

A survey of *Vibrios* associated with healthy and Yellow Band Diseased *Montastraea faveolata*

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Abstract. Bacteria of the genus *Vibrio* have been implicated in coral bleaching as well as diseases, including Caribbean Yellow Band Disease (CYBD). Four *Vibrio* species have been previously identified as causative agents of CYBD through a series of infection and re-isolation experiments. The mechanisms of pathogenesis and the dynamics of the *Vibrio* community as a whole during CYBD infection are poorly understood. In order to elucidate the role of *Vibrios* in CYBD, a survey of *Vibrio* species associated with healthy corals and CYBD-infected corals was conducted using a culture-based approach. Fragments were collected from CYBD lesions of five *Montastraea faveolata* colonies, five healthy regions of the same diseased colonies, and five entirely healthy colonies. Samples were serially diluted and plated onto TCBS agar to differentially select for *Vibrio* species. Colonies were subcultured using GASWA media and a total of 391 isolates were identified by 16S rDNA sequence analysis. Phylogenetic analysis of *Vibrio* spp. communities of healthy and diseased corals illustrated a shift from isolates taxonomically affiliated with *V. fortis* dominating in healthy corals to isolates taxonomically affiliated with *V. harveyi*, a known marine pathogen, dominating in diseased corals. There was a similar shift to isolates taxonomically similar to *Photobacterium eurossenbergii* as corals progressed to diseased states. However, our study did not find any *Vibrio* species that are always present in CYBD lesions and absent from healthy samples.

Key Words: Yellow Band Disease, *Vibrio*, *Montastraea faveolata*, coral disease

Introduction

Coral reefs worldwide are declining in response to a number of threats, including bleaching and disease (Harvell et al. 1999, 2007). The susceptibility of corals to these threats involves complex interactions among environmental conditions, the coral animal, and its associated algae and bacteria. Some coral-associated bacteria benefit their hosts by providing energy and nutrients and inhibiting pathogens, while others are implicated in coral bleaching and diseases (Rosenberg et al. 2007).

One of the major types of bacteria associated with corals is *Vibrio*. Members of the genus *Vibrio* are highly abundant in the marine environment, and are found in association with a variety of organisms (Thompson et al. 2004). Several *Vibrios* are pathogenic to marine organisms, including *V. harveyi*, which has been implicated in diseases of fish (Ishimaru and Muroga 1997), shrimp (Alvarez et al. 1998), lobsters (Diggles et al. 2000), seahorses (Alcaide et al. 2001), and echinoderms (Becker et al. 2004). *Vibrios* are consistently found in association with apparently healthy corals (Ritchie and Smith 2004; Bourne and Munn 2005; Ritchie 2006), although some have been implicated in coral bacterial

bleaching and disease. Ritchie et al. (1994) demonstrated a shift to *Vibrio* dominance in the bacterial communities of bleached corals (>60% *Vibrio*), followed by a return to initial values (~25% *Vibrio*) upon recovery. Apparently healthy non-bleached coral tissue also becomes dominated by *Vibrios* under conditions of increased temperature, suggesting that a shift to *Vibrio* dominance in response to temperature may precede bleaching (Ritchie 2006). Other studies have demonstrated that *V. shiloi* and *V. coralliilyticus* are causative agents of bacterial bleaching in the corals *Oculina patagonica* and *Pocillopora damicornis*, respectively (Kushmaro et al. 1996; Ben-Haim et al. 2003).

In addition to bacterial bleaching, a number of coral diseases have been associated with *Vibrio* species, including white pox, and white syndrome (Rosenberg et al. 2007), gorgonian disease (Hall-Spencer et al. 2007), skeletal tumors in the coral *Porites compressa* (Breitbart et al. 2005), and Caribbean Yellow Band Disease (CYBD) in *Montastraea* spp. (Cervino et al. 2004b).

