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October 2010

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Overview:

Colonies of the scleractinian coral *Acropora palmata*, listed as threatened under the US Endangered Species Act in 2006, have been monitored in Hawksnest Bay, within Virgin Islands National Park, St. John, from 2004 through 2010 by scientists with the US Geological Survey, National Park Service, and the University of the Virgin Islands. The focus has been on documenting the prevalence of disease, including white band, white pox (also called patchy necrosis and white patches), and unidentified diseases (Rogers et al., 2008; Muller et al., 2008). In an effort to learn more about the pathologies that might be involved with the diseases that were observed, samples were collected from apparently healthy and diseased colonies in July 2009 for analysis.

Two different microbial assays were performed on Epicentre Biotechnologies DNA swabs containing *A. palmata* coral mucus, and on water and sediment samples collected in Hawksnest Bay. Both assays are based on polymerase chain reaction (PCR) amplification of portions of the small rRNA gene (16S). The objectives were to determine 1) if known coral bacterial pathogens *Serratia marcescens* (Acroporid Serratiosis), *Vibrio coralliilyticus* (temperature-dependent bleaching, White Syndrome), *Vibrio shiloi* (bleaching, necrosis), and *Aurantimonas coralicida* (White Plague Type II) were present in any samples, and 2) if there were any differences in microbial community profiles of each healthy, unaffected or diseased coral mucus swab. In addition to coral mucus, water and sediment samples were included to show ambient microbial populations.

In the first test, PCR was used to separately amplify the unique and diagnostic region of the 16S rRNA gene for each of the coral pathogens being screened. Each pathogen test was designed so that an amplified DNA fragment could be seen only if the specific pathogen was present in a sample. A positive result was indicated by bands of DNA of the appropriate size on an agarose gel, which separates DNA fragments based on the size of the molecule. DNA from pure cultures of each of the pathogens was used as a positive control for each assay.

In the second test, a bacterial community profile was generated using denaturing gradient gel electrophoresis (DGGE). While highly conserved, the 16S rRNA gene has several variable regions which can be used in a comparative analysis of microbial populations. By amplifying a variable region of the gene from a mixed microbial community, it is possible to profile the community based on the DNA sequence differences between organisms. Initial PCR products are visualized on an agarose gel to ensure sufficient product for the community DGGE analysis. A positive result is indicated on the agarose gel by DNA bands of uniform intensity and size (approximately 500 bp). Because these DNA segments all have the same length, individual members of the community cannot be resolved by agarose electrophoresis, requiring a second step, denaturing gradient gel electrophoresis. DGGE takes advantage of DNA's property to separate its double strands (i.e., denature or melt) when exposed to certain chemicals, temperatures or salt concentrations. The DNA sequence dictates the conditions required for strand separation. Since sequence variations between different microbial rRNA genes result in different denaturation (melting) properties of these DNA molecules, DGGE provides a good estimate of the abundance of predominant microbial community members. Variations in the bacterial community profiles associated with corals may indicate onset of disease or a disease state.

Sample information:
Nine *Acropora palmata* coral mucus swabs were collected at Hawksnest Bay on 23 July 2009 from 1) healthy portions of three diseased colonies (1H-3H), 2) diseased portions of the same colonies (1D-3D), and 3) from three healthy colonies exhibiting no disease lesions (1C-3C). Three samples of water (W1-W3) and sediment (S1-S3) were taken within one meter of three colonies using a standard sampling protocol (Woodley, 1996). Samples were sent in a cryo shipper to the Coral Health and Disease research group at the Hollings Marine Laboratory, Charleston, SC for microbial community profiling. Samples were stored at -80°C prior to experimental analysis.

DNA extraction:
Swabs were removed from cryovials using sterile technique and placed in 2.0 ml screw-capped tubes containing silica beads (FastDNA® SPIN for Soil Kit, MP Biomedicals, LLC, Solon, OH). Water and sediment samples were thawed on ice, vortexed, and 200 µl of water or water plus sediment was added to a screw-capped tube. DNA was extracted from each sample using a modified kit protocol which included the addition of 4 µl of 50 mg/ml poly A RNA in the initial extraction volume to increase DNA yield from each sample. Elution volume for each sample was 100 µl.

