Title
Investigation of Anti-cancer Compounds in the Marine Bryozoan Bugula pacifica

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Original Goals and Objectives

The original overall project objective was to discover anti-cancer compounds in *Bugula pacifica*. *B. pacifica* is related to *B. neritina*, whose bacterial symbionts produce anti-cancer polyketides called bryostatins. Preliminary results showed biological activity in *B. pacifica* extracts. *B. pacifica* also harbors a bacterial symbiont which also may produce bioactive polyketides.

The specific goals of this project were to:

1) Collect *B. pacifica* in sufficient quantity for study
2) Isolate and characterize secondary metabolites
3) Test *B. pacifica* for biological activity in bioassays
4) Isolate biosynthetic genes involved in secondary metabolite production
5) Characterize the symbiont by its 16S ribosomal RNA genes
6) (not in original proposal) Determine the phylogenetic relationships among *Bugula spp.*

Introduction

General significance

Most of the metabolic and biochemical diversity of life resides in microorganisms: the domains Bacteria and Archaea, and unicellular members Eukarya. Hence, it is unsurprising that multicellular eukaryotes adopt microbial symbionts to gain access to their metabolic diversity.

Our lab group has evidence that the bacterial symbiont of *B. neritina*, "*Candidatus* Endobugula sertula", is the producer of the bryostatins (Davidson, Allen et al. 2001). This symbiont is a gamma-proteobacterium that resides in the pallial sinus of *B. neritina* larvae (Haygood and Davidson 1997), which can be seen by transmission electron microscopy (TEM) (Woollacott 1981). *"E. sertula"* has also been localized to the base of ovicells, which are specialized structures on maternal zooids that brood larvae till they are ready for release (Sharp, pers comm.). The presence of *"E. sertula"* in the larvae and in the ovicells suggests that these symbionts are transmitted vertically, thus ensuring each generation will be inoculated with the symbiont. We have also discovered that *B. neritina* is a species complex, with one genotype found in deep Californian waters, and another shallow genotype that has a wider distribution (Davidson and Haygood 1999). More recently, other researchers have found a third sibling species (McGovern and Hellberg, pers comm.) of *B. neritina* that is found on the Delaware coast. Lopanik and Lindquist (pers comm.) have also demonstrated that the symbiont-produced bryostatins chemically defend *B. neritina* larvae from predation by fish.

Compared to other *Bugula spp.*, *B. neritina* is extremely well-studied and serves as a good model to initiate studies of other *Bugula spp*.

The Bugula genus

The bryozoan genus *Bugula* is a relatively speciose genus, with about 80 species identified and curated at various museums around the world. However, there is probably a

smaller subset which is found in abundance. The number of species may be greater if we consider the fact that some species may be further split into sibling species as described above. Though this genus is well-described, the relationship among species is unknown (Soule, Winston, Woollacott, personal communication). Colonies of *Bugula spp.* are arborescent and are comprised of many individuals, or zooids. These colonies either have a spiral or bushy appearance, and their sizes can range from a few centimeters to 20 cm. Bryozoans feed on phytoplankton using their crown of tentacles, which is also called a lophophore. *Bugula spp.* reproduce sexually, releasing lecithotrophic larvae which swim briefly and settle on solid substrate to form a new colony.

Some members of this group of bryozoans harbor bacteria within their tissue (Woollacott 1981) as observed by TEM. Besides *B. neritina*, *B. pacifica* and *B. simplex* have morphologically similar bacteria in their larval pallial sinuses, whereas *B. turrita* and *B. stolonifera* do not. It is unknown whether these bacteria are related and this question was addressed in this study.

Results and Accomplishments

1. Collection

We have made collections of *B. pacifica* from several locations, ranging from Friday Harbor, Washington to Bamfield, British Columbia. Some of the collections were sufficient only for bioassays and molecular biology experiments, while some of them were larger, on the order of hundreds of grams, which is necessary for the isolation of bioactive molecules. It was essential to obtain several populations of *B. pacifica* as we needed to verify that the symbiotic bacterium is present in all populations.

