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BLACK BAND DISEASE OF CORALS: ECOLOGY AND PHYSIOLOGY OF THE SULFUR BACTERIA *DESULFOVIBRIO SPP.* AND *BEGGIATOA SPP.*

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ABSTRACT OF THE THESIS

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Black band disease (BBD) of corals is a pathogenic microbial consortium that exhibits an active sulfur cycle. This study is focused on two questions aimed at understanding two bacterial populations integral to the sulfur biogeochemistry of the disease consortium. First, the nature of the taxonomic identity of BBD-associated sulfate-reducing bacteria was determined by selective culturing and sequencing (16S rDNA) of samples from regional BBD infections. Second, microbial motility patterns in BBD were investigated with a focus on sulfide-oxidizing bacteria (*Beggiatoa*) to determine if patterns were comparable to non-disease microbial mat communities. Eight out of 10 cultures sequenced showed 98+% species homology to *Desulfovibrio* sp. strain TBP-1, and revealed a consistency in *Desulfovibrio* sp. between different BBD infections, coral species, and geographic areas. *In situ* studies of BBD cyanobacteria and *Beggiatoa* exposed to both natural and manipulated changes in light intensity showed the microbial vertical migration pattern to be unique.

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INTRODUCTION

Reef Disturbances

Coral reefs have historically been exposed to many natural ecological disturbances varying widely in temporal and spatial scales, from hurricanes and sea level change to pathogens and parasites. Some level of disturbance is often viewed as necessary for ecosystem stability. In particular, an intermediate level of disturbance should maintain a maximal level of species diversity, with both superior competitors and superior colonizers present (Connell and Slatyer, 1977; Aronson and Precht, 1995). Diverse ecosystems, according to the diversity-stability hypothesis, are more resistant to extinctions or shifts to alternative stable states resulting from perturbation (Tilman and Downing, 1994; Tanner *et al.*, 1994). Thus, species diversity is often used as an indicator of community and ecosystem health (Karlson and Hurd, 1993).

Coral reefs are renowned for extremely high levels of species diversity. However, coral reef monitoring programs have shown species diversity to be declining for corals on coral reefs worldwide (Dustan, 1977; Brown, 1987; Porter and Meier, 1992; Hughes, 1994; Edinger *et al.*, 1998; Porter *et al.*, 2001; Porter *et al.*, 2002). It is commonly conjectured that coral reefs are subject to a combination of human and natural disturbances (Grigg and Dollar, 1991; Ginsburg, 1994), which contribute cumulatively to degradation (Wilkinson, 1992). These detrimental influences include anthropogenic effects such as overfishing, pollution, and sediment runoff. Effects of natural versus anthropogenic impacts are often difficult or impossible to distinguish (Brown, 1987), complicated by the fact that very little to no information may exist for baseline effects of

natural or anthropogenic impacts. As well, the magnitude and consequences of patterns observed in small-scale studies can be difficult to determine due to the enormous spatial and temporal scales of coral reef dynamics (Jackson, 1992).

Coral Diseases

One important disturbance factor on coral reefs is coral disease. Diseases are considered to be a major factor influencing population dynamics in marine ecosystems (Kinne, 1980). Coral diseases directly affect coral species diversity (Aronson *et al.*, 1998; Porter *et al.*, 2001), which may in turn have a negative impact on populations of other organisms within the coral reef community. Coral species diversity on reefs in Florida and the Caribbean seem to be especially influenced by coral diseases (Dustan, 1977; Porter and Meier, 1992; Epstein *et al.*, 1998; Harvell *et al.*, 1999; Porter *et al.*, 2001; Porter *et al.*, 2002). Some postulate that, if present trends continue, coral diseases will be more detrimental to coral reef survival than all natural and anthropogenic effects combined (Hayes and Goreau, 1998).

Several coral diseases (such as black band disease, white band disease, and white plague) directly cause coral tissue lysis as the disease progresses over the coral colony. This leads to either partial or complete death of the colony. The remaining bare coral skeleton is colonized by algae within days. Not only is coral tissue destroyed directly by disease, but the probability of new coral recruitment onto the newly available substrate (exposed coral skeleton) is negligible (Edmunds, 1991; Bruckner and Bruckner, 1997a; Kuta, 2000), contributing to a reduction in coral cover (Kuta and Richardson, 1996). In addition, coral colonies with a reduced area of live tissue may have less potential for

reproduction (Szmant-Froelich, 1985). Fecundity, which generally increases with the size of the coral because it is a colonial organism (Kojis and Quinn, 1985), can decrease with stress (Brown and Howard, 1985) and with reduction in colony size (e.g. tissue loss) (Szmant, 1986).

The first coral diseases discovered (black band disease, white band disease, and white plague) were all found on reefs of the Caribbean or Florida in the 1970s (Antonius, 1973; Gladfelter *et al.*, 1977; Dustan, 1977). Almost thirty years later, new coral diseases are still apparently emerging and are now found on reefs around the world (see Green and Bruckner, 2000). The reasons for such rapid emergence and spread of multiple coral diseases are unknown. Defense mechanisms of corals may have become compromised, new pathogens may have been introduced, pathogen virulence may be increasing, or a combination of these factors may be occurring (Hayes and Goreau, 1998). As well, environmental factors could increase the stress level of corals, increasing their vulnerability to environmental microbe(s) that would normally be benign (Chet and Mitchell, 1975).

Microbial communities within the coral surface mucopolysaccharide layers may be important to coral health(Means and Sigleo, 1986) and disease prevention (Ritchie and Smith, 1997). Bacterial associations with coral appear to be related to species morphology (boulder corals, acroporids and agaricids) (Smith, pers. comm.), coral genus (Ritchie and Smith, 1997), and coral species (Rohwer *et al.*, 2001), an association which may be related to the unique mucous composition of different coral species (Ritchie and Smith, 1997). Corals increase mucous production when exposed to stressors (Ducklow and Mitchell, 1979a), including disease or bleaching (Segel and Ducklow, 1982). This

leads to changes in bacterial community composition in the coral surface mucopolysaccharide layer (Ritchie *et al.*, 1994a; Ritchie *et al.*, 1994b; Ritchie and Smith, 1995a; Ritchie and Smith, 1995b). Such a shift in the normal microbiota of healthy coral could be a factor in disease infection (Ritchie and Smith, 1997).

Levels of disease incidence vary with type of disease, coral species affected, geographic area, and expression of pathogenicity (see Weil *et al.*, in press). For example, when plague was first described in 1977 in the northern Florida Keys, it affected six species of scleractinian corals with a tissue destruction rate of approximately 3mm/day (Dustan, 1977). It continued to be present through the 1980s in the same geographic area but infected additional coral species (Dustan and Halas, 1987). In 1995-1997, plague type II appeared throughout the Florida Keys, with slightly different signs than described in type I, affected even more species (17), and had a much higher tissue destruction rate of up to 2cm/day. Up to 38% of the *Dichocoenia stokesii* population, the species most susceptible to plague, was killed in some areas surveyed (Richardson *et al.*, 1998b). In 1999, plague type III was described on the same reefs again, and appeared to have a still faster rate of tissue destruction (Richardson *et al.*, 2001), but apparently fewer susceptible species. It was documented Caribbean-wide in 2000-2001 (Richardson and Aronson, in press). Aspergillosis of sea fans, also seen Caribbean-wide, varied from a 60% prevalence at Bahamas sites in 1996, to 32-39% in 1995 – 1996 at Curacao study sites, to 90-94% prevalence in 1995 – 1996 at Saba sites (Nagelkerken *et al.*, 1997). However, in 1999, the highest prevalence documented in Caribbean-wide surveys by Weil *et al.* (in press) was 5.9%, in Puerto Rico. White pox disease, in the Florida Keys, has been reported to affect *Acropora palmata* at rates above 70%, with a tissue loss rate

(associated with circular lesions) averaging 2.5 cm/day (Patterson *et al.*, 2002). At present, only white band disease, which affects Caribbean acroporid species (*Acropora palmata* and *A. cervicornis*), has been geologically shown to have restructured coral community composition on reefs (Aronson and Precht, 1997; Aronson *et al.*, 1998). Much remains to be discovered about diseases themselves and their role in both reef degradation and natural coral population cycles.

Many coral syndromes have been described, yet to date only five diseases have been described in terms of microbial pathogens (Richardson and Aronson, in press). Koch's postulates have been satisfied for proposed pathogens of only four coral diseases (plague, aspergillosis, bacterial bleaching, and white pox). Plague type II is caused by *Aurantimonas coralidica*, a new bacterial genus (Denner *et al.*, in review ; Richardson *et al.*, 1998a; Richardson *et al.*, 1998b). This pathogen has been isolated from plague type II infections in the Florida Keys, the Virgin Islands and Dominica (Richardson and Aronson, in press). Aspergillosis, a disease affecting gorgonians in Florida and the Caribbean, is caused by the fungus *Aspergillus sydowii* (Smith *et al.*, 1996; Geiser *et al.*, 1998). *Vibrio shiloi* has been shown to cause bacterial bleaching of *Oculina patogonica* in the Mediterranean Sea and *Pocillopora damicornis* in the Red Sea (Kushmaro *et al.*, 1996; Kushmaro *et al.*, 1997; Rosenberg and Ben-Haim, 2002). In the case of bacterial bleaching, infection appears to be temperature related, with an increased incidence of bleaching at 29°C in laboratory culture (Toren *et al.*, 1998; Rosenberg *et al.*, 1999; Banin *et al.*, 2000; Banin *et al.*, in press). This agrees with documentation of increased levels of bleaching at higher seawater temperatures on coral reefs (Brown *et al.*, 1996; Brown, 1997). It has been proposed that much of the bleaching that has been attributed to

environmental factors (such as increased temperatures) may actually be due to bacterial bleaching pathogens (Rosenberg and Ben-Haim, 2002). The pathogen for white pox disease has recently been identified as *Serratia marcescens*, a bacterial species commonly found in the environment (Patterson *et al.*, 2002). Black band disease is composed of a microbial consortium, yet no individual pathogen has been described (see below) (Richardson, 1997).

Epidemiological (or epizootiological) theory proposes that infectious disease outbreaks in any species are generally caused by exposure to a common source of the etiologic agent (Ramos-Flores, 1983; Olive and Bean, 1999). However, although much field work has been done to document disease incidence and prevalence Caribbean-wide, biogeographical investigation of the specific microbial pathogens of coral disease has been extremely limited. Little is currently known about the mechanisms of dispersion and spreading of coral diseases (Richardson, 1998). One factor that has been widely proposed as important in the spread of diseases is changing environmental conditions (see Harvell *et al.*, 1999). A limited study of aspergillosis of sea fans (5 sites, 1 sample per site) suggested a correlation between disease incidence and increased levels of both dissolved inorganic nitrogen in seawater and turbidity (Kim and Harvell, 2002). Kuta and Richardson (in press) found evidence of a potential correlation between black band disease and increased nitrite levels (see below). Another hypothesized factor affecting Caribbean reef health is an increase in African dust, proposed as potentially contributing significantly to nutrient levels (i.e., N, P, and Fe) and as a vector for microbe transport, and thus influencing the increasing occurrence of diseases of coral and other invertebrates (Shinn *et al.*, 2000). Indeed, *Aspergillus sydowii,* the fungal aspergillosis pathogen, has

been cultured from spores collected in the Caribbean from airborne African dust (Weir *et al.*, in press). Although anthropogenic impact has been widely suggested as a contributing factor to coral disease, it has not been conclusively shown by any study to date. In fact, black band and other coral diseases have been found in areas both close to and remote from human influence (Edmunds, 1991).

Black Band Disease

Black band disease (BBD) is considered to be an important factor in the decline and community restructuring of some coral reefs (Porter and Meier, 1992; Bruckner and Bruckner, 1997a). First documented in 1973 (Antonius, 1973), this disease has since been found on reefs throughout the Caribbean (Rützler *et al.*, 1983; Edmunds, 1991; Bruckner and Bruckner, 1997b; Borger, in press), the Florida Keys (Kuta and Richardson, 1996), the Indo-Pacific (Antonius, 1985), the Red Sea (Antonius, 1988a; Antonius, 1988b), and the Great Barrier Reef (Dinsdale, in press). Forty-two different coral species worldwide have been observed to be susceptible to BBD (Green and Bruckner, 2000). Black band disease affects slow-growing scleractinian coral species that form the framework of reefs (Shinn *et al.* 1989) such as *Montastraea annularis, M. cavernosa, Colpophyllia natans, Diploria clivosa, D. labyrinthiformes, D. strigosa, Siderastrea siderea.* This disease has also been found on octocorals (Antonius, 1981; Antonius, 1985; Feingold, 1988).

Black band disease is generally found at low frequencies on susceptible coral colonies. Prevalence of BBD, as with other coral diseases, varies with season, year, reef, geographic area, and coral species. Some examples of this variation include BBD

prevalence rates of 0.5 – 1% in the Caribbean and Florida Keys, (Garrett and Ducklow, 1975; Edmunds, 1991; Kuta and Richardson, 1996; Kuta, 2000), 2.8% on the Great Barrier Reef (Dinsdale, in press), and 6.1% in Jamaica in 1992-93 (Bruckner and Bruckner, 1997a). A Caribbean-wide survey in 1999 found BBD prevalence to vary between 0- 6.7% at different locations (Weil *et al.*, in press).

Although there has been much speculation as to a correlation between black band disease and anthropogenic effects (Chet and Mitchell, 1975; Antonius, 1977; Taylor, 1983; Bruckner *et al.*, 1997), this has not been proven to date. Pollution, such as sewage outfalls and sedimentation, has also been conjectured to be correlated with disease incidence, although no data have been provided to support this hypothesis (Taylor, 1983; Antonius, 1988b; Bruckner *et al.*, 1997). As noted above, BBD has been found on reefs far from human influence (Edmunds, 1991). The limited research to date aimed at the relationships between BBD and the environment has revealed: 1. a much higher incidence of BBD on reefs in the Caribbean during periods of warm water temperatures (>29° C), the basis of the observed seasonality of the disease (Dustan, 1977; Taylor, 1983; Bruckner and Bruckner, 1997a; Kuta, 2000; Kuta and Richardson, in press); 2. a statistically significant relationship of BBD incidence with elevated nitrite levels, and decreased ortho-phosphate levels (Kuta, 2000; Kuta and Richardson, in press); and 3. a significant correlation between lower coral diversity and black band disease incidence (Kuta, 2000; Kuta and Richardson, in press).

Microbiology of Black Band Disease

Black band disease is characterized by a distinct black or dark red band,

approximately 1mm thick, that varies in width from less than 0.1cm to more than 6 cm (Figure 1) (Antonius, 1973; Antonius, 1981; Rützler *et al.*, 1983). It is often present in a roughly circular or semi-circular pattern. A single coral colony may have one or multiple infections. The band migrates across the surface of an infected coral colony, destroying live tissue at a rate of 0.1 to 2 cm per day (Antonius, 1981; Rützler *et al.*, 1983; Richardson, 1997). The disease is composed of a complex and distinct microbial consortium (Richardson, 1997; Bythell *et al.*, 2002), consisting of variable amounts of cyanobacteria, including *Phormidium corallyticum* (Antonius, 1973; Antonius, 1981; Rützler and Santavy, 1983; Taylor, 1983; Bythell *et al.*, 2002; Cooney *et al.*, 2002), the sulfide-oxidizing bacterium *Beggiatoa* spp. (Ducklow and Mitchell 1979), marine fungi (Ramos-Flores 1983), the sulfate-reducing bacteria *Desulfovibrio*, and many other microbes (Chet and Mitchell, 1975; Garrett and Ducklow, 1975; Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002). Although it has been proposed that *Phormidium corallyticum* is the primary pathogen (Rützler *et al.*, 1983), Koch's postulates have not been fulfilled for this (or any other) member of the disease community.

Within the BBD microbial consortium, two types of bacteria can be identified by light microscopy. These are the sulfide-oxidizing bacterial genus (*Beggiatoa*) and the cyanobacterial species (*Phormidium corallyticum).* Together, these and the additional BBD microorganisms form a vertically structured community directly analogous to laminated microbial mats (Carlton and Richardson, 1995). Few of the BBD microbes have been cultured or isolated, and only very recently have molecular techniques been applied to assess community composition (Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002). *Beggiatoa* and *Desulfovibrio* are two known members of the disease community that are

well known to be important in active sulfur cycling. These genera are not unique to the black band disease consortium, and are found elsewhere in many non-disease environments (Leadbetter, 1974; Widdel and Pfennig, 1984; Strohl, 1989). In contrast, *Phormidium corallyticum* has only been found in active BBD, and in biofilms (disease reservoirs) with the other known BBD consortium members (Richardson, 1997).

The black band microbial mat has an active sulfur cycle similar to that found in other microbial mat communities (Carlton and Richardson, 1995). Oxygenic photosynthesis by *Phormidium corallyticum* occurs simultaneously with sulfide production by sulfate-reducing bacteria, including *Desulfovibrio* spp. (Carlton and Richardson, 1995). Together, this forms a vertically fluctuating interface of oxygen and sulfide with steep concentration gradients of each. The intensity of oxygen production by photosynthesis of *P. corallyticum* is directly dependent upon light intensity (Carlton and Richardson, 1995). Microelectrode studies have shown that photosynthesis of *P. corallyticum* during the day causes supersaturated concentrations of oxygen at or near the surface of the band and extending down into the band as far as 500 μm (Carlton and Richardson, 1995; Richardson *et al.*, 2001). *P. corallyticum* is incapable of anoxygenic photosynthesis using sulfide as an electron donor; however, this species can carry out oxygenic photosynthesis in the presence of 0.5mM sulfide. This is an unusual capability among cyanobacteria and may contribute to the dominance of this species in the highly sulfidic disease band (Kuta, 2000; Richardson and Kuta, in press). Sulfide, produced by sulfate-reducing bacteria, is present throughout the day only at the very base of the band, but at night, it is present all the way to the band surface. It has been directly measured at concentrations of >800 μM (Carlton and Richardson, 1995). These highly anoxic and

sulfidic conditions, which are adjacent to the coral tissue, have been shown to be a mechanism of coral tissue death (Richardson *et al.*, 1997).

