Abstract

The occurrence of coral diseases associated with bacterial infection increases dramatically with increasing sea surface temperatures. However, the mechanism by which elevated seawater temperature actually induces or causes coral disease is poorly understood. One possibility is that increasing seawater temperature causes a change in the community structure of bacteria inhabiting healthy coral tissues. To assess the effect of increasing seawater temperature on the bacterial communities inhabiting the brain coral *D. strigosa*, colonies were collected directly from the reef and compared to colonies maintained in both heated and control flow-through aquaria. Heated aquaria were maintained at 30.5°C (2.5°C above ambient) and coral colonies were left in the aquaria for up to two weeks prior to sampling. Bacterial assemblages were compared statistically through the use of Terminal-Restriction Fragment Length Polymorphisms (T-RFLP) of 16S rRNA genes. The bacterial communities inhabiting coral colonies maintained in the unheated flow-through aquaria were statistically different from colonies sampled directly from the reef. However, colonies maintained in the heated aquaria were statistically similar to those sampled from the unheated aquaria.

Keywords
Bacteria, Coral disease, T-RFLP, Temperature

Introduction

Recent reports have highlighted the impact of coral diseases on reefs worldwide (Green and Bruckner 2000), and suggest that both the intensity and number of different diseases may be on the rise (Richardson et al. 1999). The correlation of disease occurrence to numerous environmental factors, including temperature (Gil-Agudelo and Garzon-Ferreira 2001; Kuta and Richardson 2002; Richardson et al. 1998; Rosenberg and Ben-Haim 2002), depth (Kuta and Richardson 2002), sedimentation (Bruckner et al. 1997; Taylor 1983) nutrients (Antonius 1988; Kim and Harvell 2000; Taylor 1983) and pollution, (Antonius 1988; Bruckner et al. 1997) hints at the complex interactions between disease and the environment. Of these factors, one of the most striking correlations to disease occurrence is temperature (Kuta and Richardson 2002, Rosenberg and Ben-Haim 2002). In the case of bleaching by *Vibrio shiloi* increased temperature triggers the expression of virulence factors (Rosenberg and Ben-Haim 2002). At high seawater temperatures, *V. shiloi* produces an adhesin that allows it to adhere to and then penetrate coral surfaces. Once inside the coral tissue *V. shiloi* toxins inhibit and lyse symbiotic zooxanthellae. In other coral diseases such as plague, dark spot disease and black band disease, the correlation to temperature is less absolute, and the mechanism by which elevated temperatures affect disease incidence is poorly understood. In these cases, the relationship between temperature and disease may not be entirely tied to the pathogen(s), but instead may involve the temperature response of a whole microbial community. One of the best defense mechanisms to the growth of pathogenic microbes is the maintenance of a healthy microbial community. These microorganisms can inhibit pathogens through interspecific competition and secretion of antibiotic substances (Klaus et al. 2005; Rohwer et al. 2002, Pantoas et al. 2003)

It is well established that coral tissues harbor diverse bacterial communities, primarily associated with the coral surface microlayer or coelenteron (Bythell et al. 2002; Frias-Lopez et al. 2002; Gast et al. 1998; Rohwer et al. 2001). However, the effect of seawater temperature on these communities remains poorly understood. To further understand the ecological process link between coral disease and temperature, additional information is needed on how the healthy bacterial community associated with coral tissue responds to varying temperature conditions.

The goal of the present study was to assess the effect of temperature on the bacterial communities of healthy colonies of *D. strigosa*. The bacterial community associated with naturally occurring colonies as well as those maintained in heated and unheated experimental aquaria were characterized through the use of terminal restriction fragment length polymorphisms (T-RFLP) of 16S rRNA genes. The variation in bacterial communities was analyzed quantitatively using a combination of statistical procedures, including multidimensional scaling (MDS), one-way analysis of similarity (ANOSIM), and
similarity percentages (SIMPER) (Clarke and Warwick 2001).