The bryozoans were rinsed twice in filtered seawater promptly after collection. Visible epibionts were removed and the bryozoans were either fixed in a paraformaldehyde buffer or in 100% ethanol. Larvae were collected from *B. pacifica*, *B. simplex* and *B.turrita*. The adult colonies were kept in the dark for eight hours or more and then exposed to light. The larvae are released in the response to light and swim towards light. The larvae are then collected, rinsed and used for DNA extractions.

Genomic DNA was extracted from the organisms (either adult or larvae) using the Qiagen Dneasy Kit according to manufacturer's instructions. About 30mg of animal tissue was used for each extraction. The resulting DNA contained both host and symbiont DNA.

Species	Range	Number of samples in hand	Collection Locations
B. neritina	Cosmopolitan	5	CA, NC, Chile, Namibia
B. pacifica	Alaska to Channel Islands	7	Vancouver Island, Friday Harbor, Anacortes
B. californica	British Columbia to Channel Islands	6	Vancouver Island, Friday Harbor
B. simplex	North Atlantic, Mediterranean	6	Woods Hole
B. turrita	Western Atlantic	10	Woods Hole
B. dentata	Widespread	1	South Africa
B. stolonifera	Widespread	7	CA, Woods Hole, DE, Namibia

2. Isolation and characterization of secondary metabolites

We hypothesized that since the bacterial symbionts in *B. pacifica* and *B. simplex* are closely related to *"E. sertula"*, they may also produce bryostatin-like polyketides.

Only *B. pacifica* was collected in sufficient quantities for chemical characterization. *B. pacifica* was extracted in methanol overnight and the extract was dried down. Proton nuclear magnetic spectroscopy (^{1}H-NMR) was performed on this crude extract, paying attention to any signal that could correspond to bryostatin or molecules with interesting functional groups. No bryostatin signal was observed, so the extract was fractionated further on a silica column. The fractions were analyzed by ^{1}H-NMR, however, there were no signals that corresponded to molecules with interesting functional groups. Bryostatins are present in minute amounts in *B. neritina* (Pettit, Herald et al. 1982), so it is possible that they were masked in the chemical analyses. Hence, we decided to investigate bioactive molecules by their activity.

3. Biological Activity

In our preliminary experiments we had detected activity in extracts from *B. pacifica* from Northern California. These samples were recently discovered to be *B. stolonifera*. We have tested organic extracts from our new collections of authentic *B. pacifica* from Washington and British Columbia for bryostatin activity. This is accomplished using the phorbol dibutyrate (PdBU) displacement assay, which tests for the ability of bryostatins to displace PdBUfrom protein kinase C (PKC). We tested *B. pacifica* extracts at similar concentrations as *B. neritina* extracts which have previously been shown to be active. Unfortunately, the *B. pacifica* extracts were not active at these concentrations tested. The concentrations were increased, and only slight activity was detected at a concentration of four orders of magnitude higher than that of *B. neritina*. This activity was also seen in extracts of *Crisia sp*., a distantly related bryozoan. We concluded that the slight activity is not real, but due to background noise caused by some compounds in all bryozoans. Whether the activity previously seen in N. California *B. stolonifera*

(previously identified as *B. pacifica*) is significant is still not clear. Since this assay is so specific to the ability of a molecule to bind to PKC, bryostatin-like compounds may still present in *B. pacifica*. These compounds may lack the functional groups that are needed to bind PKC, hence render the assay negative. Recent PdBU assays have shown that *B. simplex* is active. This means that *B. simplex* contains a molecule/s with similar functional groups as bryostatin which allows it to bind to PKC. *B. stolonifera* is not active in the PdBU assay.

B. pacifica and *B. simplex* extracts were also tested for their anti-cancer activity in the human colon tumor-118 (HCT-118) cell line bioassay. They were not active in this assay. Bryostatins are not active in this assay either. Hence, this assay also is selective; not all anti-cancer compounds will give a positive result in this assay.

Lastly, *B. pacifica* extracts were also tested in for their anti-microbial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus*. No activity was detected. This was surprising since previous researchers have found B. pacifica extracts to be active against Gram positive bacteria (Shellenberger and Ross 1998). They, however, did not report the concentrations they tested.

4. Biosynthetic genes

Since no bioactive metabolite was detected by various bioassays and spectroscopic methods, we focused our attention on finding biosynthetic genes instead. We hypothesized that since bryostatin is made by a modular polyketide synthase (PKS) in *"E. sertula"*, a homologous PKS may be present in the closely related bacterial symbionts of *B. pacifica* and *B. simplex*.