The extent of microbial variability of BBD between geographic areas, between infections, or as an infection develops and progresses remain unknown. Microorganisms present in BBD infections are predominantly distinct from those found on healthy coral, (Cooney *et al.*, 2002), dead coral, or seawater (Frias-Lopez *et al.*, 2002). The presence of *Phormidium corallyticum* and *Beggiatoa* in loose biofilms on sediment patches on healthy coral (Richardson, 1997) implies that naturally-occurring bacteria congregate opportunistically into the pathogenic state. The environmental cues behind this disease formation are unknown.

Black Band Disease: Microbial Migrations

Active black band disease exhibits two types of migrations, horizontal across coral colonies and vertical among the microorganisms within the band. The environmental cues for these microbial migrations are unknown (Rützler *et al.*, 1983; Richardson, 1996). It is known that black band disease contains dynamic horizontal and vertical microgradients of light, oxygen, sulfide, and organic and inorganic chemicals, each of which may elicit a microbial tactic response (Richardson *et al.* 1997). These gradients are generated and sustained by the microbial community itself.

Migrations within laminated cyanobacterial microbial mats found in naturally occurring, non-disease environments (such as sulfide-rich, marine or hypersaline areas) have been well studied (Castenholz, 1968; Jørgensen *et al.*, 1979; Castenholz, 1982; Jørgensen, 1982a; Revsbech *et al.*, 1983; Jørgensen *et al.*, 1983; Castenholz, 1984;

Richardson and Castenholz, 1987; Richardson and Castenholz, 1989; Castenholz *et al.*, 1990; Castenholz and Jørgensen, 1991; Garcia-Pichel *et al.*, 1994; Kruschel and Castenholz, 1998). Such mats are often dominated by oscillatorian cyanobacteria (a group that includes the genus *Phormidium*) that carry out vertical migrations on a diel basis. Many species of gliding filamentous cyanobacteria move downward into the mat environment in mid-morning (a negative photic response to high light) and return to the surface at late afternoon where they remain throughout the night, thus exhibiting a photo-tactic dominated dependent motility pattern (Castenholz 1968; Richardson and Castenholz 1987; Castenholz and Jørgensen 1991; Garcia-Pichel and Castenholz 1994; Bebout and Garcia-Pichel 1995, Kruschel and Castenholz 1998). Diel variations in light and resultant photosynthesis in turn contribute to steep chemical micro-gradients, which vary between a highly sulfidic and anoxic (night) micro-environment and oxygen supersaturation (day) (Jørgensen *et al.*, 1979; Jørgensen *et al.*, 1979). These micro-gradients lead to stratification of microbial populations within the mat environment as a result of chemotaxis (Revsbech *et al.*, 1983; Jørgensen, 1994).

In non-disease microbial mat environments, populations of *Beggiatoa* are typically found at the precise position of an oxygen/sulfide interface (Jørgensen 1982) (see below). *Beggiatoa* spp. can biologically oxidize sulfide at 10-1000 times the chemical oxidation rate to obtain energy (Jørgensen and Revsbech, 1983). *Beggiatoa* spp. in a typical microbial mat environment migrate vertically following oxygen/sulfide interfaces, to maintain sulfur lithotrophy. This results in a commonly observed pattern in which *Beggiatoa* is present at depth within mats during daylight hours (when oxygenic photosynthesis is occurring, which drives the oxygen/sulfide interface downwards) and

on the mat surface during darkness as sulfide concentrations accumulate and rise to the mat surface (Nelson and Castenholz, 1982; Jørgensen, 1982a; Garcia-Pichel *et al.*, 1994).

Sulfate-Reducing Bacteria

Sulfate-reducing bacteria (SRB) such as *Desulfovibrio* are commonly found in marine, estuarine and freshwater environments that are both anaerobic and reducing. Some examples of environments where SRB are found include: deep-sea oil and sulfur wells (ZoBell, 1958), marine sediments (van der Maarel *et al.*, 1996), the rhizoplane and deep cortex cells of the seagrass *Halodule wrightii* (Kusel *et al.*, 1999), sewage treatment plants (Soimajärvi *et al.*, 1978), human and animal waste (Moore *et al.*, 1976; Beerens and Romond, 1977), and spoiled foods (Werkman and Weaver, 1927; Campbell *et al.*, 1957).

Sulfate-reducing bacteria are ecologically highly significant, especially in marine or hypersaline microbial mat environments, where as the dominant anaerobes, they may account for up to 50% of oxidized total organic carbon through sulfate respiration. They are a major source of environmental sulfide (Howarth and Hobbie, 1982; Jørgensen, 1982b). Characteristically, SRB all can use the reaction $4AH_2 + SO_4^{2-} + H^+ \square 4A + HS^- + 4H_2O$ (Postgate, 1984). There are typically many different electron donors (AH_2), where A is usually an organic compound. SRB are important in the oxidation of organic matter and may oxidize fatty acids, alcohols, dicarboxylic acids and some aromatic compounds completely to CO_2. Thus they are often dependent on the main products of bacterial fermentations. In marine environments, sulfate is abundant at approximately 28mM (Hansen, 1993; Odom, 1993), providing a sufficient sulfate supply for SRB to

outcompete methanogens in an anaerobic environment and to act as terminal oxidizers the in anaerobic degradation of organic matter (Jørgensen and Fenchel, 1974). Thus, in a marine anoxic environment, the amount of organic matter available would be the limiting factor in sulfate reduction (Jørgensen, 1982a).

Although a potentially integral part of the active sulfur cycling within black band, studies aimed at characterizing the sulfate-reducing bacteria associated with black band disease have been limited to date. SRB in black band disease were first postulated to be *Desulfovibrio* in the 1970s (Garrett and Ducklow, 1975). However, it was not until the mid 1990s that an effort was made at species investigation. (Schnell *et al.*, 1996) used fluorescent *in situ* hybridization probes for sulfate-reducing bacteria and confocal laser microscopy to examine the abundance and morphology of SRB within the black band disease community. Growth of the SRB on hydrogen and lactate supported their identification as *Desulfovibrio* spp. (Schnell *et al.*, 1996).

Two very recent studies using culture-independent cloning of 16S rDNA from coral-associated microbial communities have shown the diversity within the composition of the disease consortium to be significantly greater than previously believed (Frias-Lopez *et al.*, 2002; Cooney *et al.*, 2002). In a limited survey of partial bacterial sequences cloned from three different BBD infections on different coral species in Curacao, Frias-Lopez *et al.* (2002) found bacterial strains with greater than 97% homology to *Desulfovibrio salexigens*, *Desulfovibrio* sp. strain TBP-1, and *D. aespoeensis*. Although a particular infection contained multiple species homologous to different *Desulfovibrio* species, no one particular *Desulfovibrio* species was common to multiple infections. The only other species of sulfate-reducing bacteria identified from

the disease community was *Desulfobotulus* sp. strain BG14.

Sulfate-reducing bacteria were also found on dead coral skeleton behind the black band disease infection (Frias-Lopez *et al.*, 2002; Cooney *et al.*, 2002). Two species of *Desulfovibrio* (with homology to *D. zosterae,* and *D. alaskensis,* respectively) were sequenced from the bacterial community on dead coral skeleton. Other species of SRB identified on dead coral skeleton behind the disease were *Desulfofrigus oceanense* strain Asv26, *Desulfobotulus* sp. strain BG14, *Desulfocella halophila, Desulforopalus singaporensis, Desulfofrigus fragile,* and *Desulfobacterium catecholicum* (Frias-Lopez *et al.*, 2002). Cooney *et al.* (2002) found three *Desulfovibrio* species with 95-96% homology to *Desulfovibrio* species identified from the oxic layer of marine sediment and found one species with 92% similarity to sulfate-reducing *Desulfocella halophila* found in Great Salt Lake marine sediment.

Perhaps the most obvious explanation for this difference in *Desulfovibrio* species between infections is that different BBD infections have different *Desulfovibrio* spp. present, filling an ecophysiological niche rather than an exclusively species-specific niche. However, an alternate explanation is that, given the bias inherent in the techniques used, more *Desulfovibrio* species were present in the disease band than were cloned or sequenced, thus only a subsample of species present were detected in each sample. While providing a broad survey of the composition and variability of the black band community, even the current technology employed by these studies have an inherent bias for under-representation of bacteria which may be present in low density. The question thus remains as to which, if any, components of the disease are conserved between infections in various geographic areas.

Physiology of Sulfate-Reducing Bacteria

Sulfate-reducing bacteria constitute a large, heterogeneous group of microorganisms that may use a highly diverse array of potential electron donors and acceptors (Widdel, 1988), but by definition, all are physiologically capable of utilizing sulfate as the electron acceptor during anaerobic respiration (Widdel and Bak, 1992). Dissimilatory reduction of sulfate or other oxidized sulfur species is coupled with oxidation of hydrogen gas (H_2), ethanol, or a wide variety of organic compounds such as lactate, fatty acids, ethanol, or dicarboxylic acids (Widdel and Pfennig, 1984; Postgate, 1984; Hines *et al.*, 1997). Sulfate is reduced to sulfide, using four enzymes in the cytoplasm (Voordouw, 1995). Sulfate reduction is not inhibited by sulfide concentrations of up to 5mM (Dalsgaard and Bak, 1994). Autotrophic growth on H_2, CO_2 and sulfate may occur.

SRB have been traditionally classified by such indicators as cell shape, motility, GC content of DNA, presence of the pigment desulfovirin and of the tetraheme cytochrome c_3, temperature optimum, utilizable electron donors, and complete versus incomplete oxidation of acetate (Devereux *et al.*, 1990). However, because SRB are often difficult to isolate and identify, they have been increasingly studied using molecular methods. The phylogeny of sulfate-reducing bacteria corresponds well with physiology-based taxonomy (Devereux *et al.*, 1996). 16S rRNA sequence similarity is the generally established method for analyzing phylogenetic relationships among bacteria (Fox *et al.*, 1980; Woese, 1987). Comparative gene sequencing is now widely used as a basis for establishing an association between natural relationships of bacteria and known biochemical and physiological properties (Fox *et al.*, 1980; Woese *et al.*, 1985; Woese,

1987). 16S rRNA sequence analysis has greatly expanded the known phylogeny of the diverse sulfate-reducers.

SRB are subdivided into four groups: gram negative mesophiles, gram positive spore formers, thermophilic eubacteria, and thermophilic archaea (Castro *et al.*, 2000). Comparisons of 16S rRNA sequences place the gram-negative sulfate-reducing eubacteria within the delta subdivision of the Proteobacteria along with sulfur-reducing bacteria, myxobacteria, and bdellovibrios (Woese, 1987; Castro *et al.*, 2000). Proteobacteria are characteristically phototrophs (*i.e.*, purple photosynthetic bacteria); thus, at some evolutionary point the delta subdivision diverged and members lost their photosynthetic ability, becoming heterotrophic (Woese, 1987). Two families of sulfate-reducing bacteria have been suggested within the delta subdivision of the Proteobacteria: the Desulfovibrionaceae and the Desulfobacteriaceae, which includes all genera other than *Desulfovibrio*, including *Desulfobulbus, Desulfobacter, Desulfobacterium, Desulfococcus, Desulfosarcina, Desulfomonile, Desulfonema, Desulfobotulus,* and *Desulfoarculus* (Devereux *et al.*, 1989; Widdel and Bak, 1992; Holt *et al.*, 1994)

***Desulfovibrio* spp.**

Members of the genus *Desulfovibrio* are gram-negative mesophilic SRB that have both a characteristic physiology and 16S rRNA sequence signature that distinguishes them from other lines of SRB. *Desulfovibrio* spp. are characteristically curved to straight rods ranging from 0.5-1.5 x 2.5-10.0μm with one or more polar flagella (Widdel and Pfennig, 1984). Optimum temperatures for cultures are generally 25-40°C and optimum pH is around 6.6-7.5 (Widdel and Pfennig, 1984). *Desulfovibrio* spp. were the first

nonphototrophic anaerobes shown to respire organic substances (Peck, 1962). Some potential electron donors utilized by *Desulfovibrio* spp. include H_2, lactate, pyruvate, ethanol, malate, fumarate, metallic iron (Fe^0), and various short-chain alcohols and aldehydes (Little *et al.*, 1991; Widdel and Bak, 1992; Odom, 1993; Voordouw, 1995). Organic substrates such as sugars, glycerol, choline or several amino acids are incompletely oxidized to acetate via pyruvate and acetyl-CoA (Widdel and Pfennig, 1984). *Desulfovibrio* spp. are characteristically unable to completely oxidize lactate to CO_2 or to utilize fatty acids as growth substrates (Devereux *et al.*, 1990; Widdel and Bak, 1992). H_2 is commonly utilized by *Desulfovibrio* spp as an electron donor., but requires the additional carbon source of acetate and CO_2 for chemolithotrophic growth (Widdel and Bak, 1992). Species lineages of *Desulfovibrio* are primarily defined by rRNA sequence divergence. At least five *Desulfovibrio* lineages with 16S rRNA distance measurements exist that are as distant as those of other recognized genera (Devereux *et al.*, 1990; Devereux and Stahl, 1993).

Studies on SRB, including *Desulfovibrio* spp. and *Desulfonema* spp., have shown that they are not limited to anoxic environments and some population distributions may not be restricted by the toxic effects of oxygen exposure (Dilling and Cypionka, 1990; Canfield and DesMarais, 1991; Santos *et al.*, 1993; Teske *et al.*, 1998; Krekeler *et al.*, 1998; Okabe *et al.*, 1999). In a microbial mat system from the Exportadora de Sal salt works (Guerroro Negro, Baja California Sur, Mexico), molecular probes showed *Desulfovibrio* spp. to be present both in the oxic zone (above 2mm depth) and below in the anoxic zone (7mm depth) (Minz *et al.*, 1999). A study which coupled fluorescent *in situ* hybridization with microelectrode probes to study aerobic wastewater biofilms found

Desulfobulbus and other SRB to be distributed throughout the biofilm, even at the oxic surface, although high sulfide levels were limited to the bottom of the biofilm (Okabe *et al.*, 1999). SRB in typical microbial mat communities have been shown to migrate vertically with regard to oxygen concentrations and sulfide levels during daylight hours (Krekeler *et al.*, 1998).

***Beggiatoa* spp.**

Beggiatoa species are found worldwide in both marine and fresh waters at oxygen-sulfide interfaces (Jørgensen, 1977; Jørgensen and Revsbech, 1983) and are commonly observed as white filamentous mats on the surface of anoxic sediments (Nelson *et al.*, 1989b). Some examples of fresh water habitats include sulfur springs (Jørgensen *et al.*, 1979; Revsbech *et al.*, 1983; Jørgensen and Revsbech, 1983; Richardson and Castenholz, 1987; Jørgensen, 1988; Mattison *et al.*, 1998), benthic mats in eutrophic lakes (Winogradsky, 1887; Keil, 1912; Lackey, 1960), swampy meadows, and in a microbial community with *Desulfovibrio* in rice paddies (Scotten and Stokes, 1962; Pitts *et al.*, 1972; Joshi and Hollis, 1976; Joshi and Hollis, 1977). Marine *Beggiatoa* spp. have been found in and on surface sediments of shallow seas, brackish fjords, salt marshes, the sea floor (Strohl and Larkin, 1978), intertidal sand flats (Lackey *et al.*, 1965; Ankar and Jansson, 1973; Jørgensen, 1977; Grant and Bathmann, 1987; Pinckney and Zingmark, 1991), some deep sea hydrothermal vents (2000-2500m depth) (Jannasch *et al.*, 1989), oil-soaked coral heads (Chet and Mitchell, 1975), and black band disease of corals (Ducklow and Mitchell, 1979b).

Beggiatoa are in the order Cytophagales, along with *Vitreoscilla and Thioploca*

(Leadbetter, 1974). Consituents of the genus *Beggiatoa* are motile, filamentous, gram-negative bacteria (Kowallik and Pringsheim, 1966; Leadbetter, 1974). They are generally colorless, but a defining feature of the genus is the deposition of visible globules of elemental sulfur in the periplasm when the cells are exposed to oxygen and hydrogen sulfide or thiosulfate (Kowallik and Pringsheim, 1966; Strohl, 1989). Within the genus, species were traditionally defined taxonomically by filament diameter, which varies from 1-120μm (Leadbetter, 1974; Jørgensen, 1977; Wiessner, 1981; Larkin and Strohl, 1983; Nelson *et al.*, 1989b). The emergence of molecular techniques has added further information to the taxonomy of *Beggiatoa* spp., based on 16S rRNA sequence comparisons. Phylogenetic studies have shown that narrow, nonvacuolate, freshwater, chemoheterotrophic strains appear to be evolutionarily separate from wide, vacuolated, marine *Beggiatoa* and *Thioploca,* although narrow marine strains of *Beggiatoa* were not included in the study (Teske *et al.*, 1995; Ahmad *et al.*, 1999). *Beggiatoa* spp. secrete a polysaccharide slime which surrounds the whole filament in a thin sheath that adheres to the substrate on which the filament glides in a relatively smooth and steady motion (Wiessner, 1981; Møller *et al.*, 1985). All strains that have been tested of *Beggiatoa* spp. from both marine and freshwater environments can fix nitrogen (Nelson *et al.*, 1982; Vargas and Strohl, 1985).

All studies to date have shown that *Beggiatoa* spp. exist at the interface between oxygen and hydrogen sulfide, where steep gradients of the electron donor (H_2S) and acceptor (O_2) overlap (Faust and Wolfe, 1961; Burton and and Morita, 1964; Kowallik and Pringsheim, 1966; Strohl and Larkin, 1978; Jørgensen and Revsbech, 1983; Jørgensen and Des Marais, 1986). The continuous gliding motility of *Beggiatoa* spp.

