Experimental manipulation of sponge/bacterial symbiont community composition with antibiotics: sponge cell aggregates as a unique tool to study animal/microorganism symbiosis

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Abstract

Marine sponges can harbor dense and diverse bacterial communities, yet we have a limited understanding of important aspects of this symbiosis. We developed an experimental methodology that permits manipulating the composition of the microbial community. Specifically, we evaluated sponge cell aggregates (SCA) from Clathria prolifera that had been treated with different classes of antibiotics to determine whether this system might offer novel experimental approaches to the study of sponge/bacterial symbioses. Microscopic analysis of the SCA demonstrated that two distinct morphological types of microbiota existed on the external surface vs. the internal regions of the SCA. Denaturing gradient gel electrophoresis and sequence analysis of 16S rRNA gene clone libraries indicated that we were unable to create entirely aposymbiotic SCA but that different classes of antibiotics produced distinctive shifts in the SCA-associated bacterial community. After exposure to antibiotics, some bacterial species were ‘revealed’, thus uncovering novel components of the sponge-associated community. The antibiotic treatments used here had little discernible effect on the formation of SCA or subsequent development of the adult. The experimental approach we describe offers empirical options for studying the role symbionts play in sponge growth and development and for ascertaining relationships among bacterial species in communities residing in sponges.

Introduction

Over 100 years ago, Wilson (1907a, b) created a solution of adult sponge cells by passing Clathria (Clathria) prolifera (Ellis & Solander, 1786), known then as Microciona prolifera, through a fine sieve and demonstrated that the individual cells would reaggregate to form miniature sponges with adult features. This groundbreaking work raised important questions about, and provided a model system to study, cell adhesion, self/nonself recognition, immunology, stem cell biology, regeneration, and the origins of multicellularity, among a host of other questions. Many studies have used sponge cell aggregates (SCA) to examine animal cell differentiation and general aspects of morphogenesis (Wilson, 1911; Huxley, 1912; Galtsoff, 1925a, b; Wilson & Penney, 1930; Spiegel, 1954; Humphreys et al., 1960; Curtis, 1962; Humphreys, 1963; Moscona, 1963, 1968; Borrojevic & Lévi, 1964; Loewenstein, 1967; MacLennan & Dodd, 1967; MacLennan, 1974; Buscema et al., 1980; Van de Vyver & Buscema, 1981; Amano & Hori, 1996). Other studies have used SCA to study cell motility (Gaino et al., 1985), allorrecognition (Kaye & Reiswig, 1985; Custodio et al., 2004), immune systems in sponges (Müller & Müller, 2003), secondary metabolite production (Sun et al., 2007), membrane structure (Müller, 1982), and phylogenetic trends in aggregation capability (Holmes & Blanch, 2007). More recently, researchers have employed the modern tools of molecular genetics and SCA to re-evaluate some of the questions that intrigued biologists of the early 20th century including general aspects of the control of cell proliferation and growth (Custodio et al., 1998; Müller et al.,...
microorganisms (Vogel, 2008; Webster, 2008). Precise estimates of intra-host species richness are not yet available, but sponges clearly host novel bacterial taxa, even entirely new phyla, many of which are distinct from environmental bacterioplankton (Fieseler et al., 2004; Hill et al., 2006; Taylor et al., 2007a, b; Lafi et al., 2009; To Isaacs et al., 2009; Radwan et al., 2010; Gerce et al., 2011). The ecological interactions occurring among these bacteria are poorly understood, and the structure of the intra-host bacterial communities is only now beginning to be elucidated. Schmitt et al. (2011a, b) placed bacterial operational taxonomic units into core (present in all sponges), variable (present in a sub-set of hosts), and species-specific (present in a single host) categories. This provided rough estimates of relative abundances and addressed some general questions about evenness within the community. Attempts to uncover bacterial symbiont community structure beyond this level of resolution face several practical challenges. First, defining the types of interactions that occur among symbionts and between the host and its symbiont is difficult owing to the complexity of the community harbored by sponges. Second, identifying individual species that are rare (or not so rare) is a significant challenge with available molecular approaches, although next-generation sequencing offers some promise in this regard (Lee et al., 2010; Webster et al., 2010; Schmitt et al., 2011a). Finally, it is difficult to conduct manipulative experiments (e.g. removing particular symbiont species) to monitor cause-and-effect relationships among the hosts and symbionts (e.g., Lemoine et al., 2007; Webster et al., 2008). For this reason, our knowledge of basic aspects of sponge-bacterial symbioses remains rudimentary.

