild samples. Since both wild colony samples yielded similar results and required comparable amounts of DNA from each portion, the data indicate that the DNA extraction method used for the first sample (post vacuum drying) did not result in a decrease in symbiont signal. The middle (1 ng DNA) and lower (5 ng DNA) portions of the colonies gave less symbiont signal than the upper portions (0.1 ng of DNA) of the colonies.

From the second wild collection additional samples were tested for symbiont signal. Rhizoids (stem rhizoids) growing up and around the zooids of the base were assayed separately and found to have slightly higher symbiont signal (20 ng of DNA) than the outwardly growing rhizoids (30 ng of DNA), but still much less than the base autozooids (5 ng of DNA). New growth budding from the bottom of the colony was found to have levels similar to that of the stem rhizoids, which is much lower than the symbiont levels in the new growth at the tips of the colony (data not shown).

Polyketide synthase signal:

The signal was higher in the tips than in the rhizoids when comparing a series of 50, 5 and 1 ng of DNA in PCR. The rhizoids did show amplification with only 1 ng of DNA but in all reactions the rhizoid amplification products were substantially less intense.

Discussion

In this analysis we found that the bryostatin-like activity detected by displacement of phorbol from protein kinase C is not evenly distributed through out the

colony, but concentrated in the tips and the rhizoids of colonies three months and older. We found that bryostatin activity is equivalent in both halves of divided larvae and not associated with the bacteria in the pallial sinus. This indicates that bryostatins, if produced by the symbionts, are excreted and possibly transported through the animal. The bryostatins in the larvae may even originate in the adult and be placed in the larvae by the parent as a chemical deterrent to predation upon the larvae and the newly settled ancestrula. The bryostatins in the larvae give the newly metamorphosed developing colony an initial dose of a potentially protective compound.

The evidence that bryostatins are external to the bacterial cells and may be transported in the animal makes it difficult to conclude much from the association of bryostatins with the symbiont locations in the adults. In the tips, the highest level of bryostatin activity is correlated with the highest symbiont signal. However, the pattern is not completely consistent. In the rhizoids, the symbiont DNA is barely detectable, about 30 times less in wild colonies, but this area of the colony has the highest bryostatin activity. Wild *B. neritina* showed almost four times higher PDBu displacement activity in the rhizoids than in the bottoms. There are many alternative explanations for the concentration of bryostatin activity with the symbionts in the tips, and this association does not prove synthesis by the bacteria. The mid and lower portions of the colonies consistently showed lower levels of bryostatin-like activity and lower symbiont levels than the upper most portions. These bryostatin and symbiont levels appear very similar between the middle and lowest portions of the 3 and 6 month old cultured colonies. However in the wild colonies, however, the bottom portions

yielded the lowest bryostatins activity, almost half that found in the middle portions. The symbiont signal was also much lower in the bottom portions relative to the middles of the colonies.

The finding that the symbiont levels are not directly correlated with bryostatin activity distribution does not rule out their involvement with bryostatin synthesis. Preliminary examinations of where the KSa polyketide synthase gene signal is found in the colony shows a good correlation with the symbiont location. The KSa signal correlated better with the symbiont levels than did the bryostatin activities. The tips and rhizoids show highest bryostatin, but the tips clearly have much higher numbers of symbionts and higher KSa signal relative to the rhizoids. This pattern shows that if "*E. sertula*" is producing bryostatins they are likely excreted from these cells and may be transported by the host bryozoan. It seems logical that the animal is able to move the bryostatins about the colony. If symbionts are lost from a portion of the colony, then bryostatins may be moved into that area from another part of the colony. The colony may need to concentrate bryostatins in the rhizoids, where there are not as many bacterial symbiont cells. The upper parts of the colony also contain a higher portion of actively feeding and younger zooids. The rhizoids, in contrast, are non-feeding. Possibly the symbionts are in higher numbers where the autozooids are more actively feeding, then bryostatin is distributed where necessary. The distribution of bryostatins, in this scenario, is under control of the host and not the symbiont.