(Winogradsky, 1887; Lackey, 1960; Jørgensen, 1977; Nelson and Castenholz, 1982; Jørgensen, 1982a). Potential cues for the downward movement of *Beggiatoa* filaments include a step-down photophobic response [when an increase in light results in a change in direction of movement away from light source (Castenholz, 1982)] or a negative aerotaxis (Nelson and Jannasch, 1983; Jørgensen and Revsbech, 1983; Møller *et al.*, 1985). A step-up phobic response to oxygen was demonstrated using *Beggiatoa* filaments isolated from Kalø Lagoon, Denmark (Møller *et al.*, 1985). When grown in sulfide gradient cultures (in which solid agar media with a high level of sulfide is overlain with semi-solid agar without sulfide, causing a diffusion gradient of sulfide up through the media), *Beggiatoa* spp. forms a lens-shaped growth pattern at the sulfide-oxygen interface with a characteristic sharp upper boundary which implies a chemotactic response to O_2 (Møller *et al.*, 1985). This micro-oxic aggregation has been shown to occur in gradient cultures even in the absence of added sulfide, indicating a negative aerotaxis (Nelson *et al.*, 1986b). A sharp lower boundary of the lens is present in gradient cultures with sulfide added, which suggests a negative chemotactic response to excess H_2S (Møller *et al.*, 1985). These chemotactic responses could help *Beggiatoa* find its niche (Nelson, 1992). The one laboratory study investigating light responses of *Beggiatoa* spp. found cultured *B. leptomitiformis* to have a step-up photo-phobic response to blue light. Such a photo-phobic response would be an advantageous adaptation for *Beggiatoa* in nature because full sunlight has been shown to completely inhibit *Beggiatoa* spp. growth, even when filaments are aggregated in tufts that would insulate them from unfavorable environmental conditions (Nelson and Castenholz, 1982).

Beggiatoa spp. oxidize sulfide, competing efficiently with the spontaneous

chemical oxidation of sulfide by oxygen. This effectively establishes and sustains steep, opposed gradients of oxygen and sulfide (Jørgensen and Revsbech, 1983), indicating that the bacteria may harness the chemical energy of H_2S oxidation for use in assimilation of CO_2 (Møller *et al.*, 1985). S^0 has been shown to be the first intermediate in sulfide oxidation (Nelson and Hagen, 1995). O_2 is the only electron receptor proven to sustain growth of cultured *Beggiatoa,* although other acceptors, such as nitrate, may be important as well (Nelson, 1992). A low oxygen tension has been shown to have beneficial effects on the growth and metabolism of *Beggiatoa* spp. (Strohl and Larkin, 1978; Nelson and Castenholz, 1981; Nelson *et al.*, 1982; Nelson and Jannasch, 1983; Nelson *et al.*, 1986b). The oxidation of sulfide by *Beggiatoa* spp. may contribute to alleviation of the toxicity of sulfide in the environment. In addition to being an energy source for *Beggiatoa* spp., the oxidation of sulfide may detoxify metabolically formed hydrogen peroxide, which could be harmful because many *Beggiatoa* spp. lack catalase (Schmidt *et al.*, 1987).

All *Beggiatoa* spp. that have been isolated from marine environments are chemolithoautotrophs, with the one exception being a strain that could combine utilization of CO_2 and organic carbon for growth via mixotrophy (Hagen and Nelson, 1996). Marine *Beggiatoa* spp. gain energy for lithoautotrophic growth by oxidation of sulfide through elemental sulfur to sulfate (Nelson and Jannasch, 1983; Nelson *et al.*, 1986a). The capacity for lithoautotrophy has been demonstrated by showing a functional Calvin cycle determined by diagnostic levels of ribulose-1,5-bisphosphate carboxylase/oxygenase and phosphoribulokinase activities (Nelson *et al.*, 1989a). Some *Beggiatoa* strains have been shown to increase growth when provided with a combined carbon and nitrogen source (Pringsheim and Kowallik, 1964; Vargas and Strohl, 1985).

Nitrogen sources used in the culture of *Beggiatoa* strains include nitrate, nitrite, urea, peptone, yeast extract, and a few amino acids including aspartate and asparagines; limited growth occurred with glutamate and methionine (Pringsheim and Kowallik, 1964; Strohl and Larkin, 1978; Nelson *et al.*, 1982; Mezzino *et al.*, 1984; Vargas and Strohl, 1985).

All *Beggiatoa* spp. studied from freshwater habitats are chemoheterotrophs or mixotrophs (Strohl and Larkin, 1978; Grabovich *et al.*, 1998). These may grow, in addition to via sulfide lithotrophy, as aerobic chemoheterotrophs. All freshwater strains that have been studied have a functional tricarboxylic acid (TCA) cycle with glyoxylate bypass (Nelson *et al.*, 1989a) but no enzyme activity indicative of autotrophic CO_2 fixation pathways such as RUBP-carboxylase, α-ketoglutarate synthase, or phosphoenolpyruvate carboxylase (found in the the Calvin-Benson cycle or the serine pathway) (Strohl *et al.*, 1981). Freshwater *Beggiatoa* strains have not been grown in culture in the absence of organic compounds (Strohl and Larkin, 1978; Nelson and Castenholz, 1981). A limited number of organic acids, alcohols and TCA cycle intermediates can be used as the sole source of both carbon and energy, with lactate, acetate, pyruvate, and ethanol being the most common (Nelson, 1992).

Goals, Objectives and Hypotheses

The overall goal for this research is to investigate the sulfur bacteria found in the black band disease microbial consortium. Several specific objectives relate to this goal as follows.

Objective 1. *Desulfovibrio* spp. will be cultured and identified from separate black band disease infections in geographically distant areas.

- 16S rDNA sequences of sulfate-reducing bacteria within the BBD consortium will be compared to non-disease SRB (from GenBank and the literature)
- 16S rDNA sequences will be compared between samples from different disease infections and different geographic areas.

Objective 2. The vertical migrations of *Beggiatoa* in the BBD will be investigated in response to the environmental cues of light intensity, H_2S and O_2.

The hypotheses tested during this research include:

- Hypothesis 1: *Desulfovibrio* spp. from black band disease of corals will not vary significantly from *Desulfovibrio* species from other marine environments, in a comparison between 16S rDNA sequences.
- Hypothesis 2: *Desulfovibrio* spp. from black band disease infections will not vary significantly between different infections or different geographic areas.
- Hypothesis 3: *Beggiatoa* spp. in the black band disease consortium migrate in response to light, O_2, and H_2S in a manner analogous to *Beggiatoa* spp. found in other microbial mats.

METHODS

Bacterial Sample Collection

Samples of black band disease (Figure 1) were collected using sterile syringes in the summer and fall of 2000. Syringes remained in sterile packaging until opened underwater immediately before disease collection. For studies of sulfate-reducing bacteria, BBD was sampled from Picture reef in Biscayne National Park, and Algae Reef in the Florida Keys National Marine Sanctuary and from one reef in Dominica (Figures 2a-2c, Table 1). Four infected coral species were sampled in these three locations as depicted in Table 1.

ID #	Location	Infected coral species	Collection date
BBD-2	Picture reef, BNP	*Diploria clivosa*	9/23/2000
BBD-3.1	Picture reef, BNP	same infection	9/23/2000
BBD-6	Picture reef, BNP	*D. strigosa*	9/23/2000
BBD-10	Dominica	*Siderastrea siderea* #3	8/23/2000
BBD-11.1, BBD-11.2	Dominica	same infection	8/23/2000
BBD-13.1 BBD-13.2	Dominica	*S. siderea* #5	8/23/2000
BBD-15	Dominica	same infection	8/23/2000
BBD-16	Dominica	*S. siderea* #6	8/23/2000
BBD-19	Dominica	same infection	8/23/2000
BBD-22	Algae Reef, FKNMS	*D. labyrinthiformes*	11/15/2000

Table 1: Black band disease sampling location and infected coral species sampled. The identification number of samples of sulfate-reducing bacteria is presented with the geographic location of sample collection, coral species infected with sampled black band disease, and date of sample collection.

Samples of the disease consortium taken in Florida were used immediately (within 3 hours) for bacterial isolations while samples from Dominica were used within 5 days (allowing for shipping). For studies of *Beggiatoa* spp., multiple samples were taken from BBD infections affecting various coral species on Algae reef (Figure 2b), Little Grecian, Grecian Dry Rocks, Looe Key, Horseshoe reef, and Cannonpatch within the Florida Keys National Marine Sanctuary (not included in Figure 2b), and the same disease infections as described above from Dominica (Figure 2c).

Black Band Sulfate-Reducing Bacteria

Samples of BBD were inoculated directly from collection syringes into anaerobic vials of Sulfate API Broth (Difco Laboratories, Detroit, MI) made with natural seawater. Prior to autoclaving and inoculation, air was removed from the sealed vials by alternating cycles of vacuum and nitrogen gas through a syringe apparatus (approximately 3 cycles of 7 minutes vacuum, 3 minutes nitrogen). Media was always reduced prior to inoculation with either titanium NTA or 200mM H_2S until the resazaurin in the media changed color, indicating anoxic conditions. When added as a reducing agent, H_2S caused faint precipitation of FeS. The presence of an intense black FeS precipitate in inoculated media was indicative of bacterial sulfate reduction.

All subsampling of original inoculations and plating were done within a Coy anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI) containing approximately 90% nitrogen/ 10% hydrogen gas mixture. Original inocula were subsampled into Sulfate API Broth (with NaCl doubled) several days prior to streak plating onto modified iron sulfate agar for isolation. A variety of solid agar media was

inoculated, including Sulfate API, modified iron sulfate (Postgate, 1984), and Postgate's B media (Postgate, 1979), each made with either filtered (Whatman #1) and autoclaved seawater, Instant Ocean, and distilled water with an increased concentration of NaCl (double strength). Some colonies were re-streaked onto a second modified iron sulfate plate before picked and inoculated into broth media. Isolates were obtained by cutting a plug of agar along with the colony using a glass pipet and transferring the colony and agar plug to Sulfate API Broth (double-strength NaCl) (1.0g Bacto-yeast extract; 0.1g ascorbic acid; 5.2g sodium lactate; 0.2g $MgSO_4$; 0.01g K_2PO_4; 0.1g ferrous ammonium sulfate; 10.0g NaCl per liter of seawater). Only broth cultures showing an intense black FeS precipitate, indicative of bacterial sulfate reduction, were used in further inoculations. Purity was checked by re-inoculation onto modified iron sulfite agar plates. Colonies were again cut out and transferred into Sulfate API Broth. These were subsampled into sealed anaerobic vials of Sulfate API Broth prior to removal from the anaerobic chamber.

DNA Extraction

DNA was extracted from SRB cultures obtained as described above. Cells were first centrifuged into a pellet, then DNA was extracted using a Soil DNA Isolation Kit (MoBio Laboratories, Inc., Solana Beach, CA) according to procedures included with the kit. An agarose gel (1% in TBE buffer + ethidium bromide) was used to confirm the presence and quality of high molecular weight DNA. DNA was stored at -20°C.

DNA Amplification

Polymerase chain reaction (PCR) protocols for sequencing were as follows: (in a

20µl reaction) 2µl 10x buffer; 2µl $MgSO_4$; 2µl, 0.5µM dNTPs; 0.5µl 20µM Forward Primer; 0.5µl 20 µM Reverse Primer; 0.1µl Tfl or Taq polymerase (Promega, Madison WI); 2µl 1% bovine serum albumin (BSA) and H_2O to 20µl; to 1µl DNA. Thermal cycler parameters (MJ Research PCT 2000) were: 94°C for 2 min, 30 cycles of: 94°C for 2 min; 55 °C for 15 seconds; 72 °C for 30 seconds, followed by a final extension at 72°C for 10 min. PCR primers used were universal eubacterial primers [primer sequences given 5′□3′] FD1 (AGAGTTTGATCCTGGCTCAG) and RD1 (AAGGAGGTGATCCAGCC) (Weisburg *et al.*, 1991). Negative controls lacked added DNA. Positive controls were: 1. cultures from this study which had previously worked with the given primer and 2. a known *Desulfovibrio* strain. PCR products were viewed on a 1% agarose gel in TBE buffer, stained with ethidium bromide. Appropriate binding of PCR primers was tested by nested PCR (repeated PCR, first using FD1, RD1, and repeated using product in PCR with 1 internal primer). PCR fragments were purified for sequencing using Qiagen Quiquick PCR Purification kit and included protocols (Qiagen, Valencia, CA).

Three cultures [culture 3 from *Diploria clivosa,* Picture Reef, Biscayne National Park, culture 11 from *Siderastrea siderea,* Dominica, and culture 13 from a different *Siderastrea siderea* colony, Dominica (Figure 1)] were cloned to confirm DNA integrity from direct-PCR sequencing. The 16S PCR fragment from FD1 to RD1 was cloned into *Escherichia coli* plasmid vector using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and included protocols. Two colonies from each plate were picked to grow for use in sequencing. Plasmid DNA was extracted using Qiagen Miniprep (Qiagen, Valencia, CA). An EcoR1 restriction digest viewed on an agarose gel was used to confirm the

presence of the correctly sized insert.

M13 forward and M13 reverse primers (Invitrogen Corporation, Carlsbad, CA) were used to sequence from the plasmid insert. Primers used to sequence from the PCR product included: [primer sequences given 5′□3′] FD1 (AGAGTTTGATCCTGGCTCAG), 519R (G(G/T)ATTACCGCGGC(A/T)GCTG) (Lane *et al.*, 1985), 926F (AAACTYAAAG(A/T)AATTGACGG) (Britschgi and Fallon, 1994), and RD1 (AAGGAGGTGATCCAGCC) (Weisburg *et al.*, 1991). Once initial identification of fragment identity was made, optimal primers were designed using the OLIGO Primer Analysis Software (Molecular Biology Insights, Inc., Cascade, CO). These were 514F (CACTGGGCGTAAAGC), 1040R (CTCGTTGCGGGACTT), 1056F (AAGTCCCGCAACGAG).

Cycle sequencing was done on an MJ Research PTC 2000. The Big Dye terminator Cycle Sequencing kit (TF, Kit BDT RR-100, Perkin-Elmer, Boston, MA) was used. Cycle sequencing reaction protocols for sequencing from the PCR product followed the protocol of Collins and Rawlings (1999). These were: 1µl 3.75µM primer, up to 5µl template DNA (depending on DNA quantity), 2.0µl Dye Terminator Ready Reaction Mix, 2.5x sequencing buffer, and UV-sterilized dH_2O to bring the final reaction volume to 10µl. For sequencing from the plasmid (M13 primers and PCR primers), reaction volume was adjusted to a 20µl reaction. Cycle sequencing program parameters were: 30-40 cycles of 96°C for 10 seconds, 50-60°C for 5 seconds, 60°C for 4 minutes. DNA was cleaned using sodium acetate and ethanol precipitation (for further details, see Collins and Rawlings 1999). DNA sequencing was done by the FIU Biology CORE molecular lab with an ABI Prism 377 Automated DNA Sequencer.

Gel files were viewed on Sequencing Analysis (ABI Prism, Applied Biosystems, Foster City, CA) and sequence fragments assembled on Autoassembler (ABI Prism, Applied Biosystems).

Phylogenetic Analysis

Thirty-four sequences of *Desulfovibrio* spp. and eight species of unidentified bacterial16S rDNA were extracted from GenBank (http://www.ncbi.nlm.nih. gov). These *Desulfovibrio* spp. sequences were aligned on ClustalX (Thompson *et al.*, 1997) with the three cloned sequences from this study.

On the ClustalX alignment program, the alignment gap opening penalty was assigned to be 10, and gap extension penalty was 2.5, except in highly variable regions where penalties were changed to 15 and 6.6, respectively. The transition value was 0.5 due to the relatively equal frequency of purines and pyrimidines within the sequences. Alignments were visually checked on both ClustalX and MacClade (Maddison and Maddison, 2000)

Analyses and tree construction were done using PAUP 4.0b4 (Swofford, 1999). Bootstrap support was obtained for 1000 replicates. Outgroups were assigned to be species other than *Desulfovibrio: Desulfofrigus fragile*, *Desulfobotulus* sp. BG14, *Desulfuricans ocenense* strain Asv26, *Desulfocella halophila, Besulfobacterium catecholicum, and Desulforhopalus singaporensis*. The Jukes-Cantor distance measure was used. Heuristic search parameters were as follows: starting trees were obtained via neighbor-joining, sequences were added as-is, keeping best trees only, tree-bisection-recombination (TBR) branch swapping algorithm, and steepest descent. The *Clostridium* phylogenetic tree was constructed using the same methodology, except no outgroups

were assigned.

Microbial Migration in Black Band Disease

Vertical motility patterns associated with *Phormidium corallyticum* and *Beggiatoa spp.* in 20 different black band disease infections were examined at four reefs in the Florida Keys National Marine Sanctuary (south to north: Looe Key, Little Grecian, Horseshoe, and Algae) and further north at one reef (Picture reef) in Biscayne National Park (Figures 2a-2b). Motility studies were conducted August-September, 2000. Depths at each site were between 2 and 10 m. Black band disease was studied on six species of coral; *Diploria clivosa, D. strigosa, Montastraea cavernosa, M. annularis* (species complex), *Siderastrea siderea, Colpophyllia natans.* All observations were conducted underwater using SCUBA.

Light levels (photosynthetically active radiation between 400-700nm) were measured *in situ* using a diver-operated Biospherical Instruments, Inc. (San Diego, California) QSI-140 light meter and recorded. The instrument was calibrated at Biospherical Instruments, Inc. in 2000. Changes in natural (ambient) light levels were measured in conjunction with observations of the surface bacterial populations (n=17). Light levels were also experimentally manipulated on the reef using varying layers of neutral density screen to cover the disease band for up to 2 hours (n=3). Screen layers were held in place approximately 1 cm above the coral (and band) surface using nails that were inserted into dead coral skeleton adjacent to the band. Light levels were analyzed using a Student's t-test (equal variances not assumed) and non-parametrically using a Mann-Whitney U test.

