INTER-KINGDOM SIGNALING AND CHARACTERIZATION OF A CORAL WHITE POX PATHOGEN, *Serratia marcescens*

By

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To my parents, who have always supported my academic and personal endeavors and have made this achievement possible

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The surface mucopolysaccharide layer (SML) secreted by corals is a rich environment where bacteria proliferate. The activities required for SML colonization by bacterial pathogens and commensals are unknown. *Serratia marcescens* is an opportunistic pathogen that causes white pox disease of *Acropora palmata.* To characterize mechanisms of SML colonization by *S. marcescens* PDL100, its ability for carbohydrate catabolism was characterized. A complement of enzymatic activities induced by growth on coral mucus was identified using defined chromogenic (*p*-Nitrophenyl) substrates. Pathogenic and environmental isolates of *S. marcescens* induced a suite of catabolic enzymes during growth on coral mucus. The characterization of glycosidases induced during growth on coral mucus demonstrates that *Serratia marcescens* relies on specific catabolic genes for its colonization of acroporid SML. Induction of these specific enzymes also provides insight into the types of bonds found in coral mucus. BIOLOG EcoPlates were used to characterize the ability of several isolates of *S. marcescens* to catabolize model carbon sources. *Serratia marcescens* PDL100 showed high correlation to other pathogenic isolates as compared to environmental isolates of *S. marcescens* and native coral-associated bacteria, suggesting that this coral pathogen may have originated from anthropogenic sources.

Coral larvae prefer to settle on substrates that are colonized by coralline algae or by biofilms formed by coralline algae and associated microbes, however, the perceived cue is unknown. Both bacteria and eukaryotes produce vitamin signals with newly discovered functions in QS and host-microbial interactions. The hypothesis that known signals commonly associated with microbial biofilms may function as settlement cues for larvae of stony corals was tested. These settlement experiments involved C14-homoserine lactone, 3-oxo-C6-homoserine lactone, lumichrome and riboflavin, each compound functions in bacterial cell-to-cell communication and contribute to settlement of marine organisms. Presence of AHLs, lumichrome and riboflavin in coral-associated microbes and in coralline algae was investigated. Transgenic microbial biofilms expressing AHL-lactonase were constructed to test the consequences of AHL hydrolysis in larval settlement. Chemicals were also impregnated onto C18-bonded silica resin to simulate biologically relevant release rates of the compounds into the medium during settlement experiments. *Acropora palmata* larvae appear to respond to AHLs and coralline algae, however, trends remain unclear.

CHAPTER 1
INTRODUCTION

1.1 Value of Coral Reefs

Coral reefs are among the most diverse and biologically complex ecosystems on Earth. These ecosystems are found all over the world near the equator and attract people year round with their pristine and exotic qualities. Besides the inherent value of these wonders of nature, coral reefs are valuable to the rest of the world in a number of ways. Coral reefs have been estimated to be annually worth $375 billion (Costanza et al. 1997). These ecosystems provide economic and environmental services to millions of people in over 100 countries as areas of recreation, sources of food, jobs, antibiotics, cancer-fighting medicines, novel fluorescent proteins for biotechnological applications, and shoreline protection. Polysaccharides produced and excreted by corals are a major nutrient source in reef ecosystem (Brown and Bythell 2005). Coral reefs and the neighboring coastal areas account for 38 percent of the goods and services provided by the Earth's ecosystems, which is more than terrestrial ecosystems account for (Cooper 1999). In Florida, coral reefs contribute at least $2.9 billion to local economies annually (Johns et al. 2001). The capitalized reef user value in southeast Florida is $8.5 billion (Johns et al. 2001). Unfortunately, the state of coral reefs is not so positive. The world's coral reefs currently face degradation and destruction from naturally and human induced events and are in desperate need of protection. Approximately 60 percent of coral reefs worldwide are currently threatened by human activities and nearly 10 percent of the coral reefs have been severely damaged or destroyed (Cooper 1999). Without coral reefs, the ocean's ecosystems will collapse.

1.2 Coral Biology

In order to fully understand the stressors facing coral reefs worldwide, it is necessary to understand the basic fundamental characteristics of the corals themselves. Corals are ancient in

origin, appearing nearly 400 million years before present. They are in the class Anthozoa in the phylum Cnidaria (Ball et al. 2002). Coral polyps are physiologically similar to hydroids and sea anemones.

While corals themselves are quite small, colonies of reef building coral (scleractinian corals) are able to produce massive rock-like structures with varying shapes and colors, which act as a natural barriers to coastal degradation. The rock-like structures are actually the calcium carbonate ($CaCO_3$) skeleton, which is secreted by the coral polyps. Typically the growth rate of this skeleton is rather slow (0.5 to 2 cm per year), but times of favorable conditions lead to more rapid growth (Edmunds et al. 2004, Edmunds 2007). Coral reefs are limited to specific conditions (especially temperature) and are therefore only found near the equator (Nystrom et al. 2000). Generally, no one specific factor (abiotic or biotic) determines the distribution of a single species. It has been suggested that the distribution of a species is dependent on interactions between an abiotic gradient and biotic interactions between species (Travis et al. 2006). Such species interactions are vital for coral recruitment. Trophic cascade interactions have been shown to enhance recruitment rates due to grazing fish limit the amount of macro-algae and increased substrate availability; thus facilitating coral recruitment (Mumby et al. 2007a). There are many indicators of the specific conditions surrounding a reef and the implications of changing conditions such as the diversity of the reef.

1.3 Coral Reef Decline

The health and success of a coral reef system are naturally maintained through occasional natural disturbances (e.g. storms, predators, temperature fluctuations) so that it does not become too productive. Disturbance is defined as a sudden event, which changes the nutrient status of an ecosystem. This may be an enrichment disturbance, where additional resources remove the limits on the carrying capacity, or a destructive disturbance, where part of the existing

community is destroyed, releasing nutrients for the remaining community (Harris et al. 2006). Causes of extensive coral mortality may reside in a single or often many natural factors that include low tides, volcanic eruptions, and increased temperatures. Scientists are beginning to focus their studies on five major causes of natural disasters: storms and hurricanes, coral bleaching, diseases of reef organisms, outbreaks of coral predators, and mass mortalities of reef herbivores (Brown 1997). These sources of disturbance to coral reefs have received the most attention because they have been found to often cause the most devastation to reef communities. The impacts of storms and hurricanes to a reef community are determined by a number of factors, all of which may vary with each storm and reef affected. The intensity of the disturbance, the resilience of the system to disturbance, as well as, the history of disturbance at the site are all important in measuring the damage and how quickly an ecosystem will recover (Brown 1997). For example, prior to the early 1980s, Jamaican fore reefs with high coral cover at 75% had not experienced a severe hurricane for 36 years. In 1980, coral disease and hurricane Allen reduced coral cover to 38%, however, the reefs recovered due to the presence of urchins (Mumby et al. 2007b). The long undisturbed history of these reefs and the abundance of herbivores to combat macroalgae enhanced the resilience of the system after a relatively short, yet intense disturbance.

The capability of ecosystems to deal with disturbance is determined by a variety of characteristics such as genetic variability within populations, diversity within and among functional groups (e.g. reef builders, grazers), and diversity of habitats (Nystrom et al. 2000). Ecologists now associate stability with persistence, resilience, and resistance (Nystrom et al. 2000, Walther et al. 2002, Hughes et al. 2003, Bellwood et al. 2004) and the mechanisms by which systems return to states of equilibrium (e.g. juvenile growth and gene flow (Edmunds

2007, Vollmer and Palumbi 2007)). Persistence simply refers to the existence of a community or population over a time period (Karlson 1999). The definition of coral reef resilience depends on ways of interpreting ecosystem development (Nystrom et al. 2000). Recently, ecologists have reconsidered the role of disturbance in terms of complex systems, which include multiple-equilibria, nonlinearity and phase shifts. Therefore, two prominent concepts of resilience have resulted. The traditional and most widespread view concentrates on stability near a single equilibrium state, where resistance to disturbance and the speed of return to equilibrium are emphasized (Nystrom et al. 2000). Resilience denotes the recovery of a system towards equilibrium after a disturbance that has physically altered the community structure. Highly resilient communities recover from disturbances quickly but are not necessarily indicative of high resistance. Therefore, resistance refers to the ability of the community to minimize the impact of disturbances (Karlson 1999). The second definition focuses on ecosystems in dynamic, non-equilibrium environments with multiple stable states where phase shifts may occur (Holling 1996, Mumby et al. 2007b). Resilience, in this case, refers to the amount of disturbance that can be absorbed by the system before a shift from one stable state to another occurs.

1.4 Anthropogenic Inputs to Coral Reefs

While some disturbance is essential to the maintenance of ecosystem diversity, persistent disturbances with increasing intensity may greatly influence the ability of a system to recover. Recently, awareness regarding human ability to alter natural disturbance regimes and thus influence coral reefs and their potential for recovery following disturbance has increased (Harvell et al. 1999, Nystrom et al. 2000). Human activities (both direct and indirect) often lead to disturbances equal to those that occur naturally (e.g. tropical hurricanes). Both adult corals and larvae are susceptible sedimentation, eutrophication and contamination from waterborne toxins (Minton and Lundgren 2006), which consistently occur in reef ecosystems. Coral reefs

typically are able to reassemble and recover from routine disasters (Bellwood et al. 2004) but when coupled with human disturbance, reefs show decreased resilience to disturbance. The major difference between natural and human-induced disturbances is their continuity. Natural disturbances tend to occur in a pulsed manner (e.g. tropical storms and coral predator outbreaks), while human-induced disturbances tend to continue and accumulate (e.g. nutrient enrichment and pollution) or occur so frequently that there is little time for recovery (e.g. high fishing pressure) (Nystrom et al. 2000). Over time, even a low level of such chronic stress can have severe impacts on coral reef ecosystems.

Ecosystems facing persistent disturbances often undergo an ecological phase shift resulting from a loss of resilience. Many reefs that suffer reduced stocks of herbivorous fishes and added nutrients from land-based activities have shifted from the original dominance of coral to a preponderance of fleshy seaweed (Nystrom et al. 2000, Hughes et al. 2003, Bellwood et al. 2004). It is often difficult to predict such ecological shifts, as awareness of the full consequences of human action lags far behind the impact (Western 2001, Wallentinus and Nyberg 2007, Mora 2008). This inability to detect and predict changes on a regional basis can be seen as an obstacle that prevents appropriate decisions regarding management and ecological restoration efforts (Harris et al. 2006, Vollmer and Palumbi 2007). If human modification of the marine environment continues, diversity within and among functional groups (e.g. reef builders and herbivorous fishes) may decrease. Coral reefs with decreased diversity within functional groups may maintain ecological function but additional disturbances may shift those groups into another stable state in which large-scale degradation and loss of essential ecological function may occur (Nystrom et al. 2000).

Global climate change is regarded as one of the major threats to the future of coral reefs since increases in temperature of only a few degrees induce global-scale episodes of coral bleaching and mortality (Hughes et al. 2003, Mora 2008). Temperature is the leading cause of coral reef decline (Hoegh-Guldberg 1999) and increases outbreaks of marine disease (Harvell et al. 1999). If trends continue, the levels of atmospheric carbon dioxide will increase and the pH of ocean waters will decrease (Fung et al. 2005). The increase in sea surface temperatures and decrease of pH increase cause stress to corals and increase their susceptibility to bleaching and disease.

Anthropogenic run-off and sewage pollution can lead to nutrient enrichment and eutrophication of waters reaching coral reefs. Through increased human activities, corals are exposed to growing loads of nutrients, sediments and pollutants discharged from the land (Fabricius 2005). Increased loads of nutrients and particulates may drastically alter the dynamics of the reef ecosystem, not only at the level of the symbiotic relationship between corals and zooxanthellae, but also at the community level. Massive macroalgae blooms result from nutrient enrichment of otherwise nutrient poor waters. These blooms physically outgrow sea grass and adult corals, inhibit recruitment of juvenile corals, may lead to hypoxia and/or anoxia, as well as, decreased fisheries and reduced biodiversity (Howarth et al. 2000, Lapointe et al. 2004). Corals may also be out competed by other filter feeders (e.g. sponges, bivalves, ascidians, bryozoans, and barnacles), which are more efficient at utilizing particulate organic matter (Fabricius 2005). This may occur, however, only in areas of low light where corals lack the photosynthetic advantage over other filter feeders.

Nutrient enrichment also directly influences the dynamics of the coral-zooxanthellae symbiosis. Zooxanthellae densities increase in response to high concentrations of dissolved

inorganic nutrients (i.e. nitrogen and phosphorus). The algae use the increased nitrogen for their own growth rather than growth of host tissue (Fabricius 2005). Increased densities of zooxanthellae take up more carbon dioxide than under non-enrichment conditions. This decreases the carbon dioxide available for calcification (Szmant 2002, Fabricius 2005). Increased particulate organic matter (i.e. clay and organic particles suspended in the water) also indirectly influence corals by reducing light penetration to the zooxanthellae. If prolonged, this may lead to lower carbon gain by the coral from zooxanthellae photosynthesis, slower calcification rates, and thinner coral tissue (Fabricius 2005).

Sewage pollution and contamination is of great concern in the Florida Keys. Approximately 30,000 on-site sewage disposal systems (septic tanks, cesspits, and Class V injection wells) are dispersed throughout communities in the Florida Keys, most of them positioned near boating canals (Lapointe et al. 2004). In the past, it was assumed that eutrophication from anthropogenic sources would only affect near and inshore waters (Szmant 2002), however, sewage pollution contributes to the eutrophication of not only inshore and near shore waters, but also offshore waters (Lapointe and Clark 1992, Lipp et al. 2002, Griffin et al. 2003).

Human-induced nutrient enrichment also increases the severity of diseases affecting corals. Two Caribbean corals epizootics, aspergillosis of common sea fans and yellow band disease of *Montastrea* spp. were shown to intensify during exponential nutrient increase (Bruno et al. 2003a). Potentially, the pathogens causing these diseases are able to utilize the excess nutrients, thereby increasing the fitness and virulence. These results demonstrate that minimizing nutrient pollution could be an important management tool for controlling coral epizootics (Bruno et al. 2003a).

Natural disturbances and human activities strongly impact at least 45% of the world's oceans (Halpern et al. 2008) and have severely damaged at least 30% of coral reefs (Hughes et al. 2003). They alone, however, do not explain why some systems have not recovered from disturbance but rather remain in alternate stable states (e.g. macroalgae dominant and low stony coral abundance). Coral decline is ubiquitous (Pandolfi et al. 2003) and often leaves questions as to how it is occurring at a regional level. Human influences do not explain all coral decline, especially when habitat degradation occurs on remote reefs without human presence (Ryan 2001). Observations of high levels of coral disease on reefs with no human influence require other explanations. Benchmarks, such as Caribbean-wide mortalities of acroporid corals and the urchin *Diadema antillarum* and the beginning of coral bleaching coincide with years of maximum dust influx into the Caribbean (Shinn et al. 2000).

African dust is not a new phenomenon in the Caribbean. Dust transported annually from Africa and Asia across the Atlantic and Pacific oceans has occurred for millennia, but recently researchers have put more stock into the role of the hundreds of millions of tons of dust in the decline of coral reefs (Garrison et al. 2003). Dust from African Sahara and Sahel deserts is transported to the Mediterranean, Europe and the Caribbean. Within the components of the dust, iron (Fe), silicon (Si) and aluminosilicate clays can serve as substrates for viable spores of numerous microbial species, especially the soil fungus, *Aspergillus sydowii* (Shinn et al. 2000). This soil fungus is the known pathogen that affects sea fans (*Gorgonia ventilina* and *G. flabellum*) Caribbean-wide. Besides the observation that dust may harbor opportunistic pathogens, which can lead to infection and coral disease, African and Asian dust also brings significant quantities of water-soluble nutrients to the oligotrophic waters of the Caribbean, Gulf of Mexico, and Pacific (Garrison et al. 2003). The addition of these nutrients may not only aid

bacteria transported by dust, but also may create enhanced conditions for bacteria persisting in the nutrient poor waters. These bacteria then may out-compete native bacteria, which may react adversely to the influx of nutrients. The steady transport of dust throughout the world's coral reefs may help to explain why many systems are unable to recover fully after a traumatic disturbance due to a net influx of nutrients and non-native organisms and chemicals.

1.5 The Coral Holobiont

1.5.1 Symbiosis and Nutrient Exchange

Most coral species are involved in a symbiotic relationship with dinoflagellates functionally defined as zooxanthellae (Belda-Baillie et al. 2002). Through coevolution with these dinoflagellates, corals have developed an ecological relationship leading to enhanced respiration, metabolism, waste excretion and increased growth rates in nutrient poor waters (Stanley and Swart 1995). These relationships began to form in the middle to late Triassic period roughly 200 mybp (million years before present) (Stanley and Swart 1995). In this relationship, the coral provides a protected environment for the algae as well as carbon dioxide and other wastes used in photosynthesis (Wilson and Wilson 1985). Corals are successful in low-nutrient tropical waters due to the plasticity in the modes they utilize to obtain nutrients. The polyp captures zooplankton through suspension feeding and translocates photosynthetic products from the zooxanthellae (Muller-Parker and D'Elia 1997). Animal metabolic waste products derived from holozoic feeding are retained within the coral, and provide inorganic nutrients (i.e. nitrogen and phosphorus) required by the zooxanthellae for photosynthesis. As autotrophs, zooxanthellae only require inorganic nutrients, carbon dioxide and light for photosynthetic carbon fixation (Muscatine and Porter 1977, Stanley and Swart 1995, Muller-Parker and D'Elia 1997). The zooxanthellae produce great amounts of photosynthate in excess to that utilized by the coral for direct nutrition (Ducklow and Mitchell 1979, Wild et al. 2004). The

coral secretes the excess photosynthate in the form of mucus, which bathes the coral tissue (see discussion of coral mucus below). Zooxanthellae may also benefit corals indirectly through the uptake of inorganic nutrients. Some nutrients (e.g. phosphate) act as $CaCO_3$ crystal inhibitors and their removal from calcification sites by the zooxanthellae promote calcification by the coral (Muller-Parker and D'Elia 1997).

Corals are under increasing stress due to changes in their environment. This stress increases coral's susceptibility to diseases. The zooxanthellae, *Symbiodinium*, are not the only organisms with which corals are in association. Recently, research has demonstrated that corals also contain large, diverse, and specific populations of microorganisms on their surface and within their tissues (Ritchie and Smith 2004, Wild et al. 2004, Rosenberg et al. 2007). Some of these microorganisms have been speculated to co-evolve with coral (Rohwer et al. 2002, Knowlton and Rohwer 2003, Ritchie and Smith 2004) based on continual isolation from coral and growth on mucus treated media. Those microorganisms living as part of the coral holobiont serve important roles in maintaining a functional symbiotic relationship. Such functional roles of microorganisms include nitrogen cycling, utilization of complex carbon compounds such as proteins and polysaccharides, gene expression relating to stress response, DNA repair and antibiotic resistance (Wegley et al. 2007).

1.5.2 Signal Exchange

Each partner in the coral holobiont influences the others. Corals may receive signals from their symbiotic algae and microorganisms, as well as, the external environment (e.g. conspecifics during spawning, macroalgae, and competing corals) during different stages of their life histories. Elkhorn coral, *Acropora palmata*, reproduce during annual mass spawning events, where gametes are synchronously released into the seawater for external fertilization and dispersal (Babcock and Heyward 1986, Heyward and Negri 1999). The developing larvae

generally become competent, settle out of the water, and metamorphose into juvenile coral polyps (Babcock and Heyward 1986). As energy reserves from the oocyte diminish, cilia develop and the sensory and secretory cells of the epidermis differentiate (Heyward and Negri 1999). The onset of larval competency coincides with decreased larval buoyancy, increased motility and sensory capability at the aboral end (Heyward and Negri 1999), which potentially allows the larvae to "sample" the substrate and adhere to it (Edmunds et al. 2004). Often, coral larvae may differentially select a site of permanent attachment due to external chemical cues that potentially induce metamorphosis (Morse et al. 1994, Morse et al. 1996, Heyward and Negri 1999). Coralline algae are one of the primary sources of chemical morphogens and are thought to produce high-molecular weight polysaccharides that are recognized by chemoreceptors of the planula (Webster et al. 2004). However, lipophilic compounds extracted from CCA have also been shown to induce settlement of urchin larvae (Kitamura et al. 1993).

Studies demonstrate that, in addition to coralline algae, microbial biofilms and other chemical s induce coral larvae and other invertebrate larvae metamorphosis (Tsukamoto 1999, Tsukamoto et al. 1999, Webster et al. 2004, Huggett et al. 2006). The chemical lumichrome, a derivative of riboflavin (vitamin B-12) induces larval metamorphosis in the ascidian, *Halocynthia roretzi* (Tsukamoto et al. 1999). Lumichrome has also demonstrated capabilities to enhance alfalfa root respiration and shoot growth when produced by a symbiotic bacterium, *Sinorhizobium meliloti* (Phillips et al. 1999).

1.5.3 Other Zooxanthellate Symbioses

Corals are not the only marine invertebrate to form a symbiosis with photosynthetic algae. Taxonomically, zooxanthellae belong to seven different clades, and are known to form symbiotic relationships with coral polyps, sea anemones, sea slug (*Berghia verrucicornis*) (Kempf 1991, Stanley and Swart 1995, Wägele and Johnsen 2001). Many cnidarians (e.g. sea anemones) form

such relationships. These mutualistic interactions are often species specific and can even vary with habitat location (Secord and Augustine 2000). Unlike hermatypic corals, which form interactions with obligate symbionts of zooxanthellae, sea anemones of the genus *Anthopleura* can contain both zooxanthellae and zoochlorellae. The composition of the symbiotic algae in the sea anemone host varies with latitude and intertidal height (Secord and Augustine 2000). Zooxanthellae are also found in symbiotic relationships with nudibranchs (Kempf 1991, Wägele and Johnsen 2001). The exact role of the symbionts remains somewhat unclear with suggested functions that include camouflage for the host, the photosynthetic algae may aid the host in persisting during periods of low nutrients, and inclusion of the algae may enhance reproductive output of the nudibranch as a result of shuttling energy from photosynthetically fixed carbon to the eggs (Wägele and Johnsen 2001). The zooxanthellae are acquired while the nudibranch feeds on the symbiotic sea anemone, *Aiptasia pallida*. Zooxanthellae in the tissue of the anemone are transferred to nutrient processing cells (NPCs) and are retained intracellularly in peri-algal vacuoles (Kempf 1991). Zooxanthellae also form a type of symbiosis with the giant clam, *Tridacna gigas* in which the clam houses the algae in a unique complex diverticulum of the stomach (Lucas 1994). The ability of the giant clam to utilize both the photosynthate from the zooxanthellae as well as the nutrients obtained from efficient filter feeding offers a growth advantage over other heterotrophic bivalves.