CYBD has been documented in *Montastraea* spp. around the Caribbean and manifests as pale yellow

blotches or bands that spread over the surface of the coral (Cervino et al. 2004b). Cervino et al. (2004b) identified four *Vibrio* species as causative agents of CYBD through a series of infection and reisolation experiments. The authors suggest the four *Vibrios* act together with *V. alginolyticus* to affect the corals' symbiotic algae and that degradation of zooxanthellae *in situ* results in the pale yellow bands observed on infected Caribbean and Indo-Pacific corals (Cervino et al. 2008). In this study we characterized differences in culturable *Vibrio* communities of healthy and CYBD affected corals.

Materials and Methods

Montastraea faveolata samples were harvested from five entirely healthy colonies (HEA; H1-5), five colonies with CYBD lesions (DIS; YBD6-10-D), and five healthy regions of the same diseased colonies (^HDIS; YBD-6-10-H). In addition, one diseased *M franksii* was included in this analysis (YBD-11-D). Sampling was conducted at 10-12m depth at Turrumote reef (17°56.097'N, 67°01.130'W), off La Parguera Natural Reserve on the southwest coast of Puerto Rico in July 2007. Water temperature at the time of sampling was 29.2°C. Samples were shipped overnight to Mote Marine Laboratory, Sarasota, FL, where mucus samples were removed, serially diluted and plated onto thiosulfate-citrate-bile salt (TCBS) agar to differentially select *Vibrios*. *Vibrio* colonies were then subcultured using glycerol artificial seawater agar (GASWA; Smith & Hayasaka 1982) and 391 isolates were selected for genetic analysis. DNA was extracted by boil lysing in 100 µL of sterile water at 95°C for 10 minutes. PCR amplification of the 16S rRNA gene was carried out in 50 µL reactions using 1x PCR buffer (Qiagen), 200 µM of each deoxynucleotide (Qiagen), 2 µM each of 16S rRNA primers U9F (5'-GAGTTTGATYMTGGCTC-3') and U1509R (5'-GYTACCTTGTTACGACTT-3') (Invitrogen), 1U Taq polymerase (Qiagen), and 2 µL DNA template. Thermocycling began with an initial denaturation step at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30s, annealing at 50°C for 30s, and extension at 72°C for 90s, with a final extension step at 72°C for 10 min. Amplification of a ~1500 bp fragment was confirmed by electrophoresis on a 1% agarose gel. PCR products were sequenced by Macrogen, Inc (Korea). Consensus sequences from forward and reverse strands were generated and GenBank BLAST searches were performed to demonstrate percentage identity to known bacteria. Sequences were aligned using CLUSTAL X version 1.83 and the ends were trimmed resulting in ~1325 bp sequences for phylogenetic analysis. A pairwise comparison was used to eliminate duplicate sequences. In accordance with current *Vibrio* taxonomical standards

(Kita-Tsukamoto et al. 1993, Thompson et al. 2005) sequences with >99.3% similarity were considered representatives of the same taxon and sequences were grouped by this criterion for phylogenetic analysis. Neighbor-joining dendrograms were constructed using MEGA software (v.4) with bootstrap values based on 1000 replicates. GenBank Accession numbers are as follows: DIS (EU854819-EU854889, and FJ356774-5), ^HDIS (EU517610-EU517657) and HEA (EU854890-EU854953).

Results

A total of 391 16S rRNA gene sequences were obtained from bacterial isolates, 215 from DIS samples, 108 from ^HDIS samples, and 68 from HEA samples. All sequences were taxonomically similar to five *Vibrio* taxa: *V. harveyi*, *V. fortis*, *V. brasiliensis*, *V. chagasii*, and *Photobacterium euosenbergii* (Fig. 1). Isolates similar to *V. brasiliensis* and *V. chagasii* constituted 0.47% and 3.74% of DIS isolates, respectively, but were not present in ^HDIS or HEA samples. Isolates taxonomically similar to *P. euosenbergii* made up 3% and 4% of DIS and ^HDIS samples, respectively, but were not present in HEA samples. *V. fortis*- and *V. harveyi*-like isolates were present in all three groups, with those similar to *V. fortis* constituting 69% of HEA isolates, 58% of ^HDIS isolates, and 47% of DIS isolates, and those taxonomically similar to *V. harveyi* constituting 31% of HEA isolates, 38% of ^HDIS isolates, and 45% of DIS isolates (Fig.1).