The concentration of bryostatins in the tips and rhizoids suggests its use as a chemical deterrent for predators and competitors. The tips are likely the first portion of

the colony encountered by a large predator and the rhizoids defend space in a highly competitive fouling community. The highly exposed portions of the colony contain the highest levels of PDBu displacement activity indicative of bryostatins. The colonies tend to form rounded bushes, with growing tips at the top, the first portions of the colony encountered by swimming predators such as fish. In addition, the ovicells that harbor larvae tend to form on the younger portions of the colonies, near the tips.

At the other end, the rhizoids anchor the colony and establish space on the substrate in the fouling community. These may be susceptible to grazing predators and overgrowth by other fouling organisms including settling larvae. The loading of the rhizoids with bryostatins may ensure the colony's place on the surface and protect it from being snipped off at the base. In fouling communities, competition for space is fierce and having toxins in the anchoring base likely ensures space for the next generation and protects the existing colony from detachment and overgrowth. Extracts from the adults have been shown to inhibit larval settlement but the active component has not been identified (Martín and Uriz, 1993).

In summary, the bryostatins are most concentrated in the tips of the colony which also show the highest symbiont signal. But in the larvae and the rhizoids bryostatin concentration is not associated with the presence of symbionts. The pattern of bryostatin distribution indicates that bryostatins are strategically placed to protect the colony.

Table 1: Summary of determined dose for 50 % Phorbol Dibutyrate displacement and transformation into activity per unit weight ($1/IC_{50}$), ng of bryostatin 1 equivalents as explained in Chapter 4, and ng of bryostatin 1 equivalents per zooid of a colony. The rank gives the relative comparison within a colony from the highest to lowest phorbol displacement activity, relative symbiont DNA signal and KSa gene signal with the amount of DNA required for a signal listed in parentheses. ++++, highest signal; +, lowest signal; ND, not determined.

Sample	IC_{50}	$1/IC_{50}$	ng bryostatin per μg	ng bryostatin per zooid	rank	Symbiont signal (ng DNA)	KSa gene signal (ng DNA)
Larvae (whole)	76	.0132	0.171	2.0			
Pallial sinus	82	0.0122	0.159				
metasomal sac	75	0.0133	0.173				
whole, cut	74	0.0135	0.176				
1 month adults							
tips	155	0.00645	0.0839	0.998	2	+ (3)	- (5)
middles	155	0.00645	0.0839	0.998	2	++ (3)	ND
bottoms	113	0.00885	0.115	1.38	1	+++ (3)	+(1)
3 month adults							
tips	50	0.02	0.26	3.09	1	++++(1)	++(1)
middles	120	0.0083	0.108	1.29	2	+ (5)	ND
bottoms	160	0.00625	0.0812	0.966	2	++ (3)	ND
rhizooids	50	0.02	0.26	3.09	1	+ (5)	+(5)
6 month adults							
tips	70	0.0143	0.1857	2.21	1	+++ (1 ng)	ND
middles	170	0.00625	0.08125	0.967	2	+ (5)	ND
bottoms	135	0.0074	0.0963	1.15	2	++ (5)	ND
Wild (3-5 months)							
upper tips	70	0.0143	0.186	2.21	2	+++++(0.1)	++(1)
lower tips	60	.0167	.217	2.58	2	++++ (0.1)	ND
middles	60	.0167	.217	2.58	2	+++ (1)	ND
bottoms	90	.0111	.144	1.73	3	++ (5)	ND
rhizooids	35	0.0286	.371	4.41	1	+ (30)	+(1)

