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Role of the coral surface microbiota in disease: an *in situ* test using the *Gorgonia-Aspergillus* pathosystem

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Abstract. The surface mucopolysaccharide layer (SML) is an energy rich environment that supports host-specific microbial communities. Studies have shown that the coral surface microbiota shift, in both richness and abundance, correlated with environmental perturbations and pathogens. Thus, surface microbiota may play a mutualistic role in the health of the coral host, analogous to the role of human gut microbiota. Indeed, we hypothesize that the coral surface microbiota play an integral role in disease resistance and that perturbation of the microbiota increases disease susceptibility. Here, we report on *in situ* experiments to test whether the structure of the surface microbiota of the Caribbean sea fan, *Gorgonia ventalina,* responds to perturbations in the local environment. Specifically, we tested the effects of light reduction, nutrient enrichment, antibiotic wash, and pathogen (*Aspergillus sydowii*) exposure on the microbiota as characterized using DGGE. Results indicate that the structure of the surface microbiota changes in response to perturbation and that some bacterial strains were present in untreated control corals and remained throughout all treatments. This provides experimental evidence that the surface microbiota are a link between the coral, their environment, and potentially their health. Yet the response to the perturbations appears to be complex, and most likely are the result of primary and secondary interactions between the environment, microbiota, mucus, and coral host.

Key words: Surface mucopolysaccharide layer, Microbiota, Pathogen, Perturbation, DGGE

Introduction

The correlation of incidence of coral disease with elevated seawater temperatures (Hoegh-Guldberg 2004), increased pollutants (Kim and Harvell 2002; Kaczmarsky et al. 2005), nutrient enhancement (Bruno et al. 2003), and light intensity (Richardson and Kuta 2003) suggest that environmental conditions play a role in pathogenesis. At present, the mechanisms connecting environmental factors and disease are not well understood. However, recent studies have pointed to microbial associates of corals as a possible link between environment and disease (Kaczmarsky 2006; Reshef et al. 2006).

Coral microbial associates reside primarily within or upon a surface mucopolysaccharide layer (SML) secreted by the coral host (Rohwer et al. 2002) to protect against UV light, desiccation, and to cleanse them of sediments. The SML consists of polysaccharides, proteins, lipids, and fatty acids (Meikle et al. 1988) and represents an important nutrient source in reef ecosystems (Wild et al. 2004). Microorganisms, take advantage of this with the SML supporting species-specific (Rohwer et al. 2002) and abundant microbial communities (Ritchie, Smith 1995; Brown and Bythell 2005).

However, given the paucity of information about the role of SML microbial communities of corals, hereafter referred to as surface microbiota, the vertebrate system provides a useful model for gaining insights and developing testable hypotheses. For instance, vertebrate surface microbiota act as an obstacle to pathogen colonization (Fredricks 2001; Bojar and Holland 2002). Perturbation of the microbiota from antibiotics, for example, may facilitate proliferation of less abundant and potentially pathogenic strains of bacteria, or allow non-resident microbes to colonize (Lysenko et al. 2005). This function of resistance by the microbial community is called 'colonization resistance' and is an important part of disease resistance (van der Waaij 1989).

In corals, like vertebrates, some of the surface microbes may confer disease resistance. Indeed, Reshef et al. (2006) suggest that the microbiota of corals are 'probiotic' in that the surface microbiota adapt and facilitate resistance against pathogens. Here, we hypothesize that the surface microbial communities change in response to exposure to a pathogen and environmental perturbation. If the SML microbial communities do change, then this provides evidence that surface microbiota may help mediate coral health. Shifts would indicate a stressed system

and could represent increased disease susceptibility. Consistent with this hypothesis, several studies have reported that surface microbiota of diseased corals are different from healthy ones when compared quantitatively and qualitatively but have not included environmental effects. For example, different surface microbial communities were documented on diseased areas of *Montastraea annularis* corals than on healthy (Frias-Lopez et al. 2002; Pantos and Bythell 2006), and a more diverse microbial community has been detected in diseased relative to healthy corals (Bythell et al. 2002; Pantos et al. 2003). Changes to the microbiota of corals were also found during times of environmental stress, such as during thermal bleaching events (McGrath and Smith 1999). Also, differences in the microbiota were detected on corals depending on coastal proximity and depth (Klaus et al. 2005) as well as across seasons (Guppy and Bythell 2006).

While these correlative studies suggest that the microbiota respond to environmental change, to date, no direct experimental evidence has been provided. The goal of this research is two-fold: first to document experimental perturbation of the coral surface microbiota, and second, to provide support to what we refer to as the "Coral Microbiota Disease Hypothesis." This hypothesis posits that the surface microbiota play an integral role in mediating disease susceptibility and that any perturbations to the structure of the microbiota increase disease susceptibility. Using the *Gorgonia-Aspergillus* pathosystem, coral fragments were subjected to treatments of antibiotics, light disruption, and nutrient enhancement *in situ* to document changes in coral surface microbiota.