Due to considerable variation in the relative proportions of *Vibrio* taxa among individual samples within each group (Fig. 2) there were no significant differences in mean proportions of taxa among groups. Among DIS samples, the proportion of *V. harveyi*-like isolates ranged from 0.161 to 0.788 (mean=0.447±0.096), and that of *V. fortis*-like isolates ranged from 0.212 to 0.742 (mean=0.491±0.095). *V. chagasii*-like isolates were present only in sample YBD-1-D, and *V. brasiliensis*-like isolates were only present in sample YBD-3-D. *P. euosenbergii*-like isolates were present in every DIS sample except YBD-3-D and YBD-11-D. Among ^HDIS samples, the proportion of *V. harveyi*-like isolates ranged from 0.154 to 0.522 (mean=0.330±0.071), and that of *V. fortis*-like isolates ranged from 0.435 to 0.818 (mean=0.632±0.071). *P. euosenbergii*-like isolates were present in all ^HDIS samples except YBD-7-H. Only the HEA samples showed any consistency, with *V. fortis* dominating and *V. harveyi* being the only other isolate present in all three samples. A fourth healthy colony was sampled but not included in analysis because from it only five sequences were obtained. However, it is worth noting that a sequence similar to *Vibrio coralliilyticus*, a known coral pathogen, was found in this sample.

Discussion

In this study we have addressed culturable members of the genus *Vibrio* for analysis. Although it is estimated that less than 1% of bacteria can be cultured in the laboratory, culture based work is the only way to address pathogenesis in coral diseases. Drawbacks to this work include an inability to culture various *Vibrios*, which could enter a viable but non-culturable state during infections rendering them non-detectable using these methods. We would stress that this study does not provide quantitative data of *Vibrios* present, but only an estimate of proportional abundance. When sequences are pooled into DIS, ^HDIS and HEA groups (Fig. 1), several differences are apparent. First, the proportion of *V. harveyi*-like sequences is lowest in the HEA group, higher in ^HDIS, and highest in DIS, indicating that *V. harveyi*-like isolates become more abundant as the disease progresses. *V. harveyi* has been shown to be a pathogen to many marine organ-

isms (Austin and Zhang 2006) and produces a variety of toxins including haemolysins (Zhang et al. 2001), which can lyse cells and cause tissue damage (Zhang and Austin 2005). Involvement of these haemolysins in CYBD etiology would account for tissue lesions observed on infected corals and would support indications that lysis of zooxanthellae may be a potential disease mechanism (Cervino et al. 2004a, b). However, *V. harveyi* is also found in healthy corals (present study and Ritchie 2006), thus any involvement in CYBD must be opportunistic or mediated by a mechanism such as quorum sensing (Thompson et al. 2004).

In addition to an increase in *V. harveyi*-like isolates as corals progress to diseased states, there is a concurrent decrease in *V. fortis*-like isolates, suggesting *V. fortis* may be a dominant and innocuous member of the healthy *Vibrio* community that is outcompeted as the disease progresses. However, *V. fortis* has also been shown to be a pathogen to both fish and crustaceans (Austin et al. 2005). Additionally, an isolate