1.5.4 Coral Mucus

All corals secrete mucus (Fig. 1-1). The majority of the fixed carbon found in the surface mucopolysaccharide layer (SML) originates from the symbiotic zooxanthellae (Patton et al. 1977). Fixed carbon produced by zooxanthellae is transferred to the coral host and secreted through epidermal mucus cells (Ritchie and Smith 2004). High arabinose contents in coral mucus indicates that much of the fixed carbon is released as mucus since arabinose is generally

not found in animal tissue (Meikle et al. 1988). Both hard and soft corals secrete mucus continuously and each species has a distinctive composition (Ducklow and Mitchell 1979, Meikle et al. 1988, Ritchie and Smith 2004, Wild et al. 2004), which can vary temporally and with depth (Crossland 1987). The basic structure of mucus is an insoluble, hydrated, glycoprotein secreted by the coral (Ducklow and Mitchell 1979, Meikle et al. 1988). The observation that the majority of fixed carbon is not utilized by the coral but rather secreted, indicates that mucus serves a number of roles. Continuous release of mucus aids in ciliary-mucoid feeding in the coral reef copepod *Acartia negligens* (Richman et al. 1975) and is hypothesized to protect against microbial colonization, smothering by sediment, physical damage, desiccation during air exposure at extreme low tides, space invasion by other corals, and ultraviolet radiation damage (reviewed in (Wild et al. 2004, Brown and Bythell 2005)). Mucus secretion may also serve as an indicator of coral health. Banin et al. (2001) showed that in the Mediterranean coral *Oculina patagonica*, healthy pigmented corals secrete large amounts of mucus compared to bleached and diseased colonies (Banin et al. 2001). Coral mucus may enhance resistance to disease through a number of mechanisms, including antibiotic production and inhibition of pathogenic mechanisms (Ritchie 2006). The unique composition of mucus secreted by corals may promote coral-bacterial symbionts while inhibiting potential pathogens.

The production of mucus in vertebrates systems is well documented and several cell types that contribute to mucus secretion have been described (Verdugo 1990). In coral tissues, however, the limited histological and histochemical investigations describe only one type—the mucocyte, which is found in all tissue layers (reviewed in (Brown and Bythell 2005)). Continuous production of mucus is clearly advantageous but the rate of mucus production in relation to environmental conditions may vary greatly. Among eight species of scleractinian

corals studied in the Red Sea, the average overall mucus rate of production was approximately 51 mg of particulate organic matter m^{-3} day^{-1} (Richman et al. 1975). Other studies have demonstrated that submerged species of *Acropora* released 1.7 liters of mucus m^{-2} day^{-1} (Wild et al. 2004).

Even though the exact composition of coral mucus is not certain (except for a few common carbohydrates such as arabinose), it is obvious that the coral SML is high in organic matter. Coral mucus, therefore, creates a nutrient rich environment for bacteria and other microorganisms. This is in strong contrast to the surrounding bacterioplankton environment. In fact, culturable SML bacterial populations were found to be 100 times higher than those from surrounding water mass and many orders of magnitude more metabolically active (Ritchie et al. 1996). While, the surface mucopolysaccharide layer (SML) provides ample nutrients for bacteria on the surface, approximately 56-80% of released coral mucus immediately dissolves and provides a food source for the bacterioplankton environment (Wild et al. 2004).

1.6 Coral Bleaching

Among the numerous natural occurrences that influence coral reefs (e.g. tropical storms, coral predator outbreaks, coral disease), coral bleaching is one of the most detrimental and also most mysterious. Coral bleaching has been observed all over the world and different conditions and factors have been attributed to these disturbances. Of all the possible causes for coral bleaching, one that is receiving much of the credit is climate variation through El Niño Southern Oscillation (ENSO) events (e.g. (Stone et al. 1999)). Southern Oscillation (SO) is a dramatic fluctuation of air pressure between the eastern and western Pacific, which is not associated with El Niño events (Gray Davidson 1998). Empirical evidence indicates a coral reef bleaching cycle in which major episodes are synchronized with El Niño events that occur every 3-4 years, on average (Stone et al. 1999). Coral bleaching occurs when there is a loss of color, arising from

the partial or total elimination of the *Symbiodinium* population or degradation of algal pigments (Douglas 2003). This occurs generally in times of stress, often caused by sea surface temperatures (SST) which are much higher than the tolerance level of the coral colony (Wolanski 2001), but also is attributed to solar radiation, especially ultraviolet (UV) radiation (Stone et al. 1999). The majority of coral species have adapted life histories that function within a very narrow range of conditions, which include salinity, nutrients, sediments, and temperature (Gray Davidson 1998). There are approximately one to two million algal cells per one square centimeter of coral which give the coral the vibrant colors that we see (Sapp 1999). Left without their symbiotic partners, the corals appear white or colorless (the color of the calcium carbonate skeleton) and usually die as they can not obtain the necessary nutrients without their symbionts (Gray Davidson 1998). If, however, conditions become favorable in a relatively short time, the corals may be able to acquire a new consortia of zooxanthellae (Douglas 2003, Reshef et al. 2006). The extreme sensitivity of corals to their surrounding temperatures makes them especially susceptible to coral bleaching.

Coral pathogenic bacteria have also been shown as a causative agent of coral bleaching. *Oculina patagonica* is a scleractinian coral found in the Mediterranean and is the only hard coral known to have invaded a new region (Rosenberg and Falkovitz 2004). It is believed that the coral, a known fouling organism, traveled from the Atlantic Ocean by adhering to the hull of a ship. A pathogenic strain of *Vibrio shiloi* AK1 was found to be associated with bleached *O. patagonica*. The bacteria were isolated from bleached coral tissue and Koch's postulates were fulfilled, demonstrating that the pathogenic strain is a causative agent for coral bleaching (reviewed in (Rosenberg and Falkovitz 2004)). In this model, the classical triggers of coral bleaching (Ben-Haim et al. 2003a, Douglas 2003) are still in play as laboratory studies show *O.*

patagonica is more susceptible to bleaching by *V. shiloi* during periods of elevated seawater temperatures (Rosenberg and Falkovitz 2004). With global climate change and elevated sea surface temperatures (SST), coral reefs are under high levels of stress making them susceptible to bleaching and disease.

The overwhelming evidence and support for the *Oculina patagonica* coral-bleaching model have been used as a basis to propose that bacterial pathogenesis may be one cause for global bleaching patterns. A recent study investigating the *in situ* involvement of bacteria in the bleaching of *O. patagonica* across the Israeli coastline, substantiated evidence to dismiss the notion that bacteria are involved in coral bleaching (Ainsworth et al. 2008). Corals were monitored throughout an annual bleaching event and the proposed pathogen, *Vibrio shiloi* was not detected in any tissue layers. This observation is consistent with experimental conditions in support of the coral probiotic hypothesis (Reshef et al. 2006). A change in the endolithic (natural) community of microorganisms occurs during coral bleaching (Ainsworth et al. 2008). This shift highlights the potential importance of the diverse and complicated interactions between the organisms that comprise the coral holobiont in terms of disease resistance and resilience.

1.7 Coral Diseases and Management

1.7.1 Examples of Coral Diseases

Many organisms that cause coral diseases are not dedicated pathogens, but are *opportunistic* ones. Opportunistic pathogens are those microorganisms that are normally found in the environment and are generally benign. Opportunistic pathogens invade their eukaryotic hosts only when the host's defense systems are compromised. Opportunistic pathogens may be introduced into a habitat by a variety of means. Recently, human influence and activities have received a great deal of attention. Studies have demonstrated that not only human activities, but

also human waste, has been found to contribute to a high prevalence of enteric bacteria in near shore waters and canals of the Florida Keys (Griffin et al. 1999, Nobles et al. 2000, Lipp et al. 2002). While this observation is important, conclusive evidence, that wastewater is reaching and adversely affecting the coral reef environments along the Florida Keys is limited. One study suggests that coral mucus may serve as a better record of fecal contamination in reef areas since enteric bacteria are often difficult to recover from marine waters (Lipp et al. 2002).

Coral diseases caused by opportunistic pathogens are now widespread (Rosenberg and Ben-Haim 2002, Aronson et al. 2003, Frias-Lopez et al. 2004, Sutherland and Ritchie 2004, Gil-Agudelo et al. 2006, Weil et al. 2006). Several of these opportunistic pathogens that cause devastating diseases of corals were recently identified: coral plague by *Sphingomonas sp*. (Richardson et al. 1998), white pox disease by *Serratia marcescens* (Patterson et al. 2002) (Frias-Lopez et al. 2004), black band disease by a consortium of bacteria (Richardson et al. 1997) (Richardson and Kuta 2003), aspergillosis disease by *Aspergillus sydowii* (Smith et al. 1998). Koch's postulates have been fulfilled for white plague type II, white pox, aspergillosis, *Vibrio shiloi* induced bleaching and *Vibrio coralliilyticus* induced bleaching and disease. Coral disease symptoms described as black band disease, skeletal anomalies, white band type II, skeleton eroding band, fungal-protozoan syndrome, and pink-line syndrome have hypothesized microbial causative agents but have not been confirmed (Sutherland et al. 2004, Rosenberg et al. 2007).

Serratia marcescens is one of the better characterized opportunistic pathogens of Caribbean corals (Patterson et al. 2002, Sutherland and Ritchie 2004). Koch's postulates were fulfilled using 10^9 bacterial ml^{-1} infectious dose (Patterson et al. 2002). While this infectious dose was high, similar infection studies demonstrate at the LD_{50} of the pathogen was 10^7 bacteria ml^{-1} in mice (Carbonell et al. 2000) and as little as 1355 cells per individual larvae of *C.*

zealandica larvae (Tan et al. 2006). *Serratia marcescens* is associated with the appearance of white pox disease symptoms in *Acropora palmata*, which progress rapidly at a rate of 2.5 cm^2/day (Patterson et al. 2002). Irregularly shaped, distinct white patches, devoid of coral tissue, characterize white pox disease. White pox disease can be distinguished from white band disease (both of which affect *A. palmata*) as the potential for tissue loss (necrosis) occurs throughout the coral colony with white pox. White band disease develops at the base of the coral branch and progresses upward towards the branch tip in a concentric ring (Sutherland and Ritchie 2004). The white pox pathogen is currently the major pathogen of *Acropora*, a threatened Caribbean Elkhorn coral and it is closely related to other well-characterized and genomically sequenced pathogenic *Serratia* spp. Some catabolic enzymes and regulatory switches required for virulence of pathogenic *Serratia* in plants and animals have been characterized (Kurz et al. 2003, Soo et al. 2005, Queck et al. 2006). Although microorganisms that are pathogenic to some corals have been identified, the causative agents of many coral diseases remain unknown (Richardson 1998, Weil et al. 2006).

1.7.2 Characterization of Coral Diseases

The identification of coral pathogens as causative agents of disease must include fulfillment of Koch's postulates. To demonstrate the identity of a pathogenic microorganism, the following must be carried out: (1) the pathogen must be found in abundance in all organisms with disease and not in healthy organisms, (2) the pathogen must be isolated from the diseased host and grown in pure culture under laboratory conditions, (3) the pathogen from pure culture must cause the disease when it is inoculated into or onto a healthy animal, and (4) the pathogen must be re-isolated from the newly diseased animal and identified as the same microorganism as the presumptive pathogen (Tortora et al. 2002). In the past, some bacteria were accepted as the causative agents of disease despite the fact that Koch's postulates were not fulfilled (Richardson

1998). It is important to maintain a certain level of caution when assigning a pathogen to a specific disease based on Koch's postulates. One problem with identifying a pathogen by Koch's postulates is that the changes in host susceptibility or pathogen virulence with changes in the environment are not incorporated (Lesser et al. 2007). Another problem limiting the application of Koch's postulates is the inability to grow many potential pathogens in the laboratory (Ritchie et al. 2001). Many bacteria, viruses, protozoa and fungi cannot be propagated under laboratory conditions. It is therefore difficult to conclude that the disease produced in laboratory conditions was the same present in the environment. Recent studies have also emphasized the importance of going beyond the external macroscopic signs of coral disease in order to accurately diagnose disease (Ainsworth et al. 2007, Lesser et al. 2007). Often, macroscopic symptoms or signs associated with different diseases or syndromes overlap and may lead to misdiagnosis. Utilizing other methods such as microbial diversity characteristics and cytological observations may be useful for understanding the disease process of corals and improving the basis on which diseases are diagnosed.

1.7.3 Virulence Determinants in Opportunistic Pathogens

It is not yet clear, however, how opportunistic pathogens colonize and infect corals (Richardson 1998, Foley et al. 2005). The influence of host density and variability on disease outbreak also remains unclear. Disease outbreaks could potentially increase with increased host density (Ward and Lafferty 2004). *Vibrio harveyi* is a serious pathogen of marine animals, but despite its prevalence and characterization, the mechanisms of pathogenicity have yet to be fully elucidated (Austin and Zhang 2006). Extracellular products (e.g. cysteine protease, phospholipase, haemolysin) may play a central role in the virulence of the pathogen (Austin and Zhang 2006). While many coral diseases are well characterized, the mechanisms by which the pathogens that infect them need to be elucidated before effective management can be employed.

Coral diseases caused by microorganisms generally cause one of two major symptoms: tissue necrosis or bleaching. Of the pathogens known or assumed to cause the major necrotic diseases (white band I & II, white plague I & II, white pox, black band and aspergillosis) only a few pathogens have been observed to cause diseases in invertebrates besides corals (Grimont and Grimont 1978, Rinaldi 1983, Alker et al. 2001). *Aspergillus sydowii* is a soil saprophytic fungus known to occasionally act as an opportunistic pathogen of food, invertebrates and humans (Rinaldi 1983, Alker et al. 2001). Its pathogenicity depends on the host and the duration of exposure.

In addition to corals, *Serratia marcescens* infects a wide variety of hosts and can be viewed as a model opportunistic pathogen. *Serratia marcescens* is able to cause disease (and often high mortality) in *C. elegans* (Kurz and Ewbank 2000, Kurz et al. 2003, Schulenburg and Ewbank 2004), *Costelytra zealandica* (New Zealand grass grub) (Tan et al. 2006), numerous insects, plants, vertebrates, and humans (Grimont and Grimont 1978). During infection of *C. elegans*, *S. marcescens* is capable of killing the host by a toxin-based mechanism or following the establishment of an infection. The bacteria are able to live within the digestive tract of the nematode and proliferate and spread, eventually causing a systemic infection (Kurz and Ewbank 2000). In a screen of *Serratia marcescens* mutants, specific genes involved in a two-component regulatory system, magnesium and iron transport, hemolysin production and the biosysthesis of O-antigen and lipopolysaccharides (LPS) were found to be important to the virulence of the pathogen (Kurz et al. 2003). A similar study investigating gene expression in *Pseudomonas aeruginosa* during *C. elegans* infection identified similar genes (e.g. two-component global regulation system genes) (Tan et al. 1999).

Coral pathogens can also induce bleaching during infection. The annual bleaching of the Mediterranean coral, *Oculina patagonica* has been correlated with infection by *Vibrio shiloi* during the warm summer months (Rosenberg and Ben-Haim 2002, Rosenberg and Falkovitz 2004, Reshef et al. 2006, Ainsworth et al. 2008). The pathogen adheres via a β-galactoside receptor produced by the endosymbiotic zooxanthellae (Toren et al. 1998). Once the bacteria penetrate the coral tissue they produce heat-sensitive toxins targeting the zooxanthellae and thus inhibiting photosynthesis (Banin et al. 2001). Similarly, *V. coralliilyticus* induces bleaching and tissue lysis in *Pocillopora damicornis* (Ben-Haim and Rosenberg 2002, Ben-Haim et al. 2003a, Luna et al. 2007). This gram-negative, rod-shaped, motile bacteria produces a 36 kDa extracellular protease believed to be involved in its pathogenicity (Ben-Haim et al. 2003a, Ben-Haim et al. 2003b). While several species of *Vibrio* are pathogenic to invertebrates, the unique feature of the *Vibrio* induced bleaching of corals is that the pathogen targets the zooxanthellae rather than the coral itself (Ben-Haim et al. 2003b).

1.7.4 Disease Management

Opportunistic pathogens are of tremendous threat to corals and other invertebrate hosts in freshwater and marine systems. This threat originates in the ubiquitous nature of opportunistic pathogens and their ability to persist in a variety of environments and cause infection in a wide array of hosts (i.e. when host immune systems are compromised). As discussed earlier, pathogens, especially those that co-evolved with humans (Templeton 2007), can enter marine systems through runoff and other forms of pollution. Once introduced to marine systems, some pathogens are able to persist and even flourish. For example, *Vibrio cholerae* and *Escherichia coli* are able to survive at densities of 10^6 ml^{-1} for extended periods in niches within coral reef and turtle grass ecosystems (Perez-Rosas and Hazen 1988). *Vibrio cholerae* demonstrated higher survival rates and activity as compared to *E. coli*. This observation demonstrates how

opportunistic pathogens can easily lead to bacterial contamination for fish, shellfish and corals in this environment. Although many pathogens are associated with human illness and disease, they may enter a new niche in a different environment free of negative interspecific interactions (Perez-Rosas and Hazen 1988, Bruno et al. 2003b). In these scenarios, it is often difficult to determine whether a potential pathogen isolated from coral reefs is associated with anthropogenic inputs to the system or if it had independently evolved in that environment (e.g. (Patterson et al. 2002)). Survival of opportunistic pathogens in different environments and infection of multiple hosts highlights the need to understand the mechanism of virulence and how they are able to colonize and infect their hosts.

It is logistically impossible to "cure" coral diseases. Improvements to sewage infrastructure in coastal communities are prohibitively expensive. Treating coral diseases with antibiotics and pesticides is not feasible; therefore exploring biocontrol potential of native microbial communities may offer a possibility for a new thinking about addressing the coral reef decline. Similar biocontrol strategies have been reasonably successful in agriculture and commercial aquaculture (Garrigues and Arevalo 1995, Nogami et al. 1997, Whipps 2001, Chythanya et al. 2002, Raaijmakers et al. 2002, Fravel 2005, Persson et al. 2005, Balcazar et al. 2006, Rasmussen and Givskov 2006). The mechanisms of interactions between opportunistic pathogens, beneficial bacteria and coral hosts may offer an exciting model for addressing and managing ecosystem-wide degradation resulting from sewage pollution.

1.8 Virulence Factors in Bacteria

Bacterial pathogens often interact with a wide variety of distinct hosts, ranging from simple invertebrates to vertebrates and mammals. Most pathogens cause disease in a single or a restricted number of host species. The limitations observed in host ranges are primarily a result of a long history of coevolution (Rahme et al. 2000). Pathogens must either adapt to their new

host environment or modify it to persist over the host defenses. Involved in this is the recognition of the host, colonization and exploitation of host resources. In order to do this, bacteria have an arsenal of virulence-related factors.

Bacterial and fungal pathogens rely on enzymatic degradation of extracellular biopolymers for uptake of both nitrogen and carbon. This is particularly important during infection, when microbial proteinases are virtually unregulated by host proteinase inhibitors (Travis et al. 1995). The primary function for these proteinases is to provide a source of free amino acids for survival and growth, however, they may also lead to tissue invasion and destruction and evasion of host defenses (Travis et al. 1995). Other extracellular enzymes produced by bacteria are essential to pathogenicity (e.g. phospholipase in *Yersinia enterocolitica* (Young et al. 1999)). Extracellular proteins play an important role in virulence and are transported out of the cell through various mechanisms.

Extracellular protein secretion is generally accomplished through one or more secretion systems. There are at least six secretion systems described, three of which are well characterized (Pugsley 1993, Aizawa 2001). Recent studies indicate that proteins secreted by the type III secretion system (TTSS) often influence bacterial-host interactions for pathogens of plants and animals (Hueck 1998). Conventional secretion systems may not be the only means for pathogenic bacteria to transport proteins involved in pathogenicity to the external environment. TTSS often functions only when the bacteria are in direct association with the host. Therefore, proteins transported this way can be classified as contact-dependent (Young et al. 1999). Bacteria are also able to secrete proteins extracellularly through the flagellar export apparatus, which is similar to the TTSS. Both systems consist of homologous component proteins with common physio-chemical properties (molecular size, isoelectric point, instability index, and

aliphatic index), suggesting that they may have evolved in parallel (Young et al. 1999, Aizawa 2001). Although the flagellar transport apparatus was thought to have a role only in organelle biogenesis, it appears to also be required for transport of proteins to the extracellular environment in pathogenic bacteria (Young et al. 1999). Therefore, a functional motility apparatus is not only important for bacterial movement, but also for the secretion of virulence factors during infection.

Despite the evolutionary gap between plants and animals, virulence factors of pathogens appear to not be specific to one host, but rather, common to many hosts and used by diverse bacterial species (Rahme et al. 1995, Mahajan-Miklos et al. 2000, Rahme et al. 2000). This conclusion stems from two primary observations: (1) bacterial proteinases, serving as virulence factors, are conserved in plants and animals (Travis et al. 1995), and (2) strains of specific pathogenic bacterial species have been shown to infect plants and/or animals (Rahme et al. 1995). Such universal virulence factors have been termed effectors/toxins and boast a wide range of functions including cytotoxicity, hemolysis, proteolysis, protein phosphorylation, and protein dephosphorylation (Young et al. 1999). Effector proteins are not the only common virulence factors. In *in vivo* screens of pathogen virulence factors the global response regulator, *gacA*, was identified during infection of both *Pseudomonas aeruginosa* and *Serratia marcescens* in the nematode, *C. elegans* (Rahme et al. 2000, Kurz et al. 2003). Quorum sensing systems controlling bacterial communication (Waters and Bassler 2005) have also been identified in *P. aeruginosa* during infection of both plants and animal hosts (Rahme et al. 2000). Pathogenic bacteria have evolved a complement of virulence factors in order to mount an attack on their hosts and while some may be dedicated to a specific host, many are used during infection of plant and animal hosts.

1.9 Hypotheses and Goals

In this study the general hypotheses that both corals and microorganisms perceive chemical cues and signals from each other and that specific signaling and genetic and metabolic pathways are involved in the settlement of coral larvae and the colonization of bacteria on coral mucus were investigated. The experiments within this study focus on the interactions between the coral host (*Acropora palmata*) and potentially beneficial bacteria associated with the coral, in addition to pathogenic bacteria able to cause disease. These interactions comprise the metabolic capabilities of the bacteria to utilize and grow on coral mucus, and communication via chemical signaling between bacterial cells and between the coral and bacteria. I hypothesize that specific metabolic pathways and regulatory cascades are required for colonization and growth on coral mucus and that not only do the bacteria sense the coral host but that the corals respond to chemical cues from bacteria found on coral reefs. To test these hypotheses, the metabolic capabilities of pathogenic isolates of *Serratia marcescens* and three native coral-associated (*Photobacterium mandapumensis*, *P. leiognathi*, and *Halomonas meridiana*) were assayed for carbon-source utilization and enzymatic induction. The functionality of an evolutionarily conserved two component regulatory system, GacS/GacA, in *S. marcescens* was tested. Additionally, the response of coral larvae to different bacterial and environmental cues was investigated.

