Examining the Basis for Microbial Diversity in the Marine Sponge 
*Microciona prolifera* 

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ABSTRACT

Marine sponges produce high concentrations of bioactive metabolites and accumulate large populations of bacteria within their mesohyl interior. These bacteria are believed to exist as extracellular symbionts and have been proposed to be involved in matrix stabilization, waste processing, and producing secondary metabolites for defense. Previous surveys of the microbial diversity of different sponges show the presence of bacteria from multiple phyla, suggesting that sponge accumulation of bacteria is not based on speciation. In this study, the alternative theory that bacteria are accumulated based on common physiological instead of phylogeny was examined in *Microciona prolifera*, a marine sponge native to Cape Cod waters. The hypothesis that sponges enrich for bacteria that produce or degrade secondary metabolites and hydrocarbons was tested using traditional culturing and molecular phylogeny. *Microciona* was found to harbor bacteria that can metabolize benzoate, pentane, 1-chloropentane, and 1-bromopentane in higher abundances than seawater. A greater number of colony morphotypes were observed from bacterial isolates from *Microciona* versus seawater, which is consistent with phylogenetic analyses of both environments, which recovered bacterial 16S rDNA signatures from 10 known phyla and several uncultured groups from sponge tissue. Bacteria cultured producing antimicrobial metabolites were only identified from *Microciona*. These results support the theory that marine sponges enrich for bacterial populations that can metabolize diverse chemical substrates and synthesize bioactive compounds. This is the first known report surveying the microbial diversity of the marine sponge *Microciona prolifera* and isolation of associated bacteria that produce antimicrobial compounds.

INTRODUCTION

Sponges are ancient metazoan animals with Precambrian origins that date back more than 600 million years ago (Bergquist, 1978). The complexities of host-microbe interactions are reflected in relationships sponges have with their associated bacterial communities. In sponges, amoeboid cells are contained within a mesohyl matrix and gain energy by phagocytosing bacteria filtered through an internal canal system (Bergquist, 1978). Interestingly, a second population of bacteria is accumulated extracellularly by transport from the canal into the mesohyl interior. Bacteria are accumulated to 100 to 1,000x greater concentrations than present in seawater or as much as 40% of the total sponge biomass (Henschel, 2002). The accumulated bacteria are believed to exist as sponge symbionts and are involved in stabilizing the sponge matrix, processing cellular wastes, and production of secondary metabolites (Bergquist, 1978).

The physiological behavior of accumulating microorganisms and metabolite production has drawn attention to sponges as a source of novel marine metabolites and as systems for studying microbial-host interactions. Sponges produce many diverse compounds with antimicrobial, anti-inflammatory, or cytotoxic activities (Guyot, 2000; Haefner, 2003; Kelecom, 2002). Among sponge-associated metabolites, halogenated organic compounds constitute a large proportion of the chemical diversity of metabolites. Marine cyanobacteria produce chlorinated aromatic compounds with bioactive properties (Flowers, 1998), and bromine-containing compounds have been reported to account for 7 to 12% of the total dry-weight in *Aplysina aerophoba* sponges (Turon, 2000). These bioactive compounds have been proposed to serve as a defensive mechanism against predation. However, as the majority of studies have focused on identified novel compounds with pharmaceutical activities, fundamental questions regarding their biosynthesis and effects on bacterial community dynamics are still largely unknown.
Determining the origin of secondary metabolites has been hindered by the limitations of microbial culturing and the ability to obtain sufficient sponge materials for thorough chemical analysis. Additionally, the connection between the microbial community composition and the profile of secondary metabolites produced remains unclear. Molecular and culture-based surveys of the microbial diversity of different sponges have shown accumulation of bacteria from several phyla and signatures distinctly different from seawater (Hentschel, 2002; Rappe, 2000; Taylor, 2004; Webster, 2001).

Thus, sponges do possess the ability to enrich for specific bacteria but selection does not appear to be based on phylogenetic speciation. An alternative interpretation is that enrichment is based on identifying and retaining bacteria with common physiological traits. Based on the coexistence of high bacteria concentrations and diversity of synthesized compounds, it is hypothesized that sponges are natural enrichments for bacteria that are able to produce or tolerate secondary metabolites.