The surface of each disease band was examined for the relative presence of clumps of white *Beggiatoa* spp. versus the dark reddish black color of *Phormidium corallyticum*. Bands were photographed with a Kodak DC 265 in an Ikelite housing or with a Sea and Sea Motormarine II.

Black Band *Beggiatoa* spp.

Isolation of *Beggiatoa* spp. from fresh black band disease samples was repeatedly attempted in bell jars under variable levels (varied between approximately 80-95%) of nitrogen gas, with trace levels of air and trace levels (approximately 0.5- 1%) of carbon dioxide gas (Nelson, 1992). Solid media was inoculated either on the surface or just underneath the surface of the agar with fresh (3 hours) to 5 day-old disease samples. Various types of solid agar media were used, including J2 (Nelson, 1992), J3, and DTA (Nelson 1992). J2 minimal media was made with varied levels of sulfide and on sulfide media plus acetate. J2 media consisted of: A) 500mL filtered (Whatman #1), autoclaved natural seawater; B) 200mL dH_20, 1.2% agar; C) 50 mL 15x concentrate [3.0mM K_2HPO_4, 0.5mM $Na_2MoO_4 \cdot 2H_2O$, 0.1mM $FeCl_3 \cdot 3H_2O$, 3.75mM $Na_2S_2O_5$, 0.14mM phenol red, 0.9mL/L SL8 trace element solution (Pfennig and Biebl, 1981); distilled H_2O added to volume, and pH adjusted until solution turned red]. Solutions were autoclaved separately, cooled, and mixed. The following were added from sterile stocks: 2mM $Na_2S_2O_3$ (200mM stock); 1.3mM Na_2S (200mM stock); 0.5mL of 2M HCl; 1mM NH_4NO_3 (500mM stock); 0.2mL Va vitamin solution (Nelson 1992), 20mM $NaHCO_3$. As soon as plates solidified, they were incubated under gas (described above) with dessicant to absorb H_2O vapor. Media was aged 1-3 days. Other additives to J2 media included:

30mM sodium molybdate, and varying amounts and combinations (0.1M increments) of acetate, thiosulfate, and additional sulfide. Ratios of natural (filtered and autoclaved) seawater to artificial seawater used in the media were varied.

Cultures were grown at room temperature or at 28-30°C. Plates were incubated in darkness to discourage growth of cyanobacteria. A dissecting microscope was used to watch for filaments of *Beggiatoa* spp. as they glided out of the bacterial community on agar plates. Single filaments were cut out of the agar using forceps and placed onto a new agar plate every 1-3 days. As filaments glided off of the cut agar and multiplied, the procedure was repeated (as per Nelson, pers. comm.). J2 media and single-filament isolation methods were also used in a Coy anaerobic hood and in a BBL GasPak Pouch System CO_2-enriched anaerobic environment (Fisher Scientific).

Black Band *Beggiatoa* spp. in Sulfide-Gradient Cultures

The black band disease community was inoculated into sulfide gradient media for culture purposes and to observe the effect of a sulfide-gradient upon the self-positioning of BBD community members. Sulfide gradient media was added to gradient tubes in two portions, using DTA media with agar added (Nelson, 1992). The first portion consisted of DTA media, with 1.2% agar, and 0-6mM H_2S (varied to explore growth parameters). For the second (upper) portion, DTA media made with 0.7% agar (semisolid) and without thiosulfate or H_2S, was added to the top of the solidified bottom agar plug in a two to one volume ratio. Gradient media were inoculated either on the surface or just underneath the surface of the agar with fresh (3 hours) to 5 day-old BBD samples or with BBD from aged gradients. Individual *Beggiatoa* filaments were also cut from the agar plates

(described above) and inoculated into sulfide gradient media.

The oxygen concentration throughout one sample of sulfide-gradient media with an observable white layer of *Beggiatoa* was measured using an oxygen microelectrode (Diamond General, Ann Arbor, Michigan). Measurements were taken several days after inoculation of the sulfide-gradient media with BBD. The oxygen microelectrode was mounted on a micromanipulator, which allowed high spatial resolution (<100 μm) of the microelectrode location as it was lowered into the media tubes to measure oxygen concentration. A Keithley 485 autoranging picoammeter supplied polarization voltage and measured the output signal from the microelectrode. A two point calibrations was made (as in Carlton and Richardson, 1995) by immersing the microelectrode in seawater that had been sparged with nitrogen gas for 20 minutes (for anoxic reading) and in air-equilibrated seawater (21% O_2). One control sample of uninoculated gradient media and one experimental sample of sulfide-gradient media that had been inoculated with black band disease were measured. O_2 concentrations were adjusted for salinity and temperature.

RESULTS

Black Band Sulfate-Reducing Bacteria

Enrichment cultures of anaerobic, sulfate-reducing bacteria from the black band disease community turned black after approximately 2-3 days, indicating the presence and growth of sulfate-reducing bacteria, based on the precipitation of FeS. Plates inoculated from this culture appeared to be either: 1. a monoculture of slow-growing, small, black (diagnostic for sulfate-reducers on this media), round colonies, 2. a monoculture of unknown and faster growing clear shiny colonies, or 3. a combination of both. Best results were obtained using modified iron sulfate media in natural seawater. Black precipitate from sulfate reduction was observed 2-7 days after inoculation of the plates. The black colonies were selected and replated or inoculated into liquid media. Many colonies failed to grow or produce FeS precipitate in the isolation process.

Molecular Phylogeny of Black Band Sulfate-Reducing Bacteria

Sulfate-reducing bacteria were cultured from six coral colonies (Table 1). High molecular weight DNA was extracted (Figure 3) and fragments amplified via PCR (Figures 4a-4b). The 16S rDNA sequences of the cultures were analyzed. One sample collected from a BBD-infected *Diploria clivosa* from Picture Reef, BNP was cultured two separate, independent times (BBD-2, BBD-3.1) (Tables 1, 2). Both cultures were sequenced, one partially (BBD-2) and the other was cloned and fully sequenced (BBD-3.1)(Table 2). All cultures had homology in the range of 96-99% to *Desulfovibrio* sp. strain TBP-1 and *Desulfovibrio acrylicus,* 97-100% homologous to uncultured delta proteobacterium clone CD13B10 [cloned from BBD from Frias-Lopez *et al.* (2002)], and

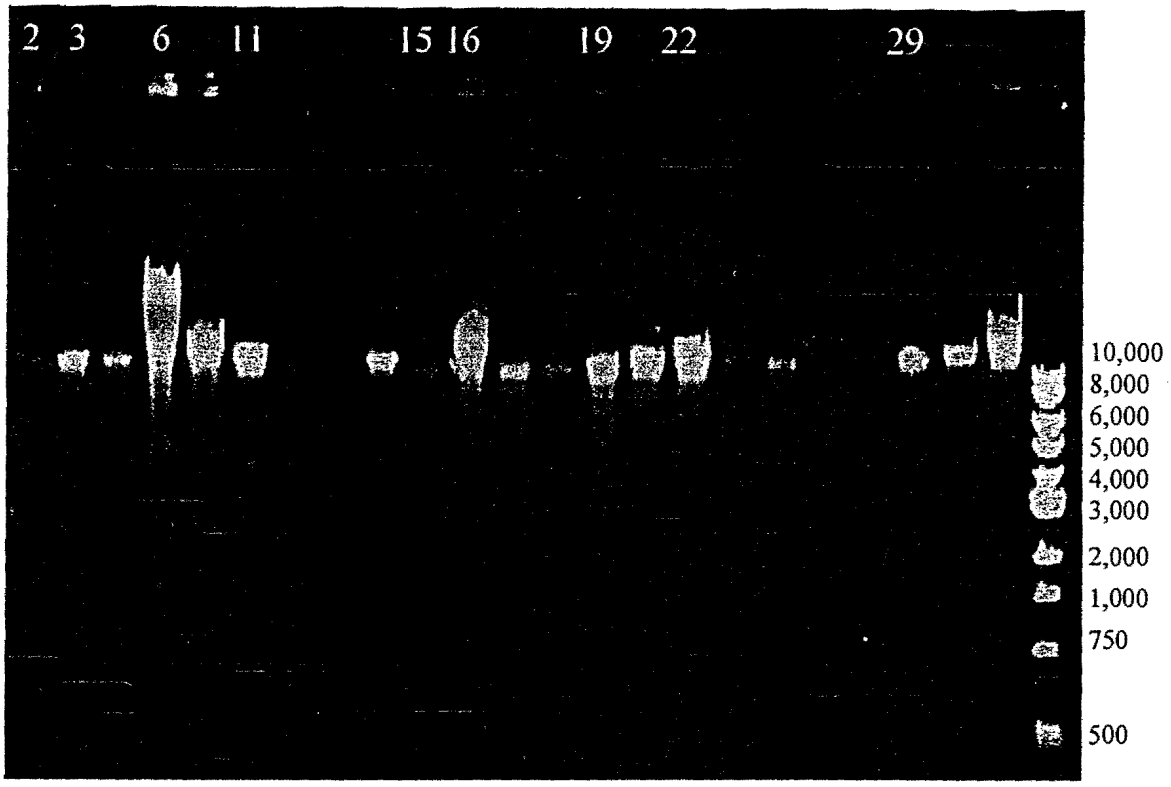


Figure 3: Photograph of high molecular weight DNA extracted from sulfate-reducing bacteria cultured from black band disease. Each vertical column that is labeled with sample numbers shows the high molecular weight DNA extracted from the particular sample. Only numbered columns contained high molecular weight DNA that was further amplified (by PCR) and sequenced. Columns that are not numbered were not sequenced. At the far right side, the Promega 1Kb ladder provides a size standard for size comparison of the extracted DNA.

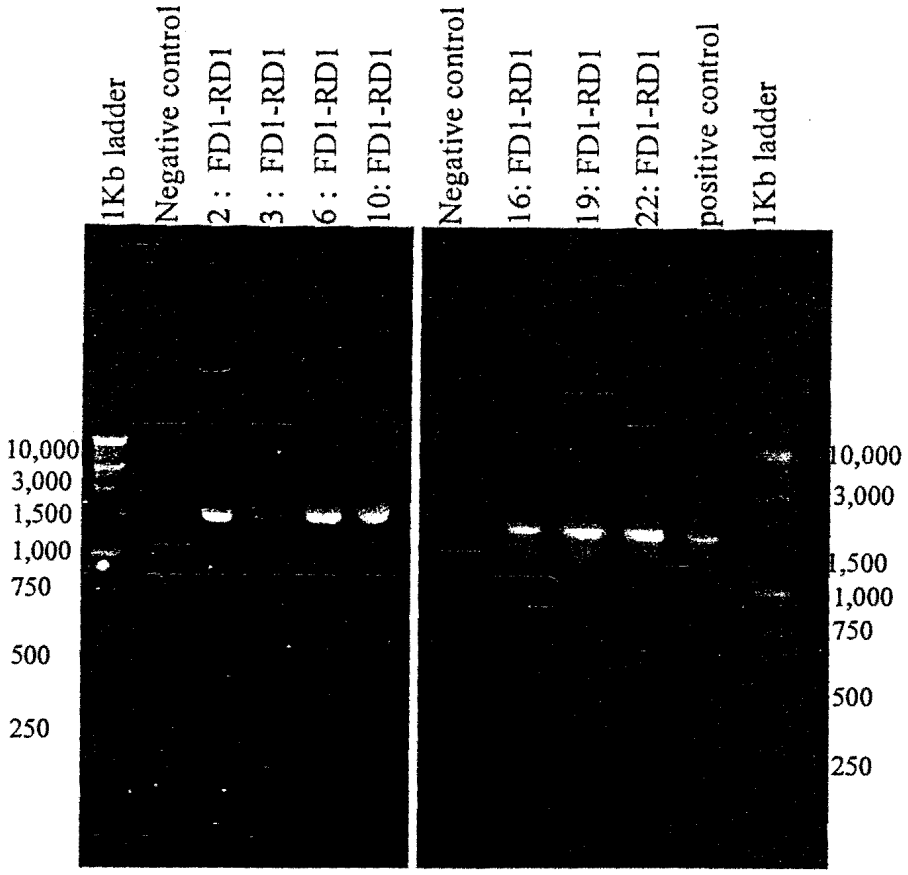


Figure 4a: Two agarose gels, stained with ethidium bromide, showing rDNA fragments that were amplified by PCR. Columns are labeled by culture number and by primers (top). Product size is confirmed against a Promega 1Kb ladder size standard (size standards given to right and left of gels). Positive and negative PCR controls are also indicated on the gel. Only cultures further sequenced are listed with sample numbers. Unidentified columns were not sequenced further. All fragments shown and labeled on these two gels were amplified using forward primer FD1 and reverse primer RD1. These two primers amplify nearly the entire 16s rDNA, resulting in a fragment of around 1500 base pairs.

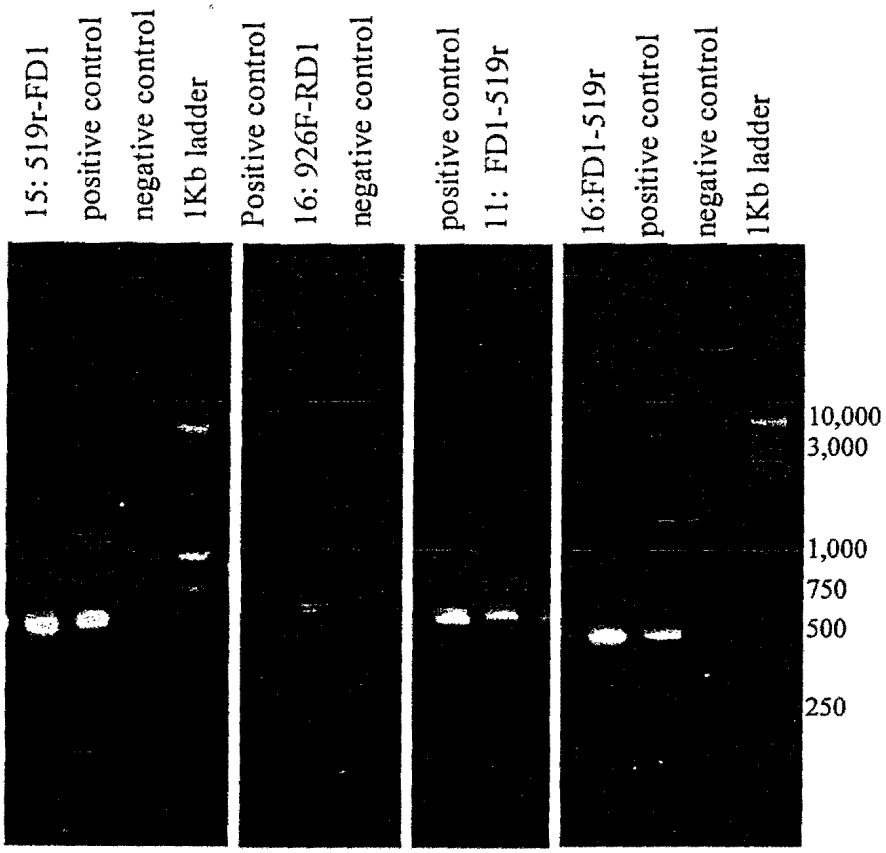


Figure 4b: Four agarose gels, stained with ethidium bromide, showing rDNA fragments that were amplified by PCR. Columns are labeled by culture identification number and by primers (top). The primer numbers indicate the expected size of the amplified fragment. This size is confirmed against a Promega 1Kb ladder size standard (standard values shown at left of gel group). Positive and negative PCR controls are also indicated on the gel.