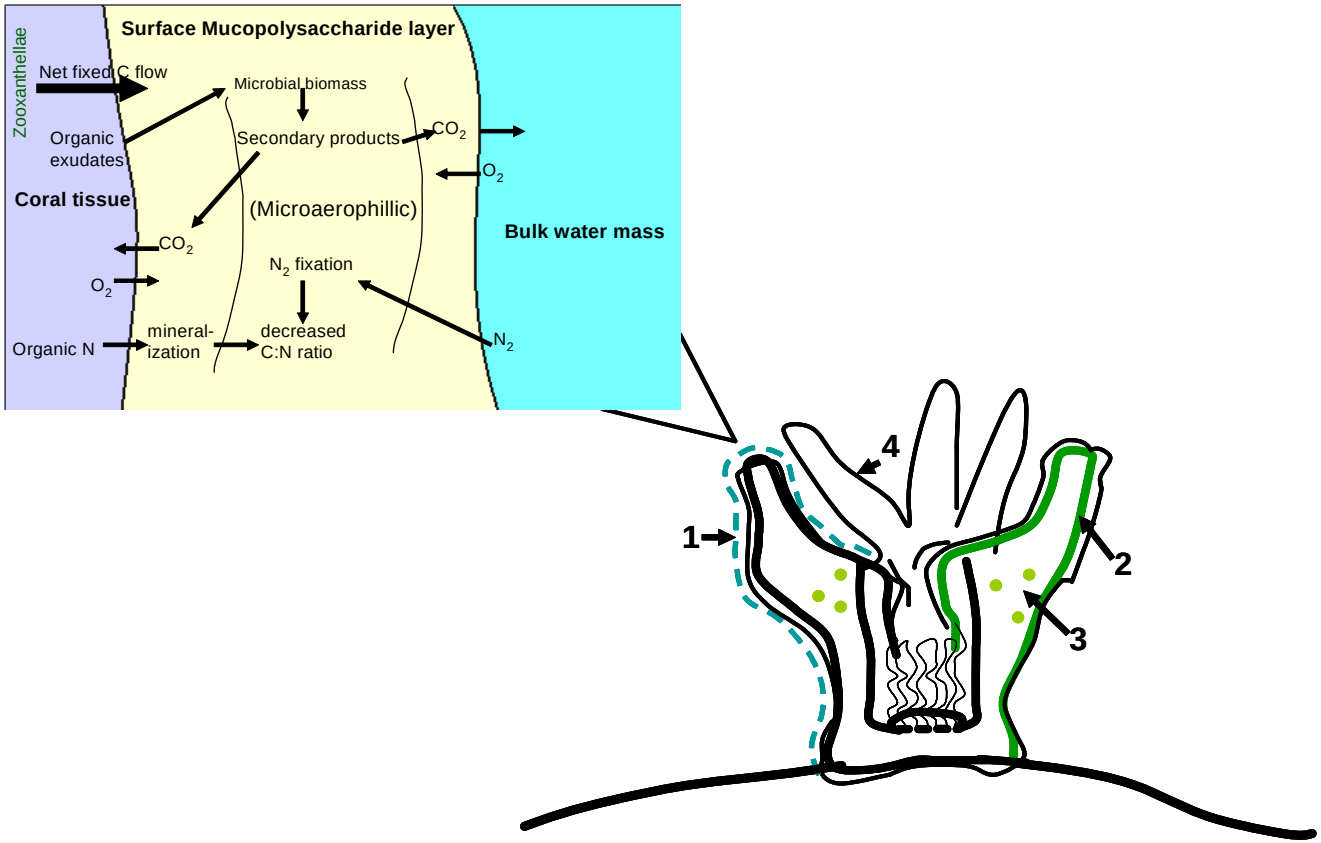

Figure 1-1. General schematic of a coral polyp with subsection of surface mucopolysaccharide layer (SML). On polyp figure (1) surface mucopolysaccharide layer (SML), which provides protection from UV, desiccation, and potentially disease resistance, (2) gastrodermis, which houses the zooxanthellae (Symbiodinium spp.), (3) zooxanthellae, photosynthetic algae, (4) feeding tentacle used for suspension feeding by coral. The subsection of the SML illustrates the complex environment within the mucus layer secreted by the coral. Photosynthates produced by the zooxanthellae leads to a net outflux of fixed carbon from the coral tissue. This and other organic exudates provide rich nutrients for the microbial population (coral residents and visitors) in addition to oxygen, thus creating a microaerophillic environment. Within the microbial population, some bacteria fix atmospheric nitrogen that can be used by the bacteria and the zooxanthellae. Adapted from (Ritchie and Smith 2004), used with permission from K.B. Ritchie.

CHAPTER 2
MATERIALS AND METHODS

2.1 Bacterial Strains, Plasmids, and Culture Conditions.

Bacterial strains and plasmids used in this study are listed in Table 2-1. Unless otherwise indicated, *Escherichia coli*, *Serratia marcescens* isolates, *Agrobacterium tumefaciens* and *Sinorhizobium meliloti* were grown in LB broth (per liter: 1.0% tryptone; 0.5% yeast extract; 0.5% NaCl Fisher Scientific, Pittsburg, PA). The coral isolated bacteria *Photobacterium mandapamensis*, *P. leiognathi*, and *Halomonas meridiana* were routinely grown in GASW broth (per liter: 356 mM NaCl; 8 mM KCl; 40 mM $MgSO_4$; 20 mM $MgCl_2 \cdot 6H_2O$; 60 µM K_2HPO_4; 7 µM $FeSO_4$; 33 µM Tris; 0.05% peptone; 0.2% yeast extract; 2.0% glycerol) or on 1.5% agar plates (Smith and Hayasaka 1982, Smith et al. 1982). *Agrobacterium tumefaciens* was also grown in AB minimal mannitol liquid media (per liter: 17 mM K_2HPO_4, 8.3 mM NaH_2PO_4, 18.7 mM NH_4Cl, 1.22 mM $MgSO_4 \cdot 7H_2O$, 1.98 mM KCl, 6.8 µM, 8.99 µM $FeSO_4 \cdot 7H_2O$, 5% mannitol) supplemented with 1% agar (Hwang et al. 1994, Shaw et al. 1997, Cha et al. 1998). Antibiotics were used in selection media at the following concentrations: for *E. coli*, Ap (100 µg/ml); Gm (30 µg/ml); Km (50 µg/ml), Cb (100 µg/ml) Tc (10µg/ml) where appropriate; for *S. marcescens* PDL100, Tc (10 µg/ml), Ap (100 µg/ml), for *Agrobacterium tumefaciens*, Gm (30 µg/ml), and for *Sinorhizobium meliloti*, Sm (500 µg/ml), Neo (50µg/ml).

Chemically competent cells are routinely made using the Inoue Method resulting in a transformation efficiency of 1.12×10^8 (Inoue et al. 1990). Briefly, overnight cultures of *E. coli* DH5α are grown in SOB broth (per liter: 2.0% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, pH 6.7-7.0, Fisher Scientific, Pittsburgh, PA) to an OD_{600} of 0.3. Cells are washed twice with TB (per liter: 10 mM PIPES; 55 mM $MnCl_2$; 15 mM $CaCl_2$; 250 mM KCl; pH 6.7 Fisher Scientific, Pittsburgh, PA) on ice and pelleted at 2500 x *g* for

10 minutes at 4˚C. Cells are then resuspended in TB and DMSO (Fisher Scientific, Pittsburgh, PA) is added to a final concentration of 7%. Cells are aliquoted and shock frozen with liquid nitrogen and stored at -80˚C.

2.2 Manipulations of DNA and Plasmid Construction

Restriction enzymes, T4 DNA ligase, and *Taq* Polymerase were purchased from New England BioLabs (Ipswich, MA) and used as recommended by the supplier. Plasmid DNA was routinely isolated using QIAprep spin mini-prep kit (Qiagen, Santa Clarita, CA). DNA restriction fragments and PCR products were eluted in DNA grade water from agarose gels by utilizing the Illustra™ DNA and gel band purification kit (GE Healthcare, Buckinghamshire, UK). All ligation reactions were conducted at 14ºC for a minimum of four hours unless otherwise specified.

Genomic DNA was prepared by standard methods as described previously (Sambrook and Russell 2001) with the following modifications for optimization. Cells from a 5 ml overnight culture were pelleted and washed with DNA grade water. Cell were lysed by vortexing with acid washed glass beads (150-212 μm in diameter, Sigma Aldrich, Atlanta, GA) with equal volumes phosphate buffer (120 mM K_2PO_4, pH 8.0) and water-saturated phenol, pH 8.0 (Fisher Scientific, Pittsburgh, PA). The mixture was vortexed for 15 seconds before centrifugation at 14, 500 rcf for 5 minutes. The aqueous phase was treated with RNAse A for 2 minutes at room temperature. One volume of saturated phenol (pH 8.0) was mixed with the DNA and centrifuged at high speed for 1 minute. The aqueous phase was mixed with one volume of phenol:chloroform:isoamyl alcohol (25:25:1, pH 8.0, Fisher Scientific, Pittsburgh, PA) and centrifuged at high speed for one minute. The aqueous phase was mixed with one volume of chloroform:isoamyl alcohol (24:1) and centrifuged for one minute yielding an aqueous containing protein-free DNA. 0.34 volumes of 3.0 M sodium acetate (pH 5.2) and 3.5 volumes of isopropanol were added and inverted until

DNA was visible. DNA was spooled with a plastic pipette tip and transferred into a new microcentrifuge tube and left to air dry. 0.1 volumes of 3.0 M sodium acetate (pH 5.2) and 3 volumes of ice cold absolute ethanol were added to dried DNA and placed at -80˚C for 30 minutes. The DNA mixture was centrifuged at 10,000 rcf for 10 minutes and supernatant was discarded. The DNA was then washed twice with ice-cold 70% ethanol in the same manner as above. After the second wash, precipitated DNA was dried completely and was stored at -20˚C until used. Genomic DNA was reconstituted in 50-100µL of DNA-grade water with incubation at 50˚C to solublize the DNA.

2.2.1 Identification of g*acA* in *Serratia marcescens* PDL100, White Pox Pathogen

The *gacA* gene was amplified from the *S. marcescens* genomic DNA using primers CJK12 and CJK18 (Table 2-2), which were designed based on the *gacA* sequence from *S. plymuthica* (NCBI GenBank: AY057388). PCR conditions included initial denaturation at 95˚C for 7 minutes, 35 cycles (95˚C, 1 minute, 53˚C, 1 minute, 72˚C, 2.5 min) and a final extension at 72˚C for 10 minutes. The resulting 957 bp product was cloned into pCR2.1 using a TOPO TA kit (Invitrogen, Carlsbad, CA), transformed into chemically competent DH5α and sequenced (Agencourt Bioscience Corp., Beverly, MA). A nucleotide BLAST in the NCBI database confirmed that the amplified sequence matched that of *S. plymuthica*.

2.2.2 Construction of a Plasmid that Contains Arabinose-Inducible g*acA*

To test whether *gacA* of *S. marcescens* PDL100 is functional, its ability to complement a *gacA* (*uvrY*) mutation in *E.coli uvrY33::kan* was tested. Therefore, a complementation construct was engineered to complement a previously engineered mutant in the *uvrY* gene of *E. coli* through a transposon (tn5) insertion (M. Teplitski, unpublished data).

To engineer a construct to complement the *uvrY* mutant in *E. coli* the *gacA* gene from p1318 was cloned into pBAD18-Ap. Plasmid p1318 was digested with EcoRI and the resulting

fragments were sub-cloned into the EcoRI site of the arabinose-inducible promoter vector, pBAD18-Ap, which yielded pCJK3 and was transformed into chemically competent *E. coli* DH5α. Transformants were selected on LB agar supplemented with Ap 200 μg/ml. Orientation of the insert was confirmed by PCR using primers MT13 and CJK18 (Table 2-2, Fig. 2-1).

2.3 Complementation Assay

To test the functionality of *gacA* in *Serratia marcescens* PDL100, an arabinose inducible promoter-based complementation assay was performed. The complementation vector pCJK3 was transformed into *E. coli* RG133 pMT41 by electroporation (25 μF, 200 Ω, 2.5 kV, 0.2 cm cuvette, 50 μL cell volume) using a Bio-Rad MicroPulser (Bio-Rad Laboratories, Hercules, CA). As vector controls, the original pBAD18-Ap vector was transformed into both the wild-type reporter *E. coli* 1655 pMT41 and its isogenic *uvrY33::kan* derivative reporter *E. coli* RG133 pMT41. Two overnight cultures of each strain were grown in LB with appropriate antibiotics at 37˚C on a rotary shaker (180 rpm). Following overnight incubation, cultures were diluted 1/100 in LB and incubated at 37˚C for 3 hours on a rotary shaker (180 rpm). Cultures were diluted to an OD_{600} of 0.3, and then diluted 1/25000 and aliquoted into a black polystyrene 96-well plate (in quadruplicate). Luminescence was measured with Victor-3 (Perkin Elmer, Shelton, CT) every hour for ten hours and the expression of the complemented mutant was compared to the wild-type reporter strain.

2.4 Carbon Source Utilization Profile Using Biolog Ecoplate Assay

Carbon-source utilization of the white pox pathogen, *Serratia marcescens* PDL100, and fifteen other isolates of *Serratia marcescens* and other coral isolated bacteria was assayed using Biolog EcoPlates. These 96-well plates are manufactured with 31 different substrates in triplicate per plate with a water control (Table 2-3). The EcoPlates rely on the tetrazolium violet dye redox reaction, which detects fermentation of sole carbon sources (Garland and Mills 1991,

Garland 1997). Assays were set up according to Choi and Dobbs (1999) with the following modifications (Choi and Dobbs 1999). Isolates were grown in either 5 ml LB broth or GASW broth overnight at 30˚C with shaking. Cells were pelleted at 10,000 x *g* using an Eppendorf table-top centrifuge 5415D (Eppendorf, Hamburg, Germany) and washed twice with filter sterilized (0.22 µm) seawater to remove any residual nutrients from the overnight media. Cells were then resuspended in 10 ml filter sterilized seawater and starved for 24 hours at 30˚C. Following the starvation period, 100 µL of cell suspension was inoculated into each well of the Biolog EcoPlate. The initial A_{590} of each plate was read on Victor-3 (Perkin Elmer, Shelton, CT) and was continuously read every 24 hours for a total of 72 hours.

2.5 Enzyme Induction during Growth on Coral Mucus

While the exact composition of coral mucus is unknown, detection of specific enzymes induced in response to growth on coral mucus can tell us certain types of bonds within the coral mucus matrix. Enzymatic induction assays using p-nitrophenyl chromogenic substrates allow for detection of individual specific enzymes induced in response to growth on a certain medium or in a specific niche. In addition to identifying specific bonds in the various components of coral mucus, the ability of different isolates and strains of bacteria to utilize the components of coral mucus may elucidate phenotypic relatedness among bacterial species and strains.

Serratia marcescens isolates from wastewater, canal water and other environments were compared with a pathogenic strain of the same species and three coral associated bacterial strains isolated from *Acropora palmata* mucus (Table 2-1). Two overnight cultures of each isolate were grown in 5 ml Luria-Bertani (LB) broth or in GASW broth to an approximate OD_{600} of 2.0 (stationary phase), which was determined spectrophotometrically. Cells were pelleted at 10,000 rcf and washed 3 times in filtered-sterilized seawater (0.22 µm) buffered with 10 mM HEPES to remove any residual nutrients and resuspended in 5 ml of buffered seawater. The cells were

starved in filter-sterilized seawater at 30˚C while shaking for three days in order to use up any internal resources. A three-day starvation period was found sufficient during preliminary studies with *S. marcescens* PDL100. Following the three-day starvation, 1 ml of cells was added to 2 ml of either 1x coral mucus (freeze dried, UV irradiated for 20 minutes and reconstituted to original volume (Ritchie 2006)) or 10 mM HEPES buffered seawater). Negative controls of coral mucus alone and buffered seawater alone were performed in parallel with the experimental treatments for each isolate. Cells were incubated in treatments for two and eighteen hours at 30˚C. Following incubation, the initial OD_{590} of each treatment was determined spectrophotometrically and recorded. Cells where then mixed with Z-buffer (1:1 v:v) and lysed with 0.1% sodium dodecyl sulfate solution and chloroform (4:1 v:v) (Miller 1972). Cell suspension was aliquoted into chloroform-resistant microcentrifuge tubes so to accommodate two biological and two technical replicates per substrate per treatment. Enzymatic substrates were prepared in HPLC-grade water and each substrate was added to the appropriate reactions to a final concentration of 0.8 μg/μL. Assays were conducted at room temperature for approximately 24 hours to allow for maximum color development. Sodium carbonate (Na_2CO_3) was added to a final concentration of 416 mM to stop the reaction and to intensify the color of each reaction. Cellular debris and unused enzymatic substrate were pelleted at 4,000 x *g* (16,000 rcf) for two minutes. The clear supernatant was transferred to a clear polystyrene 96-well plate and the A_{405} was measured on Victor-3 (Perkin Elmer, Shelton, CT). Buffered seawater and coral mucus were included in each plate as blanks.

Representative isolates from the three broad categories of *Serratia marcescens* isolates examined (human isolate, Sm 43422; environmental isolate, Sm39006; and white pox pathogen, PDL100) were also assayed with a treatment testing whether the catabolic capabilities of the

isolates could be repressed in the presence of glucose. A minimal media consisting of 10 mM HEPES buffered seawater supplemented with glucose (4 g/L) and Casamino Acids (0.1 g/L) as the sole carbon and nitrogen sources was filter sterilized through a 0.22 μm filter. This additional treatment was examined due to the observation that some enzymes appeared to be induced during starvation stress regardless of which treatment ultimately experienced by the cells. Glucose is a known catabolite repressor. In *Enterobacteriaceae*, glucose inhibits expression of catabolic and regulatory genes required for growth on most other carbon sources; glucose also inhibits expression of virulence genes and regulators (Ferenci 1996, Reverchon et al. 1997, Jackson et al. 2002, Gosset et al. 2004, Teplitski et al. 2006). As controls for this treatment, cells were also incubated with 10 mM buffered seawater supplemented with Casamino Acids (0.1 g/L) without glucose and buffered seawater alone. The enzyme induction assay was conducted as described above.

2.6 Proteinase Induction in Response to Coral Mucus

Acroporid coral mucus is made of a variety of carbon and nitrogen compounds and may consist of up to 22% protein (Ducklow and Mitchell 1979). These proteins provide serve as nutritional substrates to those bacteria able to utilize them as a food source. Therefore, it is plausible that induction of various proteinases may occur in response to growth on coral mucus. The production of proteinases during growth in rich medium was first investigated for both cell-associated and extracellular proteinase production (Demidyuk et al. 2006). A volume of 0.3 ml of either cell suspension or culture supernatant was added to 1.7 ml of azocasein solution (5 mg/ml azocasein in 0.1 M Tris Buffer pH 7.5) resulting in a solution with final azocasein concentration of 0.16 mg/ml. As a control, a blank of 0.16 mg/ml azocasein solution in water was prepared. Reactions were allowed to incubate statically at 30˚C for 60 min. Following incubation, trichloroacetic acid (TCA) was added to each reaction to a final concentration of

3.2% v:v to stop the enzymatic reaction and to precipitate any unhydrolyzed azocasein. Each reaction was pelleted at 10,000 x g for 1 min to sediment the unhydrolyzed substrate. The supernatant was carefully transferred to a new tube, to which NaOH (250 mM final concentration) was added to intensify the color. 200 µL of each reaction was transferred to a clear polystyrene 96-well plate and the A405 of each reaction was read on VICTOR-3.

2.7 Presence of Lumichrome and Riboflavin in Coralline Algae

2.7.1 Thin Layer Chromatography of Pure Compounds

In order to determine the conditions necessary for pure lumichrome and riboflavin to sufficiently migrate on the TLC plate (Whatman KC18 Silica Gel 60 with fluorescent indicator, 10 x 10 cm, 200 µm thick), saturated solutions of lumichrome and riboflavin in methanol:HCl (49:1) and in pure methanol were prepared (Phillips et al. 1999). Samples were pelleted to eliminate any particulate matter in solution as lumichrome and riboflavin have low solubility in many solvents. A total of 3 µL of each mix and the solvent (methanol:HCl) were spotted onto the TLC plate. The plate was developed with a mobile phase of chloroform:methanol:water (17.5:12.5:1.5) (Phillips et al. 1999). A total time of approximately 40 minutes was required for the mobile phase to migrate to the top of the plate.

Dilution series of the pure samples was performed in order to optimize the concentration for visualization on TLC plates. Using stock solutions of 2800 g/L lumichrome and riboflavin, 1, 10 and 50 µL were spotted onto the TLC plate in addition to 50 µL of the solvent (methanol:HCl). The TLC was developed using the same mobile phase as above.

2.7.2 Methanol Extraction of Coralline Algae

The presence of lumichrome and riboflavin in coralline algae was tested through methanol extraction (Phillips et al. 1999). Briefly, approximately 10 g of coralline algae, frozen in liquid nitrogen, were ground into a paste to which pure methanol was added and transferred to a 15 ml

plastic tube. The suspension was vortexed vigorously, and allowed to settle on ice. The contents were filtered using a Whatman 0.45 μL filter. This extraction process was performed three times. The methanol was rotary-evaporated at 45ºC at a pressure of 337 mbar, and then at 80 mbar for five hours on a Büchi Rotavapor R-200 (Büchi Labortechnik AG, Flawil, Switzerland). The final dried sample was reconstituted in 400 μL of methanol:HCl (49:1) to be used for TLC.

The methanol-extracted coralline algae samples were spotted onto the TLC plate in volumes of 1, 5, 10, and 25 μL. Five microliters of the pure lumichrome and riboflavin stock solutions were spotted as well as 25 μL of the methanol:HCl solvent. The plate was developed with chloroform:methanol:water (17.5:12.5:1.5) for 40 minutes, allowed to dry and visualized using a UV transluminator.

2.7.3 Solvent Partitioning of Lumichrome and Riboflavin

Due to the suspicion that chlorophyll is also extracted with methanol from the coralline algae, solvent portioning was attempted to separate lumichrome from chlorophyll. As a chlorophyll control, chlorophyll was extracted from grass blades with methanol. The starting solvent was methanol, which was then mixed with either ethyl acetate, isopropanol, chloroform, or tetrahydrofuran. If the two solvents were miscible then a 1:1 chloroform:water step was added. The solution was vortexed and then centrifuged to separate phases. Since lumichrome and riboflavin are yellow in solution and chlorophyll is green, simple observation on the phase color indicated the presence of each chemical. Acid (0.05 M HCl) and base (0.05 M NaOH) were added to each solvent mix to test the effect of pH on the partitioning.

Solvent partitioning was applied to the coralline algae extracts in order to separate chlorophyll from lumichrome and riboflavin and therefore result in a cleaner run on the TLC. The extracts were treated with methanol and ethyl acetate solvents mixed with chloroform and water and treated with 0.05 M NaOH. This resulted in the yellow lumichrome in the top phase

and the green chlorophyll in the bottom phase. The top phase was transferred to a new Eppendorf 1.5 ml tube and stored until used for TLC.

Solvent partitioned coralline algae extracts were separated TLC with both chloroform:methanol:water (17.5:12.5:1.5) and also methanol:water (3:2) mobile phases. The samples were running quickly with the mobile front so a more hydrophobic mobile phase of chloroform:methanol:water (35:12.5:1.5) was used.

2.8 Induction of Coral Larvae Settlement and Metamorphosis

Acropora palmata and *Montastrea faviolata* gametes were collected from Looe Key Reef, FL in August 2006 and 2007 during mass spawning events. Fertilization and rearing of larvae were conducted at Mote Marine Laboratory Tropical Research Center (Summerland Key, FL). Settlement experiments were set up in six well Petri plates to test the effects of pure lumichrome, riboflavin, microbial biofilms of coral-associated bacteria, and *N*-acyl-homoserine lactones (AHLs) have on the settlement and metamorphosis of coral larvae. Lumichrome and riboflavin were used due to the observation that lumichrome induces settlement in ascidian larvae and their involvement in inter-kingdom communication (Tsukamoto 1999, Tsukamoto et al. 1999).