Materials and Methods
Sample Collection and Experimental Setup
Twenty healthy colonies of *D. strigosa* were collected from shallow fringing reefs (~5 m water depth) approximately 75 m offshore of the island of Curacao Netherlands Antilles. Upon returning to shore, four colonies were rinsed with filtered seawater and immediately sampled for molecular microbial analysis. Samples were collected from the surface by removing a 2 cm by 2 cm portion of the uppermost 1 cm of the coral colony with a chisel and placing the sample in a sterile disposable 50 ml polypropylene centrifuge tube. The sample was immersed in 80% ethanol, crushed and homogenized, creating a slurry of coral tissue, zooxanthellae, mucus, microorganisms, and skeletal material. The remaining 16 colonies were put into heated and control aquaria housed at the Curacao Sea Aquarium. The Curacao Sea Aquarium maintains a large flow-through tank system in which seawater is pumped through from the adjacent reef (approximately 5 m water depth). The reef water was pumped directly from a small sedimentation reservoir to one of two experimental aquaria. One aquaria was maintained at ambient temperature (control = 28°C) while the other was heated 2.5°C above ambient temperature (heated = 30.5°C). Eight colonies were put in each tank and sampled as above; half after six days and half after fourteen days. Previous studies have shown very little overlap between the bacterial species detected from reef-water and coral tissues (Frias-Lopez et al. 2002; Rohwer et al. 2001). Based on these studies we chose to focus solely on the bacterial communities of *D. strigosa*.

T-RFLP Analysis
Total DNA was prepared from coral samples using the UltraClean Soil DNA Kit (Mo Bio Laboratories, Carlsbad, CA). This kit has proven to be one of the most efficient and consistent methods for obtaining DNA from coral tissues (Rohwer et al. 2001; Klaus unpublished data). Optimization of PCR was performed for each sample by adjusting the amount of genomic DNA extract used to obtain a strong band on an agarose gel, without visible nonspecific product (Blackwood et al. 2003). The genes encoding for 16S rDNA were amplified with a Mastercycler gradient thermocycler (Eppendorf) by PCR using specific 16S rRNA primers for bacteria. Primers used in the PCR amplifications were Univ 9F (5'-GAGTTTGATYMTGGCTC) and Univ 1509R (5'-GYTACTTGTACGACTT) (Integrated DNA Technologies, Coralville, IA). Univ 9F was labeled at the 5' end with phosphoramidite fluorochrome 6-carboxyfluorescein (6-FAM). PCR was performed using a reaction mixture of 0.2 mM of each deoxynucleoside triphosphate (Gibco/BRL, Rockville, Md), 200 ng each of the forward and reverse primers, 0.5 to 10 _1_ of the sample preparation, 1X Taq Master, 1X Taq Buffer (50mM KCl, 10mM Tris-HCl pH 8.3, 1.5 mM Mg(OAc)$_2$) and water to bring the total volume to 50 _1_.

To obtain an adequate mass of DNA for T-RFLP analysis, the previously described PCR reactions were performed in quadruplet. Pooled replicates were purified using the Wizard PCR prep kit (Promega, Madison, WI). DNA was eluted with 30 _1_ of sterile water heated to 65°C. Restriction digests were performed independently using three tetrameric enzymes (*Hha I*, *Msp I*, and *Rsa I*). Eight microliters of purified PCR product was added to 9 _1_ of sterile water and 3 _1_ of restriction enzyme master mix containing 10 U of restriction enzyme and 1X reaction buffer. Incubation was done at 37°C for 6 h followed by 15 min at 65°C to denature the restriction enzyme.

Prior to loading on a gel, 1 _1_ of sample was added to a "loading cocktail" (1.25 _1_ deionized formamide 0.25 _1_ blue loading dye and 0.3 _1_ size standard, TAMRA 2500), vortexed, spun down, and denatured at 95°C for three minutes. Samples were then run on a 5% Long Ranger acrylamide (BioWhittaker) and 7M urea gel for approximately 5 hours. T-RFLP profiles were obtained using an Applied Biosystems, Inc. (ABI) 377-XL sequencer. The data was analyzed using the ABI GeneScan software.

Previous studies (Klaus et al. 2005) have shown that T-RFLP profiles obtained from replicate samples have minimal variation due to potential differences in DNA extraction, PCR amplification, or enzyme digestion efficiency. These results indicate that the T-RFLP methodology applied in this study is robust, and comparisons of profiles between different samples should reflect the relative similarity of the microbial communities present in each sample.

Statistical Analyses
Sample versus T-RFLP peak data matrices were constructed using all peaks above a threshold of 50 units above background. To avoid detection of primers peaks smaller than 50 base pairs (bp) were culled from the data set. To account for variation in fragment size determination between samples, peaks were manually aligned and placed into groups. Abundance data was obtained from the relative peak height following sample standardization (Blackwood et al. 2003). The similarity of T-RFLP peak profiles among all possible pairs of samples was calculated using the Bray-Curtis (BC) similarity coefficient (Bray and Curtis 1957).