Material and Methods

Study site. All of the field collections and experiments were carried out at Pickles Reef (24°59.079' N, 80°24.978' W), located 14.5 km off Key Largo in the Upper Florida Keys during August 2006.

Experimental Design: Three fragments (~16 cm^2) were cut from the edge of *Gorgonia ventalina* sea fans and haphazardly assigned to a treatment, a control, and initial sample. The initial sample was sealed in a sterile plastic bag and transported to the lab in a cooler filled with seawater from the collection site for processing. The two additional fragments were fastened onto a PVC rack that was anchored to the reef substrate for 10 days. Using this design with the coral fragments (same genotype exposed to different treatments), the following three experiments were carried out.

Antibiotic Treatment. The coral fragments from two apparently healthy colonies were treated with an antimicrobial wash (50 μgml^{-1} gentamycin, 100 μgml^{-1} rifampicin, and 100 μgml^{-1} streptomycin in sterile seawater as per Buyer et al. (2001)). Half of the fragments were inoculated via attachment of a pathogen strip consisting of a sterile piece of gauze (1 x 2 cm) embedded in a thin layer of agar overgrown with the fungal pathogen *Aspergillus sydowii*.

Light Disruption. Apparently healthy coral fragments from three colonies were anchored to PVC frames on the reef substrate and were covered by shade cloth laid over a clear sheet of plexiglass (to provide structural support for the shade cloth). As a control, fragments were put under the plexiglass only.

Nutrient enhancement. Increased nutrients were administered to coral fragments taken from three apparently healthy or three actively diseased parent colonies and fastened to the PVC frames. This was done by placing time-release fertilizer (Osmocote Flower and Vegetable Plant Food, N-P-K: 14-14-14, Marysville) in "nutrient pillows" per Bruno et al (2003) that were secured next to each anchored fragment. Control pillows were filled with rinsed pebbles.

Mucus Collection and DNA Extraction: All coral fragments were collected after 10 days and transported to the lab. Mucus was collected from each fragment by vortexing for 1 min in 40 ml of sterile seawater. After vortexing, the coral fragment was removed and the remaining slurry was stored at -80°C for three months until molecular analysis. Mucus and seawater samples were thawed and centrifuged three times (5 min at 10,000x g) and the DNA in the mucus concentrate was extracted using protocol outlined in the Power Soil DNA Isolation kit (Mo Bio, California) and eluted to a final volume of 1000 µl. DNA concentration was determined using a spectrophotometer set at 260 nm.

PCR Amplification: A nested PCR procedure was used to amplify bacterial 16S rDNA using primer sets previously described to analyze coral bacteria (Pantos et al. 2003). The first round of PCR used primers pA (8F) and pH'(1542R) with a minimum DNA concentration of 10 ng/µl. A touch-down PCR cycle was used following protocol from (Cooney et al. 2002). Products were verified by agarose gel electrophoresis and 5µl used as template for the second round of amplification using the primers Pc (341F) with a GC rich clamp incorporated to the 5' end, and pE'(928R). The nested products were purified and concentrated to 30 µl using a QIAquick purification kit (Qiagen, Valencia).

DGGE Analysis: Denaturant gradient gel electrophoresis was conducted using The D-Code universal mutation detection system (Bio-Rad, California). The nested and purified products were resolved on 6% (w/v) bis-acrylamide gels across a 25 to 50% denaturant gradient to select for fragment

number and sizes appropriate for analysis. 10uL of each nested product was loaded into each lane and run for 12 h at 90 V at 60° C. Gels were stained with Sybr Gold (Sigma, California) for 20 min and visualized with a UV transilluminator. Gel images were captured using Quantity One D-Code imaging software (Bio-Rad, California).

Statistical and OTU analysis: Bacterial operational taxonomic units (OTUs) seen as bands were defined by band-matching analysis as part of the Quantity One D-Code imaging software (Bio-Rad, California). The inclusion of uncertain bands was based on sensitivity of > 1%, such that a band was recognized relative to background pixels color intensity. A 1kb mass ladder (Fisher Scientific, Pennsylvania), manually defined in the software was used in a marker lane for between-gel comparisons. Mean OTUs and treatment effects were analyzed across all gels by 1-way ANOVA and 2-tailed T-tests (JMP). Multivariate cluster analysis (Mean Hierarchical algorithm, JMP, Version 5) were done by comparing samples to composites of OTUs using presence/absence of bands, to determine similarity in DGGE profiles.