Sample ID	Location	Infected coral species	Primer	Base pairs seq-uenced	Percent homology to other bacterial species
BBD-2	Picture reef, BNP	*Diploria clivosa*	FD1	487	96-97% *Desulfovibrio* sp. strain TBP-1
			RD1	364	97% Uncult. delta proteobact. clone CD13B10*
					97% Uncult. delta proteobact. clone CD12A12*
					96-97% *Desulfovibrio acrylicus*
BBD-3.1**	Picture reef, BNP	same infection	various	1476	99% *Desulfovibrio* sp. strain TBP-1
					98% *Desulfovibrio acrylicus*
					98% *Desulfovibrio* sp. PCP-1
BBD-6	Picture reef, BNP	*D. strigosa*	FD1	486	97-99% *Desulfovibrio* sp. strain TBP-1
			FD1	510	97-99% *Desulfovibrio acrylicus*
			RD1	432	98-99% Uncult. delta proteobact. clone CD13B10*
			RD1	420	97-99% Uncult. delta proteobact. clone CD12A12*
BBD-10	Dominica	*Siderastrea siderea* #3	FD1	211	96-99% *Desulfovibrio* sp. strain TBP-1
			RD1	407	95-98% *Desulfovibrio acrylicus*
					99% Uncult. delta proteobact. clone CD13B10*
					99% Uncult. delta proteobact. clone CD12A12*
BBD-11	Dominica	same infection	FD1	560	96-99% *Desulfovibrio* sp. strain TBP-1
			519R	421	95-98% *Desulfovibrio acrylicus*
BBD-11.1**	Dominica	same infection	various	1517	90% *Clostridium acidiurici*
					91% Anaerobic thermophile MV 1087
					90% *Acetanaerobacter thermotolerans*
					91% *Thermotolerans berrensis*
BBD-11.2**	Dominica	same infection	various	1536	99% *Desulfovibrio* sp. TBP-1
					98% *Desulfovibrio acrylicus*
					98% *Desulfovibrio* sp. PCP-1
BBD-13.1**	Dominica	*S. siderea* #5	M13	679	96% *Desulfovibrio* sp. TBP-1
			FD1		95% *Desulfovibrio acrylicus*
					93% *Desulfovibrio* sp. D1
BBD-13.2**	Dominica	same infection	various	1513	98% *Propionigenium maris* strain DSL-1
					98% *Propionigenium maris* strain ML-1
					95% *Propionigemium modestum*
BBD-15	Dominica	same infection	519R	367	94% Delta proteobacterium RS64 *
					94% *Desulfovibrio acrylicus*
					94% *Desulfovibrio* sp. strain TBP-1
					94% *Desulfovibrio* sp. D1
BBD-16	Dominica	*S. siderea* #6	FD1	471	98-99% Uncult. delta proteobact. clone CD13B10*
			RD1	533	98-99% Uncult. delta proteobact. clone CD12A12*
			519R	238	96-99% *Desulfovibrio* sp. strain TBP-1
			926F	293	96-99% *Desulfovibrio acrylicus*
BBD-19	Dominica	same infection	RD1	664	98% Uncult. delta proteobact. clone CD13B10*
			RD1	388	97-98% Uncult. delta proteobact. clone CD12A12*
					97% *Desulfovibrio* sp. strain TBP-1
					97-98% *Desulfovibrio acrylicus*
					97% *Desulfovibrio* sp. PCP-1
BBD-22	Algae Reef, FKNMS	*Diploria labyrinthiformes*	RD1	398	98% *Arcobacter* sp. LA31B
			RD1	517	95-97% *Arcobacter* sp. clone A3b2
			FD1	241	98% Uncult. delta proteobact. clone CD13B10*
					98% Uncult. delta proteobact. clone CD12A12*
					98% *Desulfovibrio* sp. strain TBP-1
					98% *Desulfovibrio acrylicus*

Table 2: Ten sulfate-reducing bacteria culture sequences. Sample identification numbers are shown with sample collection information (as in Table 1). The cultures are further described by sequencing primers used (giving approximate location on 16s rRNA, refer to Methods) and length of the sequenced fragment. The far right column gives the range of highest species homology between the sample fragments from this study and from GenBank. The percent homology is given as a range because one sample could have multiple 16S rDNA sequence fragments (from different primers used in sequencing) in different locations on the 16S rRNA.

† indicates the full or partial sequence was cloned (rather than sequenced directly from the PCR product).

* indicates that particular homologous species was found in other black band disease infections (Cooney *et al.* 2002; Frias-Lopez *et al.* 2002).

97-99% homologous to uncultured delta proteobacterium clone CD12A12 [cloned from BBD from Frias-Lopez *et al.* (2002)](Table 2). A culture from another BBD sample from Picture reef, from an infected *Diploria strigosa* (BBD-6) was partially sequenced using primers FD1 and RD1, resulting in fragments from 420-510 bases in length on both the 3′ and 5′ ends of the 16S rRNA. These fragments ranged from 97-99% homology to *Desulfovibrio* sp. strain TBP-1, *D. acrylicus,* and to uncultured delta proteobacterium clone CD12A12 [cloned from BBD from Frias-Lopez *et al.* (2002)], and 98-99% homology to uncultured delta proteobacterium clone CD13B10 [cloned from BBD from Frias-Lopez *et al.* (2002)].

Samples of black band disease infecting *Siderastrea siderea* colonies in Dominica were also cultured for sulfate-reducing bacteria. A sample from one of these infections (on *S. siderea* colony 3, Table 1) was cultured two separate and independent times. One of these cultures (BBD-10, Table 1) was sequenced using FD1 and RD1 primers, giving fragments on both the 3′ and 5′ ends of the 16S rDNA between 211 and 407 bases in length (Table 2). These fragments had 96-99% homology to *Desulfovibrio* sp. strain TBP-1, 95-98% homology to *Desulfovibrio acrylicus*, and 99% homology to uncultured delta proteobacteria clones CD12A12 and CD13B10 [cloned from BBD from Frias-Lopez *et al.* (2002)](Table 2). A separate culture (BBD-11) from the same *S. siderea* BBD infection (*S. siderea* colony 3, Table 1) was sequenced partially using FD1 and 519R primers. DNA from this culture was also cloned. Two separate clone colonies from this sample were sequenced fully (BBD-11.1, BBD-11.2) (Table 2). The partial sequences, obtained directly from the PCR product (BBD-11.2) had 96-99% homology to *Desulfovibrio* sp. strain TBP-1 and 95-98% homology to *Desulfovibrio acrylicus* (Table

2). A full 16S rDNA sequence from one of the clone colonies (BBD-11.1) was only 91% homologous to an anerobic thermophile MV1087 and *Thermotolerans berrensis* but only 90% homologous to *Clostridium acidiurici* and *Acetanaerobacter thermotolerans* (Table 2). These homologies are lower than the threshold of 97.5% (Stackebrandt and Goebel, 1994), indicating it to be a different species. A full sequence from the other cloned colony (BBD-11.2) from the same culture and infection was 99% homologous to *Desulfovibrio* sp. strain TBP-1, and 98% homology to *D. acrylicus and Desulfovibrio* sp. PCP-1 (Table 2). This level of homology is high enough for identification purposes (Stackebrandt and Goebel, 1994).

A BBD infection from a different *S. siderea* colony (colony #5) in Dominica was also cultured for sulfate-reducing bacteria two separate (Table 1). One of these cultures was cloned, and two of these clone colonies were sequenced (BBD-13.1, BBD-13.2). BBD-13.1 (679 base pairs) showed 96% homology to *Desulfovibrio* sp. TBP-1, 95% homology to *D. acrylicus* and 93% homology to *Desulfovibrio* sp. D1 (Table 2). The other clone, fully sequenced (BBD-13.2) was 98% homologous to *Propionigenium maris* DSL-1 and ML-1 (Table 2). This level of homology is over the 97.5% required for identification purposes (Stackebrandt and Goebel, 1994). The other culture from the same *S. siderea* BBD infection was partially sequenced, directly from the PCR product, using primer 519R (Tables 1,2). This sequenced culture (BBD-15), with a fragment length of 367 base pairs, was 96% homologous to delta proteobacterium RS15, but only 94% homologous to *Desulfovibrio acrylicus, Desulfovibrio* sp. strain TBP-1, and *Desulfovibrio* sp. D1 (Table 2). Although this level of homology is too low for conclusive identification purposes, it is likely that this sample is in the genus

Desulfovibrio.

Yet another *S. siderea* BBD infection in Dominica (Table 1) was sampled once and cultured two separate times (Table 1). 16S rDNA from both cultures was partially sequenced. One of the cultures (BBD-16) was sequenced with four primers: FD1, RD1, 519R and 926F (Table 2), resulting in fragments ranging between 238 and 471 bases in length. These fragments were 98-99% homologous to uncultured delta proteobacteria clones CD12A12 and CD13B10 [cloned from BBD from Frias-Lopez *et al.* (2002)], and 96-99% similarity to *Desulfovibrio acrylicus* and *Desulfovibrio* sp. strain TBP-1 (Table 2). The other culture (BBD-19) (Table 2) was sequenced twice with the primer RD1, resulting in fragments 388-664 bases in length. These fragments were 98% homologous to uncultured delta proteobacterium clone CD12A12 [cloned from BBD from Frias-Lopez *et al.* (2002)], 97-98% homologous to *Desulfovibrio acrylicus,* and 97% homologous to *Desulfovibrio* sp. strain TBP-1 and *Desulfovibrio* sp. PCP-1 (Table 2). Such similar homology indicates that both cultures from the same *S. siderea* infection are likely the same bacterial species.

Sulfate-reducing bacteria from a black band disease infection on *D. labyrinthiformes* on Algae Reef, FKNMS (BBD-22)(Table 1) was partially sequenced using the primers RD1 and FD1. Fragment lengths were between 241 and 517 bases (Table 2). Despite the fact that these sequence fragments were from the same DNA extracted from the same culture, homologous species varied considerably. Two fragments were 98% homologous to *Arcobacter* sp. LA31B (Table 2). The other fragment (primer FD1) was 98% homologous to *Desulfovibrio* sp. strain TBP-1, *D. acrylicus,* and uncultured delta proteobacteria clones CD12A12 and CD13B10 [cloned

from BBD from Frias-Lopez *et al.* (2002)](Table 2). These results, combined with the background noise in the sequencing electropherogram, indicated that DNA from more than one organism may have been amplified. However, for each sequence fragment, only one set of DNA was dominant and readable on the sequence electropherogram. Although the fragment homologous to *Desulfovibrio* sp. strain TBP-1, *D. acrylicus,* and uncultured delta proteobacteria clones CD12A12 and CD13B10 [cloned from BBD from Frias-Lopez *et al.* (2002)](Table 2) was very short, the organisms to which it is homologous (and the degree of homology) are consistent with fragments sequenced from SRB from other BBD infections and reefs.

When DNA sequencing was done directly from the PCR product, the results were variable, often showing multiple sequence output on the electropherogram, although only one appeared dominant. Unreadable or ambiguous electropherogram sequences were not included in these results. This ambiguity indicated that DNA from multiple organisms may have been amplified. Because amplified fragments from multiple organisms were likely of similar length (due to amplification by the same primer set), the size difference was not visible on ethidium bromide-stained gels (Figures 3a-b). Indeed, cloning reactions confirmed multiple species to be present in the same SRB cultures (see below).

The fragments amplified and sequenced were from different 16S rRNA locations, containing different 16S rRNA variable regions. Location variability of the sequenced fragments explains in part the variation in homology to known species between different fragments of one sample. 16S rDNA fragments, from the same bacterial species, yet containing different variable regions, would have different levels of homology in a comparison to a full 16S rDNA sequence. Variability in fragment length adds a degree of

uncertainty to homology to known bacterial sequences.

Clones from previous molecular survey studies (Frias-Lopez *et al.*, 2002; Cooney *et al.*, 2002) were only partially sequenced. The locations (on the 16S rRNA) of sequence fragments from the Cooney *et al.* (2002) and Frias-Lopez *et al.* (2002) studies did not always overlap with the locations of fragments sequenced in this study. For example, a sulfate-reducing bacterium from a BBD-infected *S. siderea* (BBD-15) from Dominica was sequenced using primer 519R (thus sequencing the 5′ end), and thus was not comparable to the sequence fragments from (of the 3′ end) uncultured delta proteobacteria CD12A12 and CD13B10, which were cloned from BBD by Frias-Lopez *et al.* (2002). Therefore, the sample from this study was not homologous to the bacteria found in black band disease by Frias-Lopez *et al.* (2002), even though all three fragments were highly homologous to *Desulfovibrio* sp. TBP-1 and *D. acrylicus.*

The *Desulfovibrio* found in black band disease infections in this study and in studies by Cooney *et al.* (2002) and Frias-Lopez *et al.* (2002) were compared to *Desulfovibrio* sequences in GenBank. *Desulfovibrio* spp. found on the dead coral behind the black band disease infection (Frias-Lopez *et al.* 2002) were also included in this comparison, as were other sulfate-reducing bacteria found both in the BBD (Cooney *et al.* 2002; Frias-Lopez *et al.* 2002) and on the dead coral behind the disease infection Non-disease (Frias-Lopez *et al.* 2002). These bacterial sequences were also compared phylogenetically (Figure 5).

The uncultured bacteria and sulfate-reducing bacteria, and some *Desulfovibrio* spp. that were included in the alignment were homologous to species found in molecular

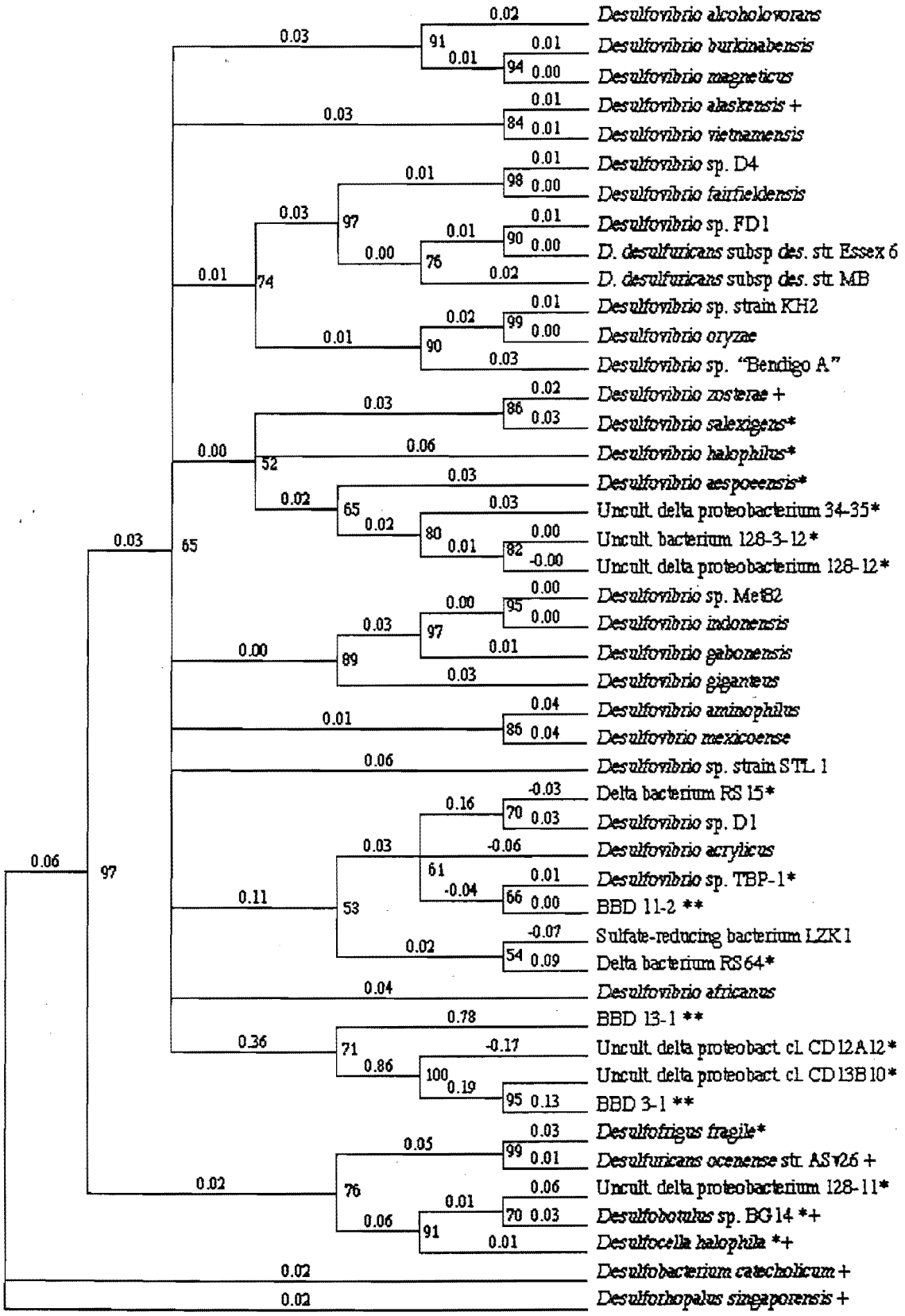

Desulfovibrio alcoholovorans
Desulfovibrio burkinabensis
Desulfovibrio magneticus
Desulfovibrio alaskensis +
Desulfovibrio vietnamensis
Desulfovibrio sp. D4
Desulfovibrio fairfieldensis
Desulfovibrio sp. FD1
D. desulfuricans subsp des. str. Essex 6
D. desulfuricans subsp des. str. MB
Desulfovibrio sp. strain KH2
Desulfovibrio oryzae
Desulfovibrio sp. "Bendigo A"
Desulfovibrio zosterae +
Desulfovibrio salexigens*
Desulfovibrio halophilus*
Desulfovibrio aespoeensis*
Uncult. delta proteobacterium 34-35*
Uncult. bacterium 128-3-12*
Uncult. delta proteobacterium 128-12*
Desulfovibrio sp. Met82
Desulfovibrio indonensis
Desulfovibrio gabonensis
Desulfovibrio giganteus
Desulfovibrio aminophilus
Desulfovbrio mexicoense
Desulfovibrio sp. strain STL 1
Delta bacterium RS 15*
Desulfovibrio sp. D1
Desulfovibrio acrylicus
Desulfovibrio sp. TBP-1*
BBD 11-2 **
Sulfate-reducing bacterium LZK1
Delta bacterium RS64*
Desulfovibrio africanus
BBD 13-1 **
Uncult. delta proteobact. cl. CD12A12*
Uncult. delta proteobact. cl. CD13B10*
BBD 3-1 **
Desulfofrigus fragile*
Desulfunicans ocenense str. ASV26 +
Uncult. delta proteobacterium 128-11*
Desulfobotulus sp. BG14 *+
Desulfocella halophila *+
Desulfobacterium catecholicum +
Desulfothopalus singaporensis +

Figure 5: Phylogenetic tree of *Desulfovibrio* from 1000 bootstrap replicates with Jukes-Cantor distance measure. *Desulfovibrio* sequenced in this study, *Desulfovibrio* spp. from the environment (GenBank), *Desulfovibrio* and other sulfate-reducing bacteria cloned from black band disease infections and from the dead coral skeleton behind black band disease infections are included. Numbers labeling branch points represent percentages of bootstrap support. Numbers along the tree branches indicate branch length.

** indicates sequences from SRB cultures of this study

* indicates bacteria from previous molecular studies on black band disease (Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002).