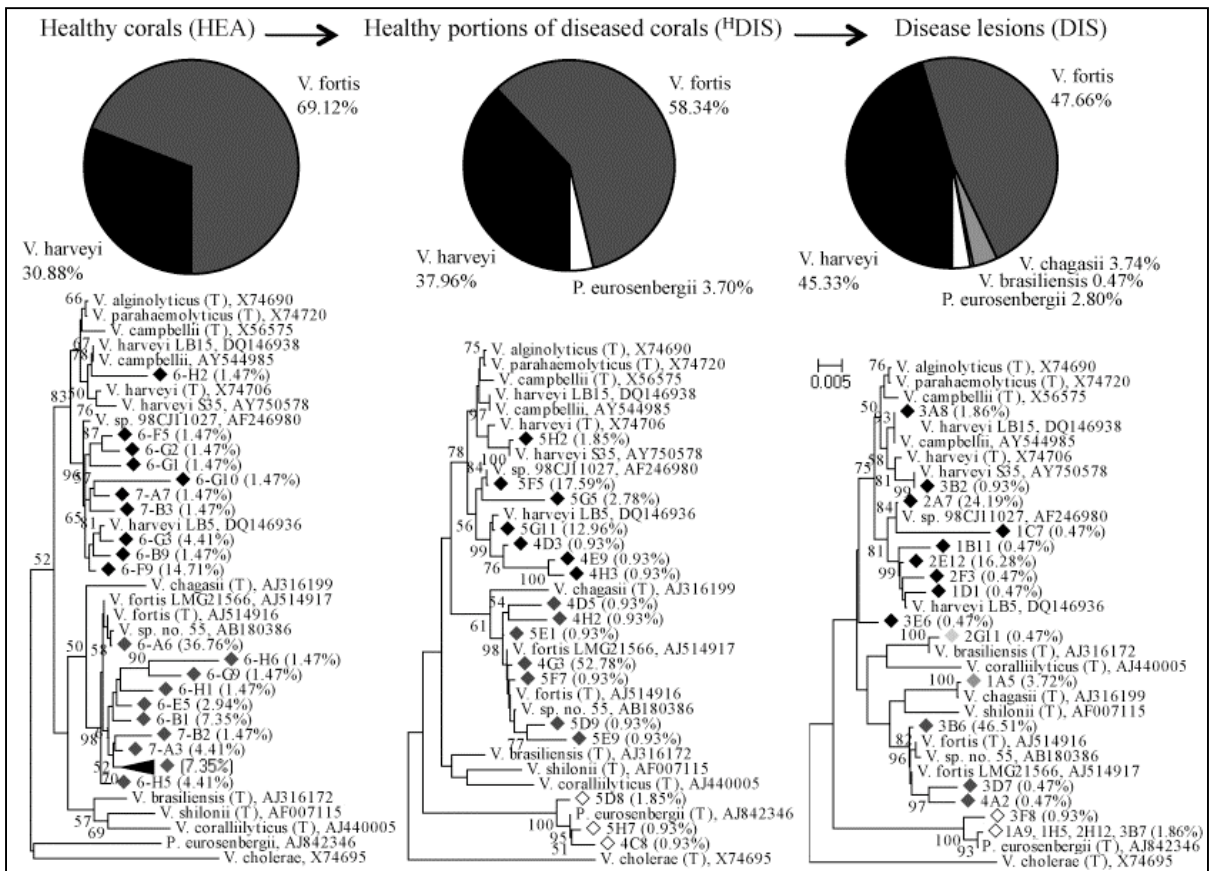


Figure 1. Phylogenetic trees and pie charts representing phylogenetic positions and relative abundances of major groups of *Vibrio* isolates identified from HEA, ^HDIS, and DIS groups by 16S rDNA sequence analysis of cultured isolates. Branches marked by diamonds represent sequences from the present study (Genbank accession numbers EU854819-EU854953). Percentages indicate the proportion of sequences in that group that are >99.3% similar. Other sequences included are the closest matches to GenBank, (T) indicating type strain. Dendrograms were created in MEGA (version 4) using the Neighbor-Joining method. Numbers at the nodes indicate the level of bootstrap support (%) from 1000 replicates. The evolutionary distances were computed using the Maximum Composite Likelihood method and the scale represents 0.005 substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). Pie charts represent the proportion of all sequences from HEA, ^HDIS, or DIS groups that match most closely to *V. harveyi*, *V. fortis*, *V. brasiliensis*, *V. chagasii*, or *P. eurosenbergii*.

with >99.5% 16S sequence similarity to *V. fortis* has exhibited algicidal activity (Imai et al. 2006), which is a proposed mechanism of CYBD infection (Cervino et al. 2004a, b). However, as *V. fortis* was dominant in healthy corals in this study, its involvement in CYBD must also be opportunistic.

