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Inoculation of *Vibrio spp.* onto *Montastraea faveolata* fragments to determine potential pathogenicity

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Abstract. *Vibrio* strains previously isolated from Caribbean yellow band (CYBD) diseased *Montastraea faveolata* from Puerto Rico were combined into metabolically similar groups based on biolog data (P1, similar to *Vibrio campbellii*; P2, similar to *V. splendidus* and P3, similar to *Aeromonas trota*). Bacterial groups were inoculated onto healthy *M. faveolata* colony fragments that were aerated in individual plastic containers exposed to two temperature regimes (28ºC and 31ºC). Coral genets were divided into two groups of five and maintained at either 28ºC or 31ºC. Three fragments were inoculated with bacterial groups P1, P2, or P3, and two were used as delivery medium or water/container controls at each temperature. Corals were monitored for ten days. After Day 1, two fragments inoculated with P1 and maintained at 31ºC showed signs of yellowing tissue similar to initial stages of CYBD. Three fragments inoculated with P2 and held at 28ºC showed similar signs of paling to yellowing tissue. Two additional fragments showed signs at Day 2 (P1 and P2 at 28ºC). At Day 5, two more P1 inoculated fragments showed disease signs (one at 31ºC and one at 28ºC). Genetic identification of isolates from each group show that members of group P1 are most similar to *V. cambellii* and members of group P2 are most similar to *Photobacterium eurosenbergii*. These preliminary results indicate that more than one type of *Vibrio* sp. may be able to initiate signs of disease and/or bleaching and no clear patterns in response were associated with temperature under the experimental conditions.

Key Words: Yellow band, *Vibrio*, *Montastraea faveolata*, coral disease

Introduction

Among the deleterious effects of climate change on coral reefs has been the increase of coral diseases (Harvell et al. 2004, 2007). Caribbean yellow band disease (CYBD) is particularly prevalent in the Caribbean (Ward et al. 2006; Weil et al. 2006; Weil and Croquer unpub. data), and a similar syndrome occurs in the Indo-Pacific. This disease is temperature influenced (Cervino et al. 2004a), appears to affect zooxanthellae (Cervino et al. 2004b), and is closely associated with species in the genus *Vibrio*. This study tested the pathogenic potential of *Vibrio* isolates obtained from CYBD-infected *Montastraea faveolata* colonies from the south coast of Puerto Rico.

Material and Methods

Bacterial isolates were obtained by SCUBA from CYBD affected *Montastraea faveolata* near La Paguera, Puerto Rico. Samples of the surface mucus and associated coral tissue were collected and *Vibrios* isolated as described in Cunning et al. (this issue). Each isolate was characterized using Biolog™ (Heyward, CA) and identified by sequencing the small subunit 16S rRNA gene as described in Cunning et al (this issue). Isolates were chosen for inoculation based on similarities to isolates previously described (Cervino et al. 2004a) and isolates that were unique to diseased colonies. Isolates fell into one of three groups, corresponding to *Vibrio harveyi/V. campbellii* (P1), *V. splendidus/Photobacterium eurosenbergii* (P2), or *Aeromonas trota/P. eurosenbergiii* (P3) based on carbon source utilization patterns obtained from Biolog™ plates or 16S rDNA sequencing BLAST results, respectively.

Two isolates were used in the P1 group inoculations (3A8 and 1B4), three in P2 (3B7, 1H5 and 3F8) and two in P3 (1A9 and 2H12). Biolog profiles, genetic identities, and GenBank Accession numbers are shown in Table 1. The inocula were made by growing each isolate overnight in GASW broth at 32°C, then combining (1.5ml of each isolate for P1 and P3 and 1.0ml of each isolate for P2) in a test tube with 1g of sterile sieved calcium carbonate sand, shaken, and allowed to absorb on the sand particles overnight (described in Patterson et al. 2002). Ten replicates were made for each inoculum (P1, P2, P3, and controls). Before inoculation, excess medium was decanted and

replaced with sterile seawater. Controls were treated similarly except sterile sand with uninoculated sterile media was used.