To fully understand the complex interactions that occur among the sponge and its symbiotic partners, we require systems that permit controlled experiments. Although the microbial symbionts appear to provide a variety of important functions to the sponge (Taylor et al., 2007a, b), teasing apart the materials and services that are exchanged among the bacterial and animal partners has proven remarkably challenging. For sponges that harbor phototrophic symbionts, several studies have shown the specific benefits the animal host receives (Wilkinson, 1979; Hill, 1996; Thacker, 2005; Weisz et al., 2010). Microorganisms with other metabolic phenotypes, however, present significant challenges to understanding these important symbiotic interactions. Evidence indicates that sponges benefit from the presence of microbial associates through enhanced metabolic capability [e.g. bacteria capable of reduction–oxidation reactions, vitamin production, nutrient transport, and utilization (Hentschel et al., 2002; Piel, 2004; Hoffmann et al., 2005; Steger et al., 2008; Thomas et al., 2010)]. One potential role for heterotrophic bacterial symbionts is the production of secondary metabolites that may provide a variety of important ecological functions [e.g. ameliorating stress (Dattelbaum et al., 2010), defense from predators and parasites (Taylor et al., 2007a)]. Hochmuth et al. (2010) found that the putative sponge-specialist bacterial lineage Poribacteria may produce methyl-branched fatty acids, and Thomas et al. (2010) found that metabolic interactions are shared between the host Cymbastella concentrica and its symbiont community, which supports previous work examining physiological integration between the partners (e.g. Arillo et al., 1993; Regoli et al., 2004). These and other studies point to the clear role that symbionts have on the sponge phenotype.

In contrast to the influence that symbionts have on host phenotype, almost nothing is known of the role that bacterial symbionts might play in normal developmental processes of the host. Vertical transmission of bacterial associates from mother sponges to their offspring indicates that developmentally, ecologically, and evolutionarily important relationships exist between these symbiotic partners (Kaye, 1991; Usher et al., 2001, 2005; Ereskovsky et al., 2005; Enticknap et al., 2006; Schmitt et al., 2007, 2008; Sharp et al., 2007). In most cases, however, the microbial community and the animal developmental system are studied as separate domains. Based on the supposed metabolic integration, the transmission of symbionts among generation, and the density of bacteria harbored by many sponges, it seems likely that sponge development may also be influenced by symbionts. An ideal system would be one that permits examination of developmental and symbiotic domains simultaneously, and as indicated by Taylor et al. (2007a), a major goal of work with sponge microbial symbioses is defining the interactions between the host and its symbionts.

While SCA provide a fascinating system to study aspects of animal development, they also provide an opportunity to manipulate bacterial symbioses in a controlled manner. Thus, SCA may serve as a useful tool and a model system to understand nuanced aspects of animal/bacterial symbioses. We used a battery of antibiotics with distinct modes of action during the creation of SCA and then monitored the effects that the different antibiotics had on microbial community structure and sponge development (see also Sipkema et al., 2003). As a proof-of-concept, we had three primary aims. The first was to determine whether it was possible to create completely axenic SCA. The second was to evaluate the effects
different classes of antibiotics had on the types of bacteria present in the SCA. The final aim was to assess whether removal of bacterial associates (through antibiotic treatment) had any discernible effect on sponge development.