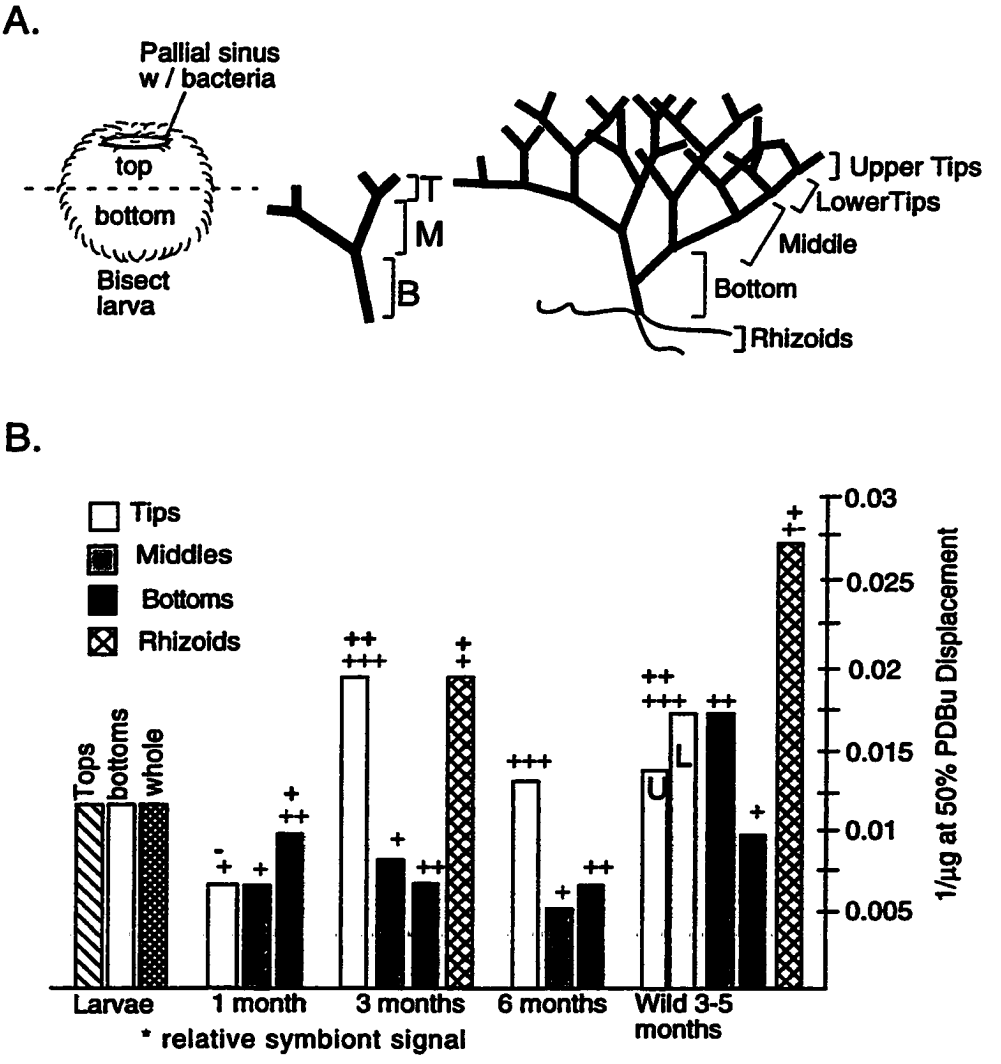

Figure 1. Distribution of bryostatin activity in *Bugula neritina* larvae and adult colonies. A, division of larvae and colonies for bryostatin assays. B, bryostatin activity expressed as activity per µg dry sample weight extracted giving 50 % phorbol dibutyrate displacement. The + symbols give relative amount of symbiont signal in symbiont-specific PCR with +++ being the strongest signal and +- being the weakest. The + symbols indicate relative amount of the KSa gene signal for samples assayed. -, no signal detected. No + indicates that this sample was not assayed.