This premise was evaluated by comparing the microbial diversity by comparing the marine sponge *Microciona prolifera* versus seawater. Traditional culturing showed *Microciona* harbor bacteria that can metabolize benzoate, pentane, 1-chloropentane, and 1-bromopentane in higher abundances than seawater. A greater number of colony morphotypes were observed from the sponge versus seawater. This diversity is consistent with phylogenetic analyses of both environments, which recovered bacterial 16S rDNA signatures from 10 known phyla and several uncultured groups from sponge tissue. Bacteria cultured from *Microciona* were the isolates that produced antimicrobial metabolites. These results support the theory that marine sponges enrich for bacterial populations that can metabolize diverse chemical substrates and synthesize bioactive compounds. This is the first known report surveying the microbial diversity of the marine sponge *Microciona prolifera* and isolation of associated bacteria that produce antimicrobial compounds.

**MATERIALS AND METHODS**

**Sample collection.** *Microciona prolifera* is a marine sponge native to the Cape Cod area found in shallow, fast-flowing waters, forming bright orange-red branches. Two *Microciona* sponges were collected near Woods Hole, MA by the Marine Resources Center of the Marine Biological Laboratory (MBL, Woods Hole, MA). Sponge A (268.8 g) was 7 in. x 4 in. x 5 in and Sponge B (591.8 g) 8 in. x 6 in. x 5 in (Figure 1). Both sponges were maintained at room temperature with a continuous flow of seawater. Two 1-L seawater samples were collected from Woods Hole Passage at a surface depth of 1 m. Seawater was stored at 4°C until processing.

**Light Microscopy.** To observe homogenized material, sponge tissue (1 g) was ground using a sterile tissue grinder with 10-mL of sterile seawater and observed under phase-contrast and epifluorescence on a Zeiss AXIOPan Imager.M1 compound light microscope with AxioVision v4.4 capture software. Dissected sponge tissue was stained in DAPI (1 ug/mL) for 10 minutes and washed with sterile seawater prior to observation.

**Scanning Electron Microscopy.** Dissected sponge tissue was fixed (2% glutaraldehyde, 1.5% formaldehyde in 0.1M sodium cacodylate buffer, pH 7.0) for 4 hours, dehydrated in acidulated 2,2-dimethoxypropane, and kept in absolute ethanol. Specimens were critical-point dried with liquid CO₂ and sputter-coated with gold. Images and micrographs were captured on a JEOL JSM-840 scanning electron microscope.
Media and growth conditions. *E. coli* used in clone libraries and antimicrobial metabolite screening were grown at 37°C on Luria-Bretani (LB) with kanamycin supplemented at 50 µg/mL and 40 µg/mL X-gal as appropriate. The marine *Bacillus* sp. and *Vibrio* sp. strains (antibiotic screening) was grown at 30°C on seawater complete (SWC) containing 5 g Bacto tryptone, 3 g yeast extract, and 3 mL glycerol, per liter (750 mL seawater with 250 mL Millipore water). LB and SWC were solidified with 1.8% (wt/vol) Bacto agar.

Sponge and seawater bacteria were cultivated on artificial seawater (ASW) at room temperature (20°C) containing 1 mM Na₂SO₄, 1.5 mM K₂PO₄, 5 mM NaNO₃, 5 mM MOPS (pH 7.2), and 1 mL of HCl-dissolved trace elements solution per liter seawater base (20 g NaCl, 3 g MgCl₂·6H₂O, 0.15 g CaCl₂·2H₂O per liter distilled water). 1-mL of the 12-vitamin solution and 2 mL of a 1% (wt/vol) cycloheximide solution were added to cooled media. Total heterotrophic bacteria were isolated using ASW complete (ASW-C), which contains 0.05% (wt/vol) each of peptone and Casamino acids. Benzoate (Ben), 2-bromobenzoate (BrBen), and 2-chlorobenzoate (ClBen) were added to a final concentration of 5 mM, and pentane (PN), 1-bromopentane (BrPN), 1-chloropentane (CIPN) provided in vapor form, accordingly. ASW-mediars were solidified with 1.8% (wt/vol) prewashed Bacto agar.

HCl-dissolved trace elements solution contains 2.1 g FeSO₄·7H₂O, 30 mg H₃BO₃, 100 mg MnCl₂·4H₂O, 190 mg CoCl₂·6H₂O, 24 mg NiCl₂·6H₂O, CuCl₂·2H₂O, 144 mg ZnSO₄·7H₂O, 36 mg Na₂MoO₄·2H₂O, 25 mg NaO₃V and 6 mg Na₂SeO₃·5H₂O in 987 mL distilled water.