N-acyl-homoserine lactones are signaling molecules and are critical components of the communication system, quorum sensing (Waters and Bassler 2005, West et al. 2007). For this experiment, 3-oxo-C6 homoserine lactone (a short-chain AHL) and C14 homoserine lactone (a long-chain AHL) were used. 3-oxo-C6 HSL is a common AHL produced by bacteria involved in quorum-sensing systems (Mohamed et al. 2008). C14 HSL was selected for these experiments based on the observation that many marine associated alpha-proteobacteria produce long-chain AHLs (Wagner-Dobler et al. 2005, Mohamed et al. 2008).

Table 2-1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
***E. coli* hosts for cloning/construction**		
DH5α	F-φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *rec*A1 *end*A1 *hsd*R17 (rk-, mk+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1	Invitrogen
S17 λ-pir	*rec*A *thi*-1 *pro*AB+ *hsd*R-M+ <RP4:2-Tc:Mu:Km:Tn7, Tp^r, Sm^r	Invitrogen
Reporter strains and plasmids		
Agrobacterium tumefaciens pZLR4	AHL reporter, cured of Ti plasmid, Gm^r	(Shaw et al. 1997)
Sinorhizobium meliloti MG32	8530 derivative, 95% *sinI* in frame deletion, ExpR+, *sinI*-, *dapA* fused with *gus* gene. Possible lumichrome reporter	M. S. Gao, unpublished data
*E. coli*MG1655 pMT41	Wild-type, Tet^r	M. Teplitski, unpublished data
E. coli RG133 pMT41	MG1655 derivative *uvrY33*::Tn5, Tet^r, Km^r	M. Teplitski, unpublished data
***Serratia marcescens* isolates**		
Serratia marcescens PDL100	isolated from *Acropora palmata* mucus, white pox pathogen, Tet^r, Ap^r, $Sucrose^r$	(Patterson et al. 2002)
Serratia marcescens MG1	previously referred to as *S. liquificians*, isolated from rotten cucumber, wild type, AHL-produces, swarmer	(Eberl et al. 1999)
Serratia marcescens ATCC# 39006	Chesapeake channel water isolate, pigmented (prodigiosin+)	ATCC
Serratia marcescens ATCC# 43422	human throat isolate, pigmented (prodigiosin+)	ATCC
Serratia marcescens ATCC# 43820	Human urine isolate	ATCC
*Serratia marcescens*EL31	Florida Keys wastewater isolate	E. Lipp (UGA)
Serratia marcescens EL34	Eden Pines canal water isolate (FL Keys), pigmented (prodigiosin+)	E. Lipp (UGA)
Serratia marcescens EL139	Florida Keys wastewater isolate	E. Lipp (UGA)
Serratia marcescens EL202	Higgs Beach isolate (FL Keys)	E. Lipp (UGA)
Serratia marcescens EL206	Mote Marine Tropical Research Laboratory canal water isolate (FL Keys)	E. Lipp (UGA)
Serratia marcescens EL266	Florida Keys wastewater isolate	E. Lipp (UGA)
Serratia marcescens EL368	seabird isolate (Key Largo, FL)	E. Lipp (UGA)
*Serratia marcescens*EL402	environmental isolate	E. Lipp (UGA)
Native coral isolates		
Photobacterium mandapamensis 33-C12	isolated from *Acropora palmata* mucus, Sp^r, Sm^r, identity confirmed by 16S rDNA	K. B. Ritchie (Mote)
Photobacterium leiognathi 33-G12	isolated from *Acropora palmata* mucus, identity confirmed by 16S rDNA	K. B. Ritchie (Mote)
Halomonas meridian 33-E7	isolated from Acropora palmata mucus, identity confirmed by 16S rDNA	K. B. Ritchie (Mote)
Plasmids		
pCR2.1 TOPO TA	cloning vector, Km^r, Ap^r	Invitrogen
pCR2.1 TOPO Zero BLUNT	cloning vector, Ap^r	Invitrogen
pZLR4	*traCDG* operon with its promoter region. *traG* is transcriptionally fused to *lacZ*. AHL reporter	(Cha et al. 1998)
p1218	*gacA* gene from *S. marcescens* PDL100 amplified with CJK12 and CJK18 cloned into pCR2.1, Ap^r, Km^r	This study
p1318	*gacA* gene from *S. marcescens* PDL100 amplified with CJK13 and CJK18 cloned into pCR2.1, Apr, Kmr	This study
pSB401	*luxRI*'::*luxCDABE* on pACYC (cloning vector), Tet^r	M. Teplitski, unpublished data
pMT41	*csrB* promoter from *E. coli* cloned upstream of promoterless *luxCDABE* of pSB401, Tet^r	M. Teplitski, unpublished data
pBAD18-Ap	arabinose inducible promoter vector to serve as complementation vector control, Ap^r	(Guzman et al. 1995)

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Sp^r, spectinomycin resistance; Sm^r, streptomycin resistance; Tet^r, tetracycline resistance, Nm^r, neomycin resistance

Table 2-2. Primers used for PCR

Primer name	Sequence	Nucleotide binding site
M13F	GTAAAACGACGGCCAG	443-448 of bottom strand of pCR2.1 (EF488744)
M13R	CAGGAAACAGCTATGAC	205-221 of top strand of pCR2.1 (EF488744)
CJK12	GGAGATTTTTCCTTGATTAGCGTTCT	413-438 of top strand of *S. plymuthica gacA* (AY057388)
CJK13	ACATCTCAGGCTATAACAGAGGCTG	367-391 of top strand of *S. plymuthica gacA* (AY057388)
CJK18	TCGTCACGCAAAAGAACATTATATC	1345-1369 of bottom strand of *S. plymuthica gacA* (AY057388)
MT13	ACTTTGCTATGCCATAGCATTTTTA	1200-1224 of top strand of pBAD18-Ap (X81838)

Table 2-3. Carbon substrates in Biolog EcoPlates

Polymers
α-cyclodextrin
glycogen
Tween 40
Tween 80
Carbohydrates
D-cellobiose[a]
i-erythritol
D-galactonic acid γ-lactone
N-acetyl-D-glucosamine
glucose-1-phosphate
β-methyl-D-glucoside
D,L-α-glycerol phosphate
α-D-lactose
D-mannitol
D-xylose[a]
Carboxylic acids
γ-hydroxybutyric acid
α-ketobutyric acid
D-galacturonic acid
D-glucosaminic acid
itaconic acid
D-malic acid[a]
pyruvatic acid methyl ester
Amino acids
L-arginine
L-asparagine[a]
glycyl-L-glutamic acid
L-phenylalanine
L-serine
L-threonine
Amines
phenyl ethylamine
putrescine
Phenolic compounds
2-hydroxybenzoic acid[a]
4-hydroxybenzoic acid[a]

[a]Indicates substrates not in GN plates.

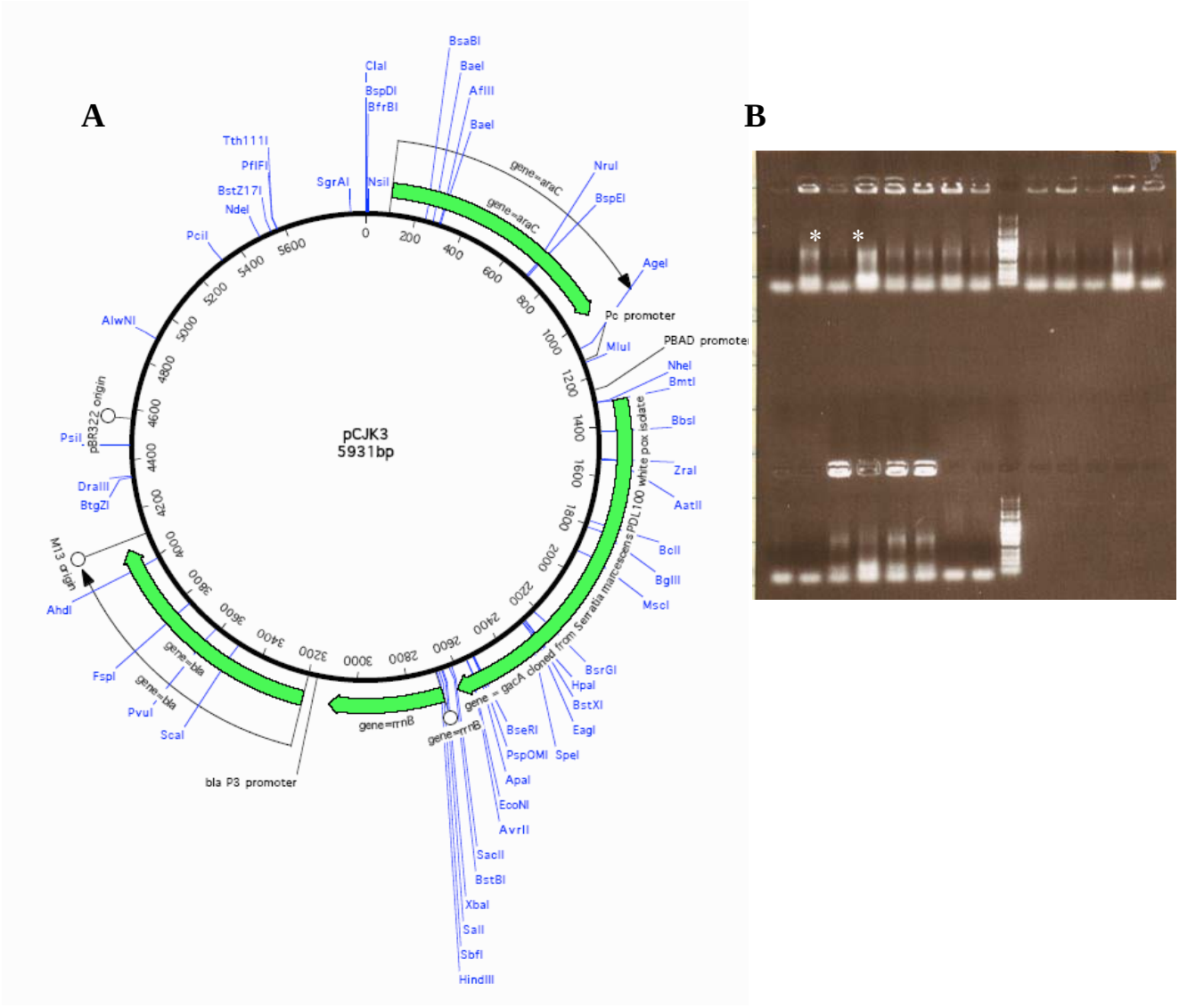

Figure 2-1. Consturction of an arabinose inducible complementation vector. (A) Complementation vector, pCJK3 plasmid map annotated with single restriction enzyme cut sites. *gacA* from *S. marcescens* PDL100 cloned downstream of the arabinose inducible promoter. (B) Gel electrophoresis of PCR of *gacA* cloning into pBAD18-Ap using primers MT13 and CJK18 (Table 2-2). Lanes 2-19 are colony PCR of *gacA* from p1318 cloned into the EcoRI site of pBAD18-Ap and transformed into DH5α and selected on LB supplemented with Amp 200 µg/ml. Lane 20 is pBAD18-Ap plasmid DNA as a template and lane 21 is the PCR master mix as a negative control. Positive reactions used for complementation assay indicated by(*). The DNA ladder used is 1 Kb Full Scale Ladder (Fisher Scientific, Pittsburgh, PA).

CHAPTER 3
PHENOTYPIC CHARACTERIZATION OF A CORAL WHITE POX PATHOGEN, *Serratia marcescens*

3.1 Introduction

Opportunistic pathogens, such as *Serratia marcescens*, rely on specific metabolic activities in order to utilize certain carbon sources present in their environment. These activities may allow the pathogen to occupy metabolic niches within the host and promote growth and ultimately infection (Berg et al. 2005, Munoz-Elias and McKinney 2006). Most of the metabolic genes and pathways involved in growth and host infection by opportunistic pathogens are uncharacterized. As most opportunistic pathogens are heterotrophs, they are capable of metabolizing a wide variety of carbon sources such as carbohydrates, lipids, glycolipids, and dicarboxylic and amino acids (Munoz-Elias and McKinney 2006). The ability of pathogenic bacteria to utilize many carbon sources also contributes to their ability to persist in a wide variety of environments and hosts. Many genera of bacteria, including *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Ochrobactrum*, *Pseudomonas*, *Ralstonia*, *Staphylococcus* and *Stenotrophomonas*, contain rhizosphere-associated bacteria that enter into interactions with plants and humans (Berg et al. 2005). This may be attributed to the rich nutrients associated with the rhizosphere due to high levels of root exudates (Campbell et al. 1997). The example of the rhizosphere as an oasis of rich and available nutrients surrounded by nutrient poor bulk soil is analogous to coral mucus surrounded by nutrient limited open water. They are able to support a different consortium of bacteria as opposed to their surrounding environments. Opportunistic pathogens may persist in these environments while in between hosts (Whipps 2001). A number of potentially pathogenic organisms, including *Aeromonas*, *Clostridium*, *Klebsiella*, *Legionella*, *Listeria*, *Pseudomonas*, and *Vibrio* are either naturally active in estuaries and oceans or able to persist in dormant states (Grimes 1991, Harvell et al. 1999). The rich nutrients of coral mucus

may also explain the relatively high abundance of microorganisms in mucus layers of corals as compared to the surrounding water (Lipp and Griffin 2004, Ritchie and Smith 2004). The use of non-host environments by opportunistic pathogens provides valuable insight to their metabolic potentials and commonalities that exist between organisms.

Many techniques are available for the identification and classification of environmental and clinically isolated bacteria including culture-based growth media, nucleic acid isolation, fatty-acid methyl-ester (FAME) analysis and fluorescence *in situ* hybridization (FISH) (rev. (Hill et al. 2000)). Metabolic profiles generated through the use of sole carbon substrate tests provide a relatively quick method to both identify and classify microbial organisms (Garland and Mills 1991, Garland 1997, Konopka et al. 1998, Hill et al. 2000, Preston-Mafham et al. 2002). The BIOLOG Microplate™ bacterial identification system was first introduced for the purpose of assessing the functional identity of microorganisms from environmental samples (Garland and Mills 1991). Rapid identification of individual isolates is based on sole-carbon source utilization of 95 individual carbon sources and a water control in a 96-well plate. Plates specific to Gram-negative and Gram-positive bacteria (GN and GP MicroPlates™, respectively) were developed with appropriate carbon sources for each group (Preston-Mafham et al. 2002). Despite their initial intentions of individual isolate characterization, GN plates were also used to analyze bacterial communities (Konopka et al. 1998). Additional plates were developed for bacterial diversity analysis at the community level. BIOLOG EcoPlates™ use the same principles as GN plates but instead of 95 individual substrates, they contain 31 substrates and a water control with intra-plate triplication (Choi and Dobbs 1999). The EcoPlates contain some substrates in common with the GN plates but also contain more complex and ecologically relevant substrates, including photosynthetic exudates, which better reflect the diversity of substrates found in the

environment (Hill et al. 2000). The rapid nature of the BIOLOG plates has contributed to their wide use, but the system is not without its limitations.

There are certain considerations that need to be accounted for when using the BIOLOG method for analysis at both the individual and community levels. The density of the inoculum is important and should be standardized if comparisons between isolates or samples will be made. If the inoculum varies between plates then the resulting patterns of carbon source utilization may be biased (Konopka et al. 1998, Hill et al. 2000, Preston-Mafham et al. 2002). Functional diversity analysis is based on the assumption that color development is a function of the proportion of organisms present that can utilize a specific substrate. This, however, may not be the case. Some substrates are simply utilized more readily than others and some organisms are more efficient at utilizing various substrates (Hill et al. 2000). A third problem associated with using the GN plates to compare isolates from different environments is that many of the substrates are not ecologically relevant and do not adequately reflect the diversity of substrates in the environment (Campbell et al. 1997, Konopka et al. 1998). The GN plates are biased toward simple carbohydrates, which are utilized by a wide variety of bacteria. This results with metabolic redundancy during comparison of metabolic profiles (Preston-Mafham et al. 2002).

With the considerations mentioned above in mind, EcoPlates were used to compare metabolic profiles of previously identified isolates of *Serratia marcescens* and coral-isolated bacteria. The EcoPlates were used due to their inclusion of more ecologically relevant substrates representing a variety of environments, which encompass those from which the isolates were collected. A comparison of the resolution of both EcoPlates and GN plates showed no significant differences in the cluster analysis (Choi and Dobbs 1999). Therefore, the lack of the common simple carbohydrates in the EcoPlates should not influence the downstream analysis.

As discussed earlier, heterotrophic bacteria are capable of utilizing a variety of carbon sources. One study positively identified upwards of twenty genes directly related to carbon compound catabolism induced in *E. coli* by growth on murine intestinal mucus (Chang et al. 2004). Specific genes involved in catabolism of N-acetylglucosamine, pentose, fucose and ribose were induced during initial colonization and growth on intestinal mucus as opposed to genes involved in degradation of ethanolamine, anaerobic respiration, and the TCA cycle were induced at a later time point (Chang et al. 2004).

Coral mucus is comprised of proteins, amino acids and carbohydrates that contain glucose, galactose, glucosamine (chitin), galactosamine, fucose and arabinose (Ducklow and Mitchell 1979, Meikle et al. 1988), although the structure of coral mucus and chemical bonds by which the individual components of mucus are held are not known. Catabolic enzymes, such as chitinase, are induced during colonization of intestinal tracts and invertebrate larvae by marine bacteria (Bassler et al. 1991, Lertcanawanichakul et al. 2004, Bhowmick et al. 2007). Chitin is the second most abundant homopolymer (repeats of the same monomers) in nature and is ubiquitous in the environment.

Production of proteinases also may enhance bacterial metabolism, allowing bacteria to persist on such a wide assortment of carbon sources. Bacteria generally obtain their carbon and nitrogen through enzymatic degradation of extracellular biopolymers by proteinases and glycosidases (Travis et al. 1995). Often these proteinases have broad substrate specificity (Travis et al. 1995, Ovadis et al. 2004). *Serratia marcescens* is known to produce both extracellular and cell-associated proteinases, which function in catabolism and virulence associated behaviors (Schmitz and Braun 1985, Ovadis et al. 2004). Just as catabolic enzymes

are induced by growth conditions; it is likely that opportunistic pathogens produce proteinases during establishment and growth on a host.

In this experiment, the metabolic capabilities and enzymes involved in coral mucus utilization of *Serratia marcescens* PDL100, human and environmental *S. marcescens* isolates, and coral-associated bacteria were investigated. I hypothesized that PDL100 should exhibit a unique metabolic profile similar to coral-associated bacteria as compared to other isolates of *Serratia marcescens*. Its ability to grow on coral mucus and cause disease may suggest that it has "evolved" specific pathways for the utilization of the nutrients comprising coral mucus. This hypothesis was tested through sole-carbon source utilization profiling with BIOLOG EcoPlates™ and enzymatic and proteinase induction by coral mucus assays. This experiment provides useful information about the types of pathways and enzymes used by *S. marcescens* PDL100 during growth on coral mucus and provides a foundation for the identification of specific genes induced during colonization of the pathogen.

3.2 Materials and Methods

3.2.1 Carbon Source Utilization Profile Using Biolog Ecoplate Assay

Carbon-source utilization of the white pox pathogen, *Serratia marcescens* PDL100, and fifteen other isolates of *Serratia marcescens* and other coral isolated bacteria (Table 2-1) was assayed using Biolog EcoPlates. These 96-well plates are manufactured with 31 different substrates in triplicate per plate with a water control (Table 2-3). The EcoPlates rely on the tetrazolium violet dye redox reaction, which detects fermentation of sole carbon sources (Garland and Mills 1991, Garland 1997). Assays were set up according to Choi & Dobbs (1999) with the following modifications (Choi and Dobbs 1999). Isolates were grown in either 5 ml LB broth or GASW broth overnight at 30˚C with shaking. Cells were pelleted at 10,000 x *g* using an Eppendorf table-top centrifuge 5415D (Eppendorf, Hamburg, Germany) and washed twice with

filter sterilized (0.22 μm) seawater to remove any residual nutrients from the overnight media. Cells were then resuspended in 10 ml filter sterilized seawater and starved for 24 hours at 30˚C. Following the starvation period, 100 μL of cell suspension was inoculated into each well of the Biolog EcoPlate. The initial A_{590} of each plate was read on Victor-3 (Perkin Elmer, Shelton, CT) and was continuously read every 24 hours for a total of 72 hours.

3.2.2 Enzyme Induction in Response to Growth on Coral Mucus

While the exact composition of coral mucus is unknown, detection of specific enzymes induced in response to growth on coral mucus can tell us certain types of bonds within the coral mucus matrix. Enzymatic induction assays using chromogenic substrates allow for detection of individual specific enzymes induced in response to growth on a certain medium or in a specific niche. In addition to identifying specific bonds in the various components of coral mucus, the ability of different bacterial isolates and strains of bacteria to utilize the components of coral mucus may elucidate phenotypic relatedness among bacterial species and strains.

Serratia marcescens isolates from wastewater, canal water and other environments were compared with a pathogenic strain of the same species and three coral associated bacterial strains isolated from *Acropora palmata* mucus (Table 2-1). Two overnight cultures of each isolate were grown in 5 ml Luria-Bertani (LB) broth or in GASW broth (Smith and Hayasaka 1982, Smith et al. 1982) to an approximate OD_{600} of 2.0 (stationary phase), which was determined spectrophotometrically. Cells were pelleted at 10,000 rcf and washed 3 times in filtered-sterilized seawater (0.22 μm) buffered with 10 mM HEPES to remove any residual nutrients and resuspended in 5 ml of buffered seawater. The cells starved in filter-sterilized seawater at 30˚C while shaking for three days in order to use up any internal resources. A three-day starvation period was found sufficient during preliminary studies with *S. marcescens* PDL100. Following the three-day starvation, 1 ml of cells was added to 2 ml of either 1x coral mucus (freeze dried,

UV irradiated for 20 minutes and reconstituted to original volume (Ritchie 2006)) or 10 mM HEPES buffered seawater). Negative controls of coral mucus alone and buffered seawater alone were performed in parallel with the experimental treatments for each isolate. Cells were incubated in treatments for two and eighteen hours at 30˚C. Following incubation, the initial OD_{590} of each treatment was determined spectrophotometrically and recorded. Cells where then mixed with Z-buffer (1:1/v:v) and lysed with 0.1% sodium dodecyl sulfate solution and chloroform (4:1/v:v) (Miller 1972). Cell suspensions were aliquoted into chloroform-resistant microcentrifuge tubes so to accommodate two biological and two technical replicates per substrate per treatment. Enzymatic substrates were prepared in HPLC-grade water and each substrate was added to the appropriate reactions to a final concentration of 0.8 µg/µL. Assays were conducted at room temperature for approximately 24 hours to allow for maximum color development. Sodium carbonate (Na_2CO_3) was added to a final concentration of 416 mM to stop the reaction and to intensify the color of each reaction. Cellular debris and unused enzymatic substrate were pelleted at 4,000 x *g* (16,000 rcf) for two minutes. The clear supernatant was transferred to a polystyrene 96-well plate and the A_{405} was measured on Victor-3 (Perkin Elmer, Shelton, CT). Buffered seawater and coral mucus were included in each plate as blanks.

