CHARACTERISTICS OF THE ELKHORN CORAL PATHOGEN PDL-100 (*SERRATIA MARCESCENS)*

by

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(Under the Direction of Erin K. Lipp)

ABSTRACT

Serratia marcescens strain PDL-100 was isolated in 1998 from a colony of *Acropora palmata* that was affected with white pox disease. Most of the research on this bacterium focuses on its prevalence in clinical infections and little is known about its occurrence in the environment, primarily the marine environment. In this study we investigated the persistence of PDL-100 in several marine environments: the surface microlayer (SML) of three coral species (*A. palmata, M. faveolata, and S. siderea)*, seawater at 30°C and 35°C, and nutrient-amended seawater and *A. palmata* SML. Our results show that the fitness of PDL-100 is heightened in *S. siderea* SML, warmer temperatures (35°C), and seawater and *A. palmata* SML amended with nutrients. We also found that natural inhibitors of PDL-100 exist in the mucus of *A. palmata* and *S. siderea*. We characterized the two antagonistic isolates as both being *Enterobacter cloacae.*

INDEX WORDS: *Serratia marcescens, Acropora palmata,* white pox disease, marine bacterial inhibitors, *Enterobacter cloacae*

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DEDICATION

To my parents, the most giving and selfless people I've ever known. Thank you for believing in a little girl who dreamed of one day becoming a marine biologist.

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CHAPTER 1

Introduction

Acropora palmata was once the most dominant reef-building coral species in the Caribbean. Loss of *A. palmata* cover in the Caribbean is attributed to many factors such as bleaching, white band disease, and hurricanes (Gladfelter 1982; Aronson and Precht 2001; Miller et al. 2002), but in the Florida Keys National Marine Sanctuary, its decimation is primarily due to white pox disease (Sutherland and Ritchie 2004). The decline of this species has led to it and *A. cervicornis* to be listed on the Endangered Species List (Diaz-Soltero 1999).

Out of the 20 coral diseases described, white pox is the 5th disease in which an etiological agent was identified through the fulfillment of Koch's postulates (Patterson et al. 2002). The identity of the bacterium which is a cause of this disease is *Serratia marcescens*, an enteric bacterium which is commonly found in clinical settings (Grimont and Grimont 1994; Miranda et al. 1996) and also in food, soil, insects, and sewage (Carbonell et al. 2000; Baya et al. 1992; Grimont and Grimont 1994). While much is known about this opportunistic bacterium in these settings, very little is known of its prevalence and persistence in the marine environment.

This thesis is an investigation into the environmental parameters that my affect the persistence and viability of *Serratia marcescens* strain PDL-100, which was isolated from an *Acropora palmata* colony affected with white pox disease in 1998. The second chapter of this thesis provides an overview of the background concerning *Serratia marcescens* and the coral species *Acropora palmata*, in which it causes white pox disease. The third chapter presents the research and findings of the microcosm studies. The fourth chapter presents the research and

finding of the inhibition experiments, and the final chapter, Chapter 5, presents the findings and conclusions of the study.

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CHAPTER 2

LITERATURE REVIEW

Introduction

Coral decline has become an increasing concern over the last 20 years. The causes for this decline are numerous and in most cases, multiple simultaneous stressors are to blame. Climate change, anthropogenic influences, natural disturbances, and pathogens are a few that either alone or together contribute to the destruction of one of the most productive ecosystems on earth.

Productivity in open sea surfaces surrounding coral reefs may be as low as 0.01 g C m^{-2} d^{-1}, while within the reef system it can be thousands of times higher (Hatcher 1998). This productivity is not only ecologically significant but it is also critical to support human populations. People living near coral reefs depend on their high productivity (Hoegh-Guldberg 1999) for fishing, tourism, and medicinal values (Carte 1996). Coral reefs also provide protection against storms, flooding, and erosion by reducing wave action (Hoegh-Guldberg 1999). For these reasons alone, understanding the factors which cause damage to reefs is crucial.

Fifty to 70% of the coral reefs worldwide are suffering from the damage due to human activities (Goreau 1992; Bryant et al. 2000). One of the most well-known pressures to reefs in the Caribbean and Pacific is sedimentation from runoff and dredging (Rogers 1985) due to construction. Corals prefer low-nutrient, clear waters, and the stress of sedimentation increases turbidity and decreases light availability, both which are not favorable for these vulnerable organisms. One other concern is wastewater treatment and disposal practices. Sewage

contamination has been recognized as a key environmental problem for some time (Banner 1974). In the Florida Keys, sewage disposal consists primarily (with the exception of Key West) of injection wells, septic tanks, and illegal cesspools (Shinn et al. 1994). Paul et al. (1995) provided evidence to show a connection between a septic tank drain field, well discharge areas, and surface marine waters. Sewage contamination in coral reef environments has implications for not only environmental health, but human health.

Coral disease has become of increasing concern for the fate of reefs as well. In 1965, the first coral disease was reported and over the next three decades only 4 new diseases were described (Sutherland and Ritchie 2004) (Table 1). By 2002, 13 new diseases had been described. This large increase in numbers is likely related to both an increase in disease itself as well an increase in recognition and reporting. As of 2004, there were 18 coral diseases described, but Koch's postulates have been successfully fulfilled for only 5 of them (Sutherland and Ritchie 2004). These five diseases are White Plaque II (WPL II), White Pox Disease (WPD), Aspergillosis (ASP), *Vibrio shiloi*-induced bleaching (VSB), and *Vibrio coralliilyticus*-induced bleaching (VCB) (Richardson et al. 1998a; Denner et al. 2003; Patterson et al. 2002; Smith et al. 1996; Geiser et al. 1998; Kushmaro et al. 1998; Kushmaro et al. 2001; Rosenberg et al. 1998; Ben-Haim et al. 2003a; Ben-Haim et al. 2003b).

The importance of attempting and fulfilling Koch's postulates is to prove a causal relationship between a causative microbe and a disease. These postulates require that: (1) the assumed pathogen must be found in diseased organisms, (2) the assumed pathogen must be isolated from a diseased organism and grown in pure culture, (3) the cultured pathogen must cause disease when induced in a healthy experimental organism (Koch 1882). The fourth postulate which wasn't a requirement of Koch, but added later is: (4) the same pathogen must be

re-isolated from the inoculated, diseased experimental organism and be identified as being identical to the original assumed causative agent.

While important for unequivocally identifying the agents of some diseases, Koch's postulates also have their limitations. For example, in the case of black band disease (BBD), Koch's postulates technically cannot be fulfilled, because it isn't a single microorganism which causes this disease, it is a consortium of microorganisms (Richardson 1997). Sutherland et al. (2004) proposed that in order to make advances in understanding coral disease etiology, other criteria for identifying disease causation should be employed and accepted in the coral disease field**.**

Acropora palmata

Acropora palmata is structurally complex and is characterized by its "elkhorn" shaped branches. This particular species is extremely environmentally sensitive and is threatened by bleaching, predation, climate change, human activity, storm damage, and disease. The habitat of *A. palmata* is shallow, high-energy reef environments throughout the Caribbean and the Florida Reef Tract (Lirman 2000). Rapid growth is another characteristic of *A. palmata*, with growth rates reaching up to 10 cm yr^{-1} (Gladfelter 1978). *A. palmata* provides high rates of calcium carbonate deposition (Adey 1978), while also providing food and shelter for other reef organisms. Acroporids reproduce by both sexual and asexual means. As broadcast spawners, they release their eggs into the water column for fertilization and development. Additionally, given their high degree of branching, they are also capable of fragmentation, which has significant influence on its survivorship and propagation (Lirman 2000). Propagation by fragmentation is especially important in *A. palmata*, because of its limited sexual recruitment and

also because fragmentation can take place year-round, whereas sexually reproduction is seasonally restricted (Szmant 1986).