The 12-vitamin solution contains 10 mg riboflavin, and 100 mg each of riboflavin, thiamine·HCl, L-ascorbic acid, D-calcium-pantothenate, folic acid, niacinamide, nicotinic acid, para-aminobenzoate, pyridoxine·HCl, lipoic acid, NAD, and thiamine pyrophosphate in 100 mL of 10 mM phosphate buffer (pH 7.2).

Isolation and enumeration of heterotrophic bacteria. 10-Gram samples of tissue from each *Microciona* sponge was homogenized using separate sterile mortar and pestles with 10-mL ASW. 100 µL of the 10² through 10⁵ dilutions of these extracts were plated in duplicate onto ASW-Ben, ASW-BrBen, ASW-ClBen, ASW-PN, ASW-BrPN, ASW-CIPN, and ASW-C agar. Two 25-mL aliquots of each seawater sample was separately filtered through a 0.2 µm Millipore membranes. Each membrane was placed in 10-mL ASW, and 100µL of 10⁻² through 10⁻⁴ dilutions were plated as described above. All plates were incubated in the dark at room temperature (20°C). ASW plates with PN, BrPN, and CIPN were placed inside sealed 5-gallon buckets containing 3-mL of the according alkane in an open glass test tube to allow for gaseous diffusion. Colony formation was monitored over the period of 10 days. Plates incubated with gaseous alkanes were vented daily and substrates added as necessary.

16S rDNA library construction. DNA was extracted from 0.5-g of sponge tissue and 0.2 µm Millipore membranes filtered with 25-mL seawater using a Mol Bio DNA extraction kit for soil according to manufacturer’s directions. 16S rDNA was PCR amplified using universal Eubacterial primers 8F and 1492 in 25 µL reactions with 0.5 to 2 µL of extracted DNA and a Promega PCR kit on an Eppendorf Mastercycler using the following program: 95°C, 5 minutes, denaturation 95°C 30 seconds, annealing 50°C 30 seconds, amplification 72°C (30 cycles total), final extension 72°C, 4°C infinity. Proper products were confirmed (~1.5 kb) and purity
checked by gel electrophoresis on 1.3% agarose TBE gels. PCR products were cloned with a TOPO-TA cloning kit according to the manufacturer’s instructions (Invitrogen).

**Sequencing and phylogenetic analysis.** *E. coli* isolates containing 48 putative clones containing plasmid inserts from sponge and seawater bacteria were picked and inoculated in 96-well blocks containing 1-mL LB-Kan within each well. Plasmids were mini-prepped and sequenced using M13 forward and reverse primers at the High Throughput Sequencing Center at the Josephine Bay Paul Center in Comparative Molecular Biology and Evolution. Phylogenetic analyses were performed with the ARB software package ([www.arb-home.de](http://www.arb-home.de)). Trees were calculated by using neighbor joining (termini filter, bacterial position variation filter, Jukes-Cantor correction) implemented in ARB. Taxonomic nomenclature was used according to Bergey’s Manual of Systematic Bacteriology (Garrity, 2001).

**Screening for antimicrobial metabolites.** 130 bacterial isolates from seawater and 170 bacterial isolates from the sponges were inoculated into 96-well microtiter dishes containing 150-µL ASW-C per well. Cultures were grown on an orbital shaker at 20°C with shaking (250 RPM). After 72 hours, cultures were harvested by centrifugation and 5 µL of clarified supernatant applied to sterile filter disks (5 mm diameter, punched out of Whatman no. 5 qualitative filters) placed on LB or SWC indicator plates freshly spread with 100 µL of overnight cultures of a marine *Bacillus* sp., *Vibrio* sp., or *E. coli* accordingly. After 24 hours, indicator plates were inspected for zones of inhibition around filter disks applied with culture supernatant compared to filters applied with 5 µL of ASW-C.

**RESULTS AND DISCUSSION**

**Microscopic survey of *Microciona* tissues.** Dense populations of cells were observed in sponge material stained with DAPI (Figures 2 and 3). Under epifluorescence illumination yellow cocci, blue cocci, and blue filaments are visible within the matrix material. Bacteria associated within sponge particles were further resolved in homogenized tissues (unstained, Figure 4). Individual sponge cells (3 µm x 4 µm) are seen as aggregates under phase contrast microscopy. Illumination with epifluorescence reveals internal pockets containing high motile coccoid-shaped cells (~ 1 µm x 1 µm). Scanning electron microscopy of a thinly sectioned piece of sponge tissue shows that the interior of matrix is covered with a dense population of coccoid, long and short rods (Figure 5). These observations clearly show high concentrations of bacteria are associated with *Microciona prolifera* sponges.