+ indicates bacteria sequenced from previous molecular studies on dead coral skeleton behind black band disease (Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002)

studies of black band disease (Frias-Lopez *et al.*, 2002; Cooney *et al.*, 2002). Only partial sequences were obtained in the molecular community surveys done by Cooney *et al.* (2002) and Frias-Lopez *et al.* (2002). Although sequences obtained in this study varied considerably in length, only cloned sequences were included in phylogenetic analysis. This avoided any potential bias from impure sulfate-reducing bacteria cultures that could contribute to misleading phylogenetic comparisons. In this way, comparisons could be made between cloned full or partial bacterial sequences (with regions overlapping partial sequences from other studies) from this study, partial bacterial sequences from community surveys by Cooney *et al.* (2002) and Frias-Lopez *et al.* (2002), and full and partial *Desulfovibrio* sequences from GenBank.

No phylogenetic relationship was apparent between the *Desulfovibrio* spp. found in black band disease (Figure 5). Bootstrap support values varied considerably between phylogenetic branchings, and the phylogenetic tree contained many unresolved polytomies. This indicates that some relationships are only weakly supported. Two disease samples from this study, BBD-3.1 and BBD 13.1 clustered with uncultured delta proteobacteria clones CD12A12 and CD13B10 (both cloned from black band disease infection on *Montastraea cavernosa* (Frias-Lopez *et al.*, 2002). This cluster had relatively high bootstrap support values (95-100%), although the relationship of BBD 2.1 with the other 3 strains was lower (71%). The other disease sample from this study, BBD 5.2, grouped with *Desulfovibrio* sp. strain TBP-1 and *D. acrylicus*. Bootstrap support for this grouping was low (61-66%), unexpected, given the high degree of sequence homology.

The phylogenetic relationships shown in the *Clostridium* tree are only weakly supported, with many unresolved polytomies and often low bootstrap support (Figure 6). No strong phylogenetic relationships were apparent between *Clostridium* homologous to bacteria cloned from other black band disease infections (Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002) or the unknown bacteria cloned in this study (BBD-11.1).

Light Migration

A distinctive pattern was present in the vertical position and migration of the dark red-colored *Phormidium corallyticum* and white colored *Beggiatoa* with varying light intensity (Table 3). Both *Beggiatoa* spp. and *Phormidium* were visibly present on the surface of the band (N=15) at relatively high (for the underwater reef environment) light levels of 191-308 $\mu E/m^2 \cdot sec$. At lower light levels (68-234 $\mu E/m^2 \cdot sec$), *Phormidium* was present on the surface of the band and there was no observable *Beggiatoa* (N=5). There was a slight overlap of light intensities where members of both populations were visible or where only *Phormidium* was present (Table 4). Microbial migration varied significantly ($z = -2.837$, $P = 0.005$) with light intensity.

Vertical migrations could easily be controlled by manipulating light intensity with neutral density filters. Figure 7 shows the surface of an *in situ* black band immediately after experimentally deployed neutral density screens were removed. The (control) left side of the band was not shaded during the experiment. It can be seen that white clumps of *Beggiatoa* are visible on the band surface. In contrast, the right side of the band (experimentally shaded) exhibits only the presence of *Phormidium corallyticum*, revealed by a noticeably darker band surface. The photograph was taken after the filters had been

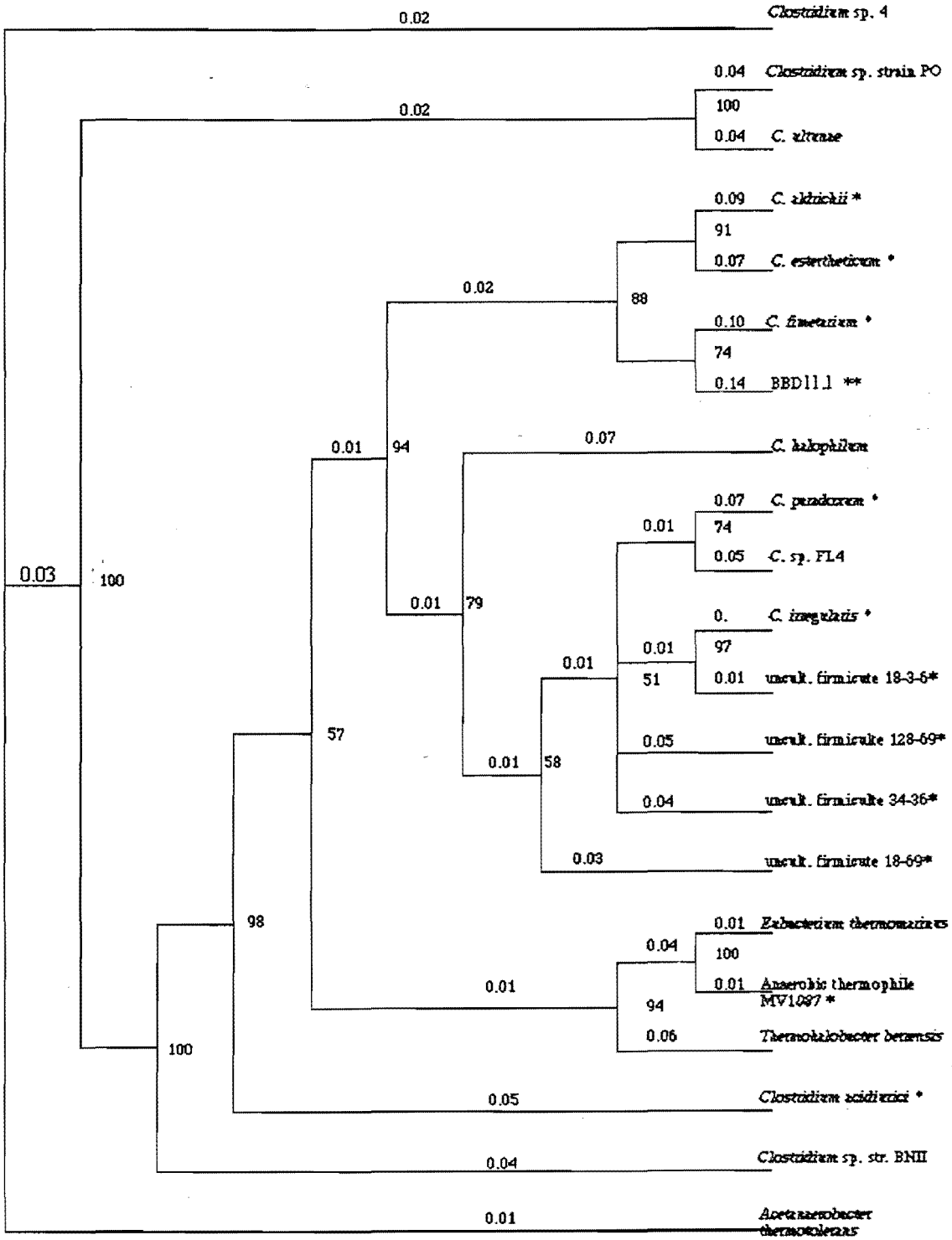

0.02
Clostridium sp. 4
0.04
Clostridium sp. strain PO
100
0.04
C. ultunense
0.02
0.09
C. aldrichii *
91
0.07
C. estertheticum *
0.02
88
0.10
C. fimetarium *
74
0.14
BBD11.1 **
0.01
94
0.07
C. kluyveri
0.07
C. pasteurianum *
0.01
74
0.05
C. sp. FL4
0.03
100
0.01
79
0.
C. irregularis *
0.01
97
0.01
51
0.01
uncult. firmicute 18-3-5*
0.05
uncult. firmicute 128-69*
57
0.01
58
0.04
uncult. firmicute 34-36*
0.03
uncult. firmicute 18-69*
0.01
Eubacterium thermoacetica
0.04
100
0.01
Anaerobic thermophile MV1097 *
0.01
94
0.06
Thermoanaerobacter brockii
98
100
0.05
Clostridium acidiurici *
0.04
Clostridium sp. str. BNII
0.01
Acetoanaerobacter thermocellanus

Figure 6 Phylogenetic tree of *Clostridium* from 1000 bootstrap replicates with Jukes-Cantor distance measure. The unidentified bacteria sequenced in this study, *Clostridium* spp. and other bacteria from the environment (GenBank), and *Clostridium* spp. and other bacteria cloned from black band disease infections and from the dead coral skeleton behind black band disease infections are included. Numbers labeling branch points represent percentages of bootstrap support. Numbers along tree branches indicate branch length. ** indicates sequences from cultures in this study (BBD-11.1).

* indicates bacteria from previous molecular studies on black band disease (Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002).

+ indicates bacteria sequenced from previous molecular studies on dead coral skeleton behind black band disease (Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002)

Light level ($\mu E/m^2 \cdot sec$)	Surface population	
	Beggiatoa	*Phormidium*
310	Both visible	
301	Both visible	
291	Both visible	
285	Both visible	
279	Both visible	
277	Both visible	
272	Both visible	
271	Both visible	
251	Both visible	
250	Both visible	
238	Both visible	
234	Not visible	Visible
232	Both visible	
231	Both visible	
231	Both visible	
224	Not visible	Visible
191	Both visible	
155	Not visible	Visible
138	Not visible	Visible
68	Not visible	Visible

Table 3: *In situ* light intensity and black band bacterial population visible on the band surface. Light data and corresponding observations of black band are presented with light intensity listed from high (top) to low (bottom). Data are included from the 20 bands investigated.

***In situ* light manipulation**	**Observed vertical migrations**
High to low light intensity	
From 231 to 155 $\mu E/m^2 \cdot sec$	*Beggiatoa* migrated down (viewed after 1 hr interval)
From 238 to 138 $\mu E/m^2 \cdot sec$	*Beggiatoa* migrated down (viewed after 2 hr interval)
Low to high intensity	
From 67 to 285 $\mu E/m^2 \cdot sec$	*Beggiatoa* migrated up (within seconds)

Table 4: Manipulation of *in situ* light levels and observations of the resulting vertical migration of *Phormidium corallyticum* and *Beggiatoa.*

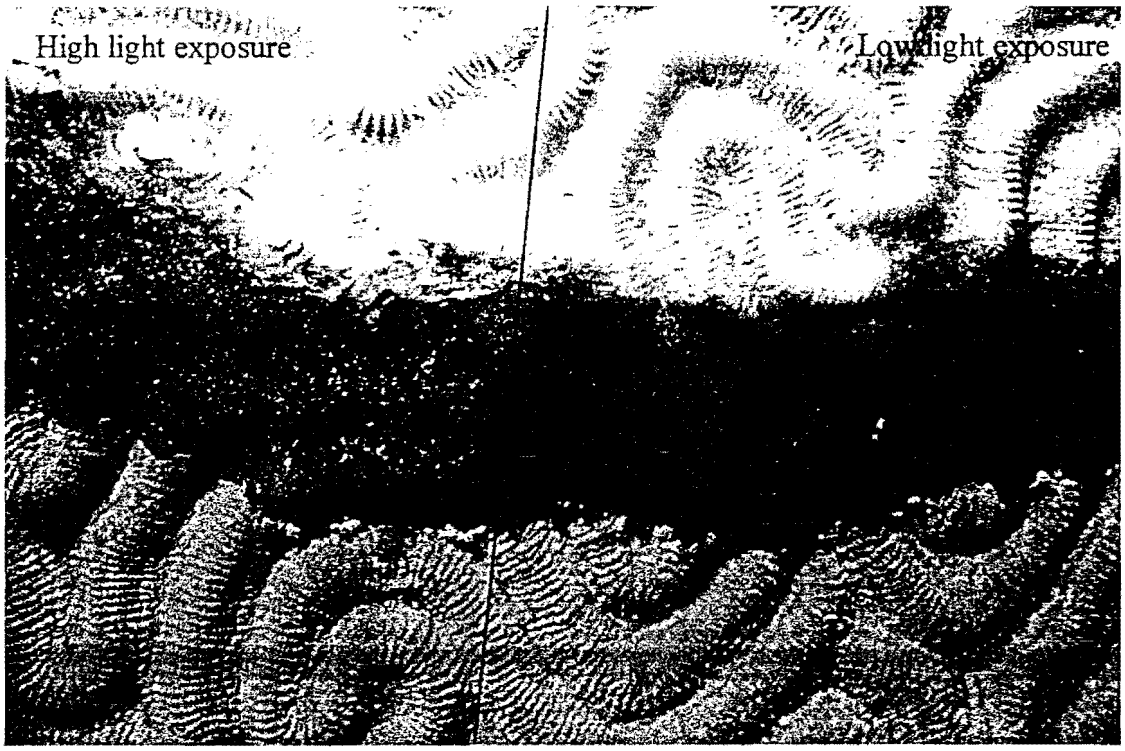


Figure 7: Vertical migration of *Phormidium corallyticum* and *Beggiatoa* in black band disease during experimental manipulation of *in situ* light intensity. The left side of the photo shows the disease band under natural (high) light. The right side of the photo shows the disease band after being shaded with four layers of neutral density screen (low light).

in place for approximately one hour. In experiments of this type, black band populations responded consistently with only dark-colored *Phormidium* present on the surface under low light and both *Phormidium* and the white flecks of *Beggiatoa* on the surface under high light.

Experimental manipulations of light intensity using neutral density screen resulted in microbial migrations that were consistent with the pattern described above, in which changes in light from low to high and high to low resulted in the appearance and disappearance of surface *Beggiatoa* respectively (Table 4). At lower light levels (68-234 $\mu E/m^2 \cdot sec$), *P. corallyticum* was present on the surface of the band with no observable *Beggiatoa* present (n=15). At higher light levels (191-308 $\mu E/m^2 \cdot sec$), both *Beggiatoa* spp. and *P. corallyticum* were present on the surface of the band (n=5). A slight overlap appeared between light intensities where either members of both populations were visible or where only *P. corallyticum* was visible (Table 3).

Microbial migration patterns visible during experimental light manipulations were also observable in the natural shading of disease infections due to the orientation of the infected colony relative to sunlight. As the sun angle changed and a naturally shaded portion of the band became exposed to sunlight, the appearance of the disease band would change in the same pattern as observed in experimental manipulations. For example, an early morning measurement was made in which one area of a band was blocked from direct sunlight as a result of shading by another part of the same coral colony. The light intensity illuminating the band surface under the shaded condition was $68 \mu E/m^2 \cdot sec$. Within the shadow, *Phormidium* covered the surface of the band, while an unshaded section of the band directly adjacent exhibited white *Beggiatoa* clumps on the

surface along with the *Phormidium*. Light intensity at this point was 285μE/m^2·sec. When the angle of the sun changed to fully illuminate the previously shaded section, white clumps of *Beggiatoa* began to appear immediately (within seconds).

Black Band *Beggiatoa* spp.

Although *Beggiatoa* spp. filaments did grow successfully on J2 media, they did not glide completely out of bacterial clumps and were overgrown and killed by unidentified vibrio-shaped bacteria. When incubated in constant darkness, BBD cyanobacteria usually died within 2-4 days. Without incubation in darkness, these cyanobacteria overgrew *Beggiatoa* filaments. Repeated attempts at single filament isolation aimed at yielding an axenic, clonal culture were unsuccessful. Some *Beggiatoa* single filaments did not multiply after being transferred to new media. *Beggiatoa* which appeared to be isolated failed to grow successfully. Agar media on both agar plates and in gradient tubes was often liquefied by unidentified agar-digesting heterotrophic bacteria. Media made with full strength seawater (2/3 NSW + 1/3 ASW) seemed to support more *Beggiatoa* spp. growth. Freshly collected disease samples were sent to the laboratory of Dr. Doug Nelson at UC Davis for isolation, where they were found to exist in a tight consortium with several species of vibrio-shaped bacteria.

Black Band *Beggiatoa* in Sulfide-Gradient Cultures

After several days of growth in the sulfide-gradient media, a visible white lens, presumably *Beggiatoa* based on morphology (light microscopy), had formed. The white *Beggiatoa* lens appears to have sharp border on both the top and the bottom (Figure 8). Such sharp boundaries appear similar to studies of other strains of *Beggiatoa* found in

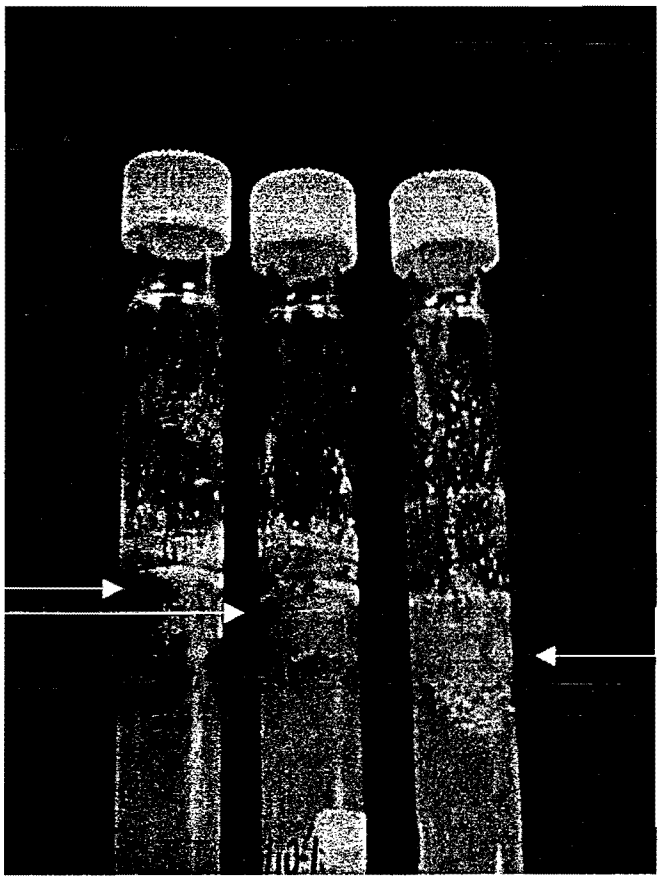


Figure 8: Three Hungate tubes prepared with sulfide-gradient media and inoculated with the black band disease microbial consortium. The thin white lens between the black layers indicates the presence of *Beggiatoa* spp.. This photo, along with the Figure 9 data show the *Beggiatoa* lens to be at the sulfide-oxygen gradient.