Acropora palmata was once the most dominant space-occupier in Caribbean reefs. Their decimation, therefore, significantly reduces coral cover (Aronson and Precht 2001). The severe decline of this species in the Caribbean has led to it and *A. cervicornis* to be listed on the Endangered Species List (Diaz-Soltero 1999). In the Florida Keys National Marine Sanctuary (FKNMS), *Acropora palmata* populations have experienced losses averaging 88% (58) (Figure 1), similar to the declines seen throughout the Caribbean (Aronson and Precht 2001; Miller et al. 2002). Loss of *A. palmata* cover in the Caribbean has been linked to many factors, such as white band disease, bleaching and hurricanes (Gladfelter 1982; Aronson and Precht 2001; Miller et al. 2002), whereas the decimation in the FKNMS was primarily due to white pox disease (Sutherland and Ritchie 2004).

Factors Affecting *Acropora palmata* Populations

Many influences have led to the demise of *Acropora palmata*. Environmental stressors such as hurricanes, cold winters, and (warm) summer bleaching are a few that have contributed to *A. palmata* decline over the past 2 decades (Dustan and Halas 1987; Porter and Meier 1992). Additionally, because this species is so environmentally sensitive, only minimal stress is required to cause severe damage. Biological controls such as predation and disease (as described in more detail below) also play a significant role in loss of coral cover either alone or in combination with environmental stressors.

Predation. *Coralliophila abbreviata* represents one biological burden on *A. palmata*. *C. abbreviata* is a corallivorous gastropod that is a predator to at least 14 species of coral (Miller

1981). Baums et al. (2003) witnessed *C. abbreviata* to be present on all *A. palmata* colonies sampled in the Florida Keys, and causing tissue loss in at least half of them. One estimate of *A. palmata* tissue loss due to consumption by *C. abbreviata* was 1.9 cm^2 $snail^{-1}d^{-1}$ to a maximum of 6.5 cm^2 $snail^{-1}d^{-1}$ (Bruckner et al. 1997). Likewise, another study conducted in the Florida Keys presented evidence that *C. abbreviata* plays a considerable role in removing coral tissue; removal of these corallivorous snails could preserve about 75% more live *A. palmata* tissue (Miller 2001). While a normal ecosystem function, snail predation could exacerbate conditions for already stressed and depleted coral populations (Knowlton et al. 1990). Furthermore, depletion of predators against these snails (or other corallivores) due to overfishing could result in population blooms, thereby producing additional stress on the local coral populations (McClanahan 1997). Published data on *C. abbreviata* predators is limited, but *Synalpheus fritzmuelleri*, the snapping shrimp (Goldberg 1971) and *Pamulirus argus*, the Caribbean spiny lobster (Baums 2003) have both been observed to feed on this snail.

White Band Disease. *Acropora palmata* populations, along with *A. cervicornis* populations, have also been affected by white band disease (WBD); this disease actually exclusively affects these two species (Gladfelter 1982; Peters 1993; Bythell et al. 2000; Aronson et al. 2002). WBD was first described by Gladfelter (1982) in the Caribbean in the mid-1970s. It is characterized by tissue that is peeling off in a consistent band starting at the base and moving up the branch (Peters 1997) and with a tissue loss of several millimeters a day, it can eventually kill entire colonies. Aronson and Precht (2001) argued that white-band disease was the most significant factor on a regional scale in reducing *A. palmata* and *A. cervicornis* populations. Gladfelter (1982) reported the effects of WBD to be devastating in Tague Bay, St. Croix; approximately 50% of *A. palmata* colonies in the shallow reef crest were actively losing

tissue, and within five years, 95% of the colonies were dead. The significance of this loss was not limited to these two species, but carried into the entire community. When the tri-dimensional spatial structure of the acroporid species collapsed, the general zonation patterns of shallow reefs changed and shifted from a coral-dominated to an algal-dominated habitat (Hughes 1994; Weil 2004).

To date, there are two varieties of WBD, Type I and Type II. WBD Type I has been found in *Acropora* species around the Caribbean, the Great Barrier Reef, the Red Sea, and the Phillippines. Type II has been found exclusively in the Bahamas. The etiology of WBD is not fully understood, although it is assumed that it is a bacterial infection of unknown origin (Antonius 1981; Peters et al. 1983). Aggregations of gram-negative bacteria were found in association with *Acropora palmata* showing signs of WBD Type I (Peters et al. 1983). Ritchie and Smith (1995, 1998) later proposed *Vibrio carchariae* as the causal agent of WBD Type II, but it was never confirmed.

White Pox Disease. White pox disease (WPD) was first described in 1996 on reefs off Key West, FL and since has been observed throughout the Caribbean (Holden 1996). The disease exclusively affects *Acropora palmata* and when the causal agent, *Serratia marcescens*, can be confirmed the disease is more accurately termed acroporid serratiosis (Patterson et al. 2002). WPD spreads rapidly and is characterized by distinctive irregular white patches of bare skeleton which can be found on the surface or undersides of branches. The lesions can grow at an average rate of 2.5 $cm^2\ day^{-1}$ and greatest tissue loss coincides with high temperature (Sutherland and Ritchie 2004). WPD is sometimes mistaken for white band disease, bleaching, and predation scars by *Coralliophila abbreviata*, although its distinct lesion characteristics can be easily discerned by trained eyes.

It is not clear when WPD first appeared (Rogers et al. 2005). Pictures taken in the 1970's at Buck Island Reef National Monument, St. Croix by Alan Robinson show lesions that look surprisingly like white pox. So the question remains: is this a new phenomenon or has this disease been around for several decades? If it is a new phenomenon, then the apparent emergence of the epizootic in the 1990s in the Florida Keys would represent the introduction of a new pathogen or a novel exploitation by a previously innocuous organism. If the disease has existed for decades then novel conditions (or new stressors) may have resulted in its reemergence and proliferation. While it may be difficult to ever know the complete history of the disease, in either case it is clear the epizootic of WPD in the Florida Keys was unparalleled in our recent history.

Serratia marcescens

To date, *Serratia marcescens* is the only confirmed agent resulting in WPD lesions in *Acropora palmata* (Patterson et al. 2002); however, other microbes could also result in similar disease patterns but are yet undescribed. Despite the fact that *S. marcescens* is a common enteric bacterium and known human pathogen, very little is known of its prevalence in or adaption to marine conditions.

Classified in the family Enterobacteriacaea, *Serratia* species are Gram-negative bacteria and are distinguished from other genera by their production of three special enzymes, DNAase, lipase and gelatinase (Giri 2004). Another special characteristic of *Serratia* spp. is their ability to produce a red pigment, prodigiosin, especially in environmental strains (Hejazi and Falkiner 1997). While prodigiosin is a common diagnostic in the identification of *S. marcescens*, most strains of *Serratia* from clinical cases are non-pigmented (Carbonell et al. 2000). The role of prodigiosin in pathogenesis is uncertain, but in at least one documented case, there was an

inverse relationship between pigment production and toxicity (Carbonell et al. 2004). In the case of white pox disease, the isolate, PDL-100 is also non-pigmented.

Once considered a harmless, non-pathogenic saprophytic aquatic microbe, *Serratia marcescens* was frequently used as a biological marker because of its characteristic red colonies (Holden 1996). This bacterium is now known as a prominent opportunistic pathogen causing, in many cases, severe nosocomial infections, including wound infections, urinary tract infections, and pneumonia (Carbonell 2004; Grimont and Grimont 1994; Miranda et al. 1996). It is also commonly found in soil, water, crops and in animal and human feces (Carbonell et al. 2000); it can also cause infection in a wide range of organisms, including insects and plants (Baya et al. 1992; Grimont and Grimont 1994).