Materials and methods

_Clathria prolifera_ was collected at dawn from pilings at the Virginia Institute of Marine Science (37°14.819′N; 76°30.052′W). SCA were created following the protocol presented in Custodio et al. (1998) with some modifications. The freshly collected _C. prolifera_ individuals were cut into 1-mm³ pieces within 4 h of collection. The sponge pieces were placed in 50-mL conical tubes with Ca- and Mg-free seawater with EDTA (CMFSW-E). The tubes were rocked on an orbital shaker at 110 r.p.m. for up to 1 h. The first wash was discarded. The second and third washes were passed through a 40-μm nylon mesh, and the dissociated cells were pelleted (500 g for 5 min). The sponge cell pellets were re-suspended in sterile-filtered seawater with antibiotics (see below). Six millilitres of the cell suspensions were placed in 50-mL conical tubes with Ca- and Mg-free seawater with EDTA (CMFSW-E). The second and third washes were discarded. The second and third washes were passed through a 40-μm nylon mesh, and the dissociated cells were pelleted (500 g for 5 min). The sponge cell pellets were re-suspended in sterile-filtered seawater with antibiotics (see below). Six millilitres of the cell suspensions were placed in 60-mm Petri dishes at densities of 10⁷ cells mL⁻¹. Petri dishes were placed on an orbital shaker at 110 r.p.m. for up to 1 h. The first wash was discarded. The second and third washes were passed through a 40-μm nylon mesh, and the dissociated cells were pelleted (500 g for 5 min). The sponge cell pellets were re-suspended in sterile-filtered seawater with antibiotics (see below). Six millilitres of the cell suspensions were plated in 60-mm Petri dishes at densities of 10⁷ cells mL⁻¹. Petri dishes were placed on an orbital shaker at 110 r.p.m. and 25 °C (i.e. RT). Within 12 h, SCA began to form. One-third of the solution in each Petri dish was replaced with fresh media daily. Once the SCA reached a diameter of 0.3 mm, they were transferred to new plates containing sterile-filtered seawater with antibiotics.

Four antibiotic treatments were used to assess the effect of antibiotics on microbial communities present within the SCA. The first antibiotic treatment involved the use of a combination of streptomycin (an aminoglycoside) and penicillin (a β-Lactam cell-wall inhibitor) at concentrations of 50 μg mL⁻¹. Three other treatments involved the use of single antibiotics. Nalidixic acid, which is a quinolone that inhibits DNA synthesis through inactivation of DNA gyrase, was added at a concentration of 50 μg mL⁻¹ (Wolfson & Hooper, 1985). Trimethoprim inhibits tetrahydrofolic acid synthesis, and thus DNA replication, and was added at 25 μg mL⁻¹. Gentamicin, administered at a concentration of 20 μg mL⁻¹, has broad effects although the precise mode of action is poorly understood. We attempted to create SCA without antibiotics as a procedural control, but aggregates did not form either owing to high levels of biological contamination (e.g. bacteria, fungi) or because micro-predators often proliferated. As is common in many cell-culture systems, we were unable to create viable SCA without antibiotics and thus were unable to employ that control.

To visualize the microbial communities present after exposure to antibiotics, several SCA were sampled from each treatment for histological work. We employed a modified vacuum-assisted microwave (Pelco Biowave™) protocol to process tissues for subsequent transmission electron microscopy (Giberson & Demaree, 2001). Primary fixation occurred in 2.5% Glutaraldehyde and 1% paraformaldehyde in seawater. After a wash with seawater, the SCA were placed under vacuum in a 1% Osmium tetroxide solution in water for secondary fixation. After washing with water, an en bloc stain with 1% uranyl acetate was performed (also under vacuum). Tissues were dehydrated (50%, 70%, 90%, and three times at 100% for 40 s each) before transferring them to a series of propylene oxide-Epon 812 (EM bed-812; Electron Microscopy Science) solutions (1 : 1, 1 : 2, and then 1 : 4 (v/v) for 30 min each). Three final incubations in full strength Epon 812 (30 min each) preceded the final embedding step, which occurred at 60 °C for 18 h. SCA were sectioned with an ultramicrotome (Leica Ultracut UCT) and examined using a JEOL 1010 TEM with an Advanced Microscopy Techniques XR-100 Digital CCD.

We also employed molecular tools to analyze the microbial communities present in SCA after exposure to antibiotics. Genomic DNA was extracted from randomly selected SCA taken from each treatment using the UltraClean™ Soil DNA Isolation Kit (MO BIO Laboratories). We also extracted DNA from tissue from freshly collected sponges, using the same methods, to compare natural symbiont communities to those from the antibiotic treatments. Metagenomic DNA was used as template for PCR employing the universal 16S rRNA gene primer pairs 1055F and 1406R-GC (Wang et al., 2007). Negative controls were included for each 16S rRNA gene amplification reaction. The cycle profile included an initial denaturing step at 95 °C for 2 min; 35 cycles of 95 °C for 1 min; 63 °C for 30 s, and 72 °C for 1 min; and a final extension step at 72 °C for 5 min. The final concentrations for primers, MgCl₂, and dNTP were 10 pmoles, 3, and 2 mM, respectively. One unit of Takara™ Taq DNA polymerase was added as was 10 ng of DNA.