**Isolation of seawater and *Microciona*-associated bacteria.** To test the hypothesis that sponges enrich for microorganisms that produce or degrade secondary metabolites and hydrocarbons, direct isolations were performed to assess the relative abundance of bacteria that can utilize halogenated alkanes and halogenated aromatic acids as the sole carbon source from *Microciona* versus seawater. Pentane, 1-bromopentane, 1-chloropentane, benzoate, 2-bromobenzoate, and 2-chlorobenzoate were selected as growth substrates as 1) their bacterial degradation pathways have been well characterized and 2) their application as starting compounds for the synthesis of many biopharmaceutical chemicals.

Colony formation was monitored over a period of 10 days, in which the number of isolate colonies were recorded and expressed as colony formation curves (Figures 6-8). ASW-C plates inoculated with *Microciona* homogenate rapidly reached their total recorded CFUs within 1
to 6 days, while seawater spread plates required 7 to 10 days to reach the total recorded CFUs. Interestingly, greater than 95% of the bacteria isolated from seawater were of a single morphology (entire, smooth, raised, white colonies) while bacteria from the sponges were of multiple morphologies. Round, white colonies were observed the first 2 days, and then a gradual accumulation of irregular edged colonies and filamentous forms appeared through the proceeding 6 days.

Hydrocarbon degrading bacteria isolated from sponges reached the maximum observed CFUs before those from seawater for all of the tested alkanes, benzoate, and 2-bromobenzoate. Isolates that can metabolize 1-chloropentane and 2-bromobenzoate were not recovered from seawater sample A, but were present in the seawater sample B. The apparent lag-times before colony formation with 2-bromobenzoate and 2-chlorobenzoate may be explained by the toxicities of these substrates compared to benzoate or bioavailability of substrate related to transport. The differences between CFU formation rate between sponge versus seawater on all 7 tested growth conditions are suggestive of different bacterial populations within the two environments.

Overall, Microciona sponges contain more bacteria compared to seawater. Sponge A contained 3.8 x 10^6 (± 3.5 x 10^4) CFU / g (wet weight) of (17% grow on pentane, 14% 1-bromopentane, 17% 1-chloropentane, 17% benzoate, < 1% 2-bromobenzoate, and <1% 2-chlorobenzoate). Sponge B contains 3.9 x 10^6 (± 1.6 x 10^6) CFU / g (wet weight) (13% grow on pentane, 16% 1-bromopentane, 12% 1-chloropentane, 21% benzoate, < 1% 2-bromobenzoate, and <1% 2-chlorobenzoate). Seawater A contained 2.7 x 10^2 (± 1.2 x 10^2) CFU / mL (1% grow on pentane, 11% 1-bromopentane, 12% benzoate, and < 1% 2-bromobenzoate). Seawater B 3.4 x 10^3 (± 1.4 x 10^3) CFU / mL (11% grow on pentane, 7% 1-bromopentane, 4% 1-chloropentane, 12% benzoate, < 1% 2-bromobenzoate, and <1% 2-chlorobenzoate).

Microciona sponges approximately accumulate 1,000 x more bacteria than the sample seawater, which is consistent with previous surveys of the abundance of bacteria in other marine sponges and surrounding seawater (Henschel, 2002). Overall, the Microciona sponges contain a greater abundance of hydrocarbon degrading bacteria than present in seawater.

Diversity of 16S rDNA bacterial signatures from seawater and Microciona prolifera. Phylogenetic analysis of the 42 16S rDNA signatures from the seawater library shows six major groupings of bacteria (Figure 9). 40% are associated with the Cyanobacteria (17 clones), 26% uncultured γ-Proteobacteria (11 clones), 10% Pseudomonas (4 clones), 10% uncultured α-Proteobacteria (4 clones), 7% uncultured Actinobacteria (3 clones), and 2% Roseobacter (1 clone).

In contrast, the 42 16S rDNA signatures belong to several groupings bacteria with no apparent phylogentic pattern (Figure 10). Sequences fall into 14 different groups of bacteria. The largest group (28%, 12 clones) did not align with any reported sequences in ARB. BLAST analysis of this group showed their presence in the NCBI database matching uncultured δ-Proteobacteria.