Representative isolates from the three broad categories of *Serratia marcescens* isolates examined (human isolate, Sm 43422; environmental isolate, Sm39006; and white pox pathogen, PDL100) were also assayed with a treatment testing whether the catabolic capabilities of the isolates could be repressed in the presence of glucose. A minimal media consisting of 10 mM HEPES buffered seawater supplemented with glucose (4 g/L) and Casamino Acids (0.1 g/L) as the sole carbon and nitrogen sources was filter sterilized through a 0.22 µm filter. This additional treatment was examined due to the observation that some enzymes appeared to be

induced during starvation stress regardless of which treatment ultimately experienced by the cells. Glucose is a known catabolite repressor. In *Enterobacteriaceae*, glucose inhibits expression of catabolic and regulatory genes required for growth on most other carbon sources; glucose also inhibits expression of virulence genes and regulators (Ferenci 1996, Reverchon et al. 1997, Jackson et al. 2002, Gosset et al. 2004, Teplitski et al. 2006). As controls for this treatment, cells were also incubated with 10 mM buffered seawater supplemented with Casamino Acids (0.1 g/L) without glucose and buffered seawater alone. The enzyme induction assay was conducted as described above.

3.2.3 Protease Induction in Response to Coral Mucus

Acroporid coral mucus consists of a variety of carbon and nitrogen compounds and may contain of up to 22% protein (Ducklow and Mitchell 1979). These proteins may serve as substrates to those bacteria able to utilize them as a food source. Therefore, it is plausible that induction of various proteases may occur in response to growth on coral mucus. The production of proteases during growth in rich medium was first investigated for both cell-associated and extracellular protease production (Demidyuk et al. 2006). A volume of 0.3 ml of either cell suspension or culture supernatant was added to 1.7 ml of water and azocasein solution (5 mg/ml in 0.1 M Tris Buffer pH 7.5) was added to a final concentration of 0.16 mg/ml. As a control, a blank of 1.6 mg/ml azocasein solution in water was prepared. Reactions were incubated statically at 30˚C for 60 min. Following incubation, trichloroacetic acid (TCA) was added to each reaction to a final concentration of 3.2% v:v to stop the enzymatic reaction and to precipitate any unhydrolyzed azocasein. Each reaction was pelleted at 10,000 x *g* for 1 min to sediment the unhydrolyzed substrate. The supernatant was carefully transferred to a new tube, to which NaOH (250 mM final concentration) was added to intensify the color. 200 µL of each

reaction was transferred to a clear polystyrene 96-well plate and the A_{405} of each reaction was read on VICTOR-3 (Perkin Elmer, Shelton, CT).

3.2.4 Statistical Analysis

Carbon utilization profile data assayed with the BIOLOG EcoPlates were analyzed by average well color development (AWCD) and principal components analysis (PCA). AWCD was calculated as $\Sigma(C - R) / n$, where C is color production within each well (optical density measurement), R is the absorbance values of the plate's control well, and n is the number of substrates (EcoPlates, $n = 31$) (Garland and Mills 1991, Choi and Dobbs 1999). Principal components analysis was performed on transformed AWCD data after 72 hours. Values from the wells of individual substrates (3 replicates for each substrate) were averaged and transformed using the formula $(C - R)$ / AWCD. PCA projects original data onto new, statistically independent axes (principal components). Each principal component accounts for a portion of the variance from the original data (Garland 1997, Choi and Dobbs 1999). Relationships among isolates were obtained by correlation analysis between the principal component values.

Enzymatic activities of each isolate were compared using correlation analysis after mean-centering the original values. Hierarchical cluster analysis was used to generate dendograms to correlation relationships among isolates.

Induction of both extracellular and cell-associated proteases among isolates was compared using a one-way Analysis of Variance (ANOVA) with type I error significance level at $\alpha = 0.05$. All data were analyzed with STATISTICA software version 6.0 and/or Microsoft Excel 2003.

3.3 Results

3.3.1 Carbon Source Utilization Profile Using BIOLOG Ecoplate™ Assay

The metabolic profiles of *Serratia marcescens* PDL100, human and environmental *Serratia marcescens* isolates, and coral-associated bacteria were identified using the BIOLOG

EcoPlates™. The EcoPlates incorporate environmentally relevant sole-carbon substrates and represent the diversity of the various habitats that these isolates were collected from (Hill et al. 2000). Based on the heterogeneity of environments of isolation, I hypothesized that isolates collected from similar environments would show a similar carbon-source utilization as compared to isolated collected from distinctly different environments. *Serratia marcescens* PDL100 was expected to show a profile similar to environmental isolates and coral-associated bacteria, distinct from the human and plant pathogenic isolates.

The average well color development (AWCD) was calculated after every 24 hour reading and was plotted over time. The color development in the EcoPlate seeded with *Serratia marcescens* PDL100 followed a linear curve through the 72-hour measurement period. Similarly, the color development of the pathogenic isolates of *S. marcescens* also followed a linear curve (Fig. 3-1A). The coral associated bacteria exhibited a rapid average well color development, which then reached a plateau after 24 hours of incubation (Fig. 3-1B). The two *Photobacterium* isolates showed the same progression of color development while the *Halomonas* showed an overall lower level of color development. The various environmental isolates of *S. marcescens* revealed the greatest variety of color development (Fig. 3-1C), which may represent the diversity of environments from which they were isolated. The differences in the AWCD among the *Serratia* isolates indicates that effectiveness of an isolate at utilizing specific carbon sources is dependent on the environment in which it is found. The pathogenic isolates all encounter similar nutrients during their respective host infections and therefore show similar AWCD. The environmental isolates were isolated from diverse environments, each with a potentially unique suite of carbon sources. This diversity between isolates shows that although

all isolates are genetically identified as *S. marcescens*, subtle but important differences in terms of their metabolic potentials exist.

The average well color development for each isolate, transformed for PCA, was compared after 72 hours of incubation. Correlation analysis was then applied to group the isolates based on their ability to utilize the carbon sources in the EcoPlates. Isolate EL139 (FL Keys wastewater isolate) proved to be an extreme outlier throughout all analyses and therefore was excluded in the final cluster analysis. The correlation analysis indicated that PDL100 has a similar carbon-utilization profile to many of the other isolates tested ($r \geq 0.5$; except for EL31, $r = 0.43$). PDL100 clustered with MG1 and the other pathogenic isolates of *S. marcescens* (Fig. 3-2). The environmental isolates not associated with wastewater clustered together, as did the coral-associated bacteria. Both isolates 39006 (Chesapeake channel water) and EL31 (FL Keys wastewater) are outliers as compared to the pool of isolates, EL31 more so than 39006. This is in line with the observations of EL139, which is also a wastewater isolate.

3.3.2 Enzyme Induction in Response to Growth on Coral Mucus

Bacteria depend on specific catabolic enzymes for the degradation and uptake of the carbon and nitrogen sources of a given environment. The ability of *Serratia marcescens* PDL100 to grow on coral mucus suggests that specific enzymes may be present in PDL100, which may not be present in other *S. marcescens* isolates. I hypothesized that *S. marcescens* PDL100 utilizes a different suite of substrates potentially present in coral mucus as compared to other pathogenic and human-associated isolates. PDL100 was expected to possess the same catabolic enzymes as the three coral-associated isolates, all of which presumably have "co-evolved" with the coral host.

In *Serratia marcescens* PDL100, α-D-glucopyranosidase, α-L-arabinopyranosidase, N-acetyl-β-D-galactosaminidase (chitinase), α-L-fucopyranosidase, and β-D-galactopyranosidase

(Fig. 3-3A) were induced following growth on coral mucus. These enzymes, except for α-L-fucopyranosidase, showed similar levels of activity in the seawater control. The native coral-associated bacteria exhibited a broader range of enzymatic induction as compared to *S. marcescens* PDL100 (Fig. 3-3B). These substrates in coral mucus induced α-D-galactopyranosidase, α-D-glucopyranosidase, N-acetyl-β-D-galactosaminidase (exo-chitinase), α-L-fucopyranosidase, β-D-fucopyranosidase, β-D-galactopyranosidase, β-D-glucopyranosidase, and β-D-xylopyranosidase in response to growth on coral mucus. While more enzymes were induced in these isolates, the levels of induction were lower on average than many of the other isolates. Some of these enzymes were also induced during incubation in seawater. Overall, the pathogenic isolates of *S. marcescens* (MG1, 43422, and 43820) all enzymes tested induced more enzymes in response to growth on coral mucus than PDL100 and the coral-associated isolates. Together, the three pathogenic isolates demonstrated induction of all enzymes in the assay, although many enzymes (α-D-xylopyranosidase, α-L-arabinopyranosidase, β-D-fucopyranosidase, β-D-glucuronidase, and β-L-arabinopyranosidase) were induced at comparatively low levels (Fig. 3-3C). An assay was conducted in parallel with coral mucus alone to serve as a baseline of enzyme activity present in coral mucus (Fig. 3-3D). Enzyme activity in the coral alone mucus treatment was significantly less than the coral mucus + isolate treatments the activities observed in the mucus alone assay were subtracted from all other treatments with isolates grown on coral mucus. Similarly, a filter-sterilized buffered seawater control was performed and was used as a baseline correction for the treatments with isolates incubated in seawater.

To validate that enzymatic activity observed in response to growth on coral mucus was indeed due to the presence of coral mucus and not simply due to constitutively produced

enzymes, enzymatic activities were assayed in response to growth on filter-sterilized buffered seawater supplemented with glucose and casamino acids as the sole carbon/nitrogen sources. A representative isolate from each of the major sub-groups of isolates was used: *Serratia marcescens* PDL100 (coral white pox pathogen), *S. marcescens* 43422 (human/pathogenic), and *S. marcescens* 39006 (environmental). As casamino acids contain carbon, the influence of casamino acids on enzyme induction was accounted for. In all three isolates, enzyme activity in response to growth on glucose was greatly attenuated (Fig. 3-4). In both *S. marcescens* PDL100 and *S. marcescens* 43422, α-D-glucopyranosidase and N-acetyl-β-D-galactosaminidase remained active in response to glucose, while all others induced on coral mucus were repressed (Fig. 3-4A & B). Enzyme activity was repressed in *S. marcescens* 39006 (Fig. 3-4C). α-D-glucopyranosidase and N-Acetyl-β-D-galactosaminidase were not induced to the same degree in *S. marcescens* 39006 in response to coral mucus. In response to growth on glucose, both of these enzymes were greatly repressed. β-D-glucopyranosidase remained slightly active in *S. marcescens* 39006 during growth on glucose, while it was significantly repressed (20 fold) as compared to the isolate grown on coral mucus.

Correlation analysis was applied to the isolates based on their enzyme induction in response to growth on coral mucus and incubation in filter-sterilized buffered seawater. After 2 hours growth on coral mucus, no clear pattern was observable as the environmental were clustered with both the pathogenic isolates and the coral-associated bacteria (Fig. 3-5A). After 18 hours of growth on coral mucus, the isolates clustered more closely with isolates from their respective groups. Just as the BIOLOG data indicated, *Serratia marcescens* PDL100 was most highly correlated with *S. marcescens* MG1 and the other pathogenic isolates (Fig. 3-5B). Cluster analysis of the enzyme induction in response to incubation on filter-sterilized seawater followed

a similar pattern as induction in response to growth on coral mucus. *Serratia marcescens* PDL100 was consistently correlated with the pathogenic isolates, and the environmental isolates and the coral-associated isolates were highly correlated together after both 2 hours and 18 hours of incubation (Fig. 3-6A & B).

3.3.3 Proteinase Induction in Response to Coral Mucus

Bacteria generally obtain their carbon and nitrogen through enzymatic degradation of extracellular biopolymers by proteinases and glycosidases (Travis et al. 1995). *Serratia marcescens* is known to produce both extracellular and cell-associated proteinases, which function in catabolism and virulence associated behaviors (Schmitz and Braun 1985, Ovadis et al. 2004). I hypothesized that all strains of *Serratia marcescens*, especially the pathogenic strains, would produce proteinases in response to growth on coral mucus. I also predicted that the coral-associated bacteria would produce proteinases due to their adapted lifestyle on coral mucus. Production of both extracellular and cell-associated proteinases were measured after two hours (exponential growth phase) and 18 hours (stationary growth phase) of growth on coral mucus. Cell-associated proteinase production after two hours of growth was low in all isolates, although statistically significant differences were found between the isolates ($F_{15,32} = 3.4549$; $p = 0.0016$; Fig. 3-7A). Although statistically significant, these differences may not be biologically significant as the majority of the isolates showed similar induction. After 18 hours of growth on coral mucus, differences in cell-associated proteinase production were statistically significant and more pronounced ($F_{15,32} = 5.628$; $p < 0.0001$; Fig. 3-7B). EL139 produced the greatest enzyme activity and *S. marcescens* PDL100 was most similar to the pathogenic strains MG1 and 43422, as well as the coral-associated bacteria and the other *S. marcescens* isolates. 43820, the human urine isolate clustered with a canal water isolate, EL34 and a seabird isolate, EL368.

Extracellular proteinase activity was induced at similar levels as the cell-associated proteinase in those isolates with the ability to utilize the general proteinase substrate, azocasein. After two hours of growth on coral mucus, isolates 43820, EL139 and EL368 exhibited the highest activity, which was statistically significantly more than the other isolates ($F_{15,32}$ = 47.4265; $p < 0.0001$; Fig. 3-8A). The same pattern was observed after 18 hours of growth on coral mucus with much variability of proteinase activity within the pathogenic and environmental isolates ($F_{15,32}$ = 27.6133; $p < 0.0001$; Fig. 3-8B).

3.4 Discussion

This study provides a glimpse at the range of metabolic capabilities of a coral white pox pathogen, *Serratia marcescens* PDL100. The results present evidence as to the types of catabolic enzymes induced during growth on coral mucus and the diversity of substrates found in nature that *S. marcescens* PDL100 could utilize. Based on the carbon-source utilization profiles and the enzyme induction assays, *S. marcescens* PDL100 is highly correlated with *S. marcescens* MG1 and other pathogenic isolates. These findings suggest that the metabolic capabilities of opportunistic pathogens are inherently broad so as to take advantage of different environments and hosts when conditions are favorable. It appears that *S. marcescens* PDL100 does not possess unique metabolic capabilities specific to growth on coral mucus that could have been characteristic of a co-evolved pathogen. Native coral-associated bacteria clustered together in all experiments and were clustered distantly from *S. marcescens* PDL100 (Fig. 3-2, 3-5, 3-6). Similar clustering was also observed in the proteinase induction assays but was less conclusive as only marginal differences between PDL100 and the coral-associated bacteria were found (Fig. 3-7 & 3-8).

The high correlation between *Serratia marcescens* PDL100 and the other pathogenic *S. marcescens* isolates may also suggest that the coral white pox pathogen may have originated

from anthropogenic sources, although source tracking analyses were not performed in this study. When the etiology of white pox disease was described it was hypothesized that *S. marcescens* PDL100 may be associated with pollution of fecal origin (Patterson et al. 2002). Since then, studies have shown that enteric bacteria and viruses from human waste do not survive long in warm, saline and transparent waters around coral reefs, but can persist in marine sediments and in coral mucus (Lipp et al. 2002, Lipp and Griffin 2004). Enteric bacterial loads were found to be significantly higher in coral mucus samples and sediments as compared to the surrounding water column (Griffin et al. 2003, Lipp and Griffin 2004). The isolation of important fecal indicator bacteria, such as *Clostridium perfringens* from coral mucus has been attributed to potentially low oxygen levels on coral heads (Lipp et al. 2002), however the dissolved oxygen levels on coral heads and surrounding water were not compared. Communities in the Florida Keys rely heavily on on-site disposal as treatment of wastewater with an estimated more than 25,000 septic tanks, cesspools and injection wells (Paul et al. 2000). Enteric bacteria and viruses are quickly transported via water currents from canals into near shore waters and onto coral reefs, often within 24 hours of introduction (Griffin et al. 2003). The exact origin of *S. marcescens* PDL100 is yet unclear and investigations are underway (Patterson et al. 2002).

Mucus is secreted by a wide array of organisms. In humans alone, mucosal surfaces are found wherever absorptive and excretive functions occur, primarily the gastrointestinal (GI), respiratory, and urinogenital tracts (Pearson and Brownlee 2005). Mucus provides a rich source of nutrients where surrounding environments may be lacking (Ritchie and Smith 2004, Kooperman et al. 2007, Sharon and Rosenberg 2008) and may serve as an oasis until surrounding conditions become favorable (Drake and Horn 2007). Human-derived mucus is made up of mucins, which are glycoproteins with a central protein core attached to a

carbohydrate side chain. Up to 80% of the molecule is comprised of carbohydrate chains including galactose, fucose and N-acetylgalactosamine, N-acetylglucosamine, sialic acid, and mannose (Pearson and Brownlee 2005). These carbohydrates and glycoproteins are also the main components of coral mucus (Ducklow and Mitchell 1979, Meikle et al. 1988, Ritchie and Smith 2004). The ability of bacteria to colonize mucosal surfaces is in large part determined by the properties of the microorganism and the conditions of the environment. Bacteria not only use specific pathways and enzymes to grow on mucosal surfaces but also use flagella for motility and pili and fimbriae for adhesion to the surface (Laux et al. 2005, Virji 2005). *Serratia marcescens* 43422 was isolated from a human throat, an environment bathed by mucus structurally similar to coral mucus (Ducklow and Mitchell 1979, Meikle et al. 1988, Pearson and Brownlee 2005). Therefore, it may not be that surprising that *S. marcescens* PDL100 showed high similarity to this human isolate and other pathogenic strains of *S. marcescens*. With its ubiquitous nature, *S. marcescens* may have simply evolved a broad range of metabolic pathways and enzymes in order to cope with survival in a variety of environments.

In addition to elucidating some basic metabolic capabilities of bacterial isolates during growth on coral mucus, the enzymatic induction assays also identified some of the types of carbohydrates and bonds/formations present in *Acropora palmata* coral mucus during the summer months. All twelve substrates used in the induction assay showed induction in at least one isolate in response to growth on coral mucus, although some were induced more than others (Fig. 3-3). Most of the substrates were assayed in two conformations, α and β. In some cases the alpha conformation appeared to be more abundant in coral mucus (α-L-arabinopyranosidse, α-D-glucopyranosidase, α-L-fucopyranosidase), while in others the β form was more abundant (β-D-galactopyranosidase, β-D-xylopyranosidase). Some enzymes appeared to be constitutively

expressed (α-D-glucopyranosidase and N-acetyl-β-D-galactosaminidase) as they were active in both cells grown on coral mucus and cells incubated in sterile seawater with no added carbon sources (Fig. 3-3). The fact that both α and β forms of the substrates were found in coral mucus makes it is difficult to conclude if one conformation occurs more in nature than the other. Surely, some organisms are better able to recognize and utilize certain conformations (e.g. (Sexton and Howlett 2006)). The presence of these carbon sources in *A. palmata* mucus is in line with previous studies regarding the composition of coral mucus. Ducklow and Mitchell (1979) found mucus from *Acropora* spp. to consist of glucose, galactose, glucosamine, galactosamine, fucose and high levels of arabinose (Ducklow and Mitchell 1979). Therefore, it is not a stretch for all of these substrates to be found in the mucus of this species of coral. It is worth noting, however, that different conformations of the same substrate are secreted into coral mucus by the host. The open chain forms of monosaccharides are quite flexible around the central carbon bonds. The reactive nature of aldehyde and ketone groups often lead to reversible cyclization of the molecule resulting in either an alpha or beta conformation. In aqueous solution, equilibrium between the conformations will exist (Pérez et al. 1996, Färnbäck et al. 2008). One form may dominate over the other depending on environmental conditions such as temperature and pH (Drickamer and Dwek 1995). These findings add to the complexity of the composition of coral mucus and only represent mucus collected at one time point. It is possible that the composition of coral mucus changes temporally and with changes in conditions (Crossland 1987). While the goal of this study was not to characterize the composition of *A. palmata* mucus, structural components were identified and may provide a foundation for further characterization.

This study has also described a new potential use for BIOLOG EcoPlates™. Although designed for community based analyses of bacterial diversity in different environments (Hill et al. 2000, Preston-Mafham et al. 2002), EcoPlates were clearly able to differentiate bacterial isolates of the same species based on their carbon-source utilization profiles. EcoPlates were used instead of the BIOLOG GN1 plates because GN1 plates are primarily used for identification of a single isolate and contain ecologically irrelevant carbon substrates. While EcoPlates contain fewer substrates, the substrates included better represent the diversity of substrates found in different environments. Therefore, they may provide a means for not only characterizing the metabolic profile of environmental isolates, but also serve as a method of comparing different environmental isolates that are genetically identified as the same species.