Despite evidence that *Serratia marcescens* is ubiquitous in a range of terrestrial and freshwater environments, little is known about its prevalence and persistence in marine environments. Most reports that mention *S. marcescens* from coastal or marine areas are anecdotal in nature. Baya et al. (1992) isolated *Serratia marcescens* from 20% of the white perch (*Morone americanus*) sampled from the sewage-polluted Back River, Maryland. Marine fishes (Austin and Austin 1999) and other marine organisms (Inglis et al. 1993) have also been found to be infected with *S. marcescens* although in some cases it is not clear if the bacterium was only isolated post-harvest (market conditions). Nearly all recent reports of a marine strain of *S. marcescens* have been in association with white pox disease.

Patterson et al. reported in 2002 that at least one causal agent of white pox disease in *Acropora palmata* was *Serratia marcescens*. The fact that this bacterium has been linked to sewage and the reports that human enteric bacteria and viruses are prevalent in nearshore,

offshore, canals, and coral surfaces in the Florida Keys led to the proposition that the bacterium which causes white pox in *Acropora palmata* originates from land-based sewage problems.

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Table 1. Borrowed from Sutherland et al. (2004). First report of coral diseases in the Caribbean and the Indo-Pacific.

Disease	Abbreviation	Year	Source
Skeletal anomalies	SKA	1965	Squires (1965)
Black band	BBD	1973	Antonius (1973)
White plague Type I	WPL I	1977	Dustan (1977)
Shut-down reaction	SDR	1977	Antonius (1977)
White band Type I	WBD I	1982	Gladfelter (1982)
Aspergillosis	ASP	1996	Smith et al. (1996)
White pox	WPD	1996	Holden (1996)
Vibrio shiloi-induced bleaching	VSB	1996	Kushmaro et al. (1996)
Yellow blotch/band	YBL	1997	Santavy & Peters (1997)
White plague Type II	WPL II	1998	Richardson et al. (1998a)
White band Type II	WBD II	1998	Ritchie & Smith (1998)
Yellow band	YBD	1998	Korrûbel & Riegl (1998)
Dark spots	DSD	1998	Goreau et al. (1998)
Skeleton eroding band	SEB	2000	Antonius & Lipscomb (2000)
Fungal-protozoan syndrome	FPS	2000	Cerrano et al. (2000)
White plague Type III	WPL III	2001	Richardson et al. (2001)
Pink-line syndrome	PLS	2001	Ravindran et al. (2001)
Vibrio coralliilyticus-induced bleaching and disease	VCB	2002	Ben-Haim & Rosenberg (2002)

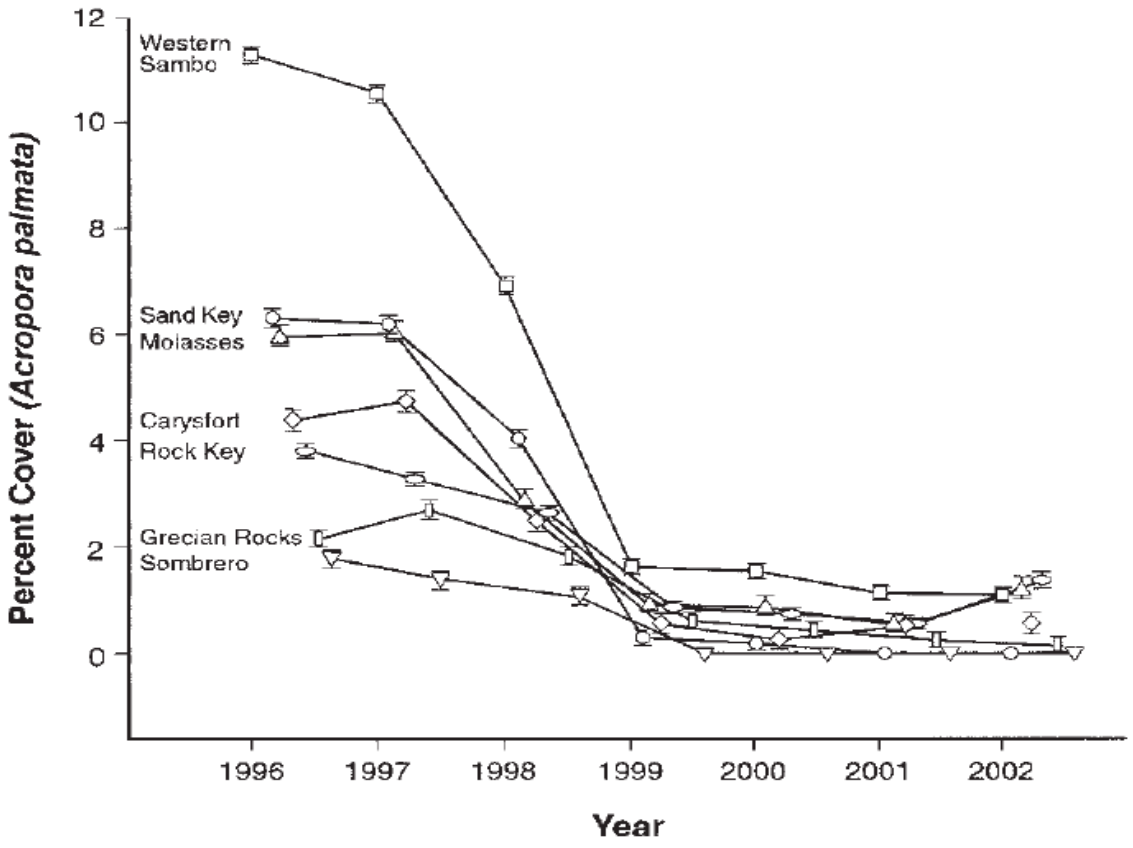

Figure 1. Borrowed from Sutherland and Ritchie (2004). Percent cover of Acropora palmata at seven reef sites in the FKNMS, 1996-2002: Western Sambo Reef (squares), Sand Key Reef (circles), Molasses Reef (triangles), Carysfort Reef (diamonds), Rock Key Reef (ovals), Grecian Rocks Reef (rectangles), Sombrero Reef (inverted triangles). Data presented as mean +/- SD

CHAPTER 3

PERSISTENCE OF THE CORAL-PATHOGENIC STRAIN OF *SERRATIA MARCESCENS* PDL-100 IN SEAWATER AND EXPERIMENTAL MICROCOSMS [1]

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Abstract

The persistence and survival of enteric bacterium *Serratia marcescens* in the marine environment is largely unknown, despite the fact that it is among only 5 identified and confirmed coral pathogens. This study investigates basic questions related to the fitness of the coral pathogenic strain, PDL-100, in seawater, coral mucus, and in nutrient amended water and mucus. PDL-100-inoculated microcosms of natural seawater, mucus from the corals *Acropora palmata*, *Montastraea faveolata*, and *Siderastrea siderea*, natural seawater amended with NO_3^-, NH_4^+, PO_4^{3-}, and dissolved organic carbon (DOC [glucose]), and *A. palmata* mucus amended with NO_3^-, NH_4^+, PO_4^{3-}, and DOC were evaluated for the survival in these matrices by fitting to first-order decay curves and decay constants (k) were compared between the experimental conditions. Median survival time for PDL-100 populations ranged from a low of 20 hours in unamended natural seawater (k=-0.173) at 30°C to a maximum of 144 hours in glucose-amended *A. palmata* mucus (k=-00320). Among unamended seawater and mucus microcosms, only PDL-100 in *S. siderea* mucus showed significantly improved survival ($p<0.0001$). In seawater, conditions resulting in a significant increase in survival, or slower decay rate, were increasing temperature (35°C vs. 30°C; $p<0.0001$) and addition of nutrients, especially DOC and PO_4^{3-} ($p<0.0001$). When nutrients were added to *A. palmata* microcosms, DOC resulted in the greatest improvement in survival ($p<0.0001$) with PO_4^{3-} resulting in similar decay rates as NO_3^- and NH_4^+. Results suggest that coral species besides *A. palmata* might act as reservoirs for this pathogen on the reef and that increasing nutrients, especially DOC, and temperatures may contribute to pathogen survival and infection.

