

Microbial Symbionts of Marine Invertebrates: Opportunities for Microbial Biotechnology

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Abstract

Marine invertebrates are sources of a diverse array of bioactive metabolites with great potential for development as drugs and research tools. In many cases, microorganisms are known or suspected to be the biosynthetic source of marine invertebrate natural products. The application of molecular microbiology to the study of these relationships will contribute to basic biological knowledge and facilitate biotechnological development of these valuable resources. The bryostatin-producing bryozoan *B. neritina* and its specific symbiont "*Candidatus Endobugula sertula*" constitute one promising model system. Another fertile subject for investigation is the listhioid sponges that contain numerous bioactive metabolites, some of which originate from bacterial symbionts.

Introduction

Most of the metabolic and biochemical diversity of life resides in microorganisms: the domains Bacteria and Archaea, and unicellular members of the Eukarya. Eukaryotic multicellular organisms, although more conspicuous, are limited in their biochemical capabilities. Thus, it is not surprising that microbial symbionts have been adopted by higher organisms time and again throughout evolution. Indeed, the eukaryotes are the product of symbiotic associations between early eukaryotic cells and bacteria that became mitochondria and chloroplasts, greatly expanding the ecological opportunities for these chimeric organisms.

Both the fact and the function of symbiosis are often relatively obvious. For example, many fishes and some squid maintain cultures of bioluminescent bacteria whose light is put to a multitude of uses (Haygood, 1993; McFall-Ngai, 1994). The hydrothermal vent vestimentiferans and bivalves that are supported by chemoautotrophic symbiotic bacteria are noteworthy examples that were readily recognized because their unique habitat and unusual morphology pointed inevitably to dependence on symbionts (Cavanaugh, 1994). Likewise, the dependence of many cnidarians such as corals on photosynthetic symbionts is well known (Buddemeier, 1994; Rowan, 1998).

It is becoming apparent, however, that symbionts can fulfill much more diverse and subtle roles. In particular, animals can gain access to the biosynthetic virtuosity of prokaryotes through symbiosis. Marine invertebrates, particularly sessile ones, are rich sources of unusual metabolites. Like terrestrial plants, they often rely upon chemical defense to discourage predation. Symbiosis with biochemically versatile microorganisms is an efficient strategy to accomplish chemical defense. Microbial symbionts have often been invoked as biosynthetic sources for natural products found in marine invertebrates. In some cases, the evidence supports an autogenous source for the compounds, while in others the data support a microbial symbiont origin. The vast majority lies in a gray area, where arguments based on structural similarity to microbial metabolites often suggest a symbiotic origin, but solid evidence is lacking. There are difficulties in relying upon structural similarities to speculate about the source of compounds. Similar and identical compounds can arise from parallel or convergent evolution. Similarities can also arise from digestive degradation of standard compounds, such as the dioxopiperazines formed by hydrolysis of peptides and proteins. Another potential problem is transfer of genes from bacteria to other organisms (Hopwood, 1997). Genes for many biosynthetic pathways are contiguous in bacteria, and it is reasonable to assume that pieces of these genes, as well as entire gene clusters, may be moved by lateral gene transfer. Thus, specialized genes encoding unusual functions could be obtained by gene transfer from bacteria to invertebrates rather than evolving *de novo*. A high frequency of plasmid transfer in the marine environment amplifies the possibility of lateral gene transfer (Dahlberg *et al.*, 1998). Finally, it is premature to declare that because a compound has only previously been found in a single source such as a species of microbe, related compounds must come from the same microbe. It is equally possible that other sources have not yet been found. Hypotheses concerning the biosynthetic source for a compound must be investigated in each instance.

Natural products from marine invertebrates greatly expand the chemical diversity available for biotechnological exploitation. The soil microbiota and terrestrial plants have proven to be extraordinary repositories of diverse compounds that can be employed directly or modified for application. The marine realm promises to be another excellent source with little overlap with traditional sources of natural products. Developing these resources can present problems, however. The organisms can be rare, slow growing or difficult to collect. Wild collection can cause environmental damage to vulnerable habitats. Aquaculture is an option in some cases, but even aquaculture can have negative environmental consequences.

Marine invertebrate natural product symbioses present a fascinating subject for basic biological and biochemical research. In addition, they present an opportunity to develop these resources in ways that circumvent environmental and supply problems. Cultivating the

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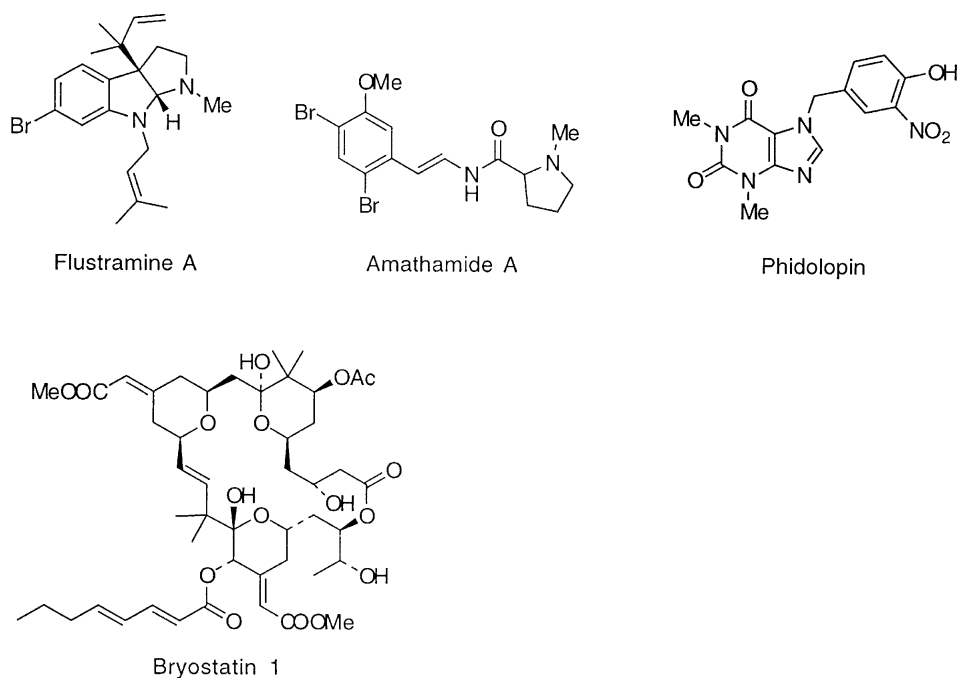


Figure 1. Bryozoan Natural Products

symbionts, understanding the regulation of compound production and optimizing biosynthesis *ex symbio* completely eliminates the problems of wild collection, and can potentially improve the yield and thus the economics of production. However, cultivation of microbial symbionts is not a trivial problem.