Table 3-1. Chromogenic substrates

Substrate number (#)	Substrate name
1	α-D-Galactopyranoside
2	α-D-Glucopyranoside
3	α-D-Xylopyranoside
4	α-L-Arabinopyranoside
5	N-Acetyl-β-D-Galactosaminide
6	α-L-Fucopyranoside
7	β-D-Fucopyranoside
8	β-D-Galactopyranoside
9	β-D-Glucuronide
10	β-D-Glucopyranoside
11	β-L-Arabinopyranoside
12	β-D-Xylopyranoside

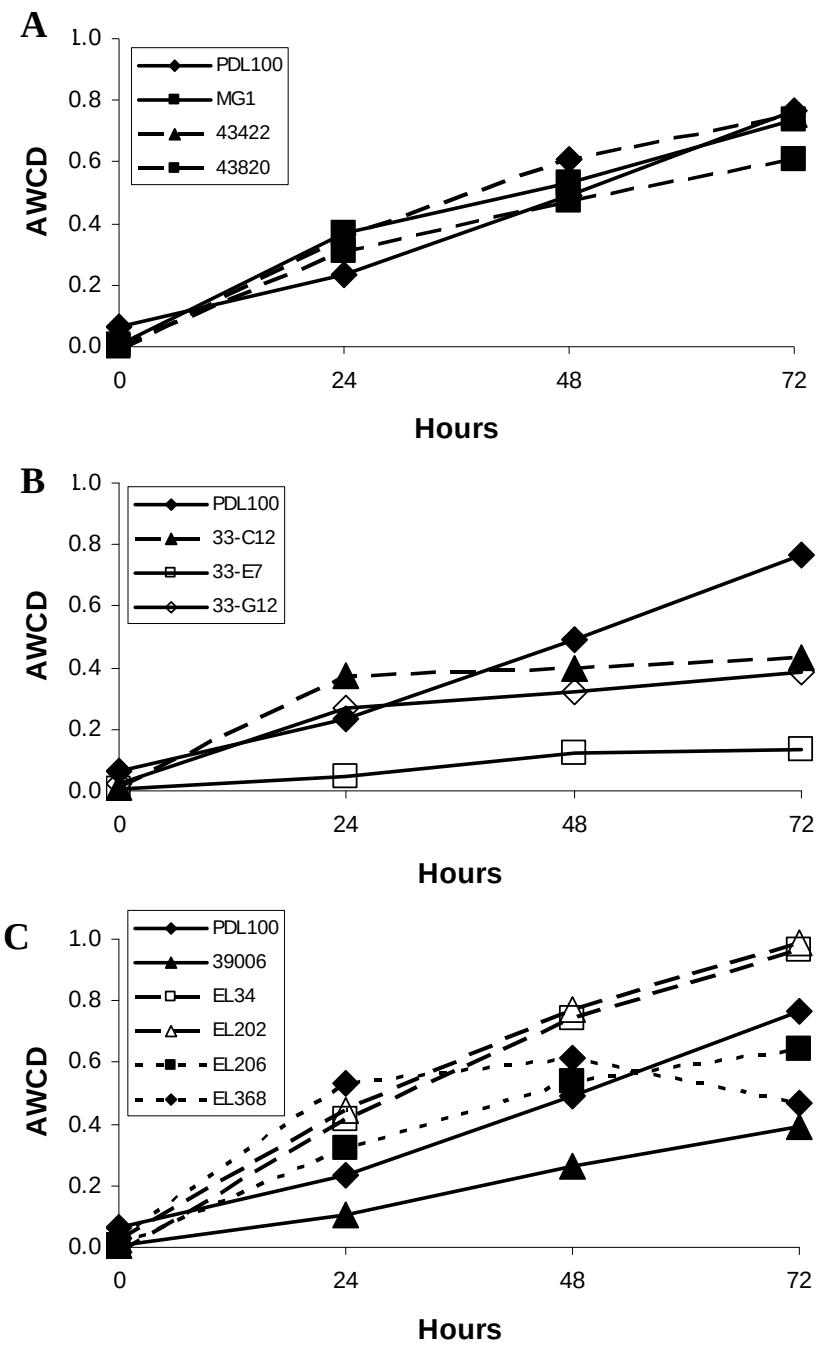

Figure 3-1. Carbon-source utilization profiles of bacterial isolates. (A) Average well color development (AWCD) for *Serratia marcescens* PDL100 and pathogenic isolates of *S. marcescens*. (B) AWCD for *S. marcescens* PDL100 and coral-associated bacteria. (C) AWCD for *S. marcescens* PDL100 and environmental *S. marcescens* isolates. AWCD = $\Sigma(C - R) / n$ where C is color production within each well (OD_{590}), R is the absorbance value of the plate's control well, and n is the number of substrates (n = 31).

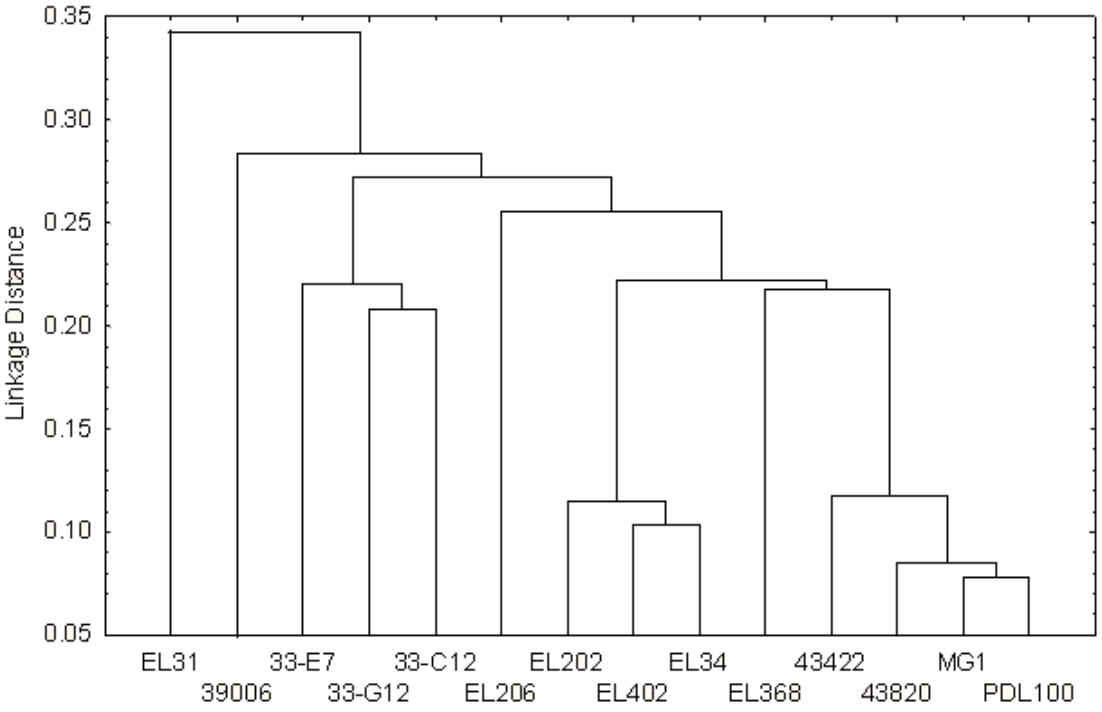

Figure 3-2. Correlation analysis of carbon-source utilization profiles of bacterial isolates. Average well color development (AWCD) values after 72 hours of incubation were subjected to correlation analysis using EXCEL and cluster diagram generated in STATISTICA. Tree-based clustering of substrate values (AWCD); [1 – Pearson's r] was used as the single linkage distance measure. Values from wells of individual substrates (3 replicates for each substrate) were averaged after 72 hour incubation. The averages for each substrate were then transformed for PCA analysis with the formula (*C* - *R*) / AWCD.

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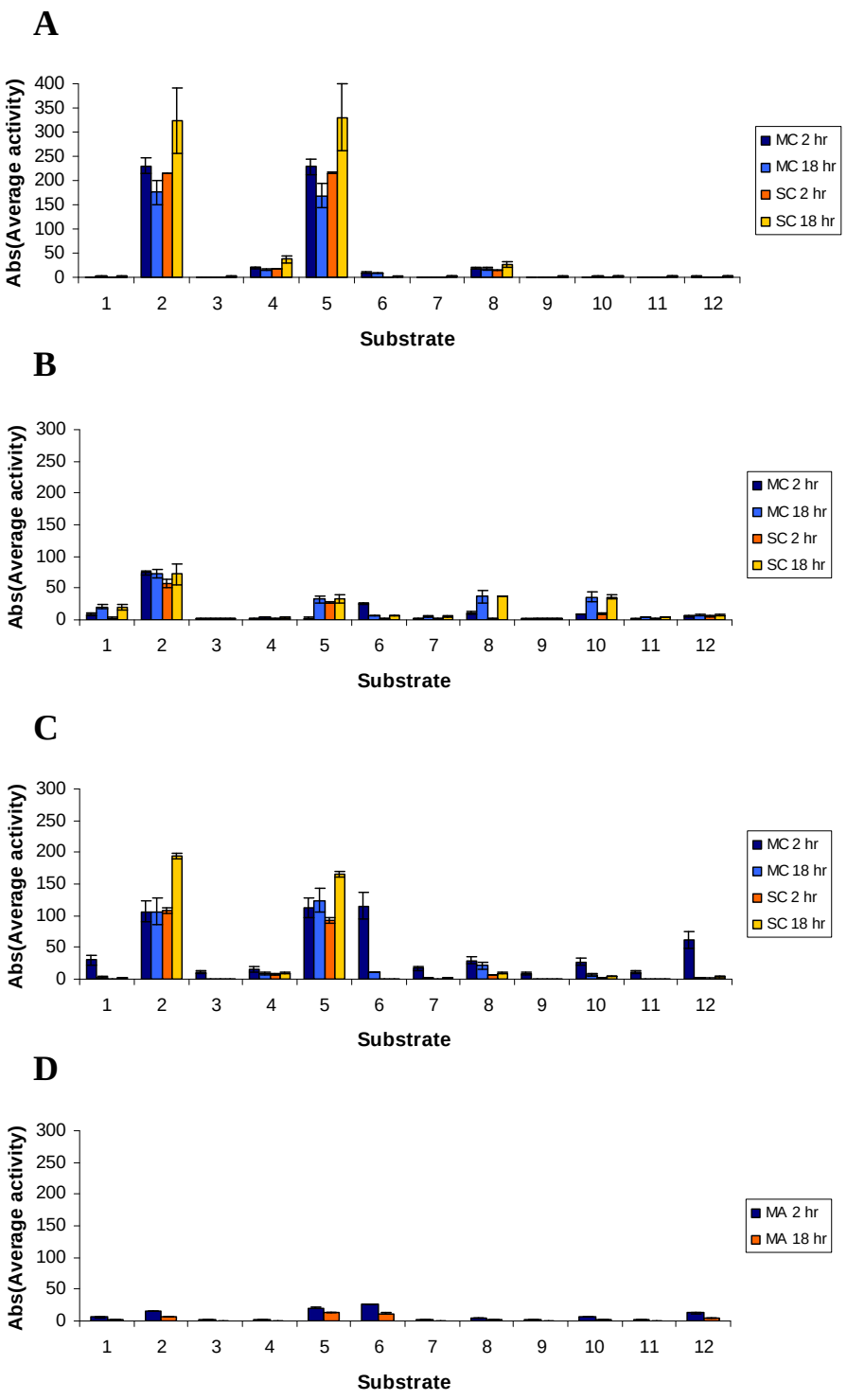

Figure 3-3. Average enzyme induction by *Serratia marcescens* PDL100 (A), coral-associated bacteria (B), pathogenic *Serratia marcescens* (C), and coral mucus alone as a negative control (D). Starved cultures grew on either coral mucus (freeze-dried/UV-irradiated) or filter-sterilized buffered seawater (10 mM HEPES, pH 6.5) for 2/18 hours, blue/orange bars, respectfully. List of substrates can be found in Table 3-1.

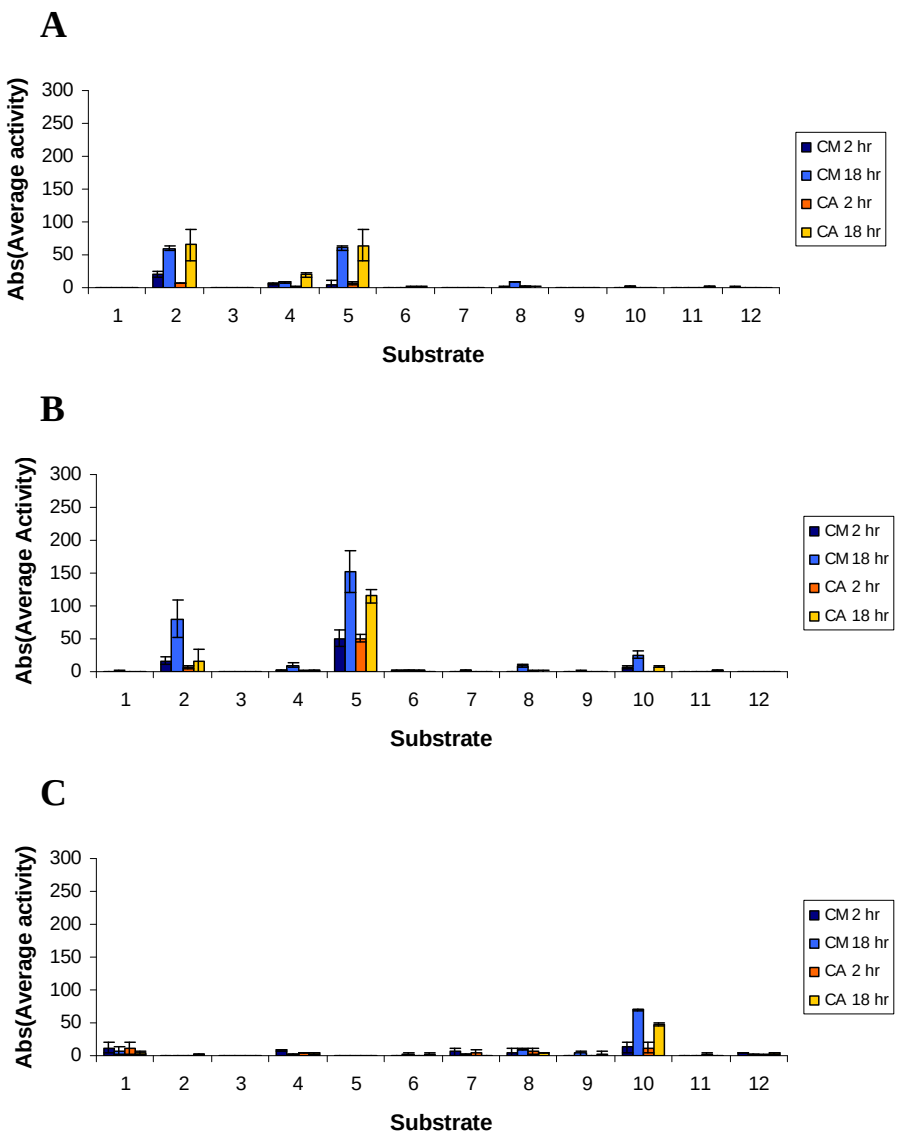

Figure 3-4. Average enzyme induction by *Serratia marcescens* PDL100 (coral white pox pathogen) (A), *Serratia marcescens* 43422 (human throat isolate) (B), and *Serratia marcescens* 39006 (Chesapeake channel isolate) (C) grown on filter-sterilized buffered seawater (10 mM HEPES, pH 6.5) supplemented with glucose (4 g/L) and Casamino Acids (0.1 g/L) or filter-sterilized buffered seawater (10 mM HEPES, pH 6.5) supplemented with Casamino Acids (0.1 g/L) for 2/18 hours, blue/orange bars, respectfully. List of substrates can be found in Table 3-1.

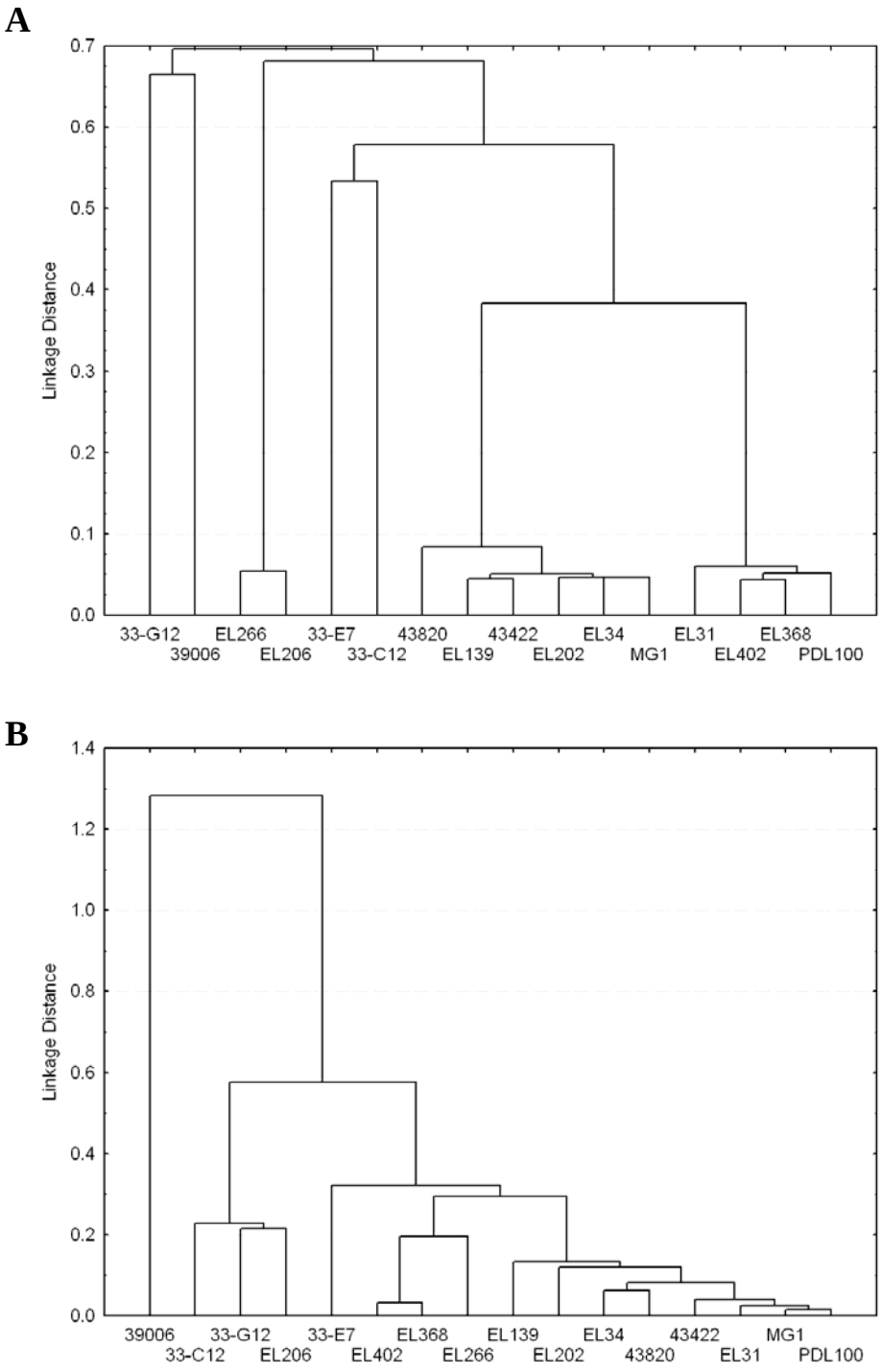

Figure 3-5. Enzyme induction for all isolates by growth on coral mucus for 2 hours (A) and 18 hours (B). Tree-based clustering of mean-centered substrate values; [1 - Pearson's r] was used as the single linkage distance measure.

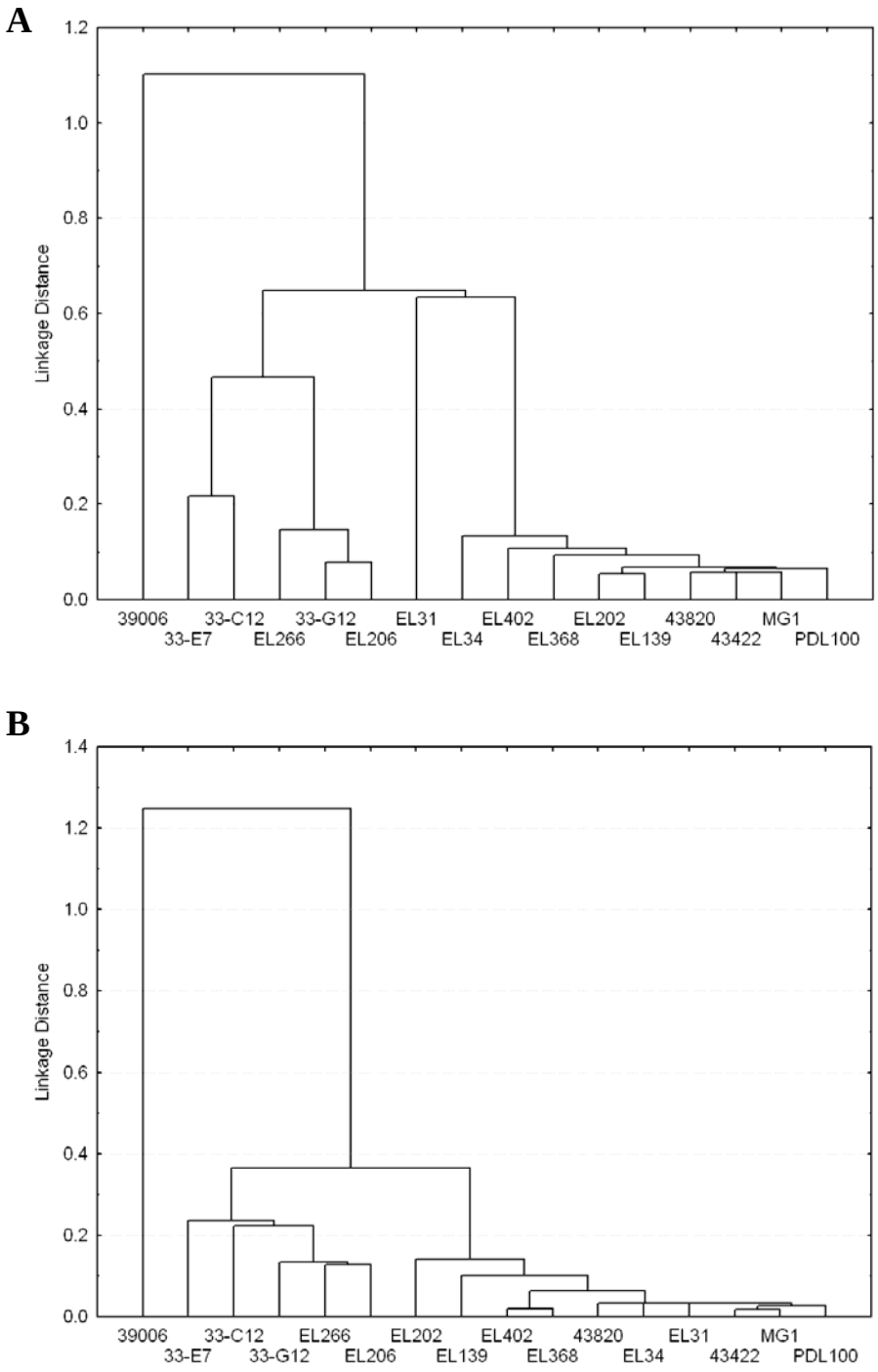

Figure 3-6. Enzyme induction negative control on filter-sterilized buffered seawater (10 mM HEPES) for 2 hours (A) and 18 hours (B). Tree-based clustering of mean centered values; [1 – Pearson's r] was used as the single linkage distance measure.

