

Development of a Novel Method for Coral RNA Isolation and the Expression of a Programmed Cell Death Gene in White Plague-Diseased *Diploria strigosa* (Dana, 1846)

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Abstract. Coral diseases are significantly contributing to the degradation of tropical coral reefs throughout the world. A majority of research on coral diseases conducted to date has focused on microbiological and ecological surveys to characterize coral associated microbiota and factors contributing to disease prevalence. Recent studies have shown that corals use innate immunity to resist microbial invasions, but the mechanisms controlling these defenses remain poorly understood. In this study we investigated molecular responses to disease manifested by corals during periods of infection. During July of 2007 in Cayos Cochinos, Honduras, surface layer mucus samples were collected from colonies of diseased and healthy *Diploria strigosa*, putatively affected by White Plague Disease. Coral RNA was isolated from condensed mucus samples stabilized in RNAlater (Ambion), and differential gene expression was analyzed by differential display PCR. PCR products were analyzed qualitatively by 2 % agarose gel electrophoresis, and candidate immunity genes were isolated, cloned, and sequenced. Derived sequences had similarities to immunity genes of other invertebrates, including programmed cell death. The results of this study contribute to the characterization of molecular mechanisms controlling coral immune responses to disease infection, and present the use of a novel and non-invasive method to collect coral RNA.

Keywords: Coral Disease, Immunity, Gene Expression, Differential Display PCR, Programmed Cell Death

Introduction

Since the first report of coral disease over three decades ago, research has been largely dominated by ecological and microbiological investigations (Pantos et al. 2003, Sekar et al. 2006, Santavy et al. 2001). Although these studies are necessary to characterize coral-associated microbiota and ecological factors influencing disease prevalence, it remains unclear how coral innate immune defenses contribute to disease resistance and susceptibility.

Studies of coral innate immunity are just beginning to reveal the repertoire of defenses against microbial invasions. In gorgonians, inflammatory responses involving the activation of amoebocyte differentiation and proliferation, melanin deposition, chitinases and antioxidants have been observed (Mydlarz et al. 2008, Douglas et al. 2007, Mydlarz et al. 2006). Programmed cell death pathways have also recently been identified to have a putative role in White Syndromes observed on the Great Barrier Reef (Ainsworth et al. 2007). Immunity genes that are conserved among different phyla have been identified in corals (Miller et al. 2007), but a characterization of

their functions in coral innate immunity has not been reported.

The coral surface mucus layer (SML) is an important component of the coral innate immune system. It is a physical barrier that is the first line of defense against potential pathogens in the water column (Sutherland et al. 2004). Periodic sloughing of the SML and the production of antimicrobial compounds are important mechanisms for regulating coral-associated microbial communities (Brown and Bythell 2005). Differential antimicrobial properties and microbial communities of the SML of diseased and healthy corals have been observed (Gil-Agudelo et al. 2006, Ritchie 2006, Pantos et al. 2003).

In this paper, we present an investigation of differential gene expression between colonies of *Diploria strigosa* which are healthy and which are putatively infected with White Plague using coral cells sampled from SML.

Materials and Methods

Field sample collection

A survey of coral disease prevalence was conducted on a shallow patch reef area of the Plantation Beach

Reef of Cayos Cochinos, Honduras (Fig. 1). Three *Diploria strigosa* colonies putatively affected by White Plague and three apparently healthy colonies were selected for sampling between 0400 and 0500 hours before civil dawn (Table 1). Samples from the SML of diseased colonies were collected from areas adjacent to the disease lesion and apparently healthy tissue at least 20 cm from disease lesion. Mucus samples were collected from central areas of healthy colonies unaffected by disease.

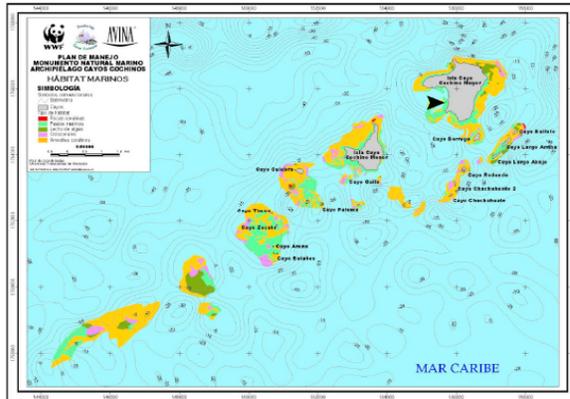


Figure 1: Map of Cayos Cochinos, Honduras. The black arrow indicates the Plantation Beach Reef area, where coral mucus samples were collected for this study (HCRF/WWF 2004).