The major challenge in microbial ecology today is that the microbes that predominate in natural environments are not well represented among laboratory strains (Hugenholtz *et al.*, 1998). We need to develop more insightful methods for making these organisms amenable to laboratory investigation. Symbioses are ideal systems for addressing these problems because the populations present are relatively stable, and the constituent organisms range in ease of cultivation. We need to expand our thinking beyond pure cultures that grow rapidly to high cell densities to include consortia and growth conditions that more closely mimic *in situ* conditions. We can obtain sequence information from ribosomal RNA (rRNA) genes directly from the symbiosis without cultivation, and design specific oligonucleotide probes from these sequences. With these tools we can monitor symbionts of interest in consortia and dissect their function in a way that was previously impossible.

Symbiotic systems are also well suited to direct investigation of biosynthesis using molecular biological techniques. Using database sequences as a starting point, we can clone biosynthetic genes directly from the symbiosis and reconstitute them in a heterologous host for expression and compound production. The fact that many bacterial pathways for secondary metabolites are organized in contiguous operons facilitates pathway reconstruction. Once the genes are in hand, combinatorial or pathway engineering approaches can be used to obtain novel structures with different activities.

Examples

Microbial symbioses have been described from most phyla of marine invertebrates: Porifera, Cnidaria, Bryozoa, Mollusca, Pogonophora, Echinodermata, Urochordata and Crustacea. Symbionts include bacteria, archaea and unicellular eukaryotes such as dinoflagellates. Interesting natural products have been found particularly in sponges, ascidians, cnidarians, bryozoans, and nudibranchs (Anthoni *et al.*, 1990). In this article, we will describe examples of both simple and complex symbiotic systems, focusing on bryozoans and sponges.

Bryozoans

Bryozoans are small, sessile colonial invertebrates. Bacterial symbionts have been discovered in several species of bryozoans, but most have not been examined for symbionts (Lutaud, 1964; 1965; 1969; 1986; Woollacott and Zimmer, 1975; Woollacott, 1981; Zimmer and Woollacott, 1983; Boyle *et al.*, 1987). The natural products most commonly found in bryozoans are alkaloids. In addition to alkaloids, bryozoans contain other classes of compounds. *Watersipora cucullata* contains sulfated ceramides (Ojika *et al.*, 1997), *Dakaria subovoidea*, contains a thiophene (Shindo *et al.*, 1993), *Alcyonidium gelatinosum* contains dimethyl sulfoxonium ion (Christophersen, 1985), and a family of macrocyclic polyketides, the bryostatins, are found in *B. neritina* (Pettit, 1991). A symbiotic origin has been suggested for several bryozoan natural products: alkaloids such as the flustramines and the amathamides, as well as the phidolopins and the bryostatins (Anthoni *et al.*, 1990) (Figure 1). Bryozoan pyrrole pigments such as the tambjamins are related to bacterial products and thus could be of dietary or symbiotic origin (Anthoni *et al.*, 1990).

Bacterial Symbionts in Bryozoans

In adult bryozoan colonies, bacterial symbionts are typically associated with the funicular cords, which are internal organs that connect individuals of the colonies (Lutaud, 1969; Woollacott and Zimmer, 1975; Zimmer and Woollacott, 1983). In some species, they reside in other structures apparently specialized for housing symbionts (Lutaud, 1964; 1965; 1986). In a few cases, bacteria have been found in modified tissues in the larvae (Woollacott, 1981; Zimmer and Woollacott, 1983). Such adaptations to ensure intergenerational transmission suggest a highly evolved association in which the presence of the bacteria is advantageous to the bryozoan. Bryozoans typically have a simple associated microbial community relative to sponges. This should make bryozoans relatively tractable model systems for the investigation of symbiosis.

Bugula neritina

B. neritina is the source of the bryostatins, a family of cytotoxic macrolides (Pettit, 1991). *B. neritina* is a case in which a symbiotic origin of the bryostatins has been proposed, but not yet proven.

B. neritina is a cosmopolitan, temperate fouling invertebrate often found on boats, piers and subtidal rocky substrates. It is considered a "weed" that grows rapidly and outcompetes the other fouling organisms. The feeding zooids extend a crown of tentacles called a lophophore that captures phytoplankton and other small particles. *B. neritina* broods its larvae in specialized zooids called ovicells (Woollacott and Zimmer, 1975). The larvae are released when mature and competent to settle. The larvae are relatively large (approx. 200-300 μm). They have no gut, and do not feed. Upon settling they rapidly metamorphose and produce the ancestrula, the first colonial unit with a feeding zooid (Woollacott, 1971; Zimmer and Woollacott, 1977; Woollacott and Zimmer, 1978). The larvae typically settle near other *B. neritina*, probably due to limited dispersal (Keough, 1989). *B. neritina* can be raised from larvae to reproductive adults in the laboratory, although growth rates are generally lower than in wild colonies. *B. neritina* larvae contain bryostatins and post-spawn colonies have less bryostatin (Thompson and Mendola, unpublished). Bryostatins appear to be stable and persistent in the colony. The highest bryostatin contents we have observed occurred in very old colonies at a site protected from winter storms, suggesting that it accumulates over time. Even completely senescent colonies that have been reduced to skeletons contain significant quantities of bryostatins, perhaps due to diffusion of bryostatins throughout the colony. Bryostatins do not originate in the diet of *B. neritina* since colonies raised on a completely artificial, bryostatin-free diet produce bryostatins (Thompson and Mendola, unpublished).