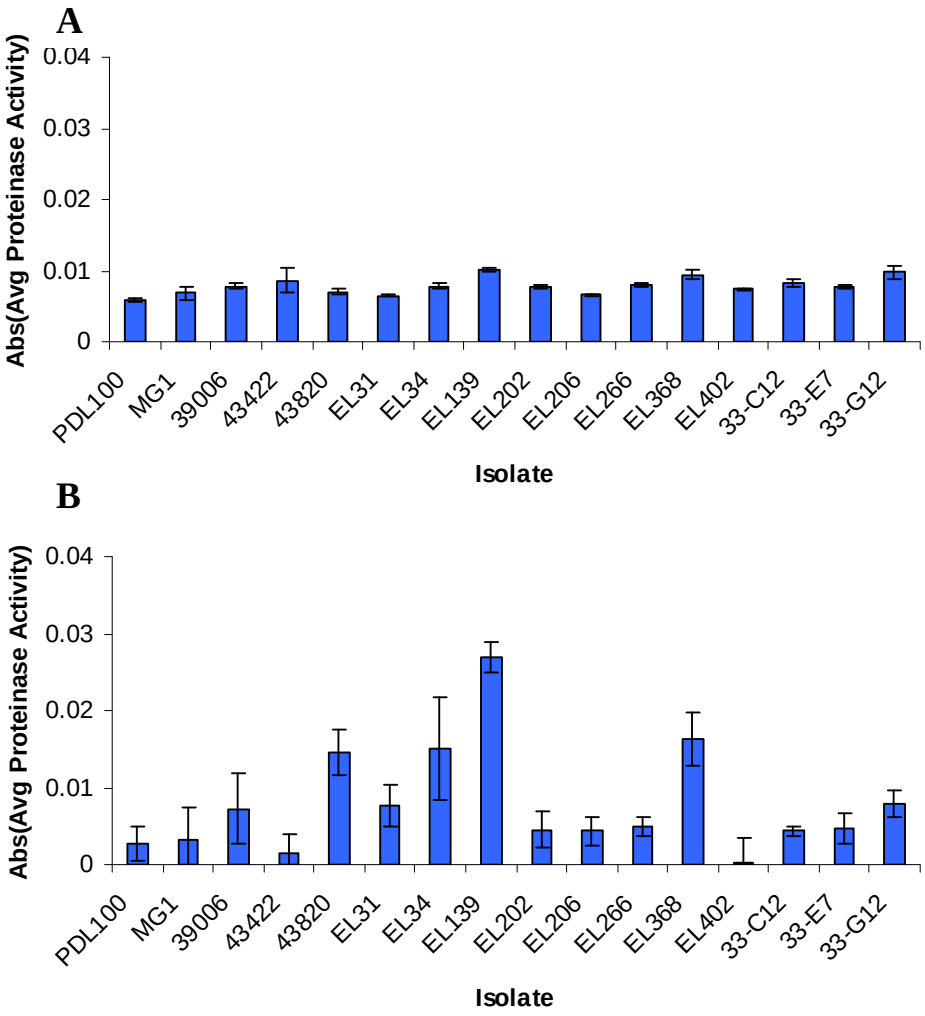

Figure 3-7. Average cell-associated proteinase induction in all isolates by growth on coral mucus for 2 hours (A) and 18 hours (B). Overnight cultures were grown in LB or GASW, washed with filter-sterilized buffered seawater (10 mM HEPES, pH 6.5) and starved for three days. Starved cells were grown on coral mucus for 2 and 18 hours before proteinase production assay.

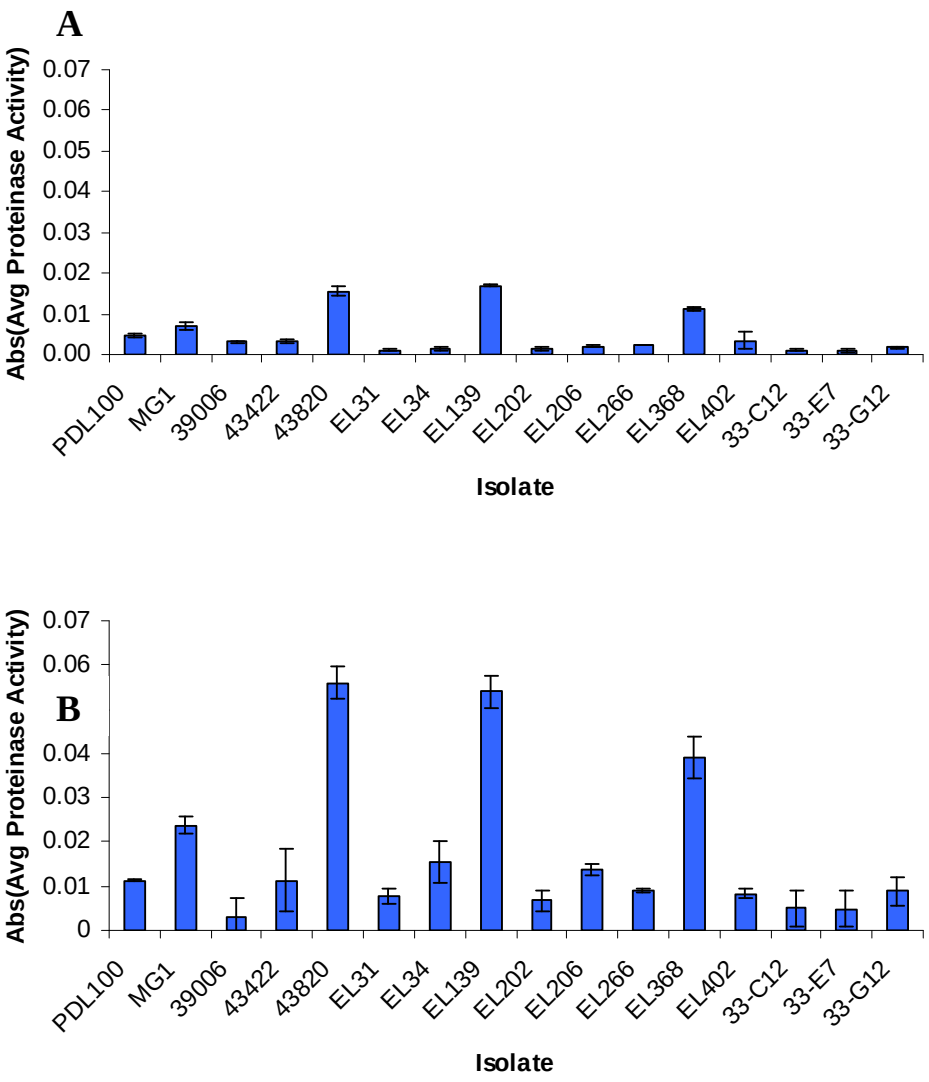

Figure 3-8. Average extracellular proteinase induction in all isolates by growth on coral mucus for 2 hours (A) and 18 hours (B). Overnight cultures were grown in LB or GASW, washed with filter-sterilized buffered seawater (10 mM HEPES, pH 6.5) and starved for three days. Starved cells were grown on coral mucus for 2 and 18 hours before proteinase production assay.

CHAPTER 4
FUNCTIONALITY OF THE RESPONSE REGULATOR gac*A* IN A WHITE POX PATHOGEN, *Serratia marcescens*

4.1 Introduction

Opportunistic bacterial pathogens, including *Serratia marcescens*, can infect a wide variety of distinct hosts ranging from plants, invertebrates, and other animals. Pathogens must either adapt to their new host environment or modify it so that they are able to overcome the host defenses. Involved in this is the recognition of the host, colonization, and exploitation of host resources. In order to recognize the host, colonize and exploit host resources, bacteria rely on an arsenal of sensors/regulators.

GacS/GacA is one of the two-component regulatory systems controlling virulence and motility in γ-proteobacteria. Orthologs of this two-component system were identified through screening of mutants defective in aspects of virulence in *Pseudomonas* spp., *Vibrio fischeri*, *E. coli*, *Salmonella enterica*, and *Legionella pneumophila* (Heeb and Haas 2001, Tomenius et al. 2005, Lapouge et al. 2008). Much of the structural information about the GacS/GacA two-component regulatory proteins have been elucidated through studies of other regulatory proteins. UvrY, the ortholog of GacA in *E. coli*, has been identified as a member of the FixJ-type class of response regulators (Pernestig et al. 2001, Pernestig et al. 2003). NarL is another FixJ response regulator that has been used as a surrogate for the structural identification of functional aspects of the proteins due to the availability of its crystal structure (Baikalov et al. 1996, Maris et al. 2005, Galperin 2006, Hussa et al. 2007).

The GacS/GacA-mediated signal transduction cascade begins when the linker domain of the N-terminal part of the membrane-associated sensor kinase GacS perceives a yet-unknown signal (Fig. 4-1). Upon interaction with the signal, a conformational change initiates an autophosphorylation cascade of the three evolutionary conserved amino acid residues (histidine-

aspartate-histidine) (Pernestig et al. 2001, Zuber et al. 2003, Kay et al. 2005, Dubuis and Haas 2007, Lapouge et al. 2008). This cascade leads to the phosphorylation of GacA's aspartate residue (D54) allowing the helix-turn-helix DNA binding domain of GacA to bind to specific promoters, such as *csrB* (Romeo 1998, Babitzke and Romeo 2007). The *csrB* gene encodes for regulatory RNA (rRNA) and upon transcription folds creating up to 22 repeated 5' leaders of mRNA (GGA) in and between loops. These repeated regions represent sites that sequester RNA-binding proteins of the CsrA family (Suzuki et al. 2002, Babitzke and Romeo 2007, Storz and Haas 2007). *csrB* is not the only small RNA molecule found to interact with the CsrA family of RNA-binding proteins. In *E. coli* and *E. carotovora*, *csrC* and *rsmB* (respectively) also act to sequester CsrA and decrease its regulatory effects (Lapouge et al. 2008). In pseudomonads, three small RNAs (*rsmX*, *rsmY*, and *rsmZ*) function together to ensure secondary metabolism and biocontrol by binding multiple CsrA/RsmA molecules (Kay et al. 2005). Similar homologs exist in *Vibrio cholerae* (*csrB*, *csrC*, and *csrD*). These three redundant RNAs have been shown to regulate quorum sensing behaviors by suppressing the activities of CsrA (Lenz et al. 2005, Babitzke and Romeo 2007, Storz and Haas 2007).

CsrA is an RNA (both messenger and regulatory RNA) binding protein. CsrA is inactivated from binding to free-floating mRNA in the cell through its binding of repeated mRNA sequences of the regulatory RNA, *csrB* and *csrC*. The small regulatory RNAs may sequester approximately 9 CsrA dimers at one time (Babitzke and Romeo 2007). Elucidation of the structure of CsrA through size-exclusion chromatography indicated that it functions as a dimer (Heeb et al. 2006). Each monomer has a β-β-β-β-β-α secondary structure. The three central strands come from one subunit and are hydrogen bonded while the two peripheral strands are from the other subunit, which are bonded within the chain of the strands from the other

subunit. The dimer is maintained through these interchain hydrogen bonding and hydrophobic interactions between the β-strands (Heeb et al. 2006). When not inhibited through binding of *csrB* and/or *csrC* or other small RNA, CsrA is free to bind to other mRNAs and either stabilize them for translation or mark them for degradation (Dubey et al. 2005). CsrA is in equilibrium between its *csrB*-bound and free-floating forms (Fig. 4-1). When bound to free-floating messages in the cell, CsrA can effectively inhibit translation by blocking the binding of the ribosome through interactions with regions upstream or overlapping the ribosome binding site (RBS) of the target transcript (Suzuki et al. 2002, Heeb et al. 2006). In *E. coli*, CsrA binds to multiple sites near the Shine-Delgarno (SD) sequences of the transcripts of *glgC* (glycogen biosynthesis) and *cstA* (carbon starvation) and prevents correct binding of the ribosome, thus inhibiting translation (Baker et al. 2002, Dubey et al. 2003).

In all γ-proteobacterial pathogens of plants and animals, orthologs of *gacS*, *gacA* play a central role in host colonization and virulence (Heeb and Haas 2001, Lapouge et al. 2008). In *S. plymuthica*, *gacA* regulates N-acyl homoserine lactone (AHL)-mediated quorum sensing, production of exoprotease and production of chitinase (Ovadis et al. 2004). Chitinases, protease, and AHL-mediated quorum sensing are typically associated with virulence and host colonization in other *Serratia* strains (Rasmussen et al. 2000, Kurz et al. 2003, Queck et al. 2006, Wei and Lai 2006). Because chitinase (N-acetyl-galactosaminadase) is induced on coral mucus (see chapter 3), it is reasonable to expect that *gacA*, *gacS* play similarly important roles in coral colonization and infection by the white pox *S. marcescens* and mutants in *gacA* will be unable to colonize the coral host.

As mentioned above orthologs of the GacS/GacA two-component regulatory system is present and evolutionarily conserved in many bacterial species[1]. Disruption of *gacA* reduces virulence in *Pseudomonas aeruginosa* (Tan et al. 1999, Parkins et al. 2001, Dubuis and Haas 2007), *Serratia* spp. (Kurz et al. 2003), *E. coli* (Pernestig et al. 2003) and *gacA* also controls the production of *N*-acyl homoserine lactone (AHL) signals, pigment, and swarming motility. A GacA ortholog in *Serratia plymuthica*, GrrS/GrrA, has been shown to regulate the production of chitinase, exoprotease, pyrrolnitrin, acyl homoserine lactones (AHLs) and biocontrol activity (Newton and Fray 2004, Ovadis et al. 2004). In many plant-associated interactions, the GacS/GacA system controls the production of secondary metabolites, extracellular enzymes involved in pathogenicity to plants, biocontrol of soil borne plant diseases, ecological fitness, or tolerance to stress (Heeb and Haas 2001, Lapouge et al. 2008). This is particularly important due to the fact that opportunistic pathogens often use a similar suit of mechanisms to invade plant and animal hosts (Rahme et al. 1995, Rahme et al. 2000).

Orthologs of GacA are present in symbiotic as well as pathogenic γ-proteobacteria. In *Vibrio fischeri*, *gacA* is necessary for colonization of the squid host (*Euprymna scolopes*) and regulates gene expression involving chemotaxis and motility (Whistler and Ruby 2003, Whistler et al. 2007). *Vibrio fischeri* is a bioluminescent bacterium that colonizers the light organ of the squid host. The light produced eliminates the shadow that the host would otherwise cast due to the moonlight; thus reducing the threat of predation (Whistler and Ruby 2003). This binary symbiosis between the bioluminescent bacterium and its squid host is an example of an association leading to accommodation and homeostasis (Whistler et al. 2007). The GacA global regulator is required for normal host tissue colonization by *Vibrio fischeri* and a recent study by

[1] An ortholog is a gene formed in two or more species, which originated in a common ancestor, but has evolved in a different way in each species.

Whistler et al. 2007 demonstrated that colonization of squid host tissue by *gacA* mutants were highly susceptible to invasion by secondary colonizers (Whistler et al. 2007). That is, mutants in *gacA* were unsuccessful in out-competing all other microorganisms colonizing the host. This suggests that targeting GacA for mutation in *Serratia marcescens* PDL100 may lead to attenuation of disease intensity and prevalence due to the inability of mutants in *gacA* to effectively establish on coral mucus.

In this experiment, a *gacA* homolog was identified in PDL100. The corresponding gene was PCR-amplified, cloned and its functionality was tested *in trans*. Based on known orthologs of *gacA* in other bacteria and within *Serratia marcescens*, I hypothesized that *gacA* is present in PDL100 and functional.

4.2 Materials and Methods

The *gacA* gene was amplified from the *S. marcescens* genomic DNA using primers CJK12 and CJK18 (Table 2-2), which were designed based on the *gacA* sequence from *S. plymutica* (NCBI GenBank: AY057388). PCR conditions included initial denaturation at 95˚C for 7 minutes, 35 cycles (95˚C, 1 minute, 53˚C, 1 minute, 72˚C, 2.5 min) and a final extension at 72˚C for 10 minutes. The resulting 957 bp product was cloned into pCR2.1 using a TOPO TA kit (Invitrogen, Carlsbad, CA), transformed into chemically competent DH5α and sequenced (Agencourt Bioscience Corp., Beverly, MA) using primer M13F. A nucleotide BLAST in the NCBI database confirmed that the amplified sequence matched that of *S. plymuthica*. The amino acid sequence for the predicted polypeptide was generated in MacVector 8.0 (Accelrys, San Diego, CA). Both the gene sequence and the hypothetical amino acid sequence were compared to those of known GacA orthologs in other bacteria.

To test whether *gacA* of *S. marcescens* PDL100 is functional, its ability to complement a *gacA* (*uvrY*) mutation in *E.coli uvrY33::kan* was tested. Therefore, a construct was engineered to

complement a mutant in the *uvrY* gene of *E. coli*. To engineer a complementation construct the *gacA* gene from p1318 was cloned into pBAD18-Ap. Plasmid p1318 was digested with EcoRI and the resulting fragments were sub-cloned into the EcoRI site immediately downstream from the arabinose-inducible promoter on pBAD18-Ap, which yielded pCJK3, which was then transformed into chemically competent *E. coli* DH5α. Transformants were selected on LB agar supplemented with Ap 200 μg/ml. Orientation of the insert was confirmed by PCR using primers MT13 and CJK18 (Table 2-2, Fig. 2-1).

To test the functionality of *gacA* in *Serratia marcescens* PDL100, an arabinose induced promoter-based complementation assay was performed. There are a wide variety of expression vectors that have been constructed in *E. coli* (de Boer et al. 1983, Brosius et al. 1985, Diederich et al. 1994). These strong inducible promoters are most often induced with a change in temperature and some are repressed better than others (Guzman et al. 1995). Some expression vectors produce high levels of the corresponding gene product and can even out-express the wild-type in addition to producing substantial levels of synthesis in uninduced or repressed conditions. In these situations, comparison to wild-type expression is difficult and evaluation of the result of a mutant or complementation is nearly impossible. The P_{BAD} vector utilized in this study satisfies two major conditions: the synthesis of the proteins can be shut off relatively rapidly and efficiently without changes in temperature (which can have deleterious effects on the host cell in terms of growth and plasmid maintenance). Also, expression before depletion of the inducer (arabinose) does not produce exceedingly high levels of protein, which in itself may give a phenotype or influence the phenotype of the depletion (Guzman et al. 1995).

The P_{BAD} promoter is regulated by the *araC* regulatory gene product. The AraC protein is both a positive and negative regulator. In the presence of arabinose, transcription from the

P_{BAD} promoter is initiated and in the absence of arabinose, transcription occurs at very low levels. The un-induced levels of transcription can be further decreased through the addition of glucose to the growth media. Glucose is a known catabolite repressor and effectively reduces the available 3', 5'-cyclic AMP. This limits the interaction between cyclic AMP and the CAP protein involved in the enhancement of transcription (Miyada et al. 1984).

The complementation vector pCJK3 was transformed into *E. coli* RG133 pMT41 by electroporation (25 μF, 200 Ω, 2.5 kV, 0.2 cm cuvette, 50 μL cell volume) using a Bio-Rad MicroPulser (Bio-Rad Laboratories, Hercules, CA). As vector controls, the original pBAD18-Ap vector was transformed into both the wild-type reporter *E. coli* 1655 pMT41 or its isogenic *uvrY33::kan* derivative reporter *E. coli* RG133 pMT41. Two overnight cultures of each strain were grown in LB with appropriate antibiotics at 37˚C on a rotary shaker (180 rpm). Following overnight incubation, cultures were diluted 1/100 in LB and incubated at 37˚C for 3 hours on a rotary shaker (180 rpm). Cultures were diluted to an OD_{600} of 0.3, and then diluted 1/25000 and aliquoted into a black polystyrene 96-well plate (in quadruplicate). Luminescence was measured with Victor-3 (Perkin Elmer, Shelton, CT) every hour for ten hours and the expression of the complemented mutant was compared to the wild-type reporter strain.

4.3 Results

4.3.1 Molecular Characterization of g*acA* in *Serratia marcescens* PDL100

As a first step in the characterization of the *gacA* gene in *Serratia marcescens* PDL100, the full gene was PCR amplified using primers designed based on the published sequence of *gacA* in *Serratia plymuthica* (NCBI GenBank: AY057388). The resulting gene was cloned, sequenced, translated *in silico*, and compared to other known *gacA* orthologs at the amino acid level (Fig. 4-2). The Clustal-W alignment of the predicted GacA protein from *S. marcescens* PDL100 to other characterized GacA orthologs indicates that all GacA orthologs share the

predicted phosphorylation site at position 54. Known residues that interact with the phosphorylation site (D54), D8-9, P58, I60, T82, E86, S103 and A107, are also conserved among all orthologs (Fig. 4-2). The central helices α8-α9 are predicted to form a helix-turn-helix motif of the DNA binding domain of GacA (Maris et al. 2005). These regions appear to be conserved in the orthologs compared.

The similarity of GacA orthologs were also compared at the DNA sequence level. A phylogenetic tree based solely on sequence maximum identity was generated using the TreeCon software. Boot-strap analysis was implemented to determine the relative similarity between sequences. The analysis indicated that sequence similarity is high even at the DNA level between orthologs of GacA (Fig. 4-3). None of the species examined demonstrated sequence differences greater than 10% based on the boot-strap analysis. The analysis did confirm that the Gram-negative bacterium, *Legionella pneumophila* is distantly related to *E. coli* and other enteric bacteria, in regards to the *gacA* gene.

Due to the high similarity between *gacA* of *Serratia marcescens* PDL100 at the DNA and amino acid levels to other characterized orthologs of GacA, the gene sequence was submitted to NCBI GenBank under the Accession number EU595544.

4.3.2 Functionality of g*acA* Through Complementation Assay

The complementation construct consisting of an arabinose-inducible *gacA* gene was compared in its ability to complement a chromosomal *uvrY33* mutant in *E. coli* to wild-type with a *csrB::luxCDABE* fusion reporter system (pMT41).

Results of the complementation assay through expression of *gacA* under (1) arabinose induction, (2) glucose repression, and (3) no-inducer induction are presented in figures 4-4 through 4-6. In each treatment, no statistically significant difference in the level of expression between the wild type (MG1655) and the wild-type with the pBAD18-Ap vector control was

observed. Under arabinose induction (0.2% arabinose supplemented media) of the P_{BAD} promoter, expression of *gacA* in the complemented mutant was statistically significantly higher than the *uvrY* mutant alone (RG133), and the mutant strain with the pBAD18-Ap vector control (Fig. 4-4). A comparison of the expression as function of luminescence at three hours under the arabinose induction treatment indicates that although the expression of the *gacA* complemented mutant was lower than the wild-type, the level of expression of the complemented mutant was statistically significantly higher than the non-complemented mutants (Fig. 4-4B).

The addition of glucose to the media was conducted to effectively shut down expression from the P_{BAD} promoter through the reduction of cyclic-AMP, which is required for transcription. Under glucose repression (0.2% glucose supplemented media), expression of *gacA* in the complemented mutant was not statistically significantly different from the *uvrY* mutant alone and the mutant carrying pBAD18-Ap (Fig. 4-5A&B). Expression in each of the mutant strains, measured as luminescence production by the reporter, remained significantly lower than the wild-type throughout the time course.

The no-inducer induction treatment tested the "leakiness" of the P_{BAD} expression system. If expression of *gacA* occurred without arabinose induction, the results of the induction treatment would be inaccurate in demonstrating the ability of *gacA* to complement the *uvrY* mutation. The no-inducer effectively demonstrates the background level of expression at the P_{BAD} promoter. Similar to the glucose repression treatment, the no inducer treatment did not result in significant expression of *gacA* in the complemented mutant as compared to the mutant controls. The level of expression was consistently lower than the wild-type (Fig. 4-6).

4.4 Discussion

The GacS/GacA two-component regulatory system has been shown to control behaviors from motility to virulence in many species of bacteria. In *Serratia* spp., *gacA* regulates N-acyl

homoserine lactone (AHL)-mediated quorum sensing, production of exoprotease and production of chitinase (Ovadis et al. 2004). Production of chitinase enzymes was found to be a significant component in the growth on coral mucus (see Chapter 3). Based on this observation and the similarity of *Serratia marcescens* PDL100 to other pathogenic strains of *S. marcescens*, the hypothesis that *gacA* was not only present in this isolate of *S. marcescens* but was also functionally tested. The PCR amplified product was cloned and sequenced and subjected to BLAST yielding a 98% match to the *grrA* (*gacA*) gene from *S. plymuthica*. The predicted amino acid sequence of GacA from *S. marcescens* PDL100 was compared to other characterized GacA orthologs from *E. coli*, *P. aeruginosa*, *P. fluorescens*, *Salmonella enterica*, *Vibrio cholerae*, *V. fischeri*, *S. plymuthica*, and *Legionella pneumophila* (Fig. 4-2 & 4-3).