Coral fragments were obtained from healthy *M. faveolata* colonies using a hammer and chisel. Fragments were left to acclimate in the field for three days after which each of the 40 fragments were retrieved, tagged, placed in individual aerated containers in a heated water bath either at 28ºC or at 31ºC (Fig.1). Fragments were acclimated in tanks for 24 hours prior to experimental onset. Salinity was checked twice a day and sterile seawater added as needed. Temperature was monitored at least three times a day. An extra five fragments were placed in each temperature, but left untreated. Treatments for each of the coral fragments used in this experiment were randomly assigned. The treated sand grains were poured directly onto the coral fragments. As sand grains were removed from the surface by the corals, inoculate was replaced twice a day using labeled droppers for two days (Fig.2). Observations on yellowing (paling) were made daily (Fig. 3) and the experiment was run for 12 days.

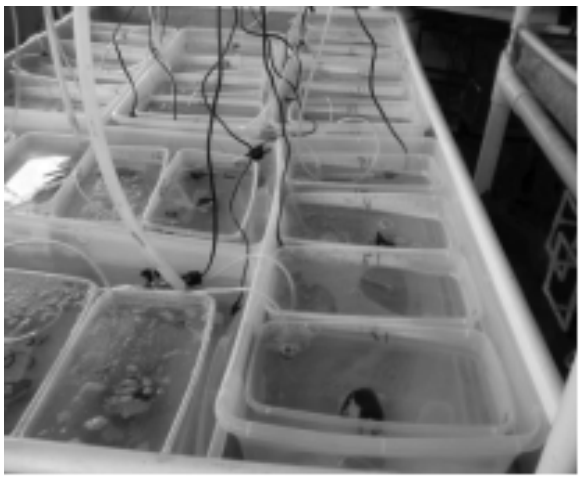

Figure 1: Experimental set up.

Results

Bacteria phylogenetically identical to *Vibrio* spp. previously implicated in YBD (Cervino et al. 2004a) were not identified in the accompyaning study (Cunning et al. this issue). Therefore, *Vibrios* unique to diseased coral that also showed similar carbon source utilization profiles to *Vibrio* species in the Cervino et al. study were subdivided into groups P1-P3 and used in inoculation experiments in this study (Table 1). P1 isolates had slightly different metabolic profiles but were identical to *V. harveyi and V. campbellii*, based on 16S rDNA sequencing results. P2 and P3 isolates were phylogenetically most similar to *Photobacterium eurosenbergii* (Table 1).

Figure 2: Replacement of carbonate inoculate.

After the first day of incubation, two fragments inoculated with group P1 showed yellowing (Fig.3) but only in those fragments incubated at 31ºC.

Conversely, three of the five replicates inoculated with P2 showed yellowing, but only with fragments incubated at 28ºC. This increased to four of the five for P2 (28ºC) on day two and one fragment from P1 (28ºC). On day three, one fragment from the P2 (31ºC) and one from P3 (31ºC) showed yellowing signs. This increased to three of the five for P3 (31ºC) and one for the control (31ºC) on day four. By day 5, all of the P2 (28C) fragments showed signs of yellowing, as well as an additional fragment from P1 (28ºC), P1 (31ºC), P3 (28ºC) and P3 (31ºC). Two additional fragments from the control (31ºC) also showed yellowing by day 5. Throughout the five day period, all fragments from the control (28ºC) remained healthy as did the untreated fragments.

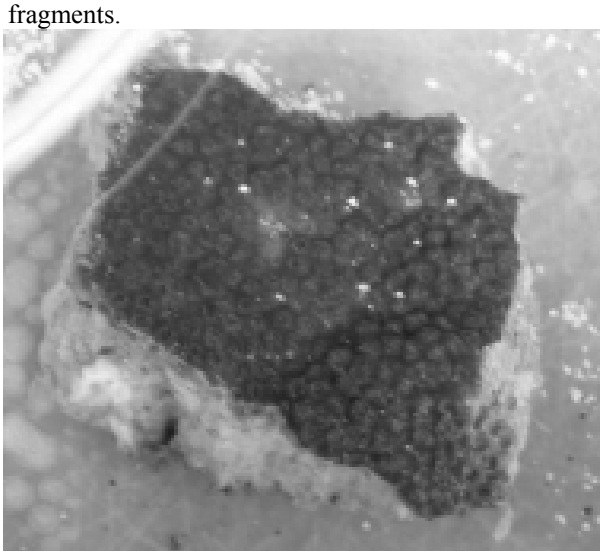

Figure 3: Potential CYBD signs three days after inoculation.