Chemical defense of *B. neritina* has not been extensively studied. A study of chemical defense of invertebrate larvae found that the larvae of *B. neritina* are strongly chemically defended, but did not find evidence for defense in the adult colony (Lindquist and Hay, 1996). It is possible that bryostatins are produced by the adult colony and sequestered in the larvae to defend the larvae.

Bacterial Symbionts of *B. neritina*

The larvae of *B. neritina* contain rod-shaped Gram-negative bacteria in the pallial sinus, a sealed circular fold in the

aboral surface of the larva (Woollacott, 1981). The bacteria appear to be extracellular by transmission electron microscopy. *B. pacifica* and *B. simplex* contain morphologically similar bacteria but *B. turrita* and *B. stolonifera* do not (Woollacott, 1981). In *B. neritina*, the bacteria can be seen in the pallial epithelium during settling and metamorphosis (Reed and Woollacott, 1983). In adult colonies, bacteria occur in the funicular cords that connect zooids and nourish the developing larvae (Woollacott and Zimmer, 1975). Whether these bacteria are the same as those in the larvae is not known, but if they are, their presence in the funicular cords would provide a mechanism for them to be transferred to the larvae and to regenerating zooids. The larval symbiont is a candidate for a microbial source of bryostatins. The elaborate adaptations to ensure transmission of symbionts from adults to new colonies could account for the universality of bryostatin production in *B. neritina*.

B. neritina is the only bryozoan for which symbiont rRNA sequence data is available (Haygood and Davidson, 1997). Small subunit (SSU, 16S) rRNA genes of the symbionts were amplified from DNA extracted from washed larvae using universal and eubacterial primers and directly sequenced. Specific oligonucleotides were synthesized based on the sequence and one was used as a probe for *in situ* hybridization. The specific probe binds to the bacteria in the pallial sinus but not to other related bacteria, confirming the authenticity of the sequence. Based on *in situ* hybridization, there are about 2500 pallial sinus symbionts per larva. Analysis of sequences from larvae from seven different West Coast populations and one from the Atlantic Ocean shows that the same symbiont is present in all cases. Amplification with specific oligonucleotides confirms the presence of this organism in all 16 populations tested. There are two strains that differ at four nucleotide sites, discussed below. Phylogenetic analysis of the sequences shows that the symbiont is a γ -proteobacterium that is not closely related to any sequence in the databases. This organism has been named "*Candidatus* Endobugula sertula" (abbreviated "*E. sertula*"). This taxonomic status is frequently used for bacteria for which a type strain is not available (Murray and Stackebrandt, 1995).

A highly specific and sensitive PCR assay for the presence of the symbionts was developed using the specific oligonucleotides. The larvae were tested to see if "*E. sertula*" could be readily cultivated by plating larval samples on standard media and then testing the strains that grew with the PCR assay. None of these strains proved to be "*E. sertula*", demonstrating that it will require a specialized method for cultivation. Bacterial DNA from seawater near *B. neritina* colonies was negative, suggesting that the symbiont is not a major component of free-living bacterial populations.

Bryostatins

Bryostatins are an important family of cytotoxic macrolides based on the bryopyran ring system (Pettit, 1991). They occur exclusively in *B. neritina*. Bryostatin 1 is now in Phase II clinical trials for treatment of leukemias, lymphomas, melanoma and solid tumors (Pluda *et al.*, 1996). Bryostatin 1 also shows promise for treatment of ovarian and breast cancer and to enhance lymphocyte survival during radiation treatment (Kraft, 1993; Lind *et al.*, 1993; Grant *et al.*, 1994; Scheid *et al.*, 1994; Sung *et al.*, 1994; Correale *et al.*, 1995;

Fleming *et al.*, 1995; Baldwin *et al.*, 1997; Lipsky *et al.*, 1997; Taylor *et al.*, 1997).

Unlike most chemotherapeutic agents that kill rapidly dividing cells, bryostatins act on signal transduction pathways by binding to the activator site of protein kinase C (Steube and Drexler, 1993; Caponigro *et al.*, 1997). Eighteen bryostatins have been described (Pettit *et al.*, 1982; 1991; 1996). These vary primarily in the substituents at C-7 and C-20. Incorporation of the octa-2,4-dienoate ester at C-20 is a unique capability of *B. neritina* from California (Pettit, 1991). Two chemotypes have been defined as follows (Davidson and Haygood, 1999). Chemotype O refers to samples that contain the octa-2,4-dienoate ester at C-20 (bryostatins 1-3, 12 and 15) as well as other bryostatins. Chemotype M lacks the octa-2,4-dienoate ester at C-20, and the presence of other bryostatins in the absence of bryostatins 1-3 is diagnostic of this chemotype. The compound in clinical trials, bryostatin 1, is only found in chemotype O and this chemotype will be the target of commercial exploitation.

The major obstacle in investigating and developing bryostatins as anti-cancer agents or for other therapeutic purposes is the difficulty of obtaining them in ample quantities. The yield of bryostatin 1 is low; in the large-scale isolation for clinical trials it was 1.4 µg per gram wet weight of *B. neritina*. (Schaufelberger *et al.*, 1991). The supply of *B. neritina* is unpredictable and harvesting has long-term negative effects on populations. The population collected for production of bryostatin 1 for clinical trials took several years to regrow. A recent study (Davidson and Haygood, 1999) showed that there are two genetic types (probably species) of *B. neritina*. Type D (deep) is found in waters deeper than 9 m in Southern California, has one strain of "*E. sertula*" (designated type D) and has chemotype O bryostatins, including bryostatin 1. Type S (shallow) is found at less than 9 m and also occurs in the Atlantic. It has another strain of "*E. sertula*" (S), and has only chemotype M bryostatins, lacking bryostatin 1. The S type of *B. neritina* is transported on boat hulls and may occur worldwide. It is not known whether the difference in chemotype is due to the genetic difference in *B. neritina* or the different strains of "*E. sertula*". The type D population containing bryostatin 1 is more limited than was previously appreciated. If the clinical trials succeed, supply from nature will be a serious problem for commercial production of bryostatin 1.