Results

Out of all the samples there was a maximum of 22 OTUs, with presence or absence of at least 1 to 8 unique OTUs that correlated with coral treatment. Arrows labeled 5 and 6 indicate unique bands present in some samples but absent in others from the DGGE gel. There were also OTUs common across all treatments, including seawater and first round amplification control samples at arrows 1-4, and 10 (Fig.1). The presence of bands in the seawater, filtered water, and first round amplification (C1) indicate microbes similar to all SML samples and seawater amplified during the first round of nesting. Yet absence of bands in lane labeled C2 indicates that there was no contamination after two rounds of amplification.

The mean richness, determined as total OTUs per mucus sample, across all treatments in all gels was 11 (SE=2.66). The minimum OTU richness was 4 with maximum of 22 and the mode was 8. Overall, OTU richness showed no significant difference among samples of the same environmental perturbation treatments (1-way ANOVA, Antibiotic: F=0.05, P=0.99; Light: F= 0.148, P=0.87; Nutrient: F= 0.60, P=0.56; 2nd & 3rd gels not shown; Figs. 2A, 3A, 4A).

The DGGE data were analyzed using a cluster analysis based on OTU presence/absence to reveal similarities among communities of SML microbiota (Figs. 2B, 3B, 4B & 5).

Antibiotic Treatments. Cluster analysis revealed OTU profiles from the initial healthy sample and the combination treatment of pathogen and antibiotic together, were more similar to each other than to the control (Fig.2B).

Light Disruption. Cluster analysis revealed profiles of the healthy and shaded samples were more similar to each other than to the control (Fig.3B). The initial samples from like colonies were more similar to the control than the shaded.

Nutrient Enhancement. Cluster analysis indicated OTU mucus profiles from initial disease, pathogen only, and the nutrient enhancement, shared 8 OTUs from a composite total of 20. Together these profiles were more similar to the control, than the initial healthy. The sample that was both diseased and nutrient enhanced was more similar to the initially healthy than the initially diseased. The two most dissimilar profiles were the initially healthy and initially diseased, with difference of 10 OTUs, yet both when subjected to increase in nutrients became more similar (Fig.4B).

All treatments. Overall, cluster analysis revealed OTU profiles were more similar based on original sea fan colony than on treatment, although more similar profiles were obtained from like treatments than not (Fig.5).

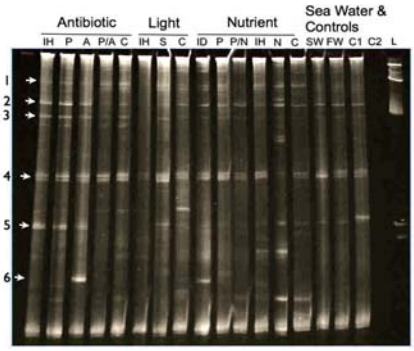

Figure 1: DGGE image depicting 16S rDNA profiles. Bands of operational taxonomic units (OTUs) represent possible unique strains of bacteria. Numbered arrows indicate OTU sites unique to some treatments (5 & 6), and those found across all treatments (1-4). Lanes are labeled by treatment given to coral fragments: IH=initial healthy, P=pathogen, A=antibiotic, C=control, S=shaded, ID=initial diseased, N=nutrients, FW= filtered water.

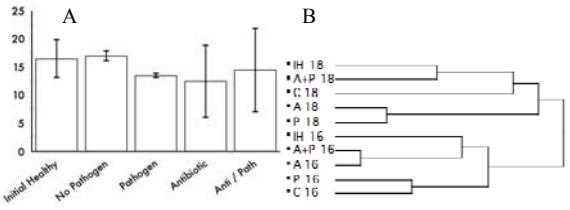

Figure 2: Mean OTU Richness (A) and Similarity of Molecular Profiles (B) from Antibiotic Treatments. Richness (± SE) is from all gels. Clusters are labeled by treatment given to coral fragments: IH=initial healthy, P=pathogen, A=antibiotic, C=control, S=shaded, ID=initial diseased, N=nutrients. Numbers indicate the original fragment colony.

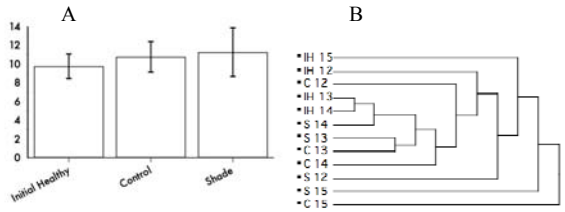

Figure 3: Mean OTU Richness (A) and Similarity of Molecular Profiles (B) from Light Disruption Treatments. Richness (± SE) is from all gels. Clusters labeled by treatment given to coral fragments: IH=initial healthy, P=pathogen, A=antibiotic, C=control, S=shaded, ID=initial diseased, N=nutrients. Numbers indicate the original fragment colony.