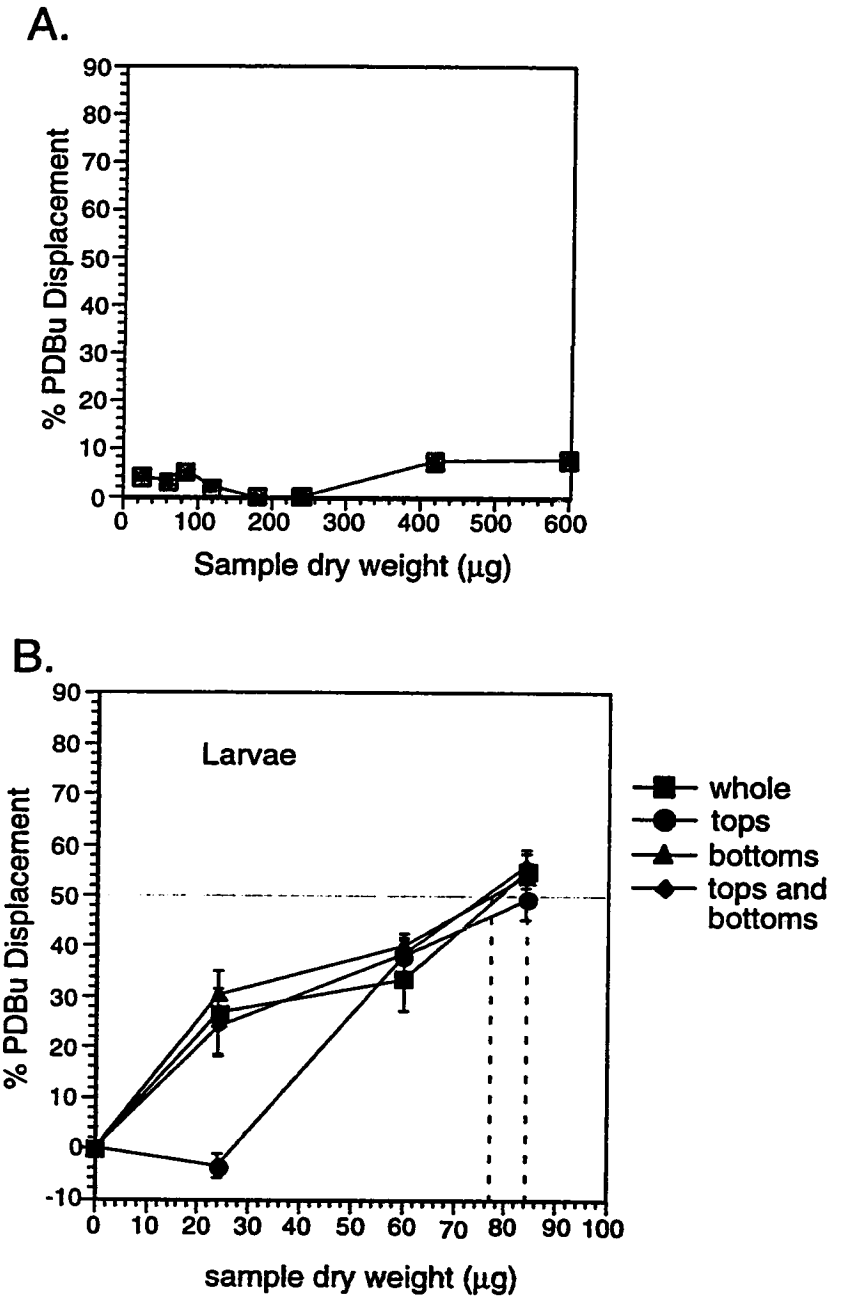

Figure 2: A, PDBu displacement activity of the bryozoan *Scrupocellaria* sp. occuring with *B.neritina*. B, displacement activity in the larvae of *B. neritina*.