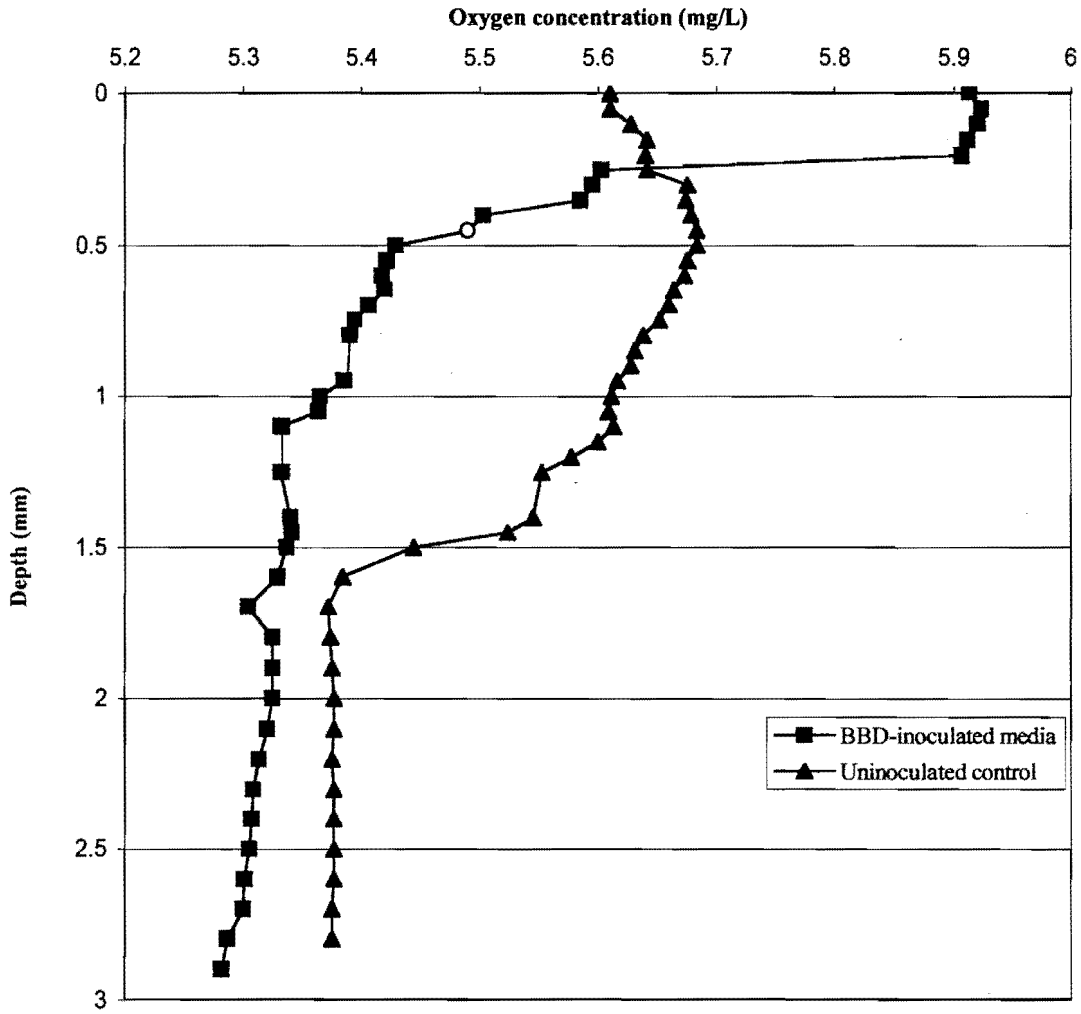


Figure 9: Microelectrode measurements of O_2 in sulfide-gradient media inoculated with *Beggiatoa* in the black band disease consortium (and uninoculated control). The open circle in the BBD-inoculated media data shows the location and oxygen concentration of the *Beggiatoa* lens in the sulfide-gradient media.

non-disease environments. The sharp upper boundary supports the step-up phobic response to oxygen demonstrated by *Beggiatoa* in other studies, and the sharp lower boundary which supports the apparent negative taxis to high sulfide concentrations has been proposed as a mechanism to help *Beggiatoa* position itself at the oxygen/sulfide interface (Møller *et al.*, 1985; Nelson *et al.*, 1986b). Oxygen microelectrode measurements showed the inoculated gradient media to have a characteristic signature distinctive to biological sulfide-oxidation (Figures 8, 9). The control sample showed gradually decreasing anoxia, indicative of chemical oxygen reduction (from sulfide diffusing up through the media). The sulfide-gradient media inoculated with black band disease showed very different pattern. The oxygen concentration dropped abruptly to anoxia, showing a high degree of biological oxygen reduction. The white *Beggiatoa* lens was located towards the bottom of this sharp decrease in oxygen concentration. This characteristic signature indicates that at least one organism is oxidizing sulfide. No adjustment was made for microelectrode drift, because the microelectrode became poisoned (and less sensitive) when immersed in highly sulfidic environments for a prolonged period of time.

DISCUSSION

Theories about the identity of the pathogen of black band disease have evolved through time along with the advancement of scientific techniques. Initially, this disease was thought to be caused by a single pathogen. Hypotheses on the identity of this pathogen focused on *Beggiatoa* spp. (Ducklow and Mitchell, 1979b) and the cyanobacterium *Phormidium corallyticum* (Rützler *et al.*, 1983). Further observation and experimentation showed that the disease was comprised of a pathogenic microbial consortium (Richardson, 1997), which agreed with the observation that a coral inoculated with the entire microbial consortium would develop black band disease (Rützler *et al.*, 1983). The complexity of the microbial community has recently been expanded using molecular techniques which have shown a high degree of microbial diversity and variability between infections (Frias-Lopez *et al.*, 2002; Cooney *et al.*, 2002). This high variability has led to further conjectures about a pathogen(s), related to microbial components common between infections (Frias-Lopez *et al.*, 2002). However, the molecular techniques used in these studies, while providing a broad survey of the microbial community, may not account for all bacteria present in low levels within the disease consortium.

Although molecular studies have elaborated on the microbial complexity of the disease consortium, the identity of primary species of cyanobacteria in the disease is still in question. The dark reddish-black color of the disease band has been attributed to high concentration of the pigment phycoerythrin in the cyanobacterium *Phormidium corallyticum* (Rützler and Santavy, 1983). The disease band is primarily composed of

cyanobacterial filaments, as shown by scanning electron microscopy (Frias-Lopez *et al.*, 2002). However, molecular community surveys identified only a small percentage (<4%) of cyanobacteria in the disease community (Frias-Lopez *et al.*, 2002), none of which were *Phormidium corallyticum*. *P. corallyticum* has not yet been detected by molecular methods in any of the microbial community studies (Frias-Lopez *et al.*, 2002; Cooney *et al.*, 2002).

The presence of a variety of heterotrophic bacteria in the black band disease consortium has been known for years (Ducklow and Mitchell, 1979a); however, only recently have any been identified. A wide variety of heterotrophic bacteria found within the disease band was documented by recent molecular surveys (Frias-Lopez *et al.*, 2002; Cooney *et al.*, 2002). None of the heterotrophic bacteria from the disease has been cultured to determine their potential contributions to chemical cycling within the disease band. As yet, the role of heterotrophic bacteria within black band disease remains unknown.

Sulfate-reducing bacteria

In this study of ten separate sulfate-reducing bacteria cultures from six different black band disease infections, three different reefs, and widely different locations (the Florida Keys and Dominica), all but two showed a high degree of species homology to *Desulfovibrio* sp. TBP-1 and *D. acrylicus* (Table 2). The other two SRB cultures, both from the same *S. siderea* infection in Dominica, were also homologous to *Desulfovibrio* TBP-1, but at a percentage under the species diagnostic threshold of 97.5% (Stackebrandt and Goebel, 1994). This is the third study to use molecular techniques aimed at

characterizing members of the black band microbial community (Frias-Lopez *et al.*, 2002; Cooney *et al.*, 2002). This study differed from the other two in that this study used enrichment cultures to select for sulfate-reducing bacteria from black band disease infections. Unlike the other two studies, this study determined that highly homologous species of *Desulfovibrio* could be cultured from different infections as well as different geographic areas.

Both Cooney *et al.* (2002) and Frias-Lopez *et al.* (2002) also included samples from healthy coral, and the water column as well as from BBD; Frias-Lopez *et al.* (2002) also included samples from dead coral skeleton behind the disease band. *Desulfovibrio* sp. TBP-1 was identified by Frias-Lopez *et al.* (2002) to be present in one black band disease infection as well as on the dead coral surface of *Montastraea cavernosa*, one of three infected coral species sampled in the Curaçao. One BBD infection yielded two separate similar clones from their clone library. Although the methods used preclude the quantification of the amount of any type of bacteria present, two separate clones from one sample roughly indicates the potential for more than a minimal presence of this SRB species in the disease band. The*Desulfovibrio* sp. TBP-1 clones were some of the few clones with more than 97.5% homology to *Desulfovibrio* spp..

While microbial culture vastly underestimates microbial community diversity (Furhman and Campbell, 1998) and possibly as few as 1% or less of bacteria types found in seawater can be cultured (Hicks *et al.*, 1992), the selective culturing techniques used in this study may have amplified growth of *Desulfovibrio* spp. naturally found in infections at background levels not necessarily detectable by limited molecular survey techniques. The presence of a species with such a high degree of homology to *Desulfovibrio* sp. TBP-

1 over such a wide geographic range, in both Florida Keys cultures and in Dominican cultures, as well as in a sample from Curaçao (Frias-Lopez *et al.*, 2002), implies that such a species may be common to black band disease infections.

Microbial community surveys determined the presence of bacterial species with varying degrees of homology to *Desulfovibrio* sp. strain TBP-1, *D. salexigens, D. aespoeensis* (Frias-Lopez *et al.*, 2002), uncultured delta bacterium 128-11, *Desulfovibrio* sp. PCP1, *Desulfovibrio* sp. LA1.2, *Desulfovibrio* sp. LA1.3, and *Desulfovibrio* sp. PR1.2 (Cooney *et al.*, 2002), in the disease band and to *D. zosterae, Desulfovibrio* sp. TBP-1, *D. aespoeensis, D. alaskensis* on the dead coral skeleton behind the disease band (Frias-Lopez *et al.*, 2002). Multiple *Desulfovibrio* species were found in one disease infection, or one sample of the dead coral skeleton behind the disease band. No single *Desulfovibrio* species was found on more than one disease infection, although multiple copies of the same *Desulfovibrio* species were cloned per infection. This mass-cloning technique does not allow precise quantification of relative species presence within the mat, due of the inherent bias of potential under- or over-estimation by PCR of DNA from species with multiple copies present (Farrelly *et al.*, 1992; Suzuki *et al.*, 1998). Thus, similar *Desulfovibrio* species may have been present, but not detected, in the three other disease infections studied by Frias-Lopez *et al.* (2002). The *Desulfovibrio* spp. detected in this study from microbial cultures may have been present in low levels in the three other disease infections studied in Curaçao by Frias-Lopez *et al.* (2002) and three disease infections in St. Croix studied by Cooney *et al.* (2002).

An alternative explanation is that *Desulfovibrio* TBP-1 and *D. acrylicus,* both of which were sequenced from samples of marine or estuarine sediment (van der Maarel *et*

al., 1996) may be ubiquitous to marine sediments. Frias-Lopez *et al.* (2002) found *Desulfovibrio* TBP-1 in one out of three samples of the sediment on the dead coral surface behind the disease band. Indeed, the cyanobacterium *Phormidium corallyticum* and sulfide-oxidizing bacterium *Beggiatoa* have been identified in sediment patches on healthy coral, suggesting sediment patches to be a BBD reservoir (Richardson, 1997). A molecular community comparison between sediment patches and BBD infections is a direction for future research.

This study did not show any phylogenetic clustering between *Desulfovibrio* species unrelated to any disease and between *Desulfovibrio* spp. either found in black band disease or highly homologous to species found in black band disease. In addition, no relationships were shown between *Desulfovibrio* species from black band disease and coral species infected. Two *Desulfovibrio* species cultured in this study, BBD-3.1 and BBD-13.1 were highly related to two clones from a BBD infection on a *Montastraea cavernosa* in Curacao (Frias-Lopez *et al.*, 2002). However, this group clustered separately (although via an unresolved polytomy) from the cluster including *Desulfovibrio acrylicus, Desulfovibrio* sp. D1, *Desulfovibrio* sp. strain TBP-1, BBD 5.2 (from this study), and delta proteobacterium RS15 (non-disease related), despite high degrees of sequence homology. *Desulfovibrio* sp. TBP-1 and *D. acrylicus* share 98.9% sequence homology to each other; however, these two species have different physiological characteristics, thus highlighting the limitations of exclusive use of 16S rRNA sequences for identification.

One reason for the contrast between high levels of species homology and low levels of bootstrap support could be the inherent bias of the Jukes-Cantor distance

analysis. This analysis has the underlying assumption that all bases are equally likely to vary at the same rate (Gillespie, 1986). Given this, phylogenetic bootstrap support and evolutionary branch lengths are likely overestimates of actual values. Parsimony-based methods also underestimate rate variation (Yang, 1996). None of the phylogenetic analyses of *Desulfovibrio* spp. (Devereux *et al.*, 1990; Rooney-Varga *et al.*, 1998; Castro *et al.*, 2000) appear to have taken into account rate variation of specific base sites with regard to rRNA structure, even though such variation considerably influences evolution (Yang 1996).

Desulfovibrio cultures used in this study were able to use lactate as a carbon source. Further studies on carbon source utilization patterns were restricted due to the culture impurity. The wide variety of carbon sources potentially used by the different *Desulfovibrio* species present in the disease consortium support the idea that decaying coral tissue under the encroaching edge of the disease band provides carbon sources for the microbial components of the band. Presumably the lysing coral tissue underlying the disease provides a sufficient organic supply for sulfate reduction. In anoxic environments, the influx of organic matter can be a controlling factor for the intensity of sulfate reduction (Jørgensen, 1982a). Further investigations of the SRB within black band disease may provide insight into the vertical migration of the band across the coral surface.

Other bacteria

A bacterium with 98% homology to *Propionigenium maris* was cloned and sequenced from a BBD infection on *Siderastrea siderea* (culture 6) from Dominica. The

lack of detection of *P. maris* in other cultures in this study does not negate the possibility this bacteria could have been present in other sampled disease infections; it simply reflects the bias inherent in selective culture techniques. Cooney *et al.* (2002) detected the presence of *P. maris* as well from non-diseased *Diploria strigosa* and *Montastraea annularis*. *P. maris* is a non-motile, fermentative, obligate anaerobe. The type strains were isolated from marine hemichordate and polychaete burrows (Watson *et al.*, 2000). Strains can ferment pyruvate and/or glucose and succinate, but cannot use inorganic terminal electron acceptors. This information about the type species shows that *P. maris*, as a naturally-occurring bacterium associated with marine sediments, can also be affiliated with black band disease.

Although the culture 5 (BBD-11.1) matched with low homology to *Clostridium acidiurici*, it appeared to be more related to *Clostridium firmetarium*. *C. firmetarium* was also homologous to a *Clostridium* species cloned from the black band disease consortium by Frias-Lopez (2002). The variable levels of bootstrap support and branch lengths do not support a close relationship between the *Clostridium* species homologous to clones from black band disease (Figure 6).

Although *Beggiatoa* has been identified to be part of black band disease for many years, and has even been proposed as a potential pathogen in the disease (Ducklow and Mitchell, 1979b), it has yet to be identified by molecular methods. Two recent microbial community surveys (Frias-Lopez *et al.*, 2002; Cooney *et al.*, 2002) did not detect any *Beggiatoa* spp. within the six disease bands sampled between the two studies. Although isolation of *Beggiatoa* spp. was not successful, bacterial species matching the physiology of *Beggiatoa* spp. were cultured repeatedly from multiple disease infections. *Beggiatoa*

spp. could not, however, be cultured from every black band disease infection sampled. The amounts of *Beggiatoa* in BBD infections could be highly variable.

Multiple efforts to isolate both *Desulfovibrio* and *Beggiatoa* spp. did not yield axenic cultures. This could be indicative of a tight consortium between specific bacteria. This type of bacterial consortium is consistent with non-disease microbial communities. For example, in fixed film growth systems, *Beggiatoa* enrichment was apparently dependent on the presence of SRB, such as *Desulfovibrio* spp. (Chung and Strom, 1997).

Microbial Migration

Black band disease appears to exhibit a unique microbial migration pattern of sulfide-oxidizing *Beggiatoa* spp. and the cyanobacterium *Phormidium corallyticum*. *Beggiatoa* is found on the surface of the mat during high light intensities but not during lower light levels. The dark colored *P. corallyticum* population is thus present alongside and below *Beggiatoa* during high light intensities, a very unusual pattern compared to other microbial mat communities. In other microbial mat systems (non-disease related), both *Beggiatoa* and migrating cyanobacteria move downward into the mat and neither is exposed to the surface environment of high light.

Variations in light are important migration cues for both *P. corallyticum* and *Beggiatoa*. Natural light variation in the coral reef environment can be dramatic due to the clarity and shallow depth of the waters. At shallow depths, black band disease infections can be exposed to wide ranges of light intensity (Kuta, 2000). *In situ* photosynthetic photon flux densities (photosynthetically active radiation) have been measured near the surface of black band disease that range from <100 (cloudy days, early

and late hours, etc.) to >2200μE m^{-2} s^{-1} on the brightest days on shallow reefs (Carlton and Richardson 1995). A correlation between shallow water and black band disease incidence has been documented by many researchers (Antonius, 1973; Antonius, 1981; Rützler *et al.*, 1983).