PCR products were examined via Denaturing Gradient Gel Electrophoresis (DGGE) using the Bio-Rad DCode™ Universal Mutation detection system (Bio-Rad Laboratories, Inc.) using a 30–70% denaturing gradient in a 10% (w/v) polyacrylamide gel in 1 x TAE. Electrophoresis was performed for 17 h at 70 V and 60 °C. The gels were stained for 30 min in 1 x TAE spiked with 1.5 μg of ethidium bromide, destained for 25 min in dH₂O, and visualized and photographed with a GelDoc System (GelDoc 2000; Bio-Rad Laboratories, Inc.). A selection of bands unique to each treatment was carefully excised using an ethanol-sterilized scalpel for subsequent sequencing. DNA was eluted from the bands overnight in 25 μL of TE buffer. The eluted DNA was re-amplified with primers 1055F and 1406R-GC using the PCR conditions described.
previously. PCR products were purified (MinElute Gel Extraction Kit; Qiagen) and cloned using the TOPO® TA Cloning Kit following the manufacturer’s instructions. In addition to the DGGE band sequences, we also sequenced 10 clones produced from the total pool of PCR amplicons recovered from each of the treatments and the control sponge. Ten insert-positive colonies were selected from each plate, and plasmids were sequenced at Virginia Commonwealth University’s Nucleic Acid Research Facility. BLAST searches were conducted to compare our results with the 16S rRNA gene in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov). All sequences were analyzed for taxonomic affiliation using the Classifier program at the Ribosomal Database Project using default parameters (Wang et al., 2007).

To assess the effects of antibiotic treatment on sponge development, two comparisons were performed. The first involved examining the number of SCA that formed in each treatment after 1 day. Plates were examined after 24 h to assess the extent of sponge cell reaggregation. Twenty-five-mm² sections were examined, and all SCA were counted. Differences between treatments in SCA formation were analyzed using one-way ANOVA followed by Tukey’s HSD post hoc test (Zar, 2009). The second involved measuring the percentage of SCA that attached to the bottom of plates after shaking was stopped. For this experiment, individual SCA were transferred to 12-well culture plates where they were allowed to undergo metamorphosis and form miniature sponges (i.e. rhagons – Fig. 1). We measured attachment as the ability of a metamorphosing SCA to avoid dislodgement when a stream of approximately 100 µL of medium was gently released from a pipette toward the SCA. Differences in the percent of SCA that remained attached to the plate (after arcsine transformation) were analyzed using one-way ANOVA followed by Tukey’s HSD post hoc test (Zar, 2009). In addition to the streptomycin/penicillin, gentamicin, nalidixic acid, and trimethoprim treatments, we added another treatment to this analysis that included a mix of all antibiotics from the treatments described above at quarter strength. The SCA produced from the combined antibiotic treatment were also used to amplify 16S rRNA gene, and the resulting PCR products were cloned and analyzed as described previously.

Results

Transmission electron micrographs showed that a morphologically distinct microbiota existed on the external surface of many SCA compared with the internal regions of the SCA (Figs 2 and 3). The external bacteria exhibited a variety of structures that appear to mediate attachment to the acellular region of SCA surfaces. In contrast to the bacteria along the outer margin of the SCA, a morphologically simple bacterial community was found in the inner portions of the SCA (Fig. 2). Regardless of the antibiotic regime employed, we were unable to create SCA that were devoid of bacteria. A number of bacteria with

Fig. 1. Clathria prolifera SCA and metamorphosed sponges derived from SCA. Surface- and trans-illuminated images are shown (left and right respectively). The red pigmentation in this sponge is primarily owing to an abundance of carotenoids in its cells. After metamorphosis, adult features were obvious as can be seen in the trans-illuminated image. Abundant clusters of choanocyte chambers are easily identified (dashed black oval), a well-defined canal system has developed (dashed white lines), and an osculum projecting upwards was often observed in the miniature adult sponges (black circle).
Fimbriae, and other unique structures projecting from their surfaces, were observed along the external margin of many SCA (e.g. Fig. 3c and d; Supporting information, Fig. S1). These fimbriae and projections may be able to facilitate interactions with the cellular cortex by forming what appear to be adhesions (Fig. 3d and e).