In addition to having more *V. harveyi*- and less *V. fortis*-like sequences, the DIS group contained sequences similar to *V. brasiliensis* and *V. chagasii* that were not present in ^HDIS or HEA samples. *V. brasiliensis*, isolated first from bivalve larvae (Thompson et al. 2003) is closely related to the oyster pathogen *V. tubiashii* and the coral pathogen *V. coralliilyticus* (Thompson et al. 2004) and has been shown to be pathogenic to fish and crustaceans (Austin et al. 2005). *V. chagasii* is a chitonolytic bacterium that has been isolated from the gut of turbot larvae (Thompson

et al. 2003) and coastal fishes (Itoi et al. 2006). The presence of these sequences only in DIS samples is consistent with increased bacterial diversity observed in other coral diseases (Pantos et al. 2003).

Another difference among the three groups was the presence of *Photobacterium euerosenbergii*, which has been associated with bleached corals on the Great Barrier Reef (Thompson et al. 2005), in DIS and ^HDIS groups, but not in the HEA group.

Although the pooled analysis shows differences among the three groups, there is inconsistency among individual samples within groups (Fig. 2). For example, in the DIS group, three samples were dominated by *V. harveyi*, and three by *V. fortis*. *V. brasiliensis* and *V. chagasii* were each only present in one out of six DIS samples. ^HDIS samples showed slightly more consistency, with four dominated *V. fortis*, and one by