PCR-Based Pathogen Screen:
DNA samples were diluted 1:10 in 10 mM Tris-1 mM EDTA (TE buffer), pH 8.0. A TaKaRa Bio, Inc. ExTaq™ Kit (Shiga, Japan) was used. Each PCR volume included 10 µl of dilute DNA sample, 5 µl 10X ExTaq buffer, 20 µM each primer, 1 µl dNTP mixture, 0.25 µl ExTaq polymerase, and sterile water to a final volume of 50 µl. Primers used in the PCR were as described previously (Polson, 2008). Amplification parameters were: initial denaturation 95°C for 5 min; 40 cycles of 95°C for 30 s, annealing for 30 s, 72°C for 60 s, and a final extension at 72°c for 10 min. Annealing temperatures for each pathogen-specific primer set were: 67°C, *V. coralliilyticus*; 63°C, *V. shiloi*, and 61°C, A. *coralicida* and *S. marcescens.* One microliter of DNA extract from a pure culture of each pathogen was used as a positive control and sterile water was used as a negative control. All PCR products (1/10 volume) were visualized on a 1.2% TAE- agarose gel (40 mM Tris-acetate-1 mM EDTA) containing 0.5 µg/ml ethidium bromide at 150 V for 45 min. A digital image was captured using the FluorChem™ 8900 fluorescent imager (Alpha Innotech, San Leandro, CA), equipped with a UV transilluminator (365nm). The agarose gel image is shown in Figure 1.

As can be seen from the pathogen screen results (Table 1), all samples were positive for *A. coralicida*. The DNA bands for samples 2H and 3H are very light. Samples S1-S3 and 1D and 2D were very bright, possibly indicating a high concentration of *A. coralicida* in those samples. Only one sample (S1) was positive for *S. marcescens*. *Vibrio coralliilyticus* was present in samples 1H, 1D-3D, 3C and S3 (very light band). Samples containing *Vibrio shiloi* were 1H, 2H, 1D (light band), 2D, 3D, S1 and S2.
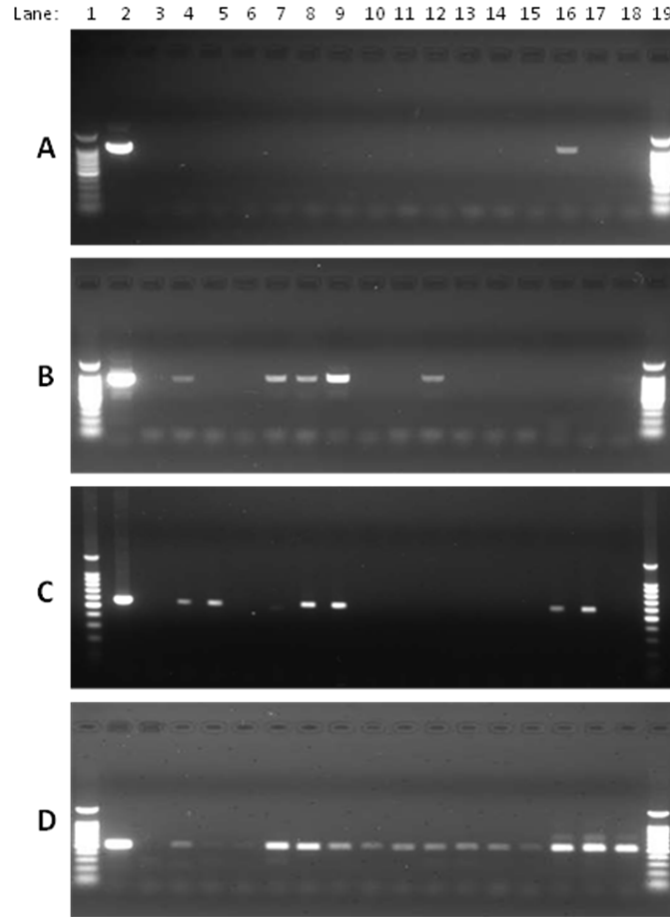
Figure 1. PCR-based screen for putative coral pathogens. Positive results are indicated with a bright DNA band in the lane. Panel A, *Serratia marcescens*; Panel B, *Vibrio coralliilyticus*; Panel C, *Vibrio shiloi*; Panel D, *Aurantimonas coralicida.* Positive control, lane 2; negative control, lane 3; Samples 1H-3H, lanes 4-6; Samples 1D-3D, lanes 7-9; Samples 1C-3C, lanes 10-12; Samples W1-W3, lanes 13-15; Samples S1-S3, lanes 16-18; Promega 100 bp DNA ladder, lanes 1 and 19.