PCR amplicons derived from bacterial DNA isolated from SCA were identified in each antibiotic treatment using DGGE (Fig. 4). This finding indicates that antibiotic treatments did not totally cure SCA of bacteria, as was also observed in the electron micrographs (Figs 2 and 3). Analysis of sequences from the excised DGGE bands (Fig. 4) and the pool of PCR product that we cloned indicated that taxonomically distinct microbiota persisted within the host depending on the antibiotic treatment used (Fig. 5; Table S1). Of particular note were the major differences we observed between sponges collected from the wild and the SCA reared under different antibiotic treatments (Fig. 5). While cyanobacteria were found to dominate the isolates we recovered from wild sponges (WS, Fig. 5), taxonomically distinct bacteria were detected in the other treatments. For example, gentamicin (G, Fig. 5) had a high percentage of taxa from the Phylum Bacteroidetes, which was not the case for any of the other antibiotic treatments; the only type of microorganisms detected in trimethoprim-treated SCA came

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**Fig. 2.** Representative transmission electron micrographs from *Clathria prolifera* SCA produced with the streptomycin/penicillin treatment. Despite treatment with antibiotics, remnant populations of bacteria were observed in each treatment. In many replicates, a morphologically variable microbiota was observed on the external surface of the SCA. These organisms were often affixed to or associated with the extracellular material surrounding the SCA (image on left). The bacteria observed deep within the SCA were always phenotypically nondescript and did not appear to be localized in any predictable pattern within the SCA.

**Fig. 3.** Representative bacteria associated with the external surface of *Clathria prolifera* SCA from the streptomycin/penicillin treatment are shown (a–d). A close up of the point of attachment in (d) is shown in (e). For comparative purposes, bacteria found in the inner portions of the SCA are shown in (f). Scale bar in a–e are 100 μm. Scale bar in (f) is 500 μm.
from the Phylum Proteobacteria. Each of the antibiotic treatments differed from one another in taxonomic composition of the SCA-associated microbiota (Fig. 5; Table S1), which was likely related to the distinct modes of action that the different antibiotics had on SCA, and the relative susceptibilities of the sponge-associated bacteria to the antibiotics.

Most of the sequences we obtained from antibiotic treated SCA shared highest affinities with samples from environmental sources. That is, BLAST searches recovered symbiont-specialist lineages, and thus putative sponge-specialist symbionts, in less than 20% of the cases. In the S-/P-treated SCA, we identified two symbiont-affiliated sequences: a *Vibrio*-like sequence (accession JQ004822; band 2, Fig. 4) showed affinity to bacteria associated with sponges from Florida (e.g. *Mycale* and *Ircinia*), and a *Planctomycetes*-like sequence (accession JQ004818) was similar to sequences derived from a *Haliclona* host in the northwest Pacific. Three sequences from the gentamicin-treated SCA retrieved sponge-affiliated bacterial taxa from GenBank during BLAST searches. The first was *Marinoscleromum* sp. (accession JQ004825), the second was *Roseivirga* sp. (accession JQ004830), and the third was a *Labrenzia* species from DGGE band 7 (accession JQ004834; Fig. 4). Three of the sequences we analyzed from the nalidixic acid-treated SCA recovered sponge-/symbiont-associated bacteria from GenBank [*Crocinitomix* (accession JQ004841); *Seohaeicola* (accession JQ004849); and *Pseudovibrio* (accession JQ004841);]

![Fig. 4. DGGE results comparing microbial community characteristics for each antibiotic treatment. C = control sponge collected from the native habitat, S/P = streptomycin/penicillin, N = nalidixic acid, G = gentamicin, and T = trimethoprim. DNA was extracted, cloned, and sequenced from bands excised from the gel (identified with numbers).](image1)

![Fig. 5. Comparison of broad taxonomic representation of bacteria identified in SCA after each of the antibiotic treatments. The first row includes a taxonomic breakdown at the rank of Phylum. The second row includes a taxonomic breakdown at the rank of Class. The numbers within some of the wedges represent the number of families that were recovered. Any wedge without a number is represented by a single family. S/P = streptomycin/penicillin, N = nalidixic acid, G = gentamicin, T = trimethoprim, M = mixed antibiotic treatment, and WS = wild-caught sponges.](image2)
None of the sequences identified in the trimethoprim or mixed antibiotic treatments, nor in the wild-caught sponge, included bacteria from putative symbiont lineages. The remaining sequences shared highest identity with bacteria from environmental sources and a variety of biofilms (Table S1). While we found different numbers of bacterial genera in the different antibiotic treatments (as predicted in Classifier; $S/P = 7; G = 11; N = 11; T = 5; M = 6$), it is unclear whether these represent significant differences.