Table 1: Isolates used in Inoculations

Group	Isolate	Biolog™ (Similarity)	% ID based on Genetic Analysis	GenBank Acc#
P1	3A8	*Aeromonas enteropelogens* (0.300) *Vibrio campbellii* (0.130)	100% *Vibrio harveyi/V. campbellii*	FJ356774
P1	1B4	*V. campbellii* (0.230) *Vibrio proteolyticus* (0.280)	100% *Vibrio harveyi/V. campbellii*	EU854881
P2	3B7	*Vibrio splendidus* (0.580) *V. splendidus* (0.770)	100% *Photobacterium eurosenbergii*	FJ356775
P2	1H5	*V. splendidus* (0.500) *V. splendidus* (0.680)	100% *P. eurosenbergii*	EU854888
P2	3F8	*V. splendidus* (0.780) *V. splendidus* (0.410)	100% *P. eurosenbergii*	EU854889
P3	2H12	*Aeromonas trota* DNA group 13 (0.200) *Aeromonas trota* DNA group 13 (0.310)	100% *P. eurosenbergii*	EU854887
P3	1A9	*Aeromonas trota* DNA group 13 (0.250) *Aeromonas trota* DNA group 13 (0.330)	100% *P. eurosenbergii*	EU854886

Although we report here results from the first five days after treatment, the trials were run for twelve days. On day six all fragments in the 31°C table appeared unhealthy (paled) so the heaters were turned off. By day twelve all fragments were unhealthy and the experiment was terminated. Comprehensive results of all inoculations are shown in Figure 4.

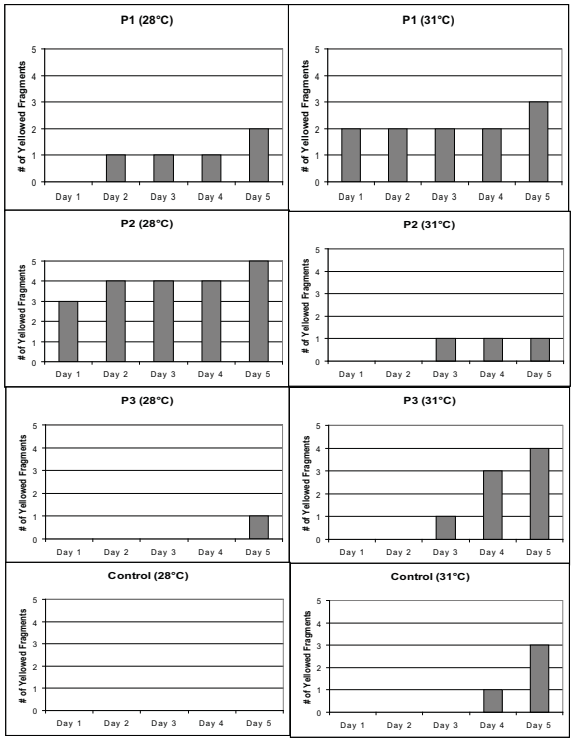

Figure 4: Number of coral fragments showing signs of yellowing over the five day incubation period. Those on the left were kept at 28°C and those on the right at 31°C. P1, P2, and P3 are bacterial inoculants.

Discussion

Of important note is that although isolates chosen for inoculation experiments were identical based on 16S rDNA sequences in the case of Group P1 (*V. harveyi/V. campbellii*) and groups P2 and P3 (*P. eurosenbergii*), however these strains were metabolically different as illustrated by Biolog results (Table 1). This suggests that phylogenetically similar isolates are different strains that could additionally have varying phenotypes with respect to pathogenic mechanisms. Results indicate that, under the experimental conditions, inoculations with one or more of the three bacterial strains in P1 results in yellowing in corals at 31ºC. However some fragments showed yellowing, although to a lesser extent, at 28ºC. A surprising result was that the bacteria in the P2 inoculants greatly hastened yellowing at 28ºC but, conversely, displayed yellowing to a lesser degree at 31ºC. P3 inoculations hastened signs of yellowing by one day at the higher temperature but did not appear significantly different from controls.

Of interest is the potential role of one or more bacteria in the P2 group which hastens yellowing at lower temperatures, but appears to arrest yellowing at higher temperatures. These results suggest that the role of temperature in *Vibrio*-coral interactions may be more complex than previously described.

Acknowledgements

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