The high abundance of Cyanobacteria sequences recovered from the seawater library is consistent with other phylogentic bacterial surveys of ocean water (Rappe 2000). The polyphyletic nature of associated Microciona bacteria is consistent in studies conducted with other marine sponges (Henstchel 2001, 2002; Taylor 2004; Webster 2001). The results show Microciona are natural enrichments for diverse bacteria that may not be present in open
seawater. The greater diversity of bacteria signatures recovered from sponge versus seawater libraries are consistent with the number of unique colony morphologies observed by culturing.

**Identification of isolates producing antibiotics.** Out of the 130 isolates from seawater and 170 isolates from *Microciona* screened for antibiotic production, 5 were able to inhibit growth of the tested indicator strains. All 5 isolates were recovered from *Microciona* sponges. SPA57 (short rods), SPB16 (vibroid), SPB30 (long-thin rods), SPB69 (coccoid), and SPB79 (short rods or coccoid) produced metabolites present in culture supernatants that inhibited the marine *Bacillus* sp. SPB69 was unique as the only isolate to inhibit the *Vibrio* sp. and *E. coli*.

SPB16, SPB30 were originally isolated on ASW-C, SPA57 and SPB69 on pentane, and SPB79 on 1-bromopentane. Thus, the connection between metabolism of halo-organic compounds and bioactive metabolite production remains unclear. The low recovery of strains that produce inhibitory compounds may reflect the need to grow cultures beyond 72 hours and entry into stationary phase.

**CONCLUSIONS**

*Microciona prolifera* contain bacteria in 1,000x higher abundance than surrounding seawater. These sponges are natural enrichments for bacteria that are able to use halogenated alkanes and aromatic acids as their sole carbon source. Using culture-based and molecular biology methods, it was determined that *Microciona prolifera* contain a higher diversity of bacteria compared to surrounding seawater. Although antimicrobial metabolites were detected only from *Microciona*-associated bacteria, the relationship between antibiotic synthesis and metabolism of halogenated hydrocarbons remains unclear.

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**REFERENCES**


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Figure 1. Two *Microciona prolifera* sponges were examined in this study (top, sponge A; bottom, sponge B).
Figure 2. A specimen of *Microciona* tissue stained with DAPI and observed under phase-contrast (top) and epifluorescence (bottom) at 200X magnification. Round clusters of cells are visible within the interior of the sponge matrix.
Figure 3. Interior of *Microciona* tissue stained with DAPI and observed under phase-contrast (left) and epifluorescence (right) at 400x magnification. Cells become defined under epifluorescence illumination (yellow clusters, blue and yellow clusters).
Figure 4. Sponge cells (3 μm x 4 μm) are clustered in a piece of tissue from homogenized sponge material under phase-contrast (top). With epifluorescence illumination (bottom), clusters of motile bacterial cells are observed. (1,000x total magnification)
Figure 5. Scanning electron microscopy of a thinly sectioned piece of sponge tissue shows that the interior of matrix is covered with a dense population of cocci, long and short rods. Scale bar is equivalent to 1 µm.
Figure 6. Colony formation curve of bacteria from *Microciona* and seawater samples on ASW-C plates. Greater than 95% of the bacteria isolated from seawater were of a single morphology (top photomicrograph; entire, smooth, raised, white colonies) while bacteria from the sponges were of multiple morphologies (bottom series of 5 photomicrographs). Round, white colonies were observed the first 2 days, and then a gradual accumulation of irregular edged colonies and filamentous forms appeared through the proceeding 6 days.
Figure 7. Colony formation curve of bacteria from *Microciona* and seawater samples on ASW plates provided different alkanes as the sole carbon source. 1-Chloropentane isolates were not retrieved from sample seawater A.
Figure 8. Colony formation curve of bacteria from *Microciona* and seawater samples on ASW plates provided different aromatic acids as the sole carbon source. 2-Bromobenzoate isolates were not retrieved from sample seawater A.
Figure 9. Phylogenetic tree of the 42 16S rDNA sequences recovered from the seawater clone library. 40% are associated with the Cyanobacteria (17 clones), 26% uncultured γ-Proteobacteria (11 clones), 10% Pseudomonas (4 clones), 10% uncultured α-Proteobacteria (4 clones), 7% uncultured Actinobacteria (3 clones), and 2% Roseobacter (1 clone).
Figure 10. Phylogenetic tree of the 42 16S rDNA sequences recovered from the *Microciona* clone library. Sequences fall into 14 different groups of bacteria. The largest group (28%, 12 clones) did not align with any reported sequences in ARB. BLAST analysis of this group showed their presence in the NCBI database matching uncultured δ-Proteobacteria.