Ordination by non-metric multidimensional scaling (MDS) (Kruskal 1964) was performed to examine differences in T-RFLP peak patterns among samples. Each ordination was run with 30 random starting configurations and proceeded through multiple iterations until the fit of a non-parametric regression of d (distances between samples on the MDS) against _ (similarities in the Bray-Curtis matrix) could not be improved. To test the significance of T-RFLP peak differences due to ecologically distinct sample groups, the Bray-Curtis similarity matrix was subjected to the analysis of similarities procedure (ANOSIM) (Clarke and Warwick 2001).
2001). This analysis is based on a non-parametric permutation procedure applied to the rank similarity matrix. If samples within a group are identical; Global R = 1. Similarity percentages (SIMPER) (Clarke and Warwick 2001) were used to determine peak contributions to the average dissimilarity of samples between different sample groups.

**Results**

A combined total of 241 different T-RF’s were identified from the sixty different T-RFLP profiles analyzed from healthy *D. strigosa* (20 *Hha* I, 20 *Msp* I, and 20 *Rsa* I). The rank abundance profile (Fig. 1) for all 241 unique T-RF’s shows that only one peak was identified from all 20 samples. An additional 9 different T-RF’s were found in at least 50 % of the samples analyzed. By comparing T-RFLP profiles with theoretical digest patterns generated from previously constructed coral tissue clone libraries, (Frias-Lopez et al. 2002) several of the dominant T-RF peaks can be tentatively identified. Based on these assessments T-RF’s *Msp*I-125, and RsaI-643 appear to be related to *Chromatium* sp. RW (AF384210) of the _-proteobacteria subdivision. Additionally, T-RF’s HhaI-210 and MspI-496 appear related to the uncultured _-proteobacterium clone 26 (AF369718), and T-RF’s HhaI-82 and MspI-453 to a magnetite-containing magnetic *Vibrio* sp. (Lo6455) of the _-proteobacteria subdivision.

![Fig. 1. Rank-abundance curve of T-RFLP peaks detected in all 20 samples of *D. strigosa* (n= 241).](image)

**Multivariate Analysis**

To test the effect of the experimental aquaria on the composition of the most abundant bacterial species, colonies of *D. strigosa* sampled directly from the reef were compared to colonies sampled from control aquaria after 6 days and 14 days. The bacterial communities of reef samples were found to be distinct from all control samples analyzed. The ANOSIM statistical test for differences in T-RF composition show reef samples to be significantly different from control samples after 6 days (R = 0.729, P < 0.029) and 14 days (R = 0.938, P < 0.029). Furthermore, the control samples collected after 6 days were significantly different from those collected after 14 days (R = 0.729, P < 0.029). These results are further supported by the MDS ordination of samples (Fig. 2) in which the reef samples and two sets of control samples all form distinct clusters. Results from SIMPER analysis reveal the major peaks responsible for the differences among sample groups (Table 1).

![Fig. 2. Nonmetric multidimensional scaling (MDS) ordination of bacterial assemblages inhabiting the tissues of *D. strigosa* (n=20).](image)

To assess the effects of temperature on bacterial communities associated with coral tissues, T-RFLP profiles from control and heat-treated colonies were compared. No statistically significant differences in T-RF composition with respect to heat treatment were detected. The ANOSIM statistical test for differences in T-RF composition did not detect any differences between control and heated samples after 6 days (R = 0.135, P < 0.171) or 14 days (R = 0.198, P < 0.171). While MDS ordination does suggest some clustering of heated and control samples, this is not outside the range of variation within sample groups (Fig. 2).

**K-dominance Curves**

While the T-RFLP method is capable of assessing phylotype richness in simple artificial communities with few members (Avaniss-Aghajani et al. 1994; Liu et al. 1997; Moeseneder et al. 1999) it has not proved successful at assessing complex natural communities (Dunbar et al. 2000; Moeseneder et al. 1999). Despite this shortcoming in assessing richness, examination of the cumulative dominance distribution generated from T-RFLP profiles can provide insight into the diversity and relative dominance of assemblages. The average k-dominance curves (Lambshead et al. 1983) for the five sample groups analyzed exhibited distinct separation. A higher dominance of abundant T-RF’s was observed in heat-treated colonies compared to both control and reef colonies (Fig. 3). The curve for the pooled reef samples had the most moderate slope of the sample groups, suggesting greater bacterial diversity.
Table 1. T-RF’s distinguishing healthy bacterial communities of *D. strigosa*.