Using primers specific to one of the β-keto synthase (KS) domains in the *"E. sertula"* PKS gene cluster, a product of the correct size was amplified by PCR from *B. pacifica*. This product was cloned and multiple clones were sequenced. None of the sequences turned out to be bacterial PKS genes. This PCR was also performed on *B. simplex*, and the product formed was of the wrong size.

Southern hybridization was also performed as this technique is usually less stringent than PCRs and is therefore able to detect weakly homologous sequences. A probe was made to the bryostatin PKS in *"E. sertula"* and used to hybridize partially digested *B. pacifica* genomic DNA. No signal was detected, however, this may be due to the low amounts of symbiont DNA in the host/symbiont DNA prep. In *B. neritina*, a positive signal was only seen when the genomic DNA prep was enriched for *"E. sertula"*.

Recently, degenerate KS primers were used to amplify *B. simplex* and a product of correct size was amplified. This product will be cloned and sequenced.

5. Characterization of symbiont 16S rRNA

We hypothesize that since the bacteria in the larvae of *B. simplex* and *B. pacifica* resemble *"E. sertula"* morphologically, and they are found in the same location in the larva, they are phylogenetically related.

Using the polymerase chain reaction (PCR) we have obtained a common bacterial ribosomal 16S small subunit (SSU) gene sequence that is present in all *B. pacifica* populations. This gene sequence was amplified using specific primers for the symbiont of *B. neritina*, *Candidatus* Endobugula sertula, but at a lower annealing temperature. All *B. pacifica* populations tested contained an identical sequence demonstrating that the same bacterium is

present, a indication of a specific symbiotic association. Denaturing gradient gel electrophoresis (DGGE) was also performed on *B. pacifica* samples. DGGE demonstrated that this symbiont was the dominant bacterium in the bacterial community of *B. pacifica*. In addition, this symbiotic bacterium is very closely related phylogenetically *to "E. sertula"*, which is the producer of bryostatin 1. They are 98% identical over 900 base pairs of the 16S rRNA gene.

Using the same method, the 16S rRNA gene of the bacterial symbiont of *B. simplex* was also sequenced. Surprisingly, a *Bugula sp.* that was previously identified as *B. pacifica* yielded another 16S rRNA sequence that is closely related to that of the other *Bugula* symbionts (bryozoans are notoriously difficult to identify). This species was only recently identified as *B. stolonifera*, which was not known to harbor bacterial symbionts (Woollacott 1981). This sample was the source of our preliminary positive results.

The sequences of the *Bugula* symbionts were aligned with other gamma-proteobacteria by eye and refined with the help of 16S rRNA secondary structure (http://www.rna.icmb.utexas.edu/). *Agrobacterium tumafaciens* was used as an outgroup. Phylogenetic trees were constructed with PAUP*. For maximum likelihood analyses, evolution models were selected using Modeltest. The parameters calculated in Modeltest were used for heuristic searches for 10 replicates with random addition under the likelihood criterion. Posterior probabilities were calculated using MrBayes. Four chains were run for one million generations, and the first two hundred trees were excluded from the analyses (Figure 1). The *Bugula* symbionts form a monophyletic group among the proteobacteria, suggesting that they radiated from a recent common ancestor.

Summary of accomplishments

- the 16S rRNA genes of three *Bugula spp.* symbionts were sequenced, and mapped on a phylogenetic tree.
- Although *B. pacifica* does not look promising as a source of anti-cancer compounds, *B. simplex* is worth investigating further. The presence of a bacterial symbiont related to *"E. sertula"*, PdBU activity, and positive PCR amplification with KS primers, are exciting results that will be followed up in our search for novel anti-cancer compounds from bryozoans.