Excess mucus production was induced at the sampling sites of the coral colonies by rapidly ejecting seawater from sterile 10 cc syringes with no needles 2 to 3 cm from coral surface. Duplicate 10 ml mucus samples were collected with sterile syringes from each sampling area. Samples were kept at ambient seawater temperature (~27°C) for no more than an hour, stored on ice, and processed upon return to shore.

Mucus samples were condensed using a hand crank centrifuge (~300 rpm) for approximately 5 min, and the resulting pellets were removed to 1.5 ml microcentrifuge tubes and stabilized in 10x volumes of RNeasy lysis buffer (Qiagen). Stabilized samples were stored in a conventional freezer for up to three weeks, transported at room temperature for 24 hours, and stored at -80°C until molecular analyses were performed at the laboratory in Sarasota, Florida.

RNA isolation and cDNA synthesis

Stabilized mucus samples were centrifuged (10,000g) for 20 min at 4°C, the RNeasy lysis buffer supernatant was removed, and the resulting mucus pellets were individually homogenized in Trizol (Invitrogen). Contaminating polysaccharides, proteins and genomic DNA were removed by centrifugation (10,000g) for

10 min at 4°C. Total RNA was extracted from the Trizol-mucus homogenate supernatant, according to the manufacturer's protocols. One µl glycogen (1 mg l⁻¹) was added to enhance the precipitation of low concentrations of RNA with 99.5% isopropyl alcohol.

Sample #	Colony #	Condition	Area of Sample Collection
7	2	WP	Disease Front
9	2	WP	Healthy Tissue
13	9	WP	Disease Front
15	9	WP	Healthy Tissue
18	4	WP	Disease Front
22	4	WP	Healthy Tissue
23	A	ND	Healthy Tissue
25	B	ND	Healthy Tissue
28	C	ND	Healthy Tissue

Table 1: Description of mucus samples collected from *D. strigosa* and analyzed in this study (WP = white plague-like disease, ND = not diseased). Matching numbers under the colony column indicate samples collected from the same *D. strigosa* colony.

For each total RNA sample, four separate SuperScript III reverse transcriptase reactions (Invitrogen) were performed with one of the following primers: anchored-dT primers with HindIII restriction sites (5' AAGCTTTTTTTTTTTT-N-3'; N = A, G, or C) and a mixture of random hexamer primers (Invitrogen). A negative control reaction with random hexamer primers and total RNA was conducted without the addition of reverse transcriptase, and the products of this reaction were used in coral gene-specific RT-PCR to ensure that there was a negligible level of genomic DNA contamination in the RNA samples (methods described below).

Coral gene-specific RT-PCR

Random hexamer primed cDNA was used as a template for coral gene-specific reverse transcriptase PCR (RT-PCR). PCR primers specific to cytochrome-B (Cyt-B) and β-tubulin (Tub) genes were designed from sequences available for *Manicina areolata* and *Diploria labyrinthiformis* (Accession # AB117305.1 and AB118249.1, respectively), which have sequences highly conserved with *D. strigosa*. RT-PCR was conducted to confirm the presence of coral RNA sequences in the mucus samples, which were likely to be dominated by microbial sequences. Tub and Cyt-B amplicons were sequenced to ensure these genes can be used effectively to confirm the presence

of good quality coral gene sequences in the mucus samples.

Differential display PCR

Anchored-dT primed cDNA was used as a template for differential display PCR (DD-PCR), modified from the methods first presented by Liang and Pardee (1992). Five combinations of arbitrary 13-base primers with Hind-III restriction sequences and the anchored-dT primer used to synthesize the cDNA template were used to amplify a subset of the transcriptome present in each cDNA sample. DD-PCR products from samples originating from disease lesions, healthy areas of diseased colonies and healthy colony controls were separated side-by-side by 2% agarose gel electrophoresis for each primer combination. Gels were stained with ethidium bromide and DNA bands that were differentially amplified were excised for cloning and sequencing.