Research has focused on bryostatin 1, but the other bryostatins have been isolated on the basis of their antileukemic activity. With the exception of bryostatins 16 and 17, all possess the structural features believed to account for the activity of bryostatin 1 (Pettit *et al.*, 1982; 1991; 1996). Other bryostatins may equal or exceed the therapeutic value of bryostatin 1. One study showed that bryostatins 5 and 8 are as effective as bryostatin 1 in treating melanoma, but with milder side effects (Kraft *et al.*, 1996). Greater availability of other bryostatins is essential to permit research to unlock the potential of this remarkable family of compounds. Although aquaculture would provide a more consistent source of bryostatins, it does not improve the low yield. Chemical synthesis of bryostatins is currently considered impractical due to their structural complexity. However, synthetic analogs have promise (Kageyama *et al.*, 1990; Wender *et al.*, 1998).

Biosynthesis of Bryostatins

Polyketides are created by sequential condensation of acetate or other simple fatty acid units as in fatty acid synthesis (Katz and Donadio, 1993). There are two types of cyclic polyketides, complex and aromatic. Bryostatins are complex polyketides.

Complex polyketides are typically microbial secondary metabolites. This raises the possibility that bryostatins are made by bacteria associated with *B. neritina* rather than by *B. neritina* itself (Anthoni *et al.*, 1990). Complex polyketides are best known in the actinomycetes, which are members of the high G+C Gram positive bacteria. Microorganisms other than actinomycetes that produce complex polyketides include cyanobacteria, mycobacteria (high G+C Gram positive), myxobacteria (delta proteobacteria), pseudomonads (γ -proteobacteria) and fungi (Katz and Donadio, 1993; Schupp *et al.*, 1995; Nowak-Thompson *et al.*, 1997).

Although complex polyketides are generally regarded as microbial secondary metabolites, some have been isolated from metazoans. Miyakolide, swinholide A, halichondrin, discodermolide and spongistatin are complex polyketides isolated from sponges (Uemura *et al.*, 1985; Hirata and Uemura, 1986; Kobayashi *et al.*, 1990; Higa *et al.*, 1992; Bai *et al.*, 1993; Pettit *et al.*, 1993; Litaudon *et al.*, 1994). However, sponges harbor many bacteria that could produce these compounds, as discussed below.

The enzymes responsible for biosynthesis of polyketides, polyketide synthases (PKS), and fatty acid synthases (FAS) are categorized as Type I, with multiple active sites on a single polypeptide, or Type II, with single active site polypeptides that form a complex. Bacterial FAS is Type II, but eukaryotic FAS is Type I. In both bacteria and fungi, complex polyketides are made only by Type I PKS (PKS-I). We postulate that the PKS responsible for synthesis of the bryopyran ring (bryopyran synthase) is a PKS-I. Since no metazoan enzymes responsible for synthesis of complex polyketides are known, we should consider the possibility is that the bryopyran synthase is a microbial enzyme.

A study of incorporation of radioactively labeled biosynthetic building blocks by *B. neritina* demonstrated that acetate is the probable precursor of the bryopyran ring, with methyl groups on the macrolactone ring system added by S-adenosyl methionine after polyketide chain assembly (Kerr *et al.*, 1996). These results are consistent with a polyketide synthase mechanism.

B. neritina as a Model System

It will be worthwhile to investigate whether the biosynthetic source of bryostatins is a microorganism, particularly "*E. sertula*". Cultivation of such a microorganism, or cloning of the genes, could provide a solution to the problem of supply of bryostatins.

In addition, the *B. neritina*/*E. sertula* system has features that are favorable for development as a model system for the study of natural product symbioses using microbiological and molecular approaches. A good model system is tractable and accessible, yet sufficiently representative to yield useful knowledge applicable to other systems. The most important advantage of this system is simplicity of the microbial community consisting of the single bacterial symbiont and relatively few other bacteria, in contrast to the complex mutualistic, commensal, and

ingested microbial populations of most sponges. In *B. neritina*, symbiont ribosomal sequences have been obtained, specific assay methods have been developed and extensive surveys carried out that demonstrate that "*E. sertula*" is universal in *B. neritina*. *B. neritina* is abundant and readily accessible. There is a large base of knowledge about *B. neritina* biology: it is the *E. coli* of bryozoan biology. It can be maintained in the laboratory, and even raised from larvae through reproductive maturity in the laboratory. The mechanism of transmission of "*E. sertula*" is well established, and is not complicated by recruitment from free-living populations. The association makes a single identified type of natural product, the bryostatins. Bryostatins are complex polyketides, a group that contains numerous molecules with a wide range of bioactivities and applications. The biosynthesis of complex polyketides is well understood and the molecular biology of complex polyketide biosynthesis is a very active area of current research (Cane *et al.*, 1998). Although it would be more convenient if "*E. sertula*" were readily cultivable by conventional techniques, if it were, it would not be representative of many natural product symbioses. The numerous virtues of the *B. neritina*/*E. sertula* association means that testing the hypothesis that "*E. sertula*" is the biosynthetic source of bryostatins should be a high priority.

Sponges

In the early Cambrian Period, before the rise of scleractinian corals, sponges thrived as reef-builders living in close association with bacterial mats (De Freitas, 1991; Brunton and Dixon, 1994; Zhang and Pratt, 1994; Riding and Zhuravlev, 1995). These simple metazoans have maintained their prokaryotic ties into the modern world, filtering bacteria from seawater as food (Turon *et al.*, 1997) and harboring large and diverse microbial populations (Wilkinson, 1984). In many sponges, the bacteria have been shown to play a role in the lives of their host, either through processing of waste products, (Beer and Ilan, 1998) transfer of nutrients to their hosts, (Schumann-Kindel *et al.*, 1997) or production of secondary metabolites (Unson *et al.*, 1994). Thus, sponges are characterized by complex microbial communities that contrast strongly with the simple symbiotic systems of bryozoans. This complexity presents opportunities for interactions that could lead to greater diversity of natural products, but can also complicate microbiological analysis. Sponges have provided more natural products than any other phylum of marine invertebrate, and many of the natural products have potent bioactivities and unprecedented molecular architectures. A microbial origin has been proposed for a number of "sponge" metabolites, but only in a few instances have these hypotheses been tested experimentally. As is the case for bryostatins, many sponge metabolites are limited by their low availability from natural sources. Studies of sponge-microbe symbioses could lead to a better supply of potential pharmaceuticals, and in addition, there is much to learn from these ancient and diverse symbioses about the formation and maintenance of relationships between bacteria and metazoans.