Table 1. Results of the coral pathogen screen. An "X" indicates presence of the bacterium in the sample.

|	1H	2H	3H	1D	2D	3D	1C	2C	3C	W1	W2	W3	S1	S2	S3
S. marcescens													X		
V. coralliilyticus	X			X	X	X			X						X
V. shiloi	X	X		X	X	X							X	X	
A. coralicida	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

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Bacterial Community Analysis by DGGE:

DNA extracts (1:10 dilution in TE buffer) from each sample were amplified using the TaKaRa Bio, Inc. ExTaq™ Kit. Primers used were 911F 5'-TCA AAT GAA TTG ACG GGG GC-3' (Maidak, 1996) and 1406R 5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CAC GGG CGG TGT GTA C-3' with GC clamp (40 bp GC clamp underlined) (Lane, 1988). The PCR mixture included 10 µl of dilute DNA sample, 5 µl 10X ExTaq buffer, 40 µM each primer, 4 µl dNTP mixture, 0.25 µl ExTaq polymerase, 4 µl 25 mM $MgCl_2$, and sterile water to a final volume of 50 µl. Amplification parameters were: initial denaturation, 94°C for 5 min; 20 cycles of 95°C for 60 s, 65°C for 60 s (-0.5°C/cycle), 72°C for 3 min; 20 cycles of 95°C for 60 s, 55°C for 60 s, 72°C for 3 min; and a final extension at 72°C for 10 min. Amplification products were verified on a 1.2% TAE-agarose gel prior to DGGE (Figure 2). A digital image was captured as detailed above.
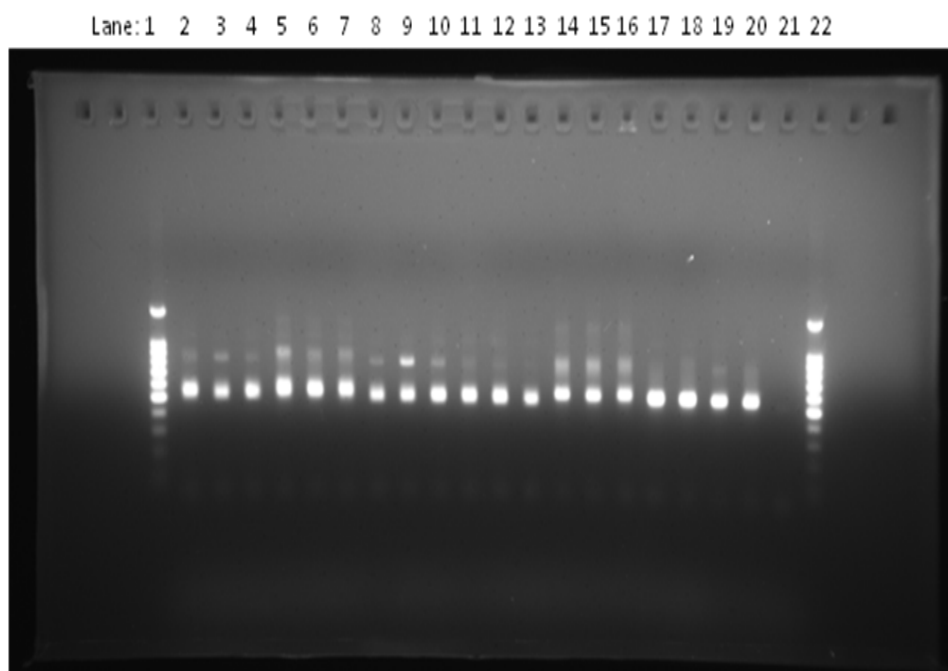
Figure 2. Agarose gel electrophoresis of DGGE PCR products. Promega 100 bp DNA ladder, lanes 1 and 22; samples 1H-3H, lanes 2-4; samples 1D-3D, lanes 5-7; samples 1C-3C, lanes 8-10; samples W1-W3, lanes 11-13; samples S1-S3, lanes 14-16, *S. marcescens*, lane 17; *V. coralliilyticus*, lane 18; *V. shiloi*, lane 19; *A. coralicida*, lane 20; negative control, lane 21.