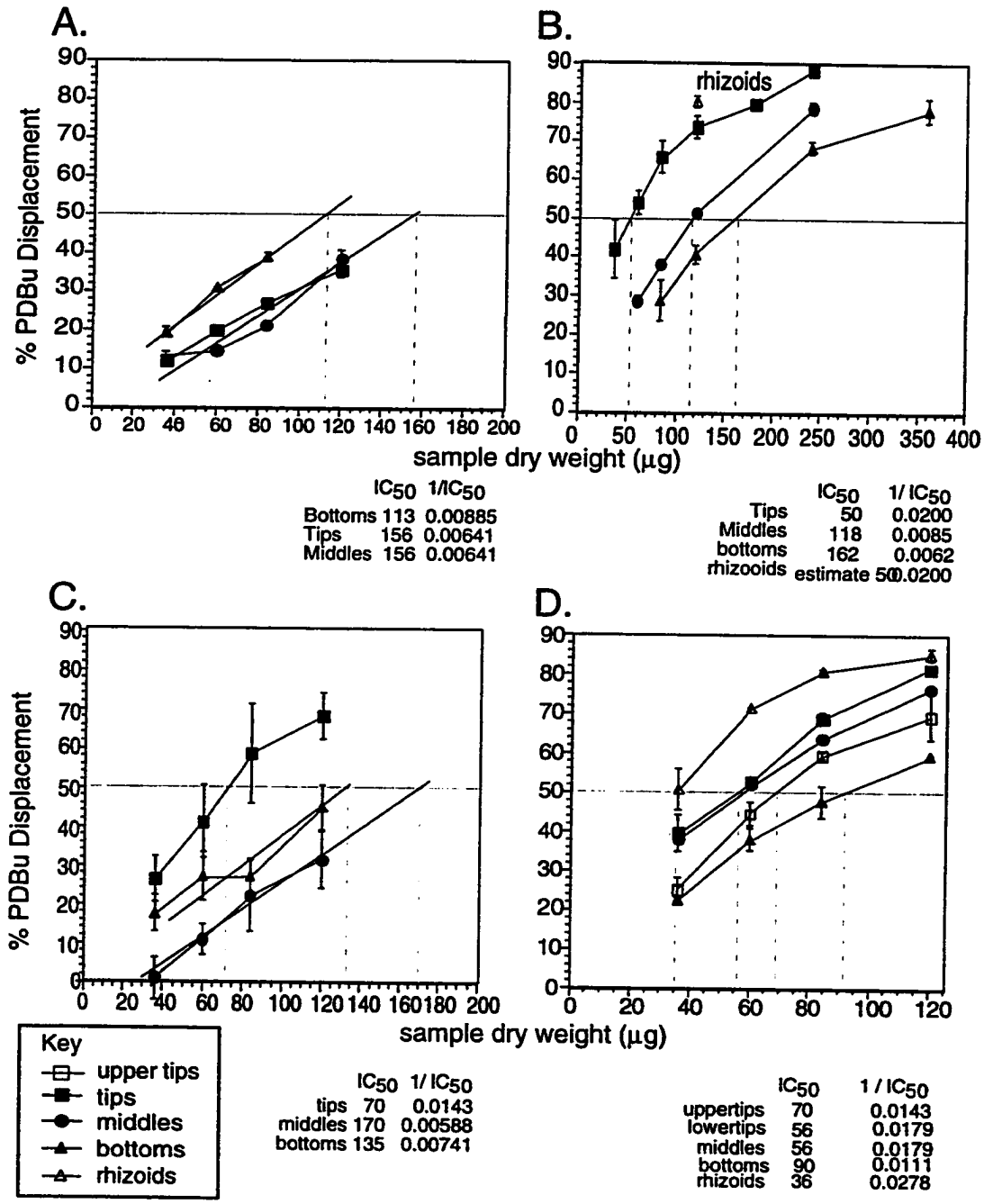

Figure 3. Percent Displacement of Tips, Middles, Bottoms, and Rhizoids in adult *B. neritina* colonies. A, 1 month old, B, 3 months old, C, 6 months old, D, wild colonies 3-5 months old.

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