Light intensity appears to directly affect the microbial vertical migration pattern found in black band disease. At very low light intensity, *Phormidium corallyticum* dominates the surface microbial community. When light intensity increases to above 190-234 μE/m^2·sec, both types of bacteria are seen on the band surface. At higher light levels, either *P. corallyticum* moves down into the band, or *Beggiatoa* moves up to the surface. Similarly, when light levels fall from this range to below (or near the bottom) of the range, either *Beggiatoa* moves down or *Phormidium* moves up.

The vertical movement of *P. corallyticum* into the disease band as light intensity increases could be explained by the well-known cyanobacterial photophobic behavior in which populations migrate down into sediments or microbial mats with high light (Garcia-Pichel *et al.* 1994). Some *Phormidium* remains visible on the surface, independent of light levels, giving the black band its characteristic dark appearance. This may be due to the thinness of the microbial mat (<1 mm) giving no place for the population to migrate down into; directly beneath the band is hard calcium carbonate coral skeleton. The migration response thus could be a combination of step-up photophobotaxis and negative phototaxis. In this manner, the population could move down as far as possible into the band and exhibit continual movement within this limited space – as filaments are attempting to move down, others are exposed to the high light, which will then cause these filaments to move to the center of the mat. Such self-shading

behavior is well documented for many naturally occurring populations of cyanobacteria (Castenholz 1982).

In situ observations on the position of *Phormidium* in relation to light intensity are consistent with laboratory studies on the photosynthetic capabilities of *P. corallyticum.* (Rützler *et al.* 1983) found *Phormidium* cultures to have good growth at 1% and 40% of full sunlight intensity. Kuta (2000) demonstrated that in culture this cyanobacterium has an extremely low threshold for attaining maximum photosynthesis (Pmax), at approximately 29$\mu E/m^2 \cdot sec$. Such a low Pmax threshold, coupled with the motility pattern of black band *P. corallyticum,* is also consistent with other studies demonstrating that some gliding filamentous cyanobacteria can physically migrate up and down in microbial mats to attain the light level optimum for photosynthesis yet avoid photosynthesis-saturating intensities (Garcia-Pichel *et al.* 1994).

Whereas the *Phormidium* migration may be explained in terms of what is known about the physiology of this species and cyanobacterial motility responses in general, the underlying reasons for the migration of black band *Beggiatoa* is more elusive. *Beggiatoa* are present on the surface of black band in an environment that is highly aerobic as well as highly illuminated, a microenvironment that is unusual for this genus. In addition to negative aerotaxis and negative phototaxis, a negative taxis to high sulfide concentrations has been proposed as a mechanism to help *Beggiatoa* position itself at the oxygen/sulfide interface (Nelson *et al.*, 1986b). A study with BBD-inoculated sulfide-gradient cultures (under fluorescent light) showed a white lens (components of which matched the physiology of *Beggiatoa* spp.) located at the sulfide-oxygen interval (Figures 8, 9), and apparently contributing to biological oxidation of sulfide. Perhaps in the case of black

band, the sulfide negative tactic response overrides the negative response to the other two (light and oxygen). Sulfide concentrations have been measured up to 800μM within black band at a depth of only a few hundred μms (Carlton and Richardson, 1995). Alternatively, perhaps other microorganisms within the band are exhibiting an influence on the migrating microorganisms that has yet to be discovered. An understanding of the *Beggiatoa* migration will require further study into the metabolic properties and tolerances of black band *Beggiatoa* spp..

Several promising lines of research arose from this work that warrants further study. It remains unknown if the microbial vertical migration pattern observed in black band is due to overriding responses by *Phormidium,* by *Beggiatoa,* a combination of both, or is influenced by other, yet unidentified microbes. The negative photic response of the cyanobacterium may override the negative photic response of the *Beggiatoa*. Or, black band *Beggiatoa* may be more sensitive to sulfide under high light. Without knowing the light regime within and towards the lower part of the band, it is difficult to tell whether light, sulfide, or oxygen provides the overriding migration cues. A direction for future study may involve insertion of light microprobes into the band to investigate whether reflected light inside the band was as bright as at the surface of the band. Further studies will require experimental manipulations designed to separate the movements and cues of one population from the movement and cues of the other. This will require laboratory experiments with individual cultures of each. Also required are further studies on the metabolism of these species. Further information on the motility cues and physiology of these organisms will lead to an increased understanding of how the black band microbial consortium can sustain such a highly structured microbial community.

Light levels clearly influence vertical migrations within the black band disease microbial community. Further research could address which wavelength(s) in light is stimulating the microbial migrations in black band disease. For example, UV wavelengths may be an important cue within the light spectrum. On disease infections in shallow clear waters, ultraviolet light levels would be high. It has been conjectured that, in microbial mats, photosynthesis is particularly vulnerable to UV photodamage (Garcia-Pichel and Castenholz, 1994). UV-A has been shown to act as an environmental cue to promote downward cyanobacterial migrations (Garcia-Pichel and Castenholz, 1994; Kruschel and Castenholz, 1998) and UV-B impairs motility in cyanobacteria (Donkor and Häder, 1991). In thermal mats formed by *Calothrix thermalis*, exclusion of UV (A and B) for a week elevated phycoerythrin levels as compared to the other photosynthetic pigments (Garcia-Pichel and Castenholz, 1994). *Phormidium corallyticum* or other cyanobacteria within black band disease may possess one or both of the two types of cyanobacterial passive photoprotection by UV-sunscreen compounds: extracellular, lipid soluble sheath pigments (ex scytonemin) and intracellular, water-soluble mycosporine-like substances (Garcia-Pichel and Castenholz, 1994).

Further studies on black band disease *Desulfovibrio* should address the response of these strains to oxygen. In this study, SRB cultures on solid media were found to be still viable after being exposed to full air for several days. *Desulfovibrio* have a complex response to oxygen that may be strain specific. SRB, including *Desulfovibrio,* are often found in the oxic zone of photosynthetic cyanobacterial mats, and yet, on a strain-specific basis, can migrate away from high concentrations of oxygen. Some *Desulfovibrio* species migrate downward into the cyanobacterial mat in the daytime (Teske *et al.*, 1998;

Krekeler *et al.*, 1998). Some strains have been found to have enzymes for oxygen tolerance, such as catalase and superoxide dismutase, (Dilling and Cypionka, 1990; Dannenberg *et al.*, 1992; Marschall *et al.*, 1993) and can use oxygen as an electron acceptor (Morris, 1976; Hatchikian *et al.*, 1977; Dilling and Cypionka, 1990; Abdollahi and Wimpenny, 1990). However, oxygen inactivates various enzymes and inhibits cell division, growth and DNA synthesis, leading to unsustainable growth in aerobic culture conditions (Fukui and Takii, 1990; Marschall *et al.*, 1993; Krekeler *et al.*, 1998). More research is required to understand the potential for aerobic tolerances or aerotaxis-related migrations of *Desulfovibrio* spp. and other sulfate-reducing bacteria found in black band disease.

More research needs to be done to quantify the presence and variability of the microbial community members within black band disease. The change in the black band microbial community composition over time is yet unknown. It seems probable that, as the band forms and develops, it becomes wider and thicker, with a constantly changing community composition. This could contribute to the variability seen between infections studied by Cooney *et al.* (2002) and Frias-Lopez *et al.* (2002). As the density and diversity of microorganisms within the mat community increases, it could become more self-sustaining. As well, the sulfur cycle and associated bacteria could become more dominant. Narrow bands, presumably younger or more subject to higher wave energy, do not exhibit the visible presence of *Beggiatoa* on the surface that is obvious on wider, thicker bands (pers. obs.).

The microbial community structure of the band may vary both horizontally and vertically; such variation has been shown in marine sediments (Risatti *et al.*, 1994). In a

non-disease microbial mat, different phylogenetic groups of SRB may be present at nonoverlapping depth intervals (Risatti *et al.*, 1994; Sass *et al.*, 1998). The available organic carbon from lysing coral tissue in all likelihood varies significantly from the front of the band to the back of the band (Richardson, 1996). This, coupled with the highly dynamic changes in light, oxygen, and sulfide within the disease band could explain the presence of multiple SRB species within BBD infections. In the black band environment, the diel variations in oxygen and sulfide levels would encourage growth of organisms with a high potential for flexible physiological adaptation and energy metabolism.

CONCLUSION

This study determined that *Desulfovibrio* species homologous to the environmental bacteria *Desulfovibrio* sp. TBP-1 and *D. acrylicus* could be cultured from different black band disease infections from two distant geographic areas. It also determined that microbial migrations (specifically *Beggiatoa* and *Phormidium corallyticum*) in the black band disease consortium are influenced by light intensity.

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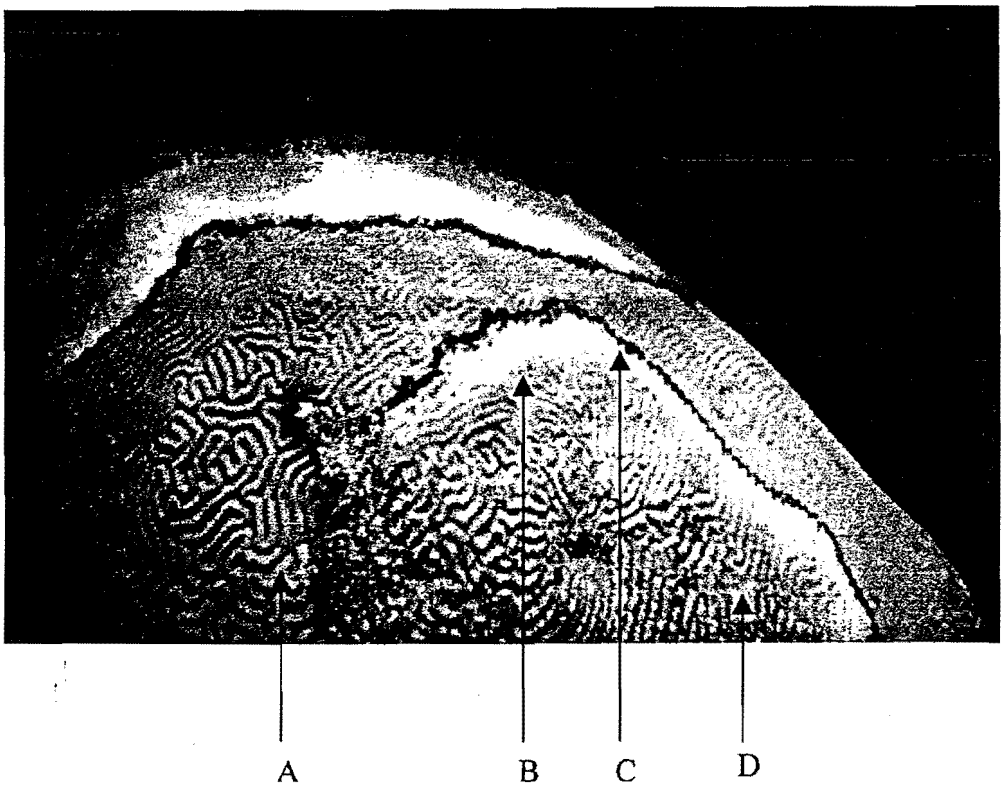


Figure 1: Black band disease infection on *Colpophyllia natans,* Algae Reef, Florida Keys National Marine Sanctuary. A shows healthy coral tissue. B shows black band disease. C shows dead skeleton, and D shows dead skeleton after having been colonized by algae.

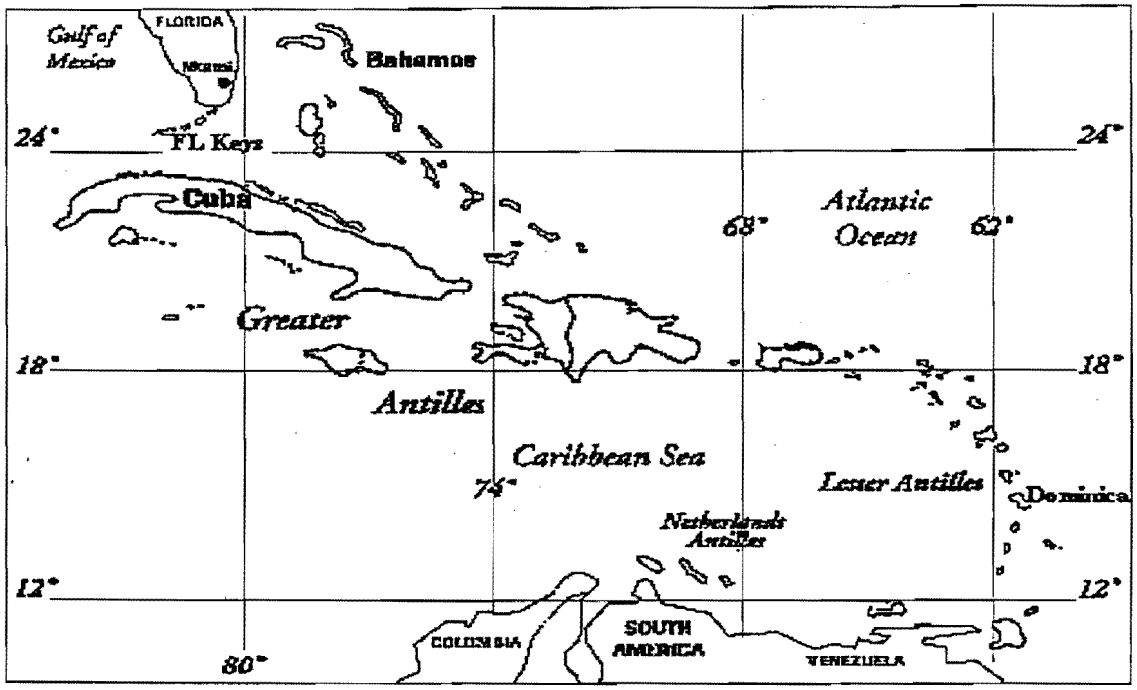


Figure 2a: Map of Caribbean showing Dominica and Florida Keys, locations where black band disease was sampled for this study.

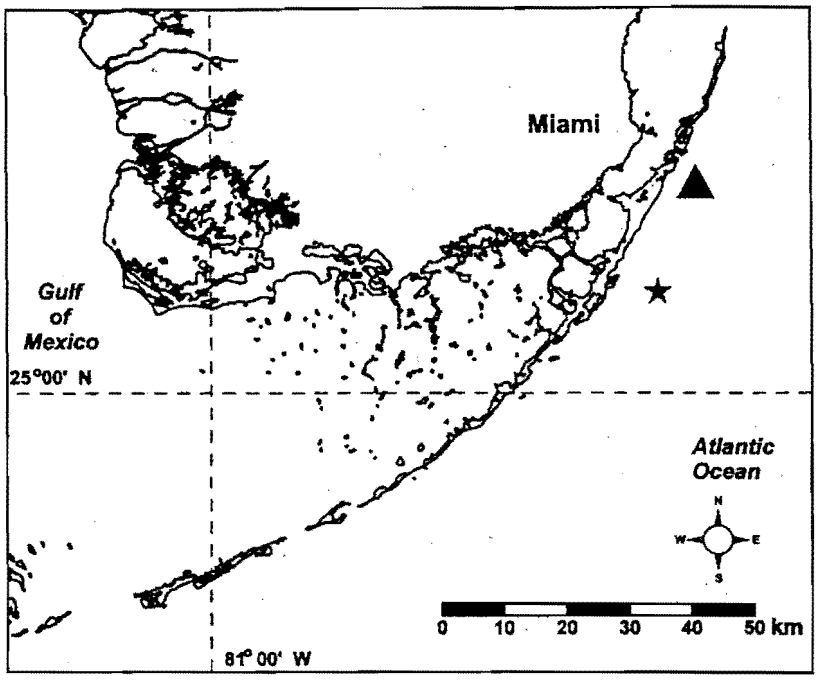


Figure 2b: Collection sites in the Florida Keys for black band disease.
★ indicates the location of Algae reef, in the Florida Keys National Marine Sanctuary.
▲ indicates the location of Picture Reef, in Biscayne National Park.

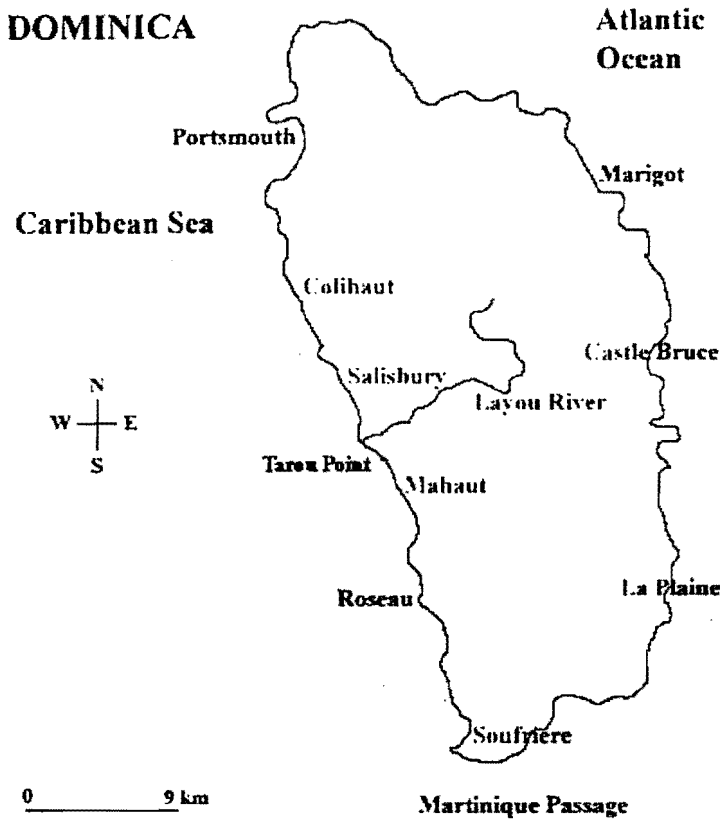


Figure 2c: Map of Dominica showing location of Tarou Point, the site of collection of black band disease from infected *Siderastrea siderea* corals.