Symbionts in Sponges

As the simplest of metazoans, sponges (Phylum: Porifera) have an extremely long evolutionary history, with a fossil

record that indicates a Precambrian origin for the phylum (Brunton and Dixon, 1994). The history of sponges is tightly coupled to microorganisms, which they filter from seawater with high efficiency (75-99%) (Turon *et al.*, 1997). In addition to their role as a primary food source for poriferans, bacteria have also interacted with sponges to produce reef-like structures (De Freitas, 1991; Brunton and Dixon, 1994; Zhang and Pratt, 1994; Riding and Zhuravlev, 1995). Sponge-microbe associations in ancient reefs have been reviewed in detail (Brunton and Dixon, 1994).

The sponges that formed ancient reefs or are well documented in the Phanerozoic Era fossil record tend to have strongly reinforced skeletons. For that reason, the major sponge fossils are hexactinellids or from the demosponge order Lithistida, both of which have strong siliceous spicules and occur widely in the deep ocean today. Unfortunately, the order Lithistida is polyphyletic, with a taxonomy based only on a spicule type (desma), and thus does not reflect the true evolutionary relationship of these sponges (McInerney and Kelly-Borges, 1998). Because of the uncertain relationship between fossil and living sponges, it is difficult to determine the importance of the early sponge-microbe associations in the evolution of living sponges.

Despite the lack of a clear co-evolution of sponges and microbes in the fossil record, numerous associations between microbes and extant sponges have been described. Sponges feed on microbes, and also harbor large populations (up to 40% of body mass) of microorganisms, both intra- and extra-cellularly (Vacelet and Donadey, 1977). Bacterial density is particularly high in massive sponges with a large mesohyl, and cyanobacteria often coat the outer layers of shallow, exposed sponges. The literature on the ecological significance of microbial sponge symbionts through 1987 has been reviewed by Wilkinson (1987). Until that time, sponges were studied primarily for their relationship with photosynthetic organisms such as cyanobacteria and dinoflagellates, and nutrient transfer relationships were demonstrated in a number of cases. Nitrogen fixation and the presence of facultative and obligate anaerobes in marine sponges were also areas of active research. In some of these studies, it was shown that sponges derive a significant amount of their nutrients from microbial symbionts, although in one recent study the transfer of photosynthate from cyanobacteria to the sponge *Theonella swinhoei* was shown to be of minor importance (Beer and Ilan, 1998). Possibly, the most novel trophic transfer from microbe to sponge is a symbiosis between the deep-sea carnivorous sponge *Cladorhiza* sp. and a methane-oxidizing bacterium (Vacelet *et al.*, 1996). The sponges live near mud volcanoes that leach methane, and they seem to derive a significant amount of nutrition from their microbial symbionts. However, without cultivable bacteria or taxonomic data derived from molecular biology, the evolutionary significance of the symbiosis is uncertain.

During the 1970s and 1980s, investigators also studied the type and specificity of sponge-microbe associations, often using microscopy to show the presence of specific symbiont morphologies within specific sponges. The molecular basis of symbiosis was also probed, albeit to a lesser extent. Bacteria which live symbiotically with sponges, can be passed through their feeding chambers without being digested. This suggested some sort of

encapsulation or recognition process (Wilkinson, 1987). In the demosponge *Halichondria panicea*, an association with the microbe *Pseudomonas insolita* may be lectin-based (Müller *et al.*, 1981). Wilkinson found an immunological basis for symbiosis in some sponges, which he claimed as evidence of a Precambrian origin for many symbioses (Wilkinson, 1984).

A major problem with the early studies on sponge-microbe symbiosis was that most microorganisms were uncultured or unculturable, so descriptions of symbioses usually relied either on morphology of symbionts or chemical measurements of nutrient transfer. Even in the cases where putative symbionts could be cultured, the ecological relevance of symbiosis could not be determined. The period following Wilkinson's review has been marked by the ascendance of molecular biological techniques in environmental microbiology, which have allowed investigators to focus on uncultured microorganisms.

The application of molecular biology to sponge-microbe symbiosis is yielding results that could not have been obtained by classical microbiological methods. The discovery of a member of the Archaea living specifically within a sponge similar to *Axinella mexicana* was a particularly exciting find (Preston *et al.*, 1997). The archaeal microorganism, *Cenarchaeum symbiosum* (P: Crenarchaeota), lives at a relatively cold 10 °C and is therefore considered psychrophilic. Subsequent *in situ* hybridization experiments showed which microorganism in the sponge was archaeal and allowed localization of the symbiont.

Another sequence-based study of demosponges aimed to elucidate the major microorganisms within the marine sponges *Chondrosia reniformis* and *Petrosia ficiformis* (Schumann-Kindel *et al.*, 1997). *In situ* hybridization probes specific to Archaea, the subclasses of Proteobacteria, Flavobacteria-Cytophaga, and sulfate-reducing bacteria was used to survey these sponges. Both sponges contained mainly γ -subclass Proteobacteria, according to the authors, with sulfate reducing (δ -subclass) Proteobacteria also being present in significant numbers. γ -Proteobacteria were the only bacterial isolates obtained using aerobic enrichment media. Sulfate-reducing bacteria were postulated to play a role in the mineralization of dead sponge tissue.