Keywords: elkhorn coral, *Acropora palmata*, *Serratia marcescens*, microcosm, nutrient amendments, temperature

Introduction

Serratia marcescens has been identified as a causal agent of white pox disease in *Acropora palmata* (Patterson et al. 2002). *A. palmata* (elkhorn coral) is an extremely environmentally sensitive species, and stressors such as hurricanes, winter cold-kills, and summer bleaching have contributed to its decline over the past two decades (Dustan 1987; Porter 1992). Because this species was once one of the most dominant space-occupiers in Caribbean reefs, its decimation has resulted in significant declines in total coral cover (Aronson and Precht 2001). From 1996 to 2002, *A. palmata* populations in the Florida Keys National Marine Sanctuary (FKNMS) experienced a loss in cover averaging 88%, primarily due to white pox disease (Sutherland and Ritchie 2004). The severe decline of this species in the Caribbean has led to it and *A. cervicornis* (staghorn coral) being listed on the Endangered Species List (Hogarth 2006).

Serratia marcescens is a Gram-negative bacterium which belongs to the family *Enterobacteriaceae*. This species is characterized by its ability to produce a red pigment, prodigiosin, which commonly occurs in environmental strains and less frequently in clinical strains (Thomson et al. 2000; Hejazi and Falkiner 1997; Carbonell et al. 2000). Virulence or resistance factors have been correlated with non-pigmented isolates (Carbonell et al. 2000). Before 1913, *Serratia* species were thought to be non-pathogenic to humans; it was later reported that *S. marcescens* caused a pulmonary infection (Woodward and Clarke 1913), and since then numerous infections have been reported such as respiratory tract infections (Cabrera 1969; Ringrose et al. 1968), urinary tract infections (Clayton and Graevenitz 1966; Magnuson and Elston1966; Taylor and Keane1962), meningitis (Rabinowitz and Schiffrin 1952; Graber et al.1965) and pneumonia (Tillotson & Finland 1969).

S. marcescens is also commonly found in soil, water, plants, and animals (Baya et al. 1992) and can cause disease in a wide range of invertebrates, vertebrates, and plants in (Grimont and Grimont 1978). Additionally, *S. marcescens* is commonly found in sewage (Sutherland et al. *in prep*).

Patterson et al. (2002) speculated that the *A. palmata* pathogen (*S. marcescens* PDL-100) might have a terrestrial origin, including a potential sewage source. Wastewater contamination has been recognized as a critical environmental problem in the Florida Keys for decades (Banner 1974; Shinn et al.1994; Lapointe et al.1990). For most of the Keys, sewage disposal consists primarily of injection wells, septic tanks, and illegal cesspools with no treatment (e.g., Shinn et al. 1994). Several studies have shown a rapid connection between septic tank effluent, groundwater, and surface marine waters using both nutrient and microbial tracers (Paul et al. 1995, 1997; Lapointe 1997; Griffin et al. 1999). Additionally, evidence of human enteric viruses (found only in human feces) has been detected throughout the Keys, especially near high population clusters (Lipp et al 2007). However, many enteric bacteria have been shown to have limited survival in warm, transparent marine waters (e.g., Craig et al 2001).

The persistence and survival of *Serratia marcescens* in the marine environment is largely unknown, despite the fact that it is among only 5 identified and confirmed coral pathogens. This study investigates basic questions related to the fitness of PDL-100 in seawater and coral mucus, which may lead to better understanding of the disease dynamics of white pox.

Materials and Methods

Field Collection. Coral mucus (the surface microlayer [SML] representing a mixture of mucopolysaccharide and water) (Paul et al 1986) was sampled from healthy colonies of *Montastrea faveolata, Siderastrea siderea,* and *Acropora palmata* on Western Sambos Reef, near Key West, FL, and Carysfort Reef, Key Largo, FL in July 2007. These were chosen based on the previous isolation of *Serratia marcescens* from these species (unpublished data & Sutherland et al. 2002). At each collection site, ~500 ml of coral SML were collected from 3 colonies of each species using sterile 60 ml syringes without a needle. Prior to collection, the coral surface was gently disturbed with the syringe to dislodge the mucus and to ensure that mucus was the primary agent collected. Seawater was also collected just below the surface in sterile 1 L bottles. All materials were stored at 4° C until further use in experiments.

Microcosm Conditions. The persistence of *Serratia marcescens* was evaluated among defined matrices to compare survival of PDL-100 among mucus type, temperature, and nutrient amendments. In experiment 1, survival was compared between natural seawater and each of the mucus types (*A. palmata, M. faveolata,* and *S. siderea)* at 30°C. In experiment 2, the effect of temperature was determined for seawater (30°C *vs*. 35°C). In experiments 3 and 4, the role of nutrient amendments was assessed in seawater and *A. palmata* mucus (glucose representing DOC, NH_4^+, NO_3^-, PO_4^{3-}, and a combination of all the nutrients). A final concentration of 10µM was added for each amended nutrient, with the exception of PO_4^{3-}, in which in the final concentration was 1µM (Bruno et al 2003; Hewson et al. 2003). Exact conditions are shown in Table 3.1.

Microcosm Technique. The non-pigmented *Serratia marcescens* strain, PDL-100 (ATCC #13880), used in this study was isolated from *A. palmata* during a white pox outbreak in

1996 by Patterson et al. (2002). Overnight cultures of PDL-100 were grown in 5 ml of Caso broth (MP Biomedicals, Solon, OH) at 30°C with shaking at 100 rpm (New Brunswick Scientific Co., Edison, NJ). The overnight culture was centrifuged (8 min, 3,500 x g, at 4°C) (Fisher Scientific, Marathon 3200R, Pittsburgh, PA), washed with sterile artificial seawater (ASW; prepared using Instant Ocean adjusted to a final salinity of 35) three times and resuspended in ASW at the original volume (5 ml). All experiments consisted of two replicate experimental flasks and one control flask. Each experimental flask contained 100 ml of the experimental water or mucus (SML), and was then inoculated with 100 µl of the washed overnight culture of PDL-100 to give an initial count of approximately 10^6 CFU (colony forming units) ml^{-1}. The control flask was not inoculated but was otherwise treated identically as the experimental flasks. The suspension was continuously mixed and a 1-ml aliquot from each flask was immediately collected, representing initial (time zero [T_0]) conditions. Flasks were held at appropriate experimental temperatures in a static, dark environment. Aliquots were collected every hour for the first 4 hours, then every 4 hours up to 24 hours, and every 24 hours until no bacteria could be cultured. In all cases, a sample was serially diluted in sterile ASW and 100 µl aliquots were spread onto duplicate MSCA (MacConkey Sorbitol Agar amended with colistin [15,000 U/ml]) plates, which supports selective and differential growth for *Serratia* spp. (Grasso et al. 1988). The plates were incubated at 37° C for 16-24 hours and colonies appearing red (positive for sorbitol fermentation and presumptive *Serratia*) were counted and reported as CFU ml^{-1}.

Analysis. The fraction of the PDL-100 population remaining at each timepoint (for all experiments) was determined by dividing the mean CFU ml^{-1} at T_n by the mean CFU ml^{-1} at T_0. First-order decay rate constants (*k*) were calculated as the slope of the line when $\log_{10}(T_n/T_0)$ was regressed against time, where T_n was the number of bacteria (CFU ml^{-1}) at time *n* and T_0

was the CFU ml^{-1} at time 0 (Crane and Moore 1986; Davies and Evison 1991). The decay constants were compared statistically using analysis of covariance (ANCOVA) (Graphpad Prism V.5 (La Jolla, CA) software for Windows). The mean time to a non-culturable population ('death') was also determined. Significance was declared at $p<0.05$.

Results

In all cases, PDL-100 decayed in the experimental conditions; no population growth was noted. Also, in all cases, there was no growth in the control flasks.