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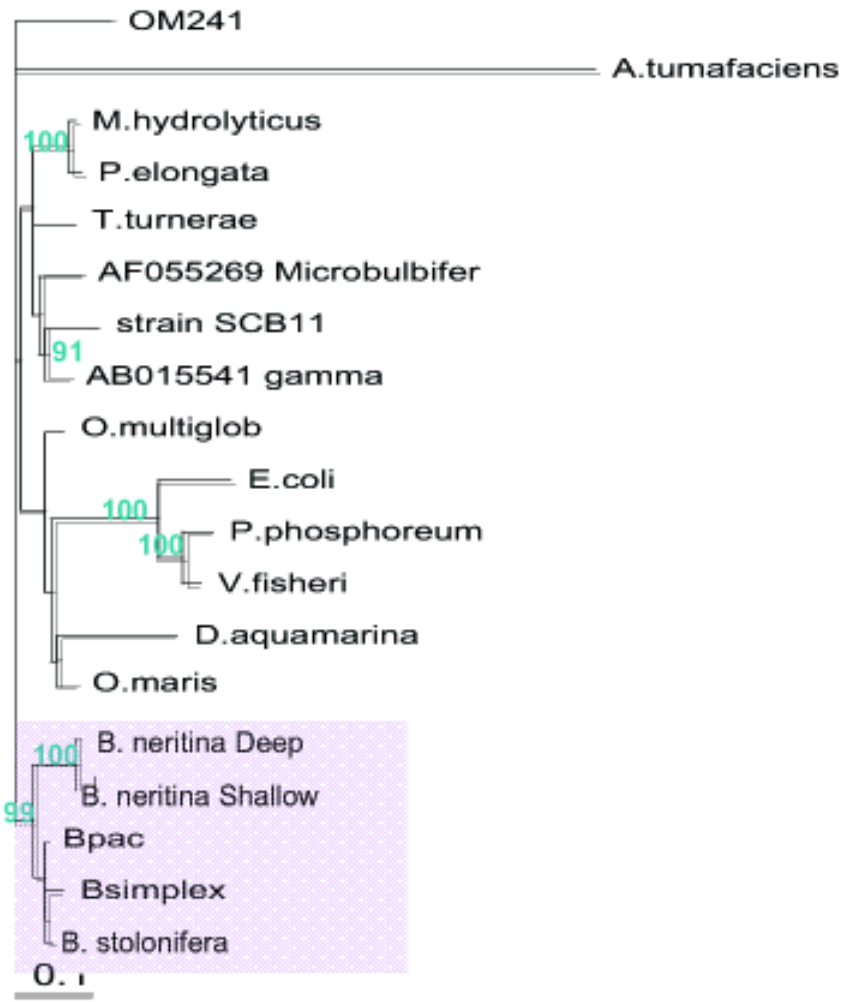

Figure 1: Likelihood tree of *Bugula* symbionts (highlighted in pink) among other proteobacteria. Posterior probabilities are shown in blue

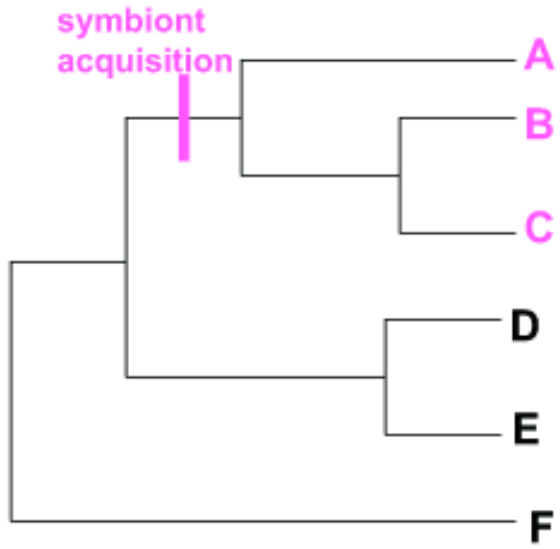

Figure 2: Hypothetical tree showing single symbiont acquisition. A, B and C are symbiotic species, while D, E and F are non-symbiotic