One-way ANOVAs indicated that significant differences existed among antibiotic treatments for SCA production (Fig. 6a; $F_{5,24} = 11.43, P < 0.001$). Tukey’s HSD test indicated that significantly lower numbers of SCA were produced in each of the antibiotic treatments compared with the streptomycin/penicillin treatment, which resulted in the highest number of SCA (Fig. 6a). The different antibiotic treatments did not differ in their effect on SCA ability to attach to the substratum (Fig. 6b; $F_{5,12} = 1.76, P = 0.21$).

**Discussion**

Our primary goal with this study was to assess the utility of SCA exposed to different types of antibiotics as a model to study animal/bacterial symbioses in general and sponge/bacterial interactions in particular. While we were incapable of creating axenic SCA, the shifts we observed in community structure were intriguing. Several observations from our work indicate that the approach we describe may be very useful for future endeavors to tease apart nuanced aspects of host/symbiont interactions as well as interactions among members of the bacterial community.

One area that will likely be of broad interest involves the distinct morphological types of bacteria that appear on the surface vs. inner regions of the SCA. Superficially, many of the externally located bacteria were often phenotypically similar to bacterial pathogens attached to mucosal membranes in animals [e.g. fimbriae of enteropathogenic and uropathogenic *Escherichia coli* (Giron et al., 1993, and Johnson, 1991, respectively)] and share characteristics of bacteria forming biofilms (Flemming & Wingender, 2010; Pamp et al., 2007). The findings we present differ from work with sponge larvae that found an absence of bacteria in the outer ciliated region of the larva (Schmitt et al., 2007). The spatial segregation that we observed may involve phenomena that are unique to SCA, but SCA appear to provide an opportunity to examine processes involved in biofilm formation. A majority, but not all, of the SCA had bacteria along the external margins. It is unclear whether the host has any control over the composition of the internal pools of bacteria that we observed, but SCA also provide a model system to explore any host-influenced community selection (if, indeed, these communities are distinct). Bacteria with certain structures may be able to attach to the outside of the SCA (which may be a prelude to entry into a host cell/SCA), whereas bacteria without those structures may remain in the inner regions of SCA. It is also possible that once internalized, the bacteria turn off expression of attachment factors because they no longer need them.

The data we report could not elucidate the taxonomic composition of these two communities, but future work could examine whether different types of bacteria reside in these two distinct habitats (perhaps with FISH). Along
these lines, our data indicate that one can enrich for rare bacterial species, many of which are not a conspicuous part of the flora associated with wild-caught sponges. This permits at least two types of analyses. The first is that antimicrobial treatment may permit detection of unusual components of bacterial taxonomic diversity. For example, our detection of three species from the order Oceanospirillales (Halomonas, Marinomonas, and Salinicola) indicates this might be a valuable tool to uncover novel diversity, which has not been commonly encountered in sponges (Sfanos et al., 2005; Li & Liu, 2006; Kaesler et al., 2008). Many of the sponge-derived 16S rRNA gene sequences we uncovered are closely related to seawater, biofilm, and sediment sequences and thus may not come from symbiosis-specific lineages. Thus, the second type of analysis that SCA permit includes an analysis of whether the microbial species affiliated with nonsymbiotic evolutionary lineages (i.e., affiliated with environmental sources) represent transient interactions, commensalisms, or more intimate ecological interactions (e.g., mutualisms).

In contrast, some of the microorganisms we encountered appear to come from symbiont-specializing lineages. For example, Crocitomix from the nalidix acid treatment (accession JQ004841), Labrenzia and Marinococcus from the gentamicin treatment [accession JQ004834 & JQ004825, respectively], and Vibrio from the streptomycin/penicillin treatment (accession JQ004813) all may belong to symbiosis-specialist lineages. SCA offer the possibility of contrasting the conditions that favor one type of host/symbiont interaction over another (e.g., the degree of specificity or the level of reciprocity between partners). In this context, SCA holds the potential to uncover the ecological factors that release rare species from the constraints on their prevalence in sponge tissue under normal circumstances.