Once PCR products were confirmed on the agarose gel, 30 microliters of sample and 5 µl of pure culture products were electrophoresed on a 35-60% denaturing gradient-7% acrylamide gel in TAE buffer. Electrophoresis conditions were 60 V for 16.5 h at 60°C constant temperature. The gel was stained for 20 min in 1.0 µg/ml ethidium bromide in 1 X TAE buffer for 20 min and destained in buffer alone for 20 min prior to imaging on a UV transilluminator using the FluorChem™ 8900. The bacterial community analysis results are presented in Figure 3.

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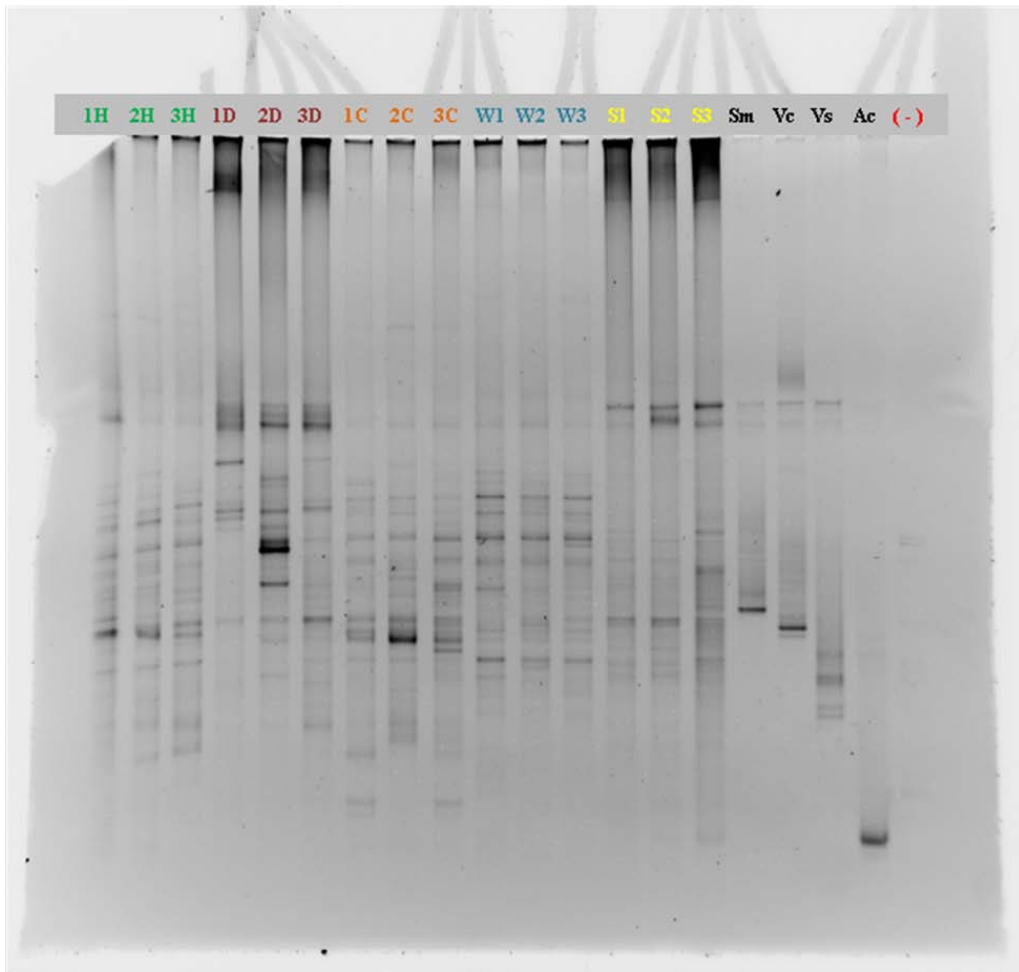
Figure 3. Bacterial community analysis by denaturing gradient gel electrophoresis (DGGE). Sample identifications are listed at the top of the gel, and include control pure culture samples: Sm, *S. marcescens*; Vc, *V. coralliilyticus*; Vs, *V. shiloi*; Ac, *A. coralicida*. Samples 1H-3H, 1C-3C and W1-W3 had similar bacterial community profiles. Bacterial profiles of sediment samples (S1-S3) are much different from groups "H" (apparently healthy), "C" (colony C, unaffected), and "W" (water), but similar to each other. The profiles of diseased samples 1D-3D appear to be unique.