In some cases, the picture of symbiosis that is emerging is markedly different from studies using cultured organisms. For instance, in the same species, *Halichondria panicea*, used by Müller *et al.* in lectin studies, sequence retrieval without cultivation led to microorganisms wholly different from the cultured *Pseudomonas* sp. described earlier (Althoff *et al.*, 1998). SSU rRNA sequences from all individuals of *H. panicea* collected led to the identification of strains of *Rhodobacter* (α -Proteobacteria) as the dominant species. A symbiotic relationship between the sponge and these microbes was suggested based on their ubiquitous occurrence in *H. panicea*. The activities of these bacteria and their effects on the host remain unknown.

Natural Products in Sponge-microbe Symbiosis

Natural products chemists have been interested in sponge-microbe symbioses because of the possibility that the diverse, bioactive chemical structures found in sponges might be produced by microorganisms (Faulkner *et al.*, 1993; Kobayashi and Ishibashi, 1993). More natural

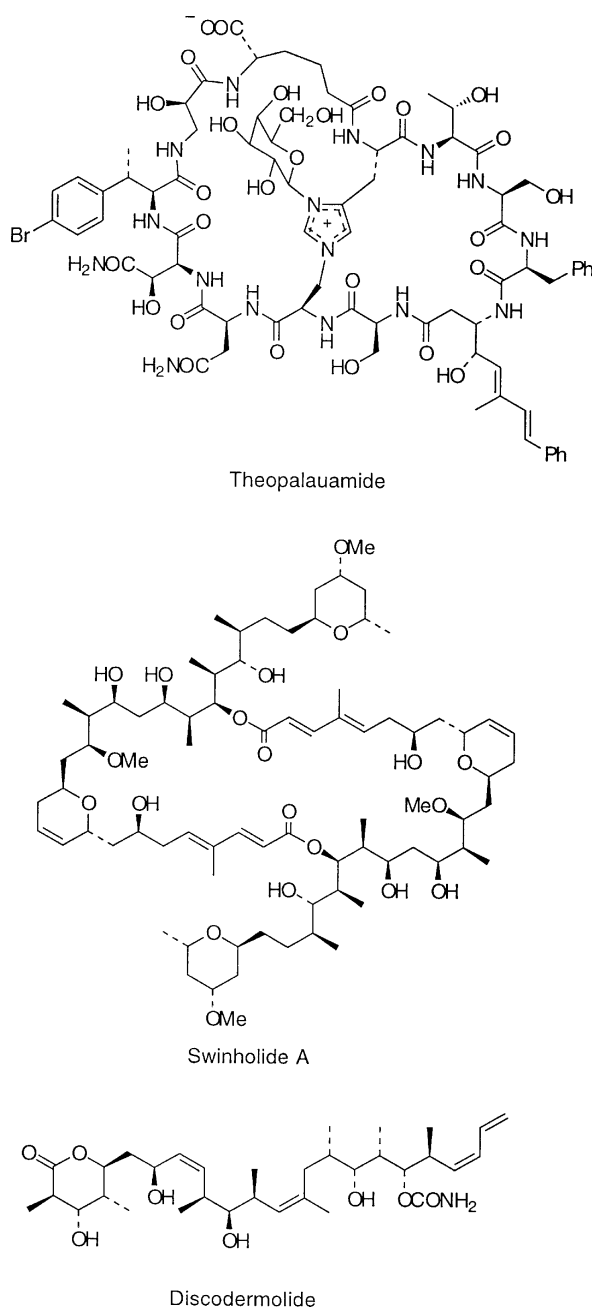


Figure 2. Sponge Natural Products

products have been reported from sponges than from any other marine invertebrate phylum, and many of the most promising pharmaceuticals and agents for cell biological research were isolated from sponges.

Unfortunately, despite the scientific and technological benefits of determining the source organisms of interesting and bioactive metabolites, a microbial origin of sponge compounds has rarely been demonstrated (Unson *et al.*, 1994; Bewley *et al.*, 1996). Most of the literature is purely speculative, based on similarities, however slight, between compounds from sponges and those from cultivated microorganisms, especially cyanobacteria (Kobayashi and Ishibashi, 1993). Several researchers have attempted to

culture microorganisms from invertebrates in the hopes of obtaining some of these bioactive compounds (Pietra, 1997). Although they have been successful in the discovery of novel natural products, this research has rarely demonstrated the presence of sponge metabolites in the microbial isolates. In one case the same compound was found in a *Hyatella* sp. sponge as in a *Vibrio* sp. cultured from that sponge (Oclarit *et al.*, 1998). These results demonstrate that traditional culturing approaches are not generally applicable to the environmental problems of sponge-microbe symbiosis. Two cases of symbiont production of sponge compounds have been clearly established. Both studies relied on cell fixation and physical separation techniques, bypassing the problem of culturing symbiotic microorganisms. Unson separated cyanobacterial symbionts from the sponge *Dysidea herbacea* by flow cytometry and showed that chlorinated amino acid derivatives could only be found in the cyanobacterial fraction, while terpenes were localized in the sponge cell fraction (Unson *et al.*, 1994). The symbiont was typed as *Oscillatoria spongelliae* based on morphological characteristics. In another study, Bewley determined the cellular locations of two major bioactive metabolites, theopalauamide and swinholide A, from the lithistid sponge *Theonella swinhoei* (Bewley *et al.*, 1996) (Figure 2). Contrary to the expectation that the metabolites would be produced by cyanobacteria, it was found that the modified peptide theopalauamide was found only in filamentous microorganisms without photosynthetic pigments, while the polyketide swinholide A was located solely in the unicellular bacterial fraction. *T. swinhoei* is a massive sponge that is packed with bacteria of many different species, so the exact microorganism producing swinholide A could not be determined, but in TEM pictures the filamentous microorganism was thought to resemble *Beggiatoa* sp., which are γ -Proteobacterial sulfide oxidizers.

Despite a good deal of speculation on the microbial origin of sponge metabolites, relatively little has been conclusively demonstrated. The number of cell separation studies on sponges is increasing, but a complete understanding of the source organisms of sponge compounds is still a distant goal. Molecular biological techniques, specifically the location of biosynthetic genes within certain cell types, are just beginning to be applied to the source question. However, no studies have addressed the evolution and ecological importance of sponge-microbe symbioses in the production of natural products. An important initial step in investigating these symbioses is to address the identity of chemically important microbes in sponges.