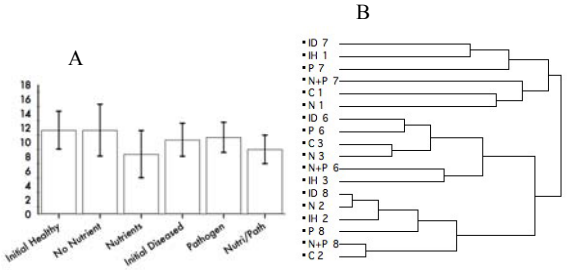

Figure 4: Mean Bacterial Richness (A) and Similarity of Molecular Profiles (B) from Nutrient Enhancement Treatments. Richness (± SE) is from all gels. Clusters labeled by treatment given to coral fragments: IH=initial healthy, P=pathogen, A=antibiotic, C=control, S=shaded, ID=initial diseased, N=nutrients. Numbers indicate the original fragment colony.

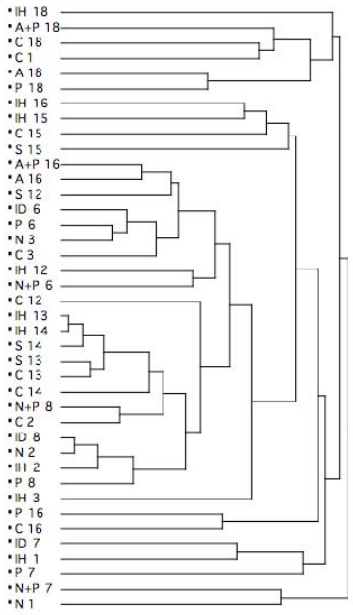

Figure 5: Similarity of Molecular Profiles across all treatments. Clusters labeled by treatment given to coral fragments: IH=initial healthy, P=pathogen, A=antibiotic, C=control, S=shaded, ID=initial diseased, N=nutrients. Numbers indicate the original fragment colony.

Discussion

Variations in molecular profiles of the coral SML microbiota were detected when sea fan fragments were infected by a pathogen, treated with antibiotics, subjected to reduced light levels, and exposed to elevated nutrient levels *in situ*. Some OTUs, potential bacterial strains, were present in control corals and remained throughout all treatments. This finding is consistent with the Coral Microbiota Disease hypothesis that posits that surface microbiota play an integral role in mediating disease susceptibility.

The shifts in the microbes demonstrated by this study may not have been statistically significant because of the DGGE gradient used. It is possible that more OTUs could have been detected because there appeared to be high concentrations of DNA the top and bottom of the gel (Fig. 1). Another consideration is that PCR bias may have caused preferential amplification of sequences that resulted in more similar OTU profiles. PCR artifacts have been shown to effect results of 16S rDNA diversity analysis (Acinas et al. 2005).

The microbes may in fact be responding to a change in mucus quantity or quality and not directly to the treatments. The microbial communities living in the SML are thought to rely on exudates from the coral-zooxanthellae association (Ducklow and Mitchell 1979; Gil-Agudelo et al. 2006). It would follow that alterations in the microbial community would occur if the coral-zooxanthellae relationship were altered. A change in nutrient and or light availability may alter the coral-zooxanthellae symbiosis and in turn affect the nature of the SML qualitatively (i.e., composition) or quantitatively. For instance, studies have also shown that copious mucus production is often the first visible sign of a generalized response by corals to environmental stress (Peters 1984).

Not to be disregarded are the potential roles of innate host response and microbial interference. Kim et al. (2000) demonstrated that the sea fan *G. ventalina* is capable of a chemically mediated inducible response against the pathogen *A. sydowii.* It may take considerable amounts of energy for the corals to produce these defensive compounds, which come at the expense of secreting the protective mucus layer (Edmunds and Davies 1989). It would follow then, that alterations in the microbial communities, which utilize the SML, would result. Alternately, diseased corals may reduce the production of these compounds so that they can maintain "normal" mucus production.

It was suggested that *G. ventalina* may respond to *A. sydowii* with a non-specific antimicrobial product regardless of whether it is fungal or bacterial (Alker et

al. 2004). The inhibitory compounds could affect non-targeted microbes. Also, some SML microbiota themselves produce antimicrobial compounds (Castillo et al. 2001; Ritchie 2006).

In the present study, host factors did appear more important to microbial diversity than direct perturbation. Particularly in the cluster analysis from all treatments that show the sea fan of origin was a better predictor of microbial similarity than the pathogen or perturbation it was exposed to. Yet, due to a lack of knowledge about microbial interactions, innate host response, SML antimicrobial products, and environmental effects, it is hard to know if the microbial shifts noted herein were affected by any or all of these factors.

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