Sequencing candidate molecular responses to disease

Excised bands were extracted from the agarose gel using Wizard SV Gel and PCR Clean-Up System according to the manufacturer's protocols (Promega). The extracted DNA was cloned into a TOPO TA plasmid and transformed into *Escherichia coli* (strain DH5 α -T1^R) with an Invitrogen kit for sequencing, according to the manufacturer's protocols. Plasmids were extracted from cultures of individual transformed *E. coli* colonies using Quick Plasmid Miniprep kit (Invitrogen), and the cloned DD-PCR inserts were sequenced using a standard automated Sanger sequencing protocol. Similarities of the derived sequences in this study to sequences available in the National Center for Biotechnology Information were found using a basic local alignment search tool (BLAST).

Results

Coral gene-specific RT-PCR

Unlike the results reported by Santiago-Vazquez and colleagues (2006), we were able to store coral nucleic acids successfully using RNAlater. Eight of nine samples analyzed in this study provided consistent positive results for the presence of coral nucleic acids, confirmed by the amplification of both Cytochrome-B and Tubulin genes (Table 2 and Figure 1). Amplicons were not observed for negative control reactions conducted with total RNA templates exposed to reverse transcriptase reaction conditions.

Sample #	Cyt-B	Tub
7	+	+
9	+	+
13	-	+
15	+	+
18	+	+
22	+	+
23	+	+
25	+	+
28	+	+

Table 2: Summary of results from coral gene-specific RT-PCR. Respectively, (+) and (-) indicate successful and unsuccessful amplification of coral Cytochrome-B (Cyt-B) and β -Tubulin (Tub) genes. Sample # refers to the original mucus sample from which the cDNA template originated.

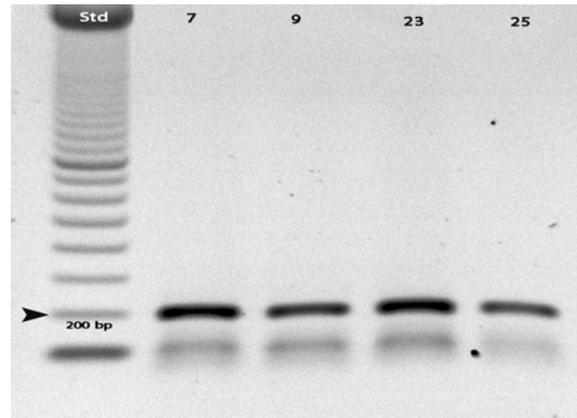


Figure 1: 1% agarose gel electrophoresis of Tubulin gene-specific RT-PCR products. "Std" lane is a 100 bp standard, and the Tub amplicons for samples 7, 9, 23, and 25 are in the expected size range of 200 bp.

Differential display PCR

A total of twenty eight differentially displayed bands were observed and excised from samples originating from diseased coral colonies (Figure 2).

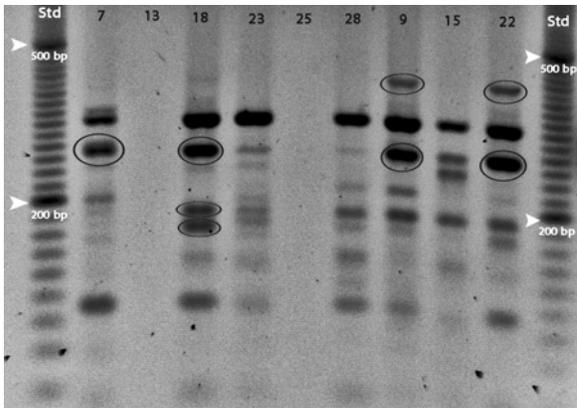


Figure 2: 2% agarose gel electrophoresis of DD-PCR products. Circled bands were excised for sequencing. Lanes “std” are standard 20 bp ladders, with white arrows indicating the 200 and 500 bp markers. The numbered lanes indicate the mucus samples from which the DD-PCR products were amplified.

Sequencing – programmed cell death gene detected at lesion boundary of White Plague-diseased corals

Sequences derived from the Cyt-B and Tub amplicons significantly matched ($E = 3e^{-30}$) sequences available for *D. strigosa* in NCBI. Sequences derived from differentially displayed bands amplified from a samples 7 and 18, originating from the lesion boundary of diseased colonies of *D. strigosa*, significantly matched ($E = 6e^{-9}$) a death domain sequence of *Nematostella vectensis* (Accession # XM001639015.1).