Another favorable aspect of SCA work is that longer-term growth experiments could begin to partition the effects particular suites of bacteria have on host performance. Sponges are one of the most prolific producers of novel natural products (Blunt et al., 2011), but the genesis of many of the compounds ultimately rests with microbial associates (Taylor et al., 2007b). For example, we know that tissue from C. prolifera contains abundant and diverse carotenoids (Fig. 1), some of which have bacterial origins (Dattelbaum et al., 2010). By monitoring the chemical profiles of the rhagons, we may be able to parse the effects that some of the sponge-associated bacteria have on host phenotype. This type of work should be especially applicable to any sponges capable of SCA formation and would be particularly attractive for sponges that produce pharmaceutically interesting compounds (Taylor et al., 2007b).

The shift in banding patterns that we observed in our DGGE analysis and the changes in the composition of the metagenome uncovered in our sequence analysis were compelling. Our data clearly indicate that it is possible to change the composition of the sponge-associated bacterial community. The observed shift had no effect on one aspect of host development (i.e., attachment to the substratum), which was surprising given that we anticipated the loss of bacterial associates to compromise sponge performance in some noticeable manner (see Sipkema et al., 2003). Other antibiotic regimes might produce different outcomes, and additional work in this area is clearly required before definitive statements can be made about the effects symbionts have on sponge development. Given the long-standing interest in linking symbiont and host performance, however, SCA offer useful experimental approaches to teasing apart symbiont contributions to phenotypic features of the holobiont.

Gerce et al. (2011) recently identified differences between the bacterial communities associated with the surfaces of eight Mediterranean sponges and those found within the mesohyl. Their analysis of DGGE banding patterns identified two main clusters that could be distinguished as containing samples from the mesohyl or surface of their sponges. SCA may provide an experimental mechanism to explore how these distinctions arise during sponge development given our observations of unique microbiota associated with the external surfaces of SCA. As indicated previously, the spatial segregation of bacterial associates that occurred in our experiments indicates that the host may have some selective capacity. If this is so, SCA provide an unparalleled opportunity to examine aspects of host-symbiont specificity.

Recently, To Isaacs et al. (2009) found that wild-caught C. prolifera harbored a dense and diverse microbiota dominated by cyanobacteria, actinobacteria, and spirochetes. Those authors did not find a strong congruence between DGGE profiles and the clone libraries, whereas our dominant DGGE band (band 1 in Fig. 4) matched the abundance of this sequence type in the clones we analyzed that were derived from the total pool of PCR amplicons recovered from the SCAs. Of particular importance for the work reported here, however, was To Isaacs et al.’s (2009) documentation of a shift in bacterial community composition upon rearing in aquaria. Similarly, Taylor et al. (2005) reported significant geographic variability in the composition of microbial communities harbored by C. concentrica. Combined with our findings, these data raise important questions about the nature of the association between C. prolifera and the bacteria harbored in and on its tissues. Furthermore, To Isaacs et al. (2009) documented the loss of actinobacteria in aquaria-reared sponges, but we only recovered this type of
bacteria when SCA were exposed to a mix of antibiotics. Thus, aquaculture procedures looking to produce particular bacterially derived bioactive compounds (Hill, 2004) may consider our findings particularly intriguing.

The diversity of microbial communities, and the challenges working with fastidious bacteria, present significant empirical challenges to ecologists interested in understanding how the communities are structured. For example, recent work has indicated that the symbiont communities found in high and low microbial abundance sponges (Weisz et al., 2008) may have distinct characteristics (e.g. Schlappy et al., 2010; Erwin et al., 2011; Gerce et al., 2011; Schmitt et al., 2011b). The SCA we produced using different antibiotics opens the possibility of exploring nuanced aspects of the bacterial communities harbored by sponges. While C. prolifera continues to be a model to study aspects of developmental and cellular biology, we introduce this sponge, and SCA, as a new model for examining microbial symbioses. Employing antibiotics permits a classic ‘species removal’ type of ecological experiment that has been difficult to perform to date. Although the antibiotics we used represent blunt instruments and more precise tools for manipulating symbiotic bacterial community remain to be developed, our study demonstrates the practical utility of SCA to enhance manipulative studies of microbial symbioses.

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the surface or tissue of Mediterranean sponge species. 


Sponge cell aggregates as a model to study symbiosis


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Electron micrographs of bacteria with unusual morphological characteristics from each antibiotic treatment.

**Table S1.** Aggregate data on DNA sequences isolated from clone libraries and DGGE bands.

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