Experiment 1: The effect of coral mucus on the persistence of PDL-100 relative to seawater. The decay rate of PDL-100 among seawater and the three SML types were significantly different ($p<0.0001$). Mean times to reach a non-culturable population ('death') ranged from 20 hours in seawater to 108 hours in *S. siderea* (Figure 1a). Survival of PDL-100 in *S. siderea* was significantly greater than in all other mucus types or seawater with a decay constant of -0.0374 ($p<0.0001$); there was no significant difference between seawater, *A. palmata* or *M. faveolata* with decay constants ranging between -0.1729 and -0.2031 (Table 3.2)

Experiment 2: The effect of temperature on the persistence of PDL-100 populations in seawater. PDL-100 persistence in seawater was enhanced by the warmer temperature (35°C vs. 30°C) ($p<0.0001$). The mean survival time was 20 hours in seawater held at 30°C, and 72 hours in seawater held at 35°C (Figure 1b). Similarly, PDL-100 decayed faster in 30°C seawater ($k= -0.1729$) than in 35°C seawater ($k=-0.05916$) (Table 3.3).

Experiment 3: The effect of individual and combined nutrients on the persistence of PDL-100 in seawater. There was a significant difference in PDL-100 among nutrient amendments ($p<0.0001$). The greatest increase in survival time of PDL-100 occurred in glucose-

amended seawater, which resulted in a mean survival of 120 h (5 days), a 100 hours longer than the mean survival of 20 h survival in unamended seawater (30°C) (Figure 1c). Survival of PDL-100 was enhanced with the addition of any the nutrients (glucose, NH_4^+, NO_3^-, or PO_4^{3-}), especially glucose, PO_4^{3-}, and the combined addition of all nutrients; however, the combined effect showed no improvement in decay rate or survival time over the addition of glucose alone. The decay constants (k) ranged from -0.1729 in unamended seawater to -0.01553 in seawater enriched with glucose (Table 3.4).

Experiment 4: The effect of individual and combined nutrients on the persistence of PDL-100 in *A. palmata* mucus. PDL-100 survival varied significantly between all nutrient treatments ($p<0.0001$). While the addition of any nutrient resulted in marginal improvement in persistence, the addition of glucose and the addition of the combined nutrients had the largest effect with a mean survival time of 144 h (decay constant of -0.03204 and -0.03345 respectively), 120 h longer than its survival in unamended *A. palmata* mucus (decay constant of -0.2031) (Figure 1d). When added to *A. palmata* mucus, PO_4^{3-} resulted in similar levels of persistence as the nitrogen amendments (Table 3.5).

Discussion

While much is known about *Serratia marcescens* in clinical environments (Davis et al. 1970; Hamilton and Brown 1972; Okuda et al. 1984; Miranda et al. 1996; Carbonell et al. 2000), very little is known about this bacterium in the marine environment. This study was designed to evaluate the persistence of *S. marcescens* among seawater and mucus of common corals under varying environmental temperatures and nutrient conditions.

The role that mucus plays in corals is an important one, as it aids as a defense mechanism against many environmental stresses and frees the coral surface of sedimentation (Brown and Bythell 2005). It also acts as a reservoir of microbial growth in reef ecosystems and provides an organic matrix for both beneficial (commensal) and potentially pathogenic bacteria (Brown and Bythell 2005; Banin et al. 2001; Lipp et al. 2002). Given the relatively higher levels of organic and inorganic nutrients in coral mucus compared to seawater, we hypothesized that PDL-100 would show improved persistence, and possibly growth, in these environments. While population growth of PDL-100 was not observed in any of the experimental conditions, mucus did appear to contribute to its survival. We found that the only coral mucus that made a significant difference in the survival time of PDL-100 was that of *S. siderea*, in which culturable bacteria were detected for 108 h on average, with a decay constant of -0.03739. This was significantly longer than PDL-100 survival in any other coral mucus environment or in seawater, and approached the persistence seen with organic carbon enrichments. Decay rates were similar between seawater, *Montastraea faveolata* and *Acropora palmata* microcosms. *S. marcescens* has been found in mucus samples from healthy colonies of both *M. faveolata* and *S. siderea* (unpublished data), as well as in WPD lesions of *A. palmata* (Patterson et al. 2002). Given the long persistence of PDL-100 in *S. siderea* mucus, in particular, this species might act as a reservoir; however, additional research is needed to investigate how the physical, chemical, and biological (i.e., microbial community) properties of mucus between these species varies.

Elevated temperatures increased PDL-100 survival in seawater. Experimental temperatures included 30°C (representing high temperature during summer months in the Florida Keys when WPD incidence increases [Patterson et al. 2002]) and 35°C, which was higher than expected sea surface temperatures but may be reached during extreme conditions for short

periods of time. Survival of PDL-100 populations was significantly enhanced in the warmer seawater conditions. This finding is contrary to previous work on other enteric bacteria, which indicate that in the absence of organic matter, high temperatures promote faster decay (e.g., Craig et al. 2001), suggesting that this strain of *Serratia marcescens* responds differently to elevated temperatures. This response is, however, consistent with the reported trends in WPD incidence (Patterson et al. 2002) and coral disease in general. Most, if not all, coral diseases occur at high seawater temperatures (Rosenberg and Ben-Haim 2002; Hoegh-Guldberg 1999); it is hypothesized that pathogenic bacteria can take advantage of a coral's susceptibility to infection if the coral is under thermal stress due to prolonged exposure to elevated temperatures (Goreau and Hayes1994). One study compared the growth of *Aurantimonas coralicida*, the pathogen which causes white plague type II in a wide range of coral hosts at temperatures between 25° and 42°C, and found that the optimal growth occurred between 30° and 35°C, with the fastest growth occurring at 35°C (Remily and Richardson 2006). Similarly, elevated temperatures in laboratory experiments caused optimal growth of the microbes that cause gorgonian aspergillosis and contribute to black band disease; in each of these cases disease coincides with elevated reef temperatures (Alker et al. 2001; Richardson and Kuta 2003). Although, not addressed in this study, for many pathogens virulence is linked to their growth potentials, which may in turn be associated with one or more environmental cues, such as temperature (Cheng and Chen 1998).

In addition to elevated temperatures, coral diseases, in general, have also been correlated with elevated nutrients. Using *in situ* nutrient enrichments via time released fertilizer packets, Bruno et al. (2003) found that both aspergillosis and yellow band diseases increased with elevated concentrations of nutrients. Additionally, Kline et al. (2006) have shown that direct

addition of dissolved organic carbon (DOC) significantly increased microbial growth and coral mortality. Therefore, we amended both seawater and *A. palmata* mucus with nutrients to determine their effect on the relative survival of PDL-100. Amendments focused on the inorganic nutrients nitrate, ammonium, and phosphate. While phosphate is often not limiting in marine waters, it is typically associated with increased fitness of heterotrophic bacteria. For organic carbon enrichments, glucose was used as a simple DOC source (Kline et al. 2006). Consistent with those findings for increase in disease with increasing nutrients, all nutrient amendments resulted in increased persistence of PDL-100 relative to unamended seawater and *A. palmata* mucus. For both water and mucus, the addition of glucose resulted in the largest increased survival. The addition of phosphate also contributed to increased persistence in seawater but in mucus there was no difference between nitrogen and phosphate amendments. This likely reflects the higher level of nutrients already present in the mucus compared to seawater (data not shown). Low nutrient concentrations have been proposed to considerably reduce the survival rates of fecal bacteria in the aquatic environment (Gauthier et al. 1989). Likewise, Craig et al. (2001) inferred from their observations that nutrient availability may be a very powerful factor in microbial survival.