Lithistid Sponges

In comparison to bryozoans, much more is known about the biology and natural products chemistry of sponges. This presents great opportunities for symbiosis research, but also potentially complicates the search for a model group of sponges from the large number of described taxa. One such model group can be found in the polyphyletic order Lithistida. In this taxon, natural products may be used, in concert with biological tools, to probe the evolution of bacterial-poriferan symbiosis. The order contains a large number of diverse, bioactive molecules that have been ascribed microbial origins, and in one case a bacterial source has been proven (Bewley *et al.*, 1996). Because it

is relatively easy to determine the presence of known compounds in a sponge, the distribution and specificity of metabolites in this order can be mapped. By comparing this data with sponge taxonomy and molecular phylogeny of sponge symbionts, hypotheses of the nature of these symbioses can be advanced.

In cases in which a link between a metabolite and specific bacteria has been proven, natural products can provide a convenient signal of bacterial-sponge symbiosis. Compounds from the order Lithistida are particularly good markers, since they are usually present in relatively large amounts, often have unusual bioactivities, and can be relatively easily purified or identified in crude extracts. Many lithistid compounds are probably of microbial origin (Fusetani and Matsunaga, 1993), and in the case of *T. swinhoei* there is strong evidence supporting bacterial production (Bewley *et al.*, 1996). In some cases, production of certain compounds seems species-specific, while other compounds are found in a number of unrelated lithistids, or even in other sponge orders. This opens the possibility of a large number of types, ages, and plasticities of symbiotic interactions. Physical or biochemical factors common to unrelated lithistid sponges, rather than a species-specific factor, may promote the growth of certain types of bacteria. By attempting to construct gene probes for the bacteria that produce lithistid compounds, symbiosis in this group of sponges can be characterized and also provide a model for other sponge groups.

From the biotechnological perspective, lithistids also make good subjects for symbiosis research because they often contain highly bioactive compounds with pharmaceutical or research potential. Supreme among lithistid metabolites is the polyketide discodermolide from the sponge *Discodermia dissoluta* (Figure 2). The antitumor activity of discodermolide has been compared to that of taxol. Polyketide metabolites swinholide A from *T. swinhoei* and calyculin A from *Discodermia calyx* are valuable agents for research in cell biology because of their potent and specific activities. Swinholide A stabilizes tubulin dimers, while calyculin A is a potent protein phosphatase II inhibitor. Modified peptides, most of which are probably produced non-ribosomally by peptide synthetases, are also known for their potent bioactivities. The antitumor cyclotheonamides, from *Theonella* sp., exemplify these compounds. The status of chemical research in the order Lithistida as of early 1997 has been reviewed (Bewley and Faulkner, 1998).

Lithistid compounds often share characteristics with microbial metabolites, particularly with those of the cyanobacteria (Fusetani and Matsunaga, 1993; Bewley and Faulkner, 1998). Once Bewley *et al.* (1996) showed that the major metabolites of *Theonella swinhoei*, a lithistid sponge, were localized in symbiotic bacteria, the hypothesis that many compounds in the order are microbe-derived gained some experimental weight. Although it would require a massive series of cell-separation experiments on a large number of lithistid sponges to test the hypothesis, it is nonetheless a reasonable assumption when choosing targets for molecular biological research. It must be noted that the authors only showed the existence of the compounds in the bacteria and not bacterial production, but it is reasonable to presume that these large metabolites are not completely transferred from one cell type to another.

Bewley hypothesized that lithistid peptides containing

ω -phenyl- β -amino acids were produced by filamentous microorganisms, similar to the producer of theopalauamide in *T. swinhoei*. This hypothesis was based on her observation of the correlation between the two properties in a large number of lithistid sponges (Bewley, 1995). Thus, gross morphology of the symbiont as well as chemistry could be used in the analysis of theopalauamide origin. However, peptide-producing sponges do not always contain filamentous microorganisms. Samples of *Aciculites* sp. containing compounds in the aciculitin family do not contain filamentous microorganisms, and representatives of *Microscleroderma* sp. containing microsclerodermins sometimes lack filamentous microorganisms. It will be interesting to see whether these peptides are found in bacteria, and if so, how they may be related to the theopalauamide-containing filaments.

The origin of other metabolites (mainly polyketides) has been more complicated to sort out. The prime example in this group of compounds is swinholide A, which was localized by Bewley *et al.* (1996) in unicellular bacterial fractions of *T. swinhoei* containing a mixture of bacteria. Thus it was impossible to identify a specific bacterial source. In addition, the compound and a related chemical were subsequently found in two sponges that are unrelated: *Ircinia* sp. and *Tedania diversiraphidiphora* from the order Dictyoceratida and *Lamellomorpha strongylata* from the order Epipolasida (Dumdei *et al.*, 1997). In the absence of lateral transfer of biosynthetic genes or in the unlikely event that the complex structure could be reached by convergent evolution, these results indicate that the symbiosis leading to the production of swinholide is not species- or even order-specific. Unfortunately, it is difficult to track down which bacterial species is actually producing swinholide because of the large number of unicellular bacteria present in these filter-feeders. This illustrates the problems inherent in investigating the complex microbial communities found in sponges. Another instance of polyketide metabolites found in different orders occurs with the structurally similar calyculins (Kato *et al.*, 1986) and clavosines (Fu *et al.*, 1998) from *Discodermia calyx* (O: Lithistida) and *Myriastria clavosa* (O: Choristida), respectively. These similar compounds and related metabolites are found in apparently unrelated sponges. It would be interesting to determine if these and other similar compounds are always produced by similar bacteria, or if convergent evolution or lateral gene transfer are possibilities. Studies of these hypotheses become accessible through the combination of chemical and biological data. Ultimately, such studies could lead to a better understanding of the evolutionary importance of these compounds and to better culturing methods for non-specific symbionts, by comparison of common factors in the host environment.