<table>
<thead>
<tr>
<th>Reef</th>
<th>Control 6 days</th>
<th>Heated 6 days</th>
<th>Control 14 days</th>
<th>Heated 14 days</th>
</tr>
</thead>
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<tr>
<td>Rsal-643</td>
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<td>0.97</td>
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<td>3.24</td>
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</tr>
<tr>
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<td>1.39</td>
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</tr>
<tr>
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</tr>
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<td>0</td>
<td>9.88</td>
<td>5.82</td>
</tr>
</tbody>
</table>

* Average % abundance per locality.

T-RF contribution to the mean Bray-Curtis % similarity at each locality.

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Fig. 3. Average k-dominance curves (x-axis logged) of T-RF peaks obtained from reef samples (*n*=4), control aquaria samples after 6 days (*n*=4) and 14 days (*n*=4), and heated aquaria samples after 6 days (*n*=4) and 14 days (*n*=4).

Discussion

In a recent review, Knowlton and Rohwer (2003) describe the coral host as a habitat. The physical and chemical conditions of this habitat are in part controlled by the coral itself and in part by the surrounding marine environment. In addition, microorganisms manipulate the physical and chemical conditions via their metabolic activities and physical presence. Thus habitat stability is maintained through a complex set of interactions. Within this habitat, the response of the coral-zooxanthellae symbiosis to thermal stress has been actively studied for decades (Brown 1997; Fitt et al. 2001; Baker et al. 2004; Rowan 2004). Here we provide a first attempt to understand the affect of thermal stress on bacterial communities.

Results of the ANOSIM statistical tests indicate that there was no significant change in bacterial communities due to the temperature treatment. This negative response suggests that, at least on short time scales, bacterial communities are stable in composition despite the physiologic responses known to occur in corals subjected to similar temperature treatments (Brown 1997). However, the current data set is characterized by a large shift in the bacterial communities found in control samples from those collected directly from the reef. It is possible that the factors responsible for the shift in control samples might also be obscuring the real signal of community change due to temperature.

There are several factors that could have affected the colonies maintained in the aquaria. While the reef samples were collected from approximately 5 m depth and exposed to normal diurnal light cycles, the control samples were maintained in shallow aquaria shaded in both the early morning and the late afternoon. The more intense yet shortened light cycles may have had an impact on the structure of bacterial communities. It is also possible that the water quality inside the aquaria was different from that found on the natural reef. Before being pumped into the aquaria, the seawater was channeled into sedimentation tanks and further passed through sedimentation filters. However, the elimination of suspended particulate matter (SPM) may have had an impact on the coral associated bacterial communities. Recent studies have shown that SPM can strongly alter the trophic environment of symbiotic corals (Anthony 2000; Anthony and Fabricius 2000). The colonies of *D. strigosa* used in this study were collected from shallow, high sediment, nearshore environments, and possibly acclimated to sustain a positive energy balance through heterotrophic suspension feeding. Therefore, removal of SPM from the flow-though aquaria may have forced a shift towards a more phototrophic energy balance. Lastly, to maintain the temperature of the heated aquaria, the flow rate had to be kept fairly low. It is therefore possible that the long residence time of seawater within the aquaria would result in a shift in available nutrient concentrations.
concentrations. These factors were not adequately controlled for in the experimental setup, and the extent of their control on bacterial communities remains largely unknown. Future experiments should directly target these potentially important factors.

While the multivariate statistical techniques applied in the present study did not detect significant differences between the control and heated aquaria samples, a greater dominance of abundant T-RF’s was observed in the heat-treated colonies compared to the control colonies. Furthermore, a greater dominance of abundant T-RF’s was observed in the control aquaria colonies than on the reef colonies. An increased dominance of specific bacterial strains is a common response observed in disturbed environments (Haack et al. 2004; Salyers and Whitt 1994; Torsvik et al. 2002). Recent surveys of bacterial communities inhabiting *D. strigosa* showed an increased dominance of the most prevalent T-RF’s at impacted localities versus control localities, as did communities associated with diseased corals versus healthy corals (Klaus et al. 2005).

Given the problems with the experimental setup, and the somewhat conflicting results found between the multivariate analyses and the dominance analyses, further studies are needed to confirm the community-level response of bacteria associated with coral tissues.

**Acknowledgements**
This work was supported by research grants from the Office of Naval Research (N00014-00-1-0609), the Geological Society of America, and the American Association of Petroleum Geologists. We acknowledge the support of CARMABI with field efforts and Heather Hentchel with sample preparation.

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