Elevated temperatures and nutrient pollution have long been considered important factors contributing to coral reef decline. In 1985 (Cortes and Risk), it was hypothesized that increasing nutrient inputs had a potential to seriously impact coral reefs. The most common notion is that nutrients feed nutrient-limited algae, which causes an increase in growth (Hughes 1994) and shift in the benthic ecosystem from coral dominated to sponge-algae dominated. With continued overfishing, there is also a significant decrease in herbivores to control the increase of algae (Done 1992). Additionally, under expected global climate change scenarios sea surface

temperatures will rise and could increase the incidence of thermal stress and bleaching. However, the role of nutrients and temperatures can also indirectly affect the coral reef ecosystem by their indirect effects on microbial populations within the coral holobiont (Reshef et al. 2006) and potential coral pathogens. Increased availability of nutrients specifically increases survival rates of enteric bacteria in seawater (Carlucci et al. 1959, Munro et al. 1989). Additionally, Kline et al. (2006) found that DOC loading caused a significant increase in microbial growth rates and coral mortality, suggesting that microbes living in the mucus of corals are carbon limited.

While coral mucus appears to have some inherent ability to promote survival among this coral pathogenic strain of *Serratia marcescens*, it does not support the growth of this bacterium. This suggests that the pathogen may need to colonize coral tissue to elicit growth and symptomatic infections. However, persistence patterns of PDL-100 in these *in vitro* studies confirms the potentially important role of both temperature and nutrients, especially dissolved organic carbon, in disease dynamics of white pox as these conditions could allow sufficient survival time to allow effective colonization of the host.

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TABLES

Table 3.1. Microcosm Conditions

Experiment	**Matrix**	**Amendment**	**Temperature**
1	Seawater	----------	30° C
	Acropora palmata	----------	
	Siderastrea siderea	----------	
	Montastrea faveolata	----------	
2	Seawater	----------	35° C
	Seawater	----------	30° C
3	Seawater	NO_3 (10µM) NH_3 (10µM) PO_4 (1µM) glucose (10µM) ALL (NO_3, NH_3, PO_4, glucose)	30° C
4	*Acropora palmata*	NO_3 (10µM) NH_3 (10µM) PO_4 (1µM) glucose (10µM) ALL (NO_3, NH_3, PO_4, glucose)	30° C

Table 3.2. Decay rate constants (*k*) for *Serratia marcescens* (strain PDL-100) among mucus and seawater; decay constants were significantly different from each using ANCOVA ($p<0.0001$)

Microcosm	***K***	**R^2**	**P**
Seawater	-0.1729	0.9618	0.0008
Acropora palmata	-0.2031	0.8150	0.0009
Montastrea faveolata	-0.1945	0.7663	0.0002
Siderastrea siderea	-0.03739	0.7585	0.0002

Table 3.3. Decay rate constants (*k*) for *S. marcescens* in each temperature microcosm; rates were significantly different ($p<0.0001$).

Microcosm	***K***	**R^2**	**P**
Seawater (30° C)	-0.1729	0.9618	0.0008
Seawater (35° C)	-0.05916	0.6981	0.0026

Table 3.4. Decay rate constants (*k*) for *S. marcescens* in each nutrient amended seawater microcosm; rates were significantly different ($p<0.0001$).

Microcosm	***K***	**R^2**	**P**
Seawater	-0.1729	0.9618	0.0008
Seawater + NH_4	-0.1183	0.9773	<0.0001
Seawater + NO_3	-0.1088	0.9756	<0.0001
Seawater + ALL	-0.03068	0.8414	<0.0001
Seawater + PO_4	-0.01853	0.4262	0.0214
Seawater + DOC	-0.01553	0.4135	0.0241

Table 3.5. Decay rate constants (*k*) for *S. marcescens* in each nutrient amended *A. palmata* microcosm; rates were significantly different ($p<0.0001$).

Microcosm	***K***	**R^2**	**P**
A. palmata	-0.2031	0.8150	0.0009
A. palmata + NH_4	-0.07943	0.9824	<0.0001
A. palmata + PO_4	-0.06224	0.8150	<0.0001
A. palmata + NO_3	-0.05897	0.9435	<0.0001
A. palmata + ALL	-0.03345	0.993	<0.0001
A. palmata + DOC	-0.03204	0.9733	<0.0001

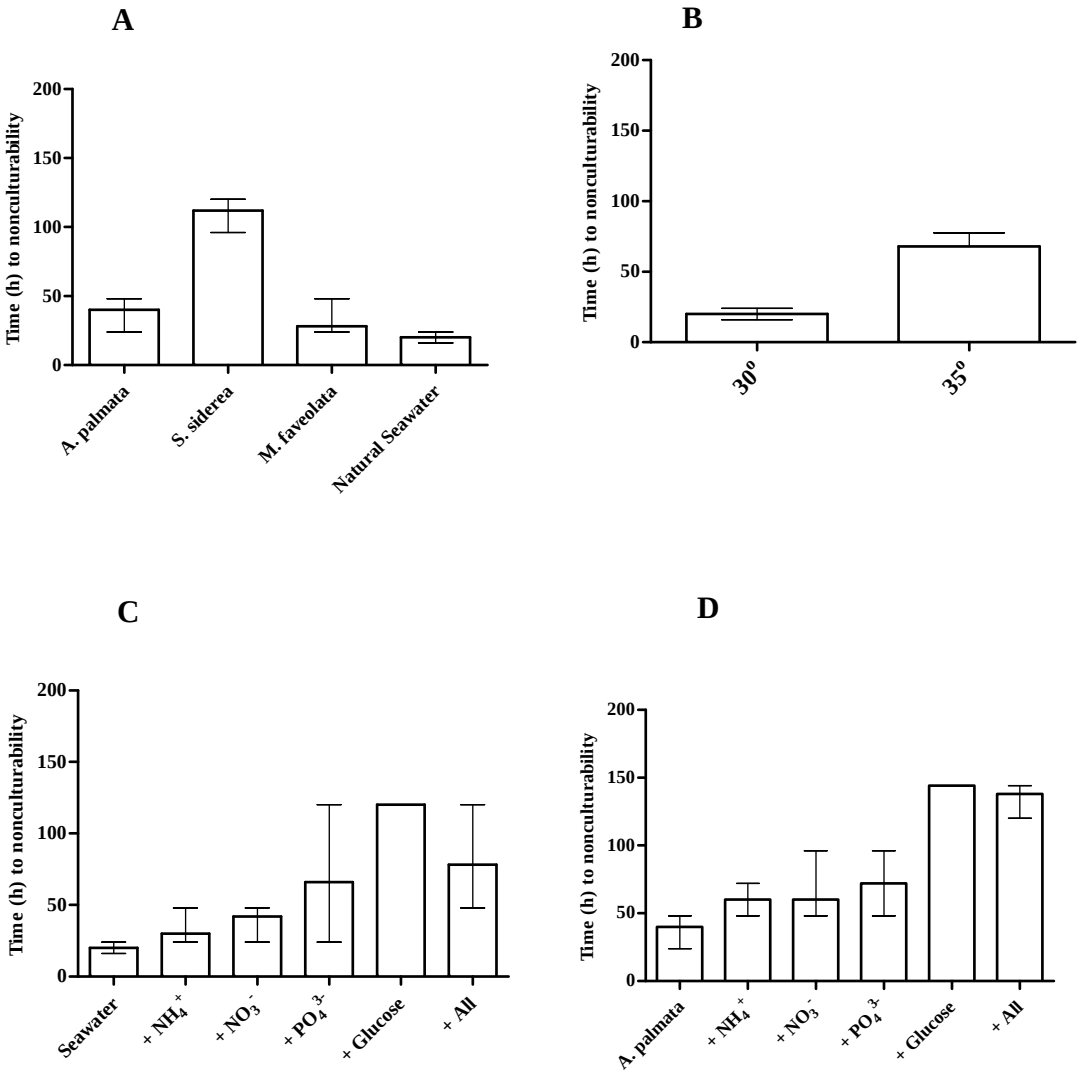

Figure 1. Mean survival times of PDL-100 in each microcosm condition (N=6).