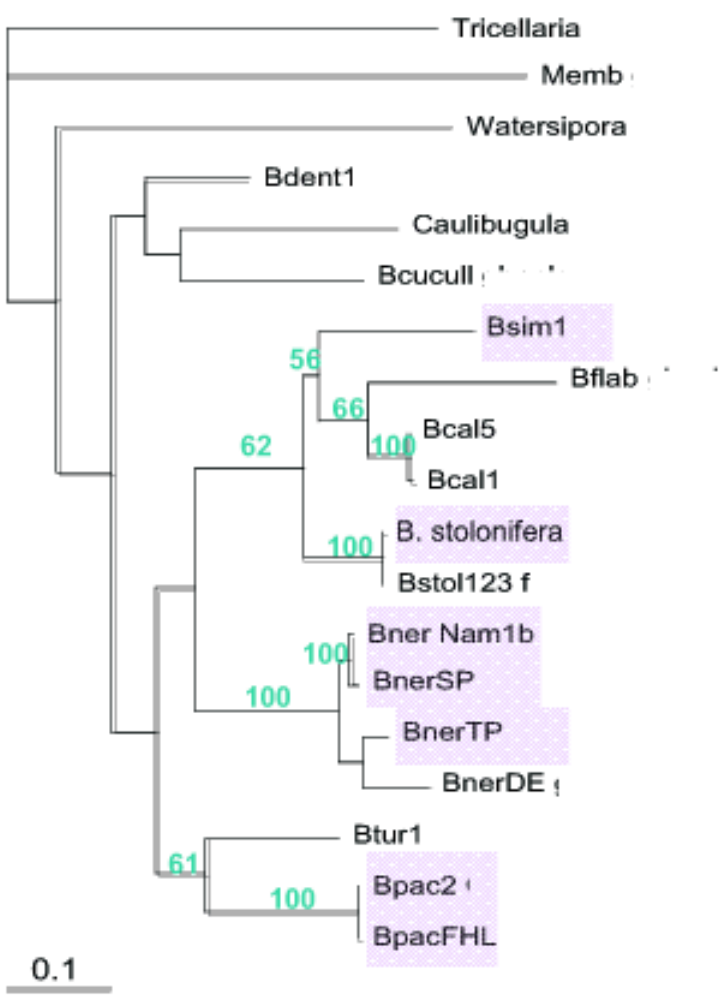

Figure 3: COI likelihood tree of *Bugula* spp. (symbiotic species highlighted in pink). Posterior probabilities are shown in blue

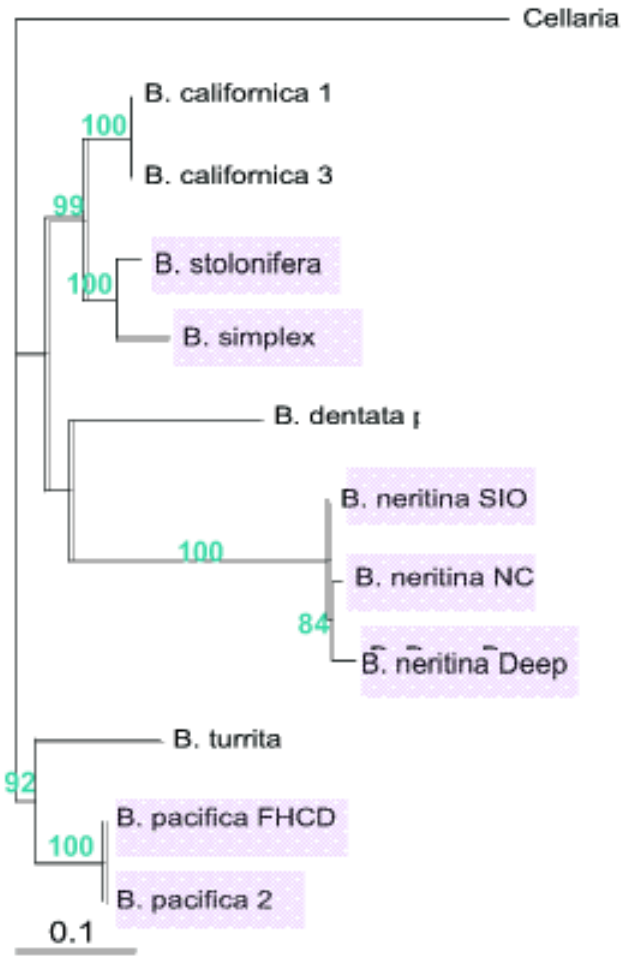

Figure 4: Mito16S likelihood tree of *Bugula* spp. (symbiotic species highlighted in pink). Posterior probabilities are shown in blue