Discussion

Coral innate immunity is not well understood, and to date, few molecular studies have been conducted to elucidate coral immune responses to disease (see Vollmer and Kline 2008 for a recent study). In this paper, results from a preliminary study on coral molecular responses to disease are discussed.

Most molecular analyses conducted on corals to date used invasive methods of collecting nucleic acids. These methods include the collection of entire coral colonies, large fragments or core samples that can reduce coral defenses on reefs that are already degraded (Edge et al. 2005, Smith-Keune and Dove 2007, Klueter et al 2006). The isolation of coral nucleic acids from condensed coral mucus is a novel method that can be used for PCR-based molecular analyses. The cellular sources of coral nucleic acids in the surface mucus layer of corals were not investigated in this study. However, mucocytes and nematocytes may release coral RNA and DNA into the surface mucus layer when the epidermis is stimulated. Amoebocytes, which are immunity-related motile cells that migrate throughout coral tissue (Meszaros and Bigger 1999), may also be shed into

the mucus layer when physical irritation to the coral surface is applied.

Genomic DNA can compete with cDNA in PCR during primer annealing steps, and contamination of RNA samples with genomic DNA can significantly reduce the efficiency of DD-PCR to identify putative differentially expressed genes (Liang and Pardee 1995). Results from the negative control coral gene-specific RT-PCR reactions conducted in this study indicate a level of genomic DNA contamination undetectable by ethidium bromide staining. For that reason, an additional DNA digestion step was not performed, which would otherwise reduce the RNA concentration by a second precipitation step before cDNA synthesis.

The presence of Tub amplicons (200 bp) in all of the β -tubulin-specific RT-PCR reactions indicated that coral nucleic acids were present in each sample. However, a Cyt-B amplicon (500 bp) was not present for sample 13, which suggests that the sequence was degraded to a length significantly shorter than 500 bp. The collection of replicate mucus samples can ensure that at least one sample will have sufficient quality for DD-PCR. Variable intensity of DNA bands of the same base pair length in Figures 1 and 2 may show variable concentrations of coral RNA present in the samples. The number of coral cells and concentration of RNA excreted into the mucus may differ between diseased and healthy corals, and were unknown in this study. The starting concentration of total RNA used for reverse transcriptase reactions and DD-PCR can be normalized for samples from diseased and healthy corals, if an average concentration of coral RNA excreted into the mucus is determined by quantitative PCR amplification of the Cyt-B and Tub house-keeping genes.

Twenty eight differentially amplified bands were excised from DD-PCR products in this study. The sequencing of these amplicons is still underway, but preliminary results from the analysis of five candidate immunity genes have provided significant data upon which future investigations will be conducted. BLAST searches revealed sequence similarities of differentially amplified bands from samples 7 and 18 to a death domain sequence in *Nematostella vectensis*. Both of these samples were collected from the lesion boundary of *D. strigosa* colonies affected by putative White Plague infections.

Death domains are genes translated into conserved proteins that have functions in programmed cell death pathways (PCD) (Stehlik 2007). PCD has been investigated for its roles in mediating the destruction of cells in immune-compromised organisms, and it is activated by receptors such as Toll and Toll-like receptors (TLRs), which recognize conserved microbial ligands (Govind 2008, Ameisen 2002).

Gene sequences for TLRs have been identified in *Acropora millepora* and *N. vectensis* (Miller et al. 2007), and a recent study by Ainsworth and colleagues (2007) confirmed the presence of PCD in coral tissues adjacent to lesions characteristic of white syndromes on the Great Barrier Reef. The identification of a coral death domain as a putative immune response in *Diploria strigosa* colonies affected by putative White Plague infections suggests that PCD may have conserved functions in corals as a final line of defense against pathogenic microbial infections.

Acknowledgements

Support was received from the New College Foundation, New College Alumni Association, New College Council of Academic Affairs, New College Natural Sciences Division, Nellie Mae Foundation, the Explorers Club, and the New College Pritzker Marine Laboratory. MPederson assisted with initial field collections. Plantation Beach Resort provided logistic support for the field studies. We gratefully acknowledge NSF grant #0520607 that allowed us to acquire the RT-PCR apparatus and Li-COR GEMF grant that allowed us to acquire the sequencer. We thank the Honduras Coral Reef Fund and the Cayos Cochinos National Marine Monument for access to corals used in this study. We also thank anonymous reviewers for improving this manuscript.

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