CHAPTER 4

BACTERIAL INHIBITORS OF A CORAL PATHOGEN, PDL-100 (*SERRATIA MARCESCENS)*, IN THE CORAL REEF ENVIRONMENT

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Abstract

Serratia marcescens, strain PDL-100, was found to cause white pox disease in *Acropora palmata* in the Florida Keys in 1998. After isolating 379 marine bacteria from the coral surface microlayer of *A. palmata, Montastrea faveolata,* and *Sidereastrea siderea*, two strains of *Enterobacter cloacae,* isolated from two distinct reefs, were found to directly inhibit PDL-100. These findings suggest that the microbial community within the coral surface may play a role in regulating the propagation of coral pathogens.

Introduction

Serratia marcescens is classified in the family Enterobacteriacaea and is a Gram-negative bacterium. It is a well-known opportunistic pathogen which causes severe infections in clinical settings, such as urinary tract infections (Clayton and Graevenitz1966; Magnuson and Elston1966; Taylor and Keane1962), pneumonia (Tillotson and Finland1969), and respiratory tract infections (Cabrera 1969; Ringrose et al.1968). It also has been found associated with fishes and other organisms in brackish environments (Austin and Austin1999; Inglis et al.1993). Recently it was implicated as a causal agent in white pox disease of the Caribbean coral *Acropora palmata*, a disease which has decimated populations of this once common coral throughout the Florida Keys and Caribbean (Patterson et al. 2002).

The coral surface microlayer (SML) is thought to act as a potential barrier to foreign bacteria and other microorganisms, and data have shown that each coral species hosts a unique consortium of bacteria, even across wide geographical distances (Rowher et al. 2002). Ritchie and Smith (2004) noted that coral SML contains up to 100 times the number of culturable bacteria than that of seawater; therefore, it is plausible that bacteria in this matrix might aid in either the colonization of certain bacteria or inhibit growth of introduced bacteria, keeping the environment surrounding the coral somewhat constant and homogeneous. The coral probiotic hypothesis, proposed by Rosenberg et al. (2007), suggests that corals have a symbiotic relationship with microorganisms associated with their mucus and tissues that protects them during changing environmental conditions and further benefits the coral host by facilitating in nitrogen fixation, photosynthesis, and developing resistance to pathogens. This hypothesis proposes that corals are able to select for the most advantageous coral holobiont (coral + zooxanthellae + resident microbial community) to adapt to the changing environment. Many

marine bacteria have been reported to inhibit the growth of other bacteria through the production of antibacterial substances (Ritchie 2006; Geffen and Rosenberg 2005) and such bacteria may also be part of the coral holobiont and act as a first line defense against possible pathogens. The purpose of the present study was to determine if bacteria found in the SML of different coral species could inhibit the growth of *Serratia marcescens*, strain PDL-100, which was originally isolated from a white pox lesion in 1998 (Patterson et al. 2002).

Sampling. Coral mucus (the surface microlayer [SML] representing a mixture of mucopolysaccharide and water) (Paul et al. 1986) was sampled from *Montastrea faveolata, Siderastrea siderea,* and *Acropora palmata* on Western Sambos Reef, near Key West, FL and Carysfort Reef near Key Largo, FL in July 2007 and again in September 2007 at Looe Key Reef and Jaap Reef. At each sampling point, SML was collected by SCUBA divers from 3 apparently healthy colonies of each species using 60 ml syringes. SML was transferred to 50 ml centrifuge tubes (VWR #21008-178) and transported to the lab. Within 4 h after collection, 100 µL from each sample was spread directly onto Marine Agar (Difco #2216) plates and incubated at 30°C for 24 hours. Colonies (~20) were randomly picked from each plate and transferred to Marine Agar stabs until further use. All cultures were streaked for isolation 5 times to ensure pure strains. Pure strains were grown overnight in Marine Broth, and 1 ml aliquots were mixed with glycerol (25% final concentration) and stored at -80° C.

Antagonism assays. Cultures of PDL-100 (ATCC #13880) and marine bacterial isolates were grown overnight at 30°C in Marine Broth (Difco #2216). Two microliters of undiluted overnight growth from each culture were spotted in a four-by-four grid onto firm Marine Agar plates (1.5% agar) in triplicate and incubated at 30°C, and 35°C for 24 hours. Soft agar overlays containing PDL-100 (3 ml molten Marine Agar tempered at 45°C plus 1 ml of an overnight

culture of PDL-100) were poured onto the plates with the marine bacterial colonies after 24 h of growth. These plates were then re-incubated at 25°C, 30°C, and 35°C for up to 2 days. Plates were examined for zones of inhibition, noted by a clear halo around the tester marine bacteria isolates. The diameter of each halo was measured and recorded. Assays were performed 3 times, and only when inhibition was observed in all three assays were isolates scored as positive for inhibition.

Identification Methods. Isolates with obvious inhibitive effects were tested for Gram-reaction, catalase activity, and oxidase production. For phenotypic characterization, Biolog GN Microplate (Biolog, Hayward, CA) test panels containing 96 different carbon sources were used.

Additionally, identifications were also made by partial sequencing of the GyrB gene, which is reported to provide improved analysis of the Enterobacteriacae family relative to 16S rRNA sequencing (Dauga 2002). Briefly, DNA was extracted from overnight cultures of the inhibitor isolates prepared in 5 ml of Marine Broth at 30° C with shaking (130 rpm; New Brunswick Scientific, Edison, NJ). The cells were pelleted by centrifugation (3,500 x g for 3 minutes) on a tabletop centrifuge (Scientific, Marathon 3200R, Pittsburgh, PA). The supernatant was decanted and the pellet re-suspended in 1 ml of 1 X PBS then transferred to a sterile 1.5 ml tube. The tubes were floated in a beaker with water and boiled for 10 minutes. After placing on ice for 5 minutes, the tubes were centrifuged again in a microcentrifuge (Eppendorf 5417 C, Hamburg, Germany) (8,000 x g for 3 minutes) and the supernatant containing the DNA was transferred to a new sterile tube. DNA was subjected to PCR for the amplification of *gyrB.* The sequence of each primer is as follows: forward primer, 320F (5'-TAARTTYGAYGAYGAYAACTCYTAYAAAGT); reverse primer, 1260R (5'-CMCCYTCCACCARGTAMAGTTC) (Dauga 2002). 1 µL of each the forward and reverse

primers was added to PCR beads (GE Healthcare illustra puReTaq Ready-To-Go PCR beads; Little Chalfont, Buckinghamshire, UK) and PCR-grade water (Fisher Scientific; Fair Lawn, NJ) was added to a final volume of 24 µL; 1 µL of sample DNA was added for a final volume of 25 µL, and the master mix provided 2.5 units of puReTaq DNA polymerase, 10mM Tris-HCl (pH 9.0 at room temperature), 50mM KCl, 1.5mM $MgCl_2$, 200µM dATP, dCTP, dGTP, and dTTP and stabilizers including bovine serum albumin (BSA). An initial denaturation-hot start of 4 min at 94°C was followed by 40 cycles 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min using a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Watertown, MA). PCR products were separated by electrophoresis on a 1.5% agarose gel (Acros Organics Agarose LE; NJ, USA), stained with ethidium bromide, and visualized on a UV transilluminator. Column-purified PCR products were sequenced by primer extension using the forward primer (320F) primer by Northwoods DNA, Inc. (Solway, MN). The sequences were compared with those in the GenBank database using a BLAST search (Altschul et al.1990) and identification to the species level was determined.