The polyphyletic nature of the order Lithistida may confound early attempts at rationalizing the evolution of symbiosis. A clear picture of sponge phylogeny is necessary to fully investigate chemical and symbiont specificity, and elucidate the evolution of natural product symbioses. Some preliminary results of phylogenetic analysis of lithistids are available (McInerney and Kelly-Borges, 1998). For instance, sponges from the genera *Plakinalpha* and *Theonella*, formerly thought not to be closely related, are actually closest relatives: some *Theonella* spp. are more closely related to certain *Plakinalpha* spp. than to other theonellids. This clarifies chemical observations that some

members of both *Theonella* and *Plakinalpha* contain aurantosides and mozamides or related peptides as their major bioactive metabolites. The compound classes are very similar to compounds produced by streptomycetes and cyanobacteria, respectively. It would be interesting to determine whether or not the compounds are actually produced by bacteria and whether such a symbiosis holds in members of both sponge genera.

The lithistid sponges have inherent disadvantages: complex and variable microbial populations, difficulty in maintaining them in captivity, and lack of knowledge about symbiont transmission. Nonetheless, they have important advantages as a model system complementary to a more tractable model such as *B. neritina*. The advantages of sponges include proof that microorganisms are the biosynthetic source of two natural products, and high levels of natural products, facilitating chemical analysis. In addition, studies on the genetics of biosynthesis of swinholide A, a polyketide, and theopalauamide, a non-ribosomal peptide, will benefit from the substantial body of research available on the biosynthesis of these classes of compounds. Finally, sponges are a rich source of compounds, a number of which may be made by microorganisms. Despite their microbiological complexity, it is imperative to develop appropriate models for study of these symbioses.

Research Opportunities

Surveying and identifying microbial symbionts present in host invertebrates is fundamentally important in cases in which biosynthesis of a natural product by a symbiont is hypothesized. Techniques for molecular survey are now standard in microbial ecology, and can be applied to symbiotic systems. Ribosomal RNA genes are the most frequently used because of the large databases available and the inherent phylogenetic utility of these genes. Amplification of rRNA genes followed by cloning or denaturing gradient gel electrophoresis can be used to evaluate the relative abundance of particular bacteria, and comparison between samples can distinguish consistently associated organisms from transient colonizers. Sequences of the genes amplified reveals the phylogenetic groups to which the organisms belong and can provide specific probes for *in situ* hybridization, allowing sequences to be linked to specific cells. This approach has been used successfully in *B. neritina*, and has great potential for improving our understanding of sponge symbioses.

Complementary approaches are required for evaluating the biosynthetic source of natural products. Cell dissociation and chemical analysis can be very powerful, as shown by the discovery of sponge natural products in bacterial cells (Unson *et al.*, 1994; Bewley *et al.*, 1996). In cases in which gross dissociation is not feasible, it will be important to develop *in situ* techniques for localizing specific natural products to bacterial or host cells, or co-localizing with particular bacterial populations using specific rRNA probes. Antibodies might be used for this purpose, or chemical techniques might be developed. An important caveat is that highly mobile compounds or those that are actively transported within the host could result in no strong localization and make conclusions about the biosynthetic source difficult.

Natural products chemistry investigations assist in

formation of hypotheses about sponge-microbe symbioses. By positively correlating certain metabolites and metabolite classes with microbial symbionts, the distribution of metabolites can provide clues about the nature of the symbiosis. For instance, swinholide A has been isolated from a mixed bacterial fraction in *T. swinhoei*, and related compounds are found in a number of unrelated sponges. Therefore, it could be proposed that the symbiont prefers a sponge host, but it is probably present in seawater and can be transmitted between sponges of various species. Since sponges are efficient filter feeders, any bacterium that can resist the sponge digestive process and immune response can successfully colonize a variety of sponges. Using a combination of molecular biological and chemical techniques, the identity of this microorganism could be determined, and isolation of swinholide from unrelated sponges could serve as a starting point for detailed studies on the symbiosis. In another example from *T. swinhoei*, theopalauamide and related compounds have only been found in theonellid sponges. Therefore, it could be proposed that the symbiosis is probably more species-specific, involving long-term co-evolution. In depth investigation of evolution of invertebrate symbioses and natural product biosynthesis will require careful phylogenetic analysis of both symbiont and host genes combined with chemical studies.

Microbial ecology in general, as well as this area of research, will benefit greatly from the development of novel methods of cultivation of symbionts and other environmental organisms. The hallmark of the bacteria that are most commonly cultivated and studied in the laboratory is their great adaptability to different conditions. We do not know the reasons why the majority of symbionts (such as "*E. sertula*" in *B. neritina*), and environmentally dominant bacteria are difficult to cultivate, but requirements for highly specific conditions are likely to be part of the problem. We need to develop effective methods to bring these organisms into, if not traditional pure cultures, at least stable means of propagation that allow laboratory study. Symbioses are excellent model systems for developing such approaches because of stable, specific environmental conditions provided by the host.

Even in cases in which natural product localization is not feasible and the bacteria are resistant to cultivation, elucidation of the biosynthetic pathway through biochemical approaches may be possible and can reveal a likely symbiotic origin through comparative sequence analysis. Cloning of biosynthetic genes, based on inferences about biosynthesis based on chemical structure, is likely to be more straightforward for bacterial symbionts than host invertebrates, because of the way bacterial genes are organized. This may lead to a satisfactory solution to the problem of supply without cultivating the organism. Logical targets for isolation of biosynthetic genes include polyketide synthases for bryostatins in *B. neritina* and swinholides in lithistids, and non-ribosomal peptide synthases for theopalauamide-like peptides in lithistids. Research on the regulation of biosynthesis will reveal the intimate details of the symbiotic relationship and can be exploited for improving yield. Finally, when biosynthetic genes from symbionts become available, this will facilitate the creation of novel compounds with potentially valuable bioactivities by manipulating and combining genes from symbionts and other organisms.

Conclusion

Researchers have just begun to investigate the role of symbionts in the biosynthesis of natural products in marine invertebrates. The potential for molecular microbiology to produce fundamental biological discoveries is tantalizing, and the possibilities for enhancing the availability of these otherwise inaccessible compounds would be a major accomplishment for microbial biotechnology. The study of invertebrate natural product symbioses is poised to emerge as an exciting new field for molecular microbiology.

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