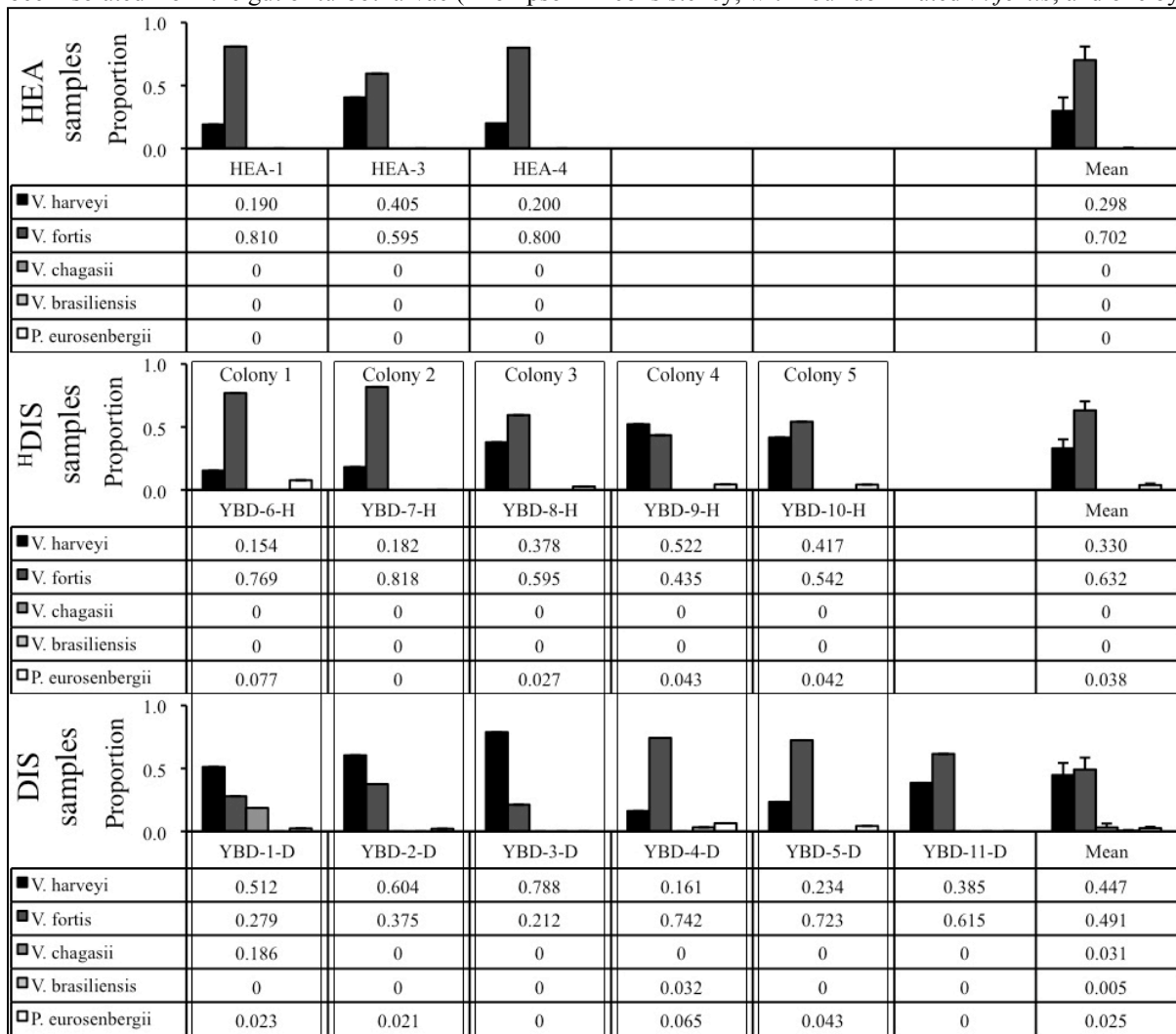


Figure 2. Relative proportions of major *Vibrio* taxa in each sample within groups (HEA (n=3), ^HDIS (n=5), and DIS (n=6)). Means represent the average proportion of each *Vibrio* taxon in all samples within each group, and error bars represent standard error of the mean. Rectangles surround samples from ^HDIS and DIS groups that originated from the same coral colony. Note that only 3 entirely healthy colonies resulted in 16S sequences derived from cultured *Vibrios*. Results in this figure include the individual *M. franksii* YBD sample, YBD-11-D.

V. harveyi. *P. eurossenbergii* was present in all but one of the DIS and ^HDIS samples. The HEA group showed more consistency, each with *V. fortis* dominating. This suggests healthy corals have a more stable *Vibrio* community that subsequently becomes unstable and variable during CYBD infection.

Despite variability in their constituents and proportions, the *Vibrio* communities in healthy and diseased portions of the same coral colonies show a consistent difference from one another. Colonies in which disease lesions were dominated by *V. harveyi* had a higher proportion of *V. fortis* in the healthy portions. Conversely, colonies with more *V. fortis* in the CYBD lesions had a higher proportion of *V. harveyi* in the healthy portions. Thus, the only common difference between healthy and diseased portions within a colony is that relative proportions of *Vibrio* taxa have changed. Thus, a disruption of the *Vibrio* community is always associated with the disease, but the magnitude and direction of this disruption is not consistent.

Previous studies on CYBD have suggested a specific group of *Vibrio* isolates not found in healthy corals are the causative agents of CYBD (Cervino et al. 2004a, b). However, our study did not find any *Vibrios* that are always present in CYBD lesions and always absent from healthy samples. Instead, we show that there is considerable variation in the community of *Vibrios* associated with the disease. A study of bacterial communities associated with white plague has shown similar variability, and has suggested there may be several different potential pathogens (Pantos et al. 2003). Our results suggest a variety of bacteria may be able to induce disease symptoms, and that the mechanism of disease may be a toxin that is widespread among *Vibrios*, such as haemolysins (Wang et al. 2007).

The variation in the *Vibrio* community associated with CYBD may also suggest that *Vibrio* taxa are working together as pathogens, and may do so regardless of their relative proportions. Supporting this is the fact that relatedness among bacteria leads to an increase in virulence (Foster 2005). Also lending support to this theory is the fact that in the CYBD infection experiments conducted by Cervino et al. (2004b), disease symptoms were only induced when multiple *Vibrio* strains were inoculated as a group. Understanding the dynamics that may cause disease symptoms, especially by groups of opportunistic microbes, will be an important new area of research. The identification of opportunistic pathogens requires establishing both pathogenicity and the conditions which facilitate opportunistic colonization. Future studies should include quantitative studies of bacterial abundance by methods such as Real Time PCR technology, pathogenicity exposure experiments and devel-

opment of novel tools to diagnose pathogenic strains of CYBD.

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