Summary of tested isolates. A total of 379 isolates were tested for inhibition against PDL-100. 129 of these were isolated from *A. palmata*, 116 were isolated from *S. siderea*, and 124 were isolated from *M. faveolata.* After initial screening, 11 isolates showed possible inhibition, but only 2 were noted to consistently result in inhibition of PDL-100. Both isolates were Gram-negative rods (designated FK180 and FK247) and each were oxidase-negative and catalase-positive. Isolate FK180 was collected from *S. siderea* on Western Sambos Reef on July 25, 2007. Isolate FK247 was collected from *A. palmata* on Carysfort Reef on July 27, 2007.

Inhibition effects were primarily noted when tester bacteria were grown at 35°C and PDL-100 overlays were grown at 30°C (Table 1). Growing both FK180 and FK247 at 35°C and

then incubating the PDL-100 overlay at 30°C resulted in maximum inhibition for both strains (inhibition diameters of 1.2 cm and 2.0 cm for FK180 and 247, respectively).

Identification of inhibitor bacteria. Based on the 95 tests determined on the Biolog GN Microplates, taxonomic determinations were performed and the result for each FK180 and FK247 showed them to be *Enterobacter cloacae* (98% and 99% probability respectively). BLAST searches against the partial sequence of *gryB* DNA amplified in the two isolates also resulted in the identification of these bacteria as *E. cloacae*. FK180 had a maximum identity of 90% for 420/463 bases (accession # EF64828) and FK247 had a maximum identity of 99% for 457/458 bases (accession # DQ386885) (Figure 1).

Microorganisms are known to inhabit the coral surface mucus layer, and under normal conditions, these bacteria aid the coral in nutrition (Sorokin 1973) and response to stress (Ducklow and Mitchell 1979) and are thought to be beneficial to the coral. Additionally, the Coral Probiotic Hypothesis (Reshef et al. 2006) suggests the multitude of bacteria and symbiotic dinoflagellates associated with the coral make the coral holobiont very adaptive to changing environmental stresses, including physical stress (temperature and bleaching) and well as disease. The hypothesis predicts that corals become resistant to certain pathogens by increasing the presence of one or more coral bacterial strains that have the ability to inhibit the growth and colonization of the pathogen (Reshef et al. 2006). The purpose of this study was to identify marine bacteria that have antagonistic properties against PDL-100. The two isolates which showed inhibition of PDL-100 were collected from 2 different species of coral, *S. siderea* and *A. palmata*, and both were identified as *Enterobacter cloacae*.

E. cloacae is part of the family Enterobacteriaceae and like most members in this family, is an opportunistic pathogen that causes infections in hospitalized patients (Flynn et al. 1987;

Gaston 1988). It is an enteric bacterium that is most frequently isolated from animals, including humans (Salas and Geesey 1983). In 1991, Nelson and Craft found that *E. cloacae* was a successful inhibitor of *Sclerotinia homoeocarpa*, the cause of dollar spot, a disease of turfgrasses. The ability of FK180 and FK247 to inhibit PDL-100 increased at lower temperatures (25° and 30°C) and decreased at higher temperatures (35°C), possibly explaining the increased competitiveness of PDL-100 at higher temperatures. High temperatures correlate with white pox disease incidence and tissue loss in *A. palmata* (summer months) (Patterson et al. 2002). Elevated temperatures are reported to accelerate growth of those coral pathogens that have been studied (Kushmaro et al. 1996; Kushmaro et al. 1998). The expression of crucial bacterial virulence genes can also be temperature-dependent (Ben-Haim et al. 2003; Rosenberg et al. 2004).

Ritchie (2006) found that 20% of cultured bacteria from *A. palmata* displayed antibiotic activity against PDL-100. The bacterial community which inhabits the SML of corals seems to be well-defined, as it has been documented that the same species of coral in different geographical regions have the same bacterial composition (Rohwer et al. 2001; 2002), rather than a random assortment of bacteria which happens to be in the water column (Ritchie and Smith 1995; Santavy 1995). Further research is needed to determine if some species harbor these antagonistic bacteria as an ecological defense against potentially harmful organisms.

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Table 1. Tester isolates grown at 30° C and 35° C; PDL-100 overlay grown at 25° C, 30° C, and 35° C. The clearest signs of inhibition occurred at first incubating tester isolates at 35° C and overlay at 30° C.

		Growth Temperatures for PDL-100		
Growth Temperatures for Tester Isolates		**25˚C**	**30˚ C**	**35˚ C**
30˚C		_____	FK180 (1.0 cm diameter) FK247 (1.5 cm diameter)	_____
35˚C		FK180 (1.0 cm diameter) FK247 (1.8 cm diameter)	FK180 (1.2 cm diameter) FK247 (2.0 cm diameter)	_____

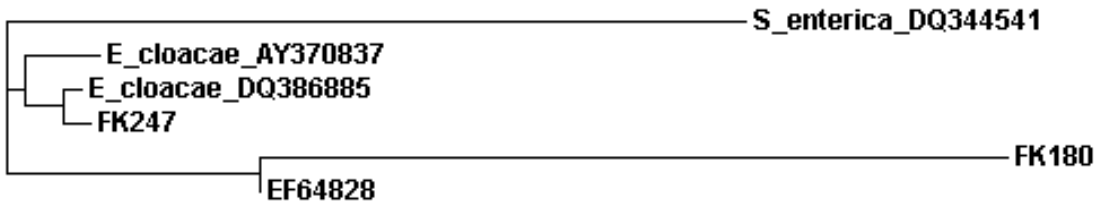

Figure 1. Phylogram of inhibiter isolates FK 180 and 247. Both are consistent with *Enterobacter cloacae gyrB* sequences and more distantly related to *Salmonella enterica*.

CHAPTER 5

Conclusions

Coral cover in the Caribbean and worldwide is declining at an alarming rate and steps need to be taken to understand the causes that adversely affect these valuable species. Among other problems such as overfishing, warming temperatures, sedimentation, and hurricanes, pathogens have become an increasing concern to coral health. The pathogen that was the focus of this study, *Serratia marcescens* strain PDL-100, has been found in many environments, but little is known of this bacterium in the marine environment, despite the fact that it is the 5th etiological agent to be identified as causing a coral disease (white pox). *Acropora palmata*, the species in which it affects, was once the most dominant space-occupier in Caribbean reefs, so decimation of this species significantly reduces overall coral cover. White pox disease is the primary cause of *A. palmata* decline in the Florida Keys National Marine Sanctuary, so it is important to understand the characteristics of the pathogen which causes this disease.

We focused first on evaluating PDL-100 persistence in the following environments: seawater, mucus from *Acropora palmata, Sidereastrea siderea,* and *Montastrea faveolata*, warm and extreme temperatures (30°C and 35°C), and nutrient-amended seawater and *A. palmata* mucus (NO_3^-, NH_4^+, PO_4^{3-}, and DOC). We found that while there was no growth of PDL-100 in any of these environments, survival was heightened in the mucus of *S. siderea,* extreme temperatures (35°C), and both seawater and *A. palmata* mucus amended with glucose (DOC). These results are important when considering the possible origin of PDL-100. It has been implicated that this bacterium may originate from land-based sewage. Under ideal conditions such as nutrient-low, average temperature seawater, this pathogen has less of a chance of

surviving the course from land to reef, and therefore less opportunity to cause disease. But if there is an overload of nutrients caused from any type of pollution and sea-surface temperatures are much higher, this pathogen has the ability to persist much longer, possibly causing disease in organisms such as *Acropora palmata.*

The second part of this study focused on biotic factors which affect PDL-100. Bacteria were isolated from *Acropora palmata, Sidereastrea siderea,* and *Montastrea faveolata* to test inhibition against PDL-100. Through phenotypic and genotypic characterization, we found that one bacterium (*Enterobacter cloacae*), isolated from 2 separate species from 2 geographically separate reefs, showed antagonistic properties against PDL-100. These results suggest that there are abiotic and biotic factors such as increased temperatures, presence of organic matter, and the presence of antagonistic bacteria affect the persistence of bacteria such as PDL-100. However, additional research addressing combinations of these factors could lead to a better understanding of disease dynamics.