Conclusions and Significance:
The results of the coral bacterial pathogen screen show that *A. coralicida* was present in all samples. The brighter DNA bands in the 1D and 2D samples are indicative of a heavier bacterial load in those swabs, and the data suggest that *A. coralicida* may be an opportunistic pathogen of *A. palmata*. All of the "D" (diseased) samples were positive for both *Vibrio* species, implying a disease state in those colonies may be associated with this bacterium. Half of the "H" samples and one of the "C" samples were positive for *Vibrio shiloi and V. coralliilyticus*. For the "H" samples, this could indicate early infection in an area of widespread coral disease. Only one sample (S1) was positive for *S. marcescens*. Three of the sediment samples also were positive for vibrios, however, as it has been estimated that the bacterial load is as much as three orders of

magnitude higher in marine sediments as compared to water (Tibbles, 1992), we would expect a greater diversity of microorganisms in those samples. It is reasonable to expect *S. marcescens* and both *Vibrio* species to be present in a random sediment sample. The data also indicate that *Serratia marcescens* is excluded as involved in the lesions from these *A. palmata* colonies.

The bacterial community analysis by DGGE resulted in similar profiles for two *A. palmata* colonies. While identical bacterial profiles are not expected from any two samples, those with little variation are indicative of a stable bacterial community (Muyzer and Smalla, 1998). As seen in Figure 3, coral swabs in groups "H" (healthy portion of diseased coral colony) and "C" (healthy colony) had bacterial DGGE profiles similar to each other, and also to those of the water samples (W1-W3). It is reasonable to expect similar microbial profiles for coral mucus and seawater samples, as microbes may diffuse freely from one medium to the other. There was much higher variability (i. e., differences in DNA banding patterns) in the swabs from group"D" (diseased samples), indicating changes in the microbial community for each of those corals as compared to non-diseased coral mucus samples (Muyzer and Smalla, 1998). Sediment samples, while quite different from groups "H", "C" and "W", were very similar to each other, indicating a stable bacterial population in the sediment. Identifications of the dominant bacterial community members have not been made, however the possibility exists for sequence analysis of the darker bands in these samples. The conspicuous absence of *Aurantimonas coralicida* in samples analyzed by DGGE (this organism was detected in all samples using the PCR pathogen screen), indicates a deficiency for specific detection of a particular bacterial organism using this methodology, resulting from the degeneracy of the oligonucleotide primers.

These data support the view that coral diseases are complex and may result from many interactions among the organism, putative agents of disease and the host's environment. Further, the lesions observed on *A. palmata* at Hawksnest Bay, USVI are likely not simply the result of a single pathogenic bacterial species, but rather a mechanism yet to be discovered.

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