From the comparison of GacA orthologs at the protein level, it is clear that specific regions and domains are well conserved. These conserved regions provide the essential structural features of the GacS/GacA two-component system (Heeb and Haas 2001). Comparison of *gacA* genes at the DNA level between enteric bacteria and pseudomonads also indicate a high similarity between the orthologs in each organism. Boot-strap analysis demonstrates that over a 90% similarity was found between all Gram-negative species analyzed. *Legionella pneumophila* represented an out-group with the greatest dissimilarity and was most distantly related to the other species. This supports the observation that the *gacA* ortholog of *Legionella* was unable to complement a similar mutation in the *uvrY* gene in *E. coli* (Hammer et al. 2002), while a similar complementation experiment demonstrated that the *gacA* from *Enterobacter* successfully complemented a *uvrY* mutant (Saleh and Glick 2001).

GacS belongs to a class of histidine sensor kinases that carry a phosphoryl transmitter, a receiver, and a histidine phosphotransfer output domain (Perraud et al. 2000, Zuber et al. 2003).

This is similar to the structures of the sensor kinases ArcB found in *E. coli* (Kwon et al. 2000) and BvgS in *Bordetella pertussis* (Perraud et al. 2000). The N-terminal part of GacS is the sensing domain and consists of two potential transmembrane segments separated by a periplasmic loop. This loop is a common feature of many two-component systems involving histidine kinases (Dutta et al. 1999, Neiditch et al. 2006). A linker domain is adjacent to the second transmembrane domain. The linker domain contains two amphipathic sequences, which are proposed to interact with each other in response to environmental signals. This interaction activates the protein, causes a conformation change at the C-terminal region, which favors autophosphorylation (Robinson et al. 2000). A primary transmitter domain with a conserved autophosphorylatable histidine residue is important for dimerization of sensor kinases due to alternating α-helices and β-sheets that occur in this region (Dutta et al. 1999). Sensor kinases may function as a dimer; therefore, conservation of the primary transmitter is crucial to correct functionality of the protein.

For GacS to function as a dimer, the substrate domain for autophosphorylation itself may function as the dimerization domain, forming a four-helix core. Both of the catalytic CA domains within the dimer flank this central core such that the ATP-binding pocket faces the histidine-presenting α helix of the twin subunit (Dutta et al. 1999, Robinson et al. 2000). A recent study measured the activity of the histidine kinase, LuxQ in *Vibrio harveyi* and found that the protein functions *in vivo* as a dimer. LuxQ is a sensor kinase involved in quorum sensing in *Vibrio harveyi* and is associated with the periplasmic binding protein, LuxP. When bound to an environmental signal (or in this case Autoinducer-2), LuxP undergoes a conformational change that stabilizes a quaternary structure in which two LuxPQ monomers are asymmetrically associated. The sensor kinase, LuxQ only functions as a dimer as demonstrated by the decreased

activity of wild-type LuxQ with the coexpression of a truncated LuxQ protein (Neiditch et al. 2006).

After autophosphorylation following stimulation by an environmental signal, a phosphate group is transferred to an aspartate residue followed by another histidine residue. This histidine phosphotransfer (Hpt) output domain serves a secondary transmitter and transfers a phosphate group to a conserved aspartate residue on the response regulator, GacA (Tomenius et al. 2005). The phosphorylated GacA protein contains a helix-turn-helix DNA binding domain motif that directly binds the promoter of small RNAs, including *csrB*, which encodes a global regulator RNA (Kay et al. 2005, Babitzke and Romeo 2007). The activated GacA response regulator is suspected to bind to a conserved upstream element termed the GacA box (consensus $TGTAAGN_6$ CTTACA, where N is any nucleotide) in the promoter regions of the sRNA genes (*csrB*, *csrC*, *rsmB*, *rsmX*, *rsmY*, *rsmZ*) (Lenz et al. 2005, Lapouge et al. 2008).

Essential structural features that are conserved in GacA include the active site in the N-terminal receiver domain (residues 1-148). Within this active site lies the phosphorylatable aspartate residue and its conserved contacts Asp-8 and Asp-9, Thr (Ser)-82, and Lys-104. The helix-turn-helix motif of the DNA binding domain (residues 149 to end) occurs in the C-terminal region (Heeb and Haas 2001, Maris et al. 2005). Much of the information regarding the roles of the specific conserved regions of the response regulator, GacA, has been elucidated through investigations of response regulator, NarL, in *E. coli* (Baikalov et al. 1996, Galperin 2006). Mutations in the predicted phosphorylation site has demonstrated two distinct phenotypes. One mutation led to a constitutive ON phenotype by mimicking the phosphorylated state of GacA, independent of the sensor kinase (Baikalov et al. 1996, Smith et al. 2004). In most cases, mutation of the phosphorylation site leads to an inability to accept the phosphate group (Smith et

al. 2004, Tomenius et al. 2005). Similarly, insertions or deletions of the response regulatory genes in *Vibrio fischeri* resulted in null phenotypes with the regulatory proteins unable to accept the phosphate group from the respective sensor kinase and transcription of downstream genes is repressed (Hussa et al. 2007). This suggests that transcription of regulatory RNA genes is dependent on the phosphorylation of GacA and will not occur without proper activation of the protein. Mutations in the amino acid residues that are proposed to interact with the phosphorylation site have also resulted in altered functional proteins, the majority leading to a constitutively activated response regulator protein (Smith et al. 2004).

Mutations in GacS and GacA have resulted in differences in the levels of transcription of downstream regulatory genes. While GacA is usually dependent on GacS for phosphorylation and therefore, full functionality, recent work has demonstrated that GacA may still function (albeit at a lower level compared to wild type) and lead to transcription of regulatory RNA genes even if GacS is mutated. In *E. coli*, a mutation in BarA resulted in 40% of downstream transcription as compared to wild-type, while a mutation in UvrY failed to produce any downstream activation (Tomenius et al. 2005). The same observation was found in *Salmonella enterica* sv. Typhimurium. Mutations in the response regulator *sirA* yielded less downstream activation as compared to mutation in the sensor kinase *barA* (Altier et al. 2000, Lawhon et al. 2002). Similarly, in *Vibrio cholerae*, a mutation in the sensor kinase VarS resulted in decreased but detectable transcription of regulatory RNA genes, while a mutation in VarA, the response regulator resulted in a completely null phenotype (Lenz et al. 2005). These observations suggest that GacA may have function independent of GacS and may receive a phosphate group from elsewhere in the cell. In *Pseudomonas aeruginosa* there are two sensors, RetS and LadS, in addition to GasS that may determine the activity of GacA (Dubuis et al. 2007). RetS is thought

to act as a GacA antagonist by removing a phosphate group from the already phosphorylated GacA, while LadS appears to activate GacA using the same type of C-terminal histidine kinase and response regulator receiver domains as GacS (Ventre et al. 2006, Dubuis et al. 2007). It appears that the LadS/RetS pathways function in parallel and also regulate the same small RNAs as GacS/GacA. This overlap in function may allow for the activation/inhibition of GacA. The demonstration that GacA remains somewhat functional after GacS mutation provides insight as to why specific targeting of GacA for mutagenesis instead of GacS may lead to a more effective disruption of the downstream virulence and regulatory factors controlled by the two-component system.

Activities and mechanisms within a functional domain (e.g. aspartate phosphorylation site) are largely conserved, as are the structures themselves. The ways in which the domains interact in terms of regulatory consequences may differ among response regulators (Gao et al. 2007b). The genes encoding for the sensor kinase and response regulator pair of proteins are often next to each other on the chromosome. This, however, is not the case with *gacS* and *gacA*. In many organisms, *gacA* lies directly upstream of an ortholog of the *E. coli uvrC* gene, which is involved in nucleotide excision repair. Despite the close proximity of *gacA* and *uvrC*, no evidence supports that GacA contributes to UV repair (Heeb and Haas 2001).

The results of the complementation assay not only demonstrate that *gacA* from *Serratia marcescens* PDL100 is structurally similar to *uvrY* from *E. coli* and is able to functionally complement the mutation, but supports the features of the P_{BAD} expression system that are favorable for physiological studies. The P_{BAD} vector utilized in this study satisfies two major conditions: the synthesis of the proteins are able to be shut off rapidly and efficiently without changes in temperature (which can have deleterious effects to the host cell in terms of growth

and maintenance). In addition, expression before depletion does not produce exceedingly high levels of protein, which in itself may give a phenotype or influence the phenotype of the depletion (Guzman et al. 1995).

Based on the alignment of the GacA protein from *Serratia marcescens* PDL100 with other orthologs, comparison of *gacA* orthologs at the DNA level and the complementation assay, it is reasonable to conclude that the *gacA* gene is not only present but functional in this white pox pathogen. Therefore, mutations disrupting *gacA* will presumably attenuate the ability of the pathogen to colonize and grow on coral mucus. The observation that GacA may remain functional even if GasS is mutated also provides rationale for the specific targeting of GacA for mutagenesis as opposed to other components of the regulatory system.

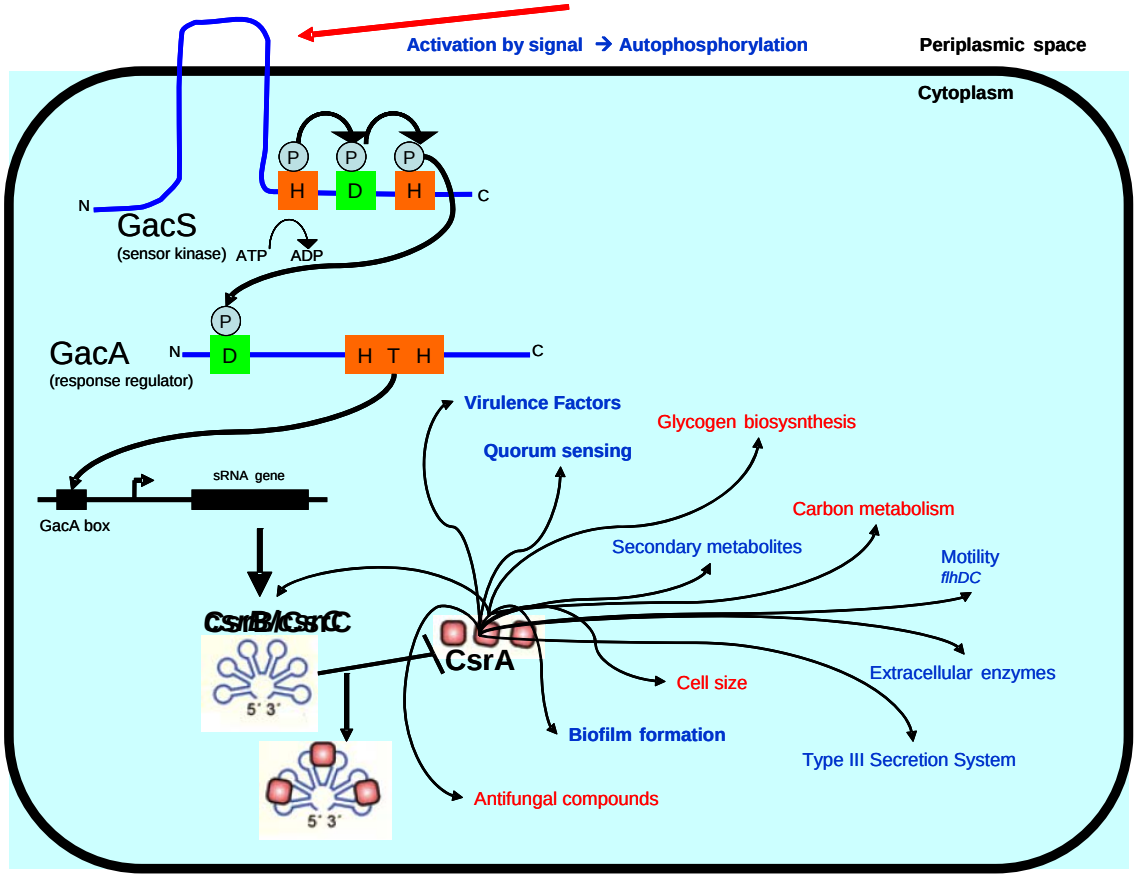

Figure 4-1. Model of regulatory pathways leading from GacS/GacA to downstream genes. Thick arrows indicate direct interactions and thin arrows indicate interactions that may be either direct or indirect. Blunt end lines (⊤) represent inhibitory or negative effects. Of the behaviors/activities regulated (either directly or indirectly) by CsrA, CsrA represses those in blue and those in red are regulated.

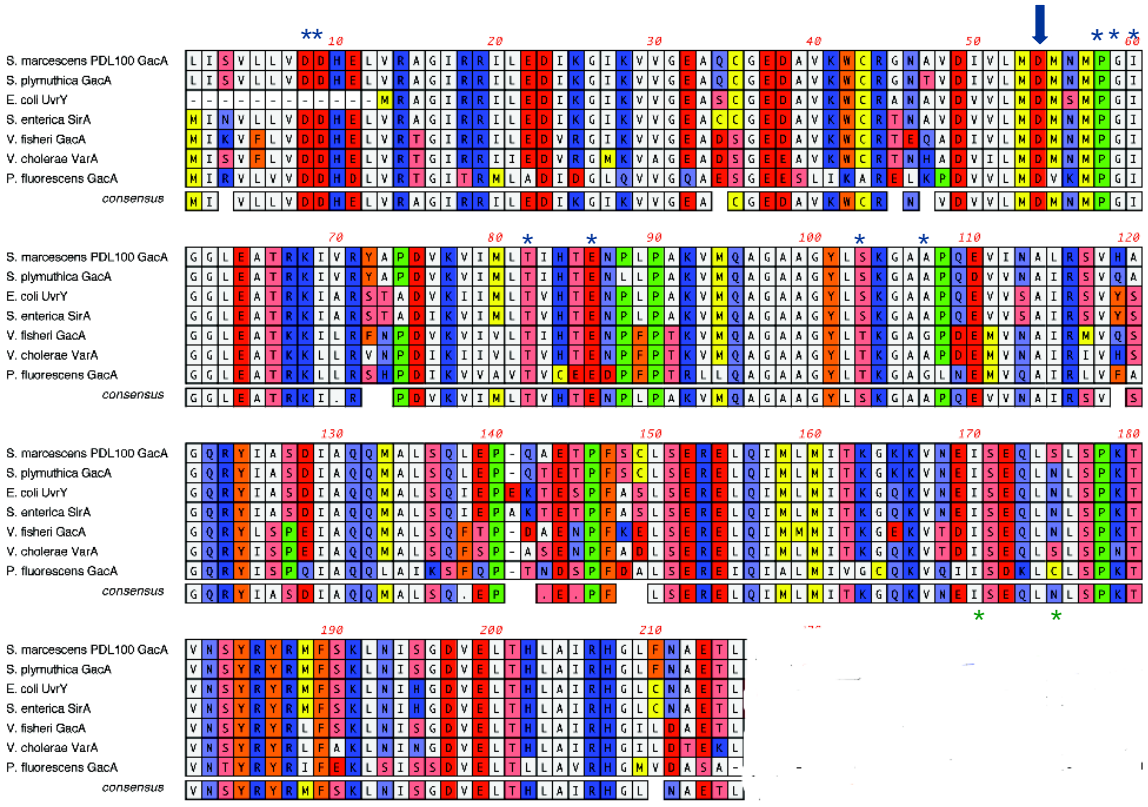

Figure 4-2. Clustal-W alignment of the deduced GacA protein from the white pox pathogen, *S. marcescens* PDL100 (top row) and other characterized GacA orthologs. All GacA orthologs share the predicted phosphorylation site (D54, blue arrow), residues that interact with the phosphorylation site (D8-9, P58, G59, I60, T82, E86, S103, A107, blue asterisks) and I170-L175 region (green asterisks) that anchors the α8-α9 of the helix-turn-helix DNA binding domain (Teplitski and Ahmer 2005, Tomenius et al. 2005).

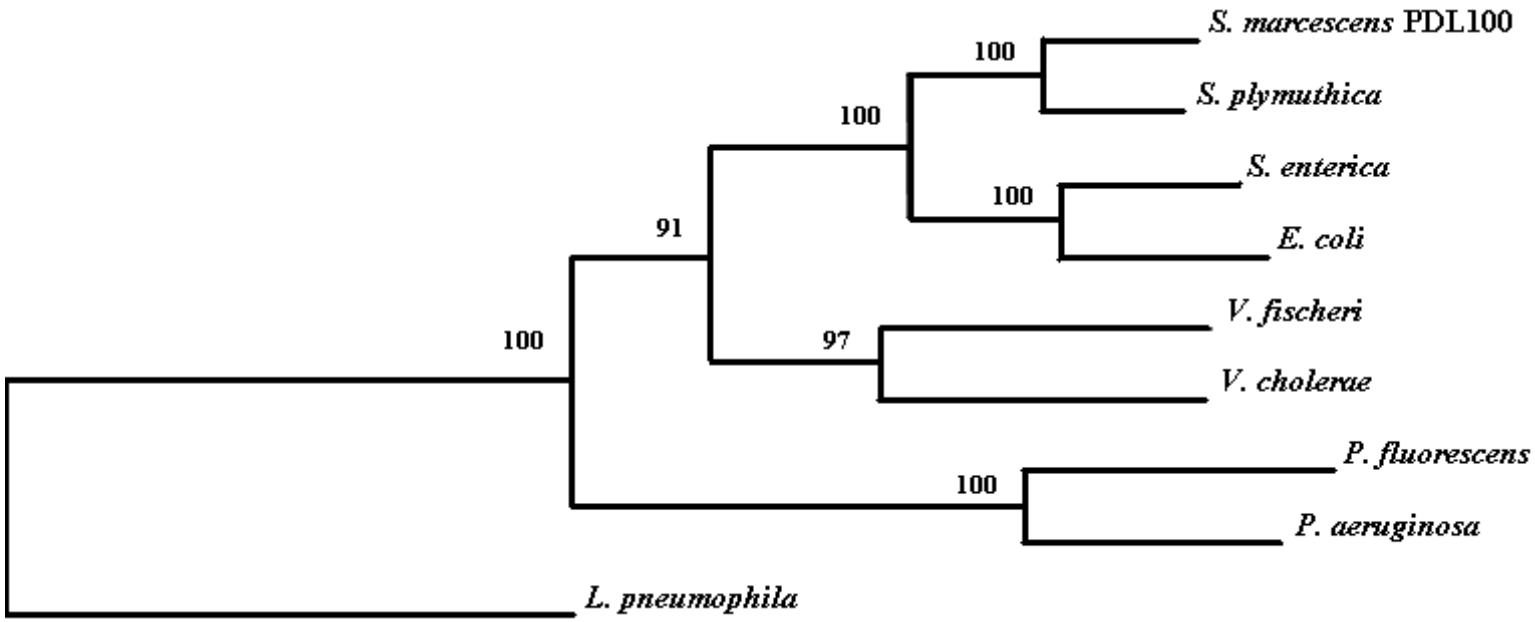

Figure 4-3. Phylogenetic tree comparison based on the *gacA* DNA sequence in common bacteria. Orthologs of *gacA* were obtained through a BLAST search of the NCBI GenBank database. DNA sequences were compared using TreeCon software with Boot-strap analysis to indicate the relative similarity between sequences. The Gram-positive bacteria, *Legionella pneumophila* served as an out-group to form the rooted-tree comparison.

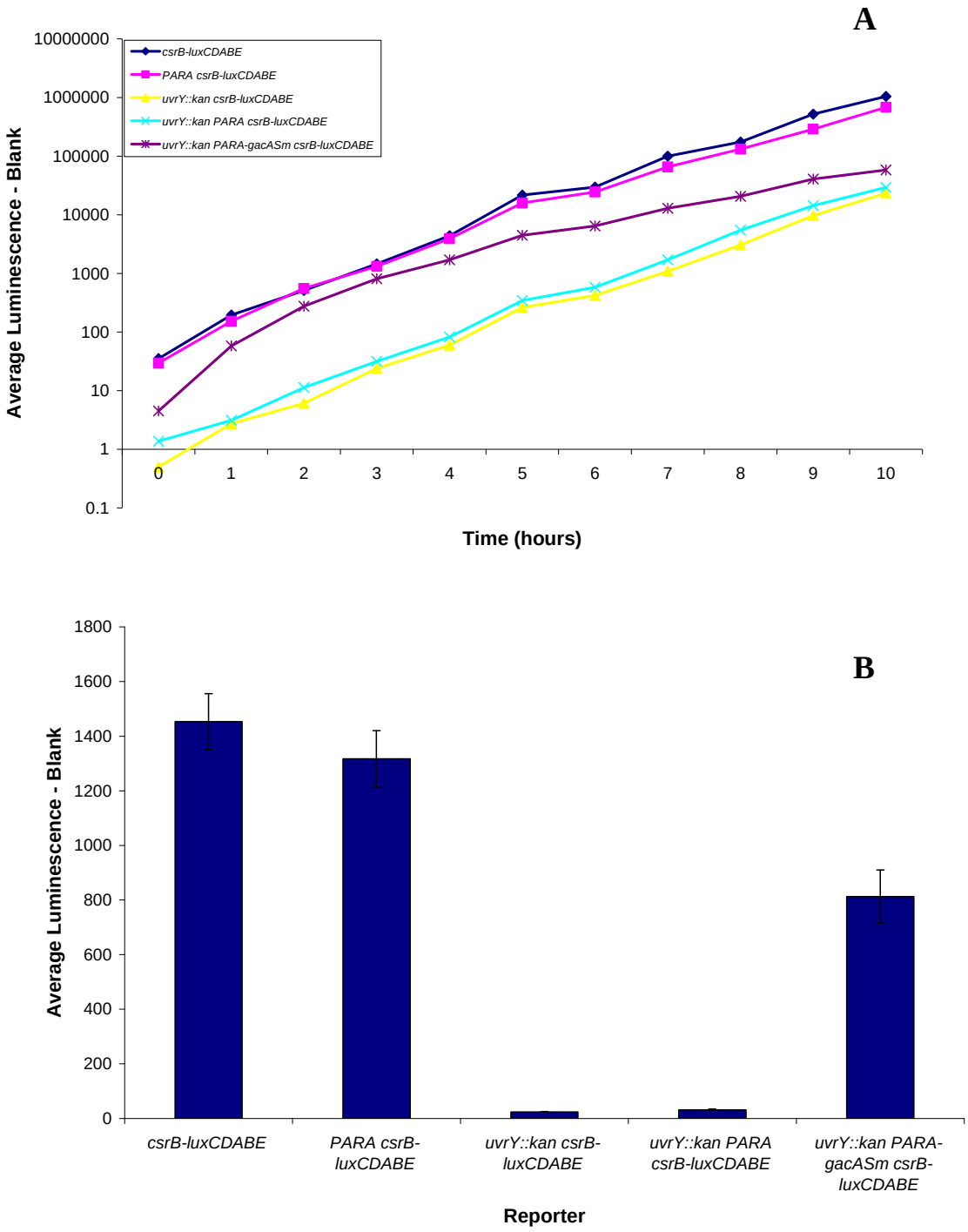

Figure 4-4. Complementation of *uvrY* mutant in *E. coli* by *gacA* with Arabinose induction of pCJK3 compared to wild-type luminescence production (A) and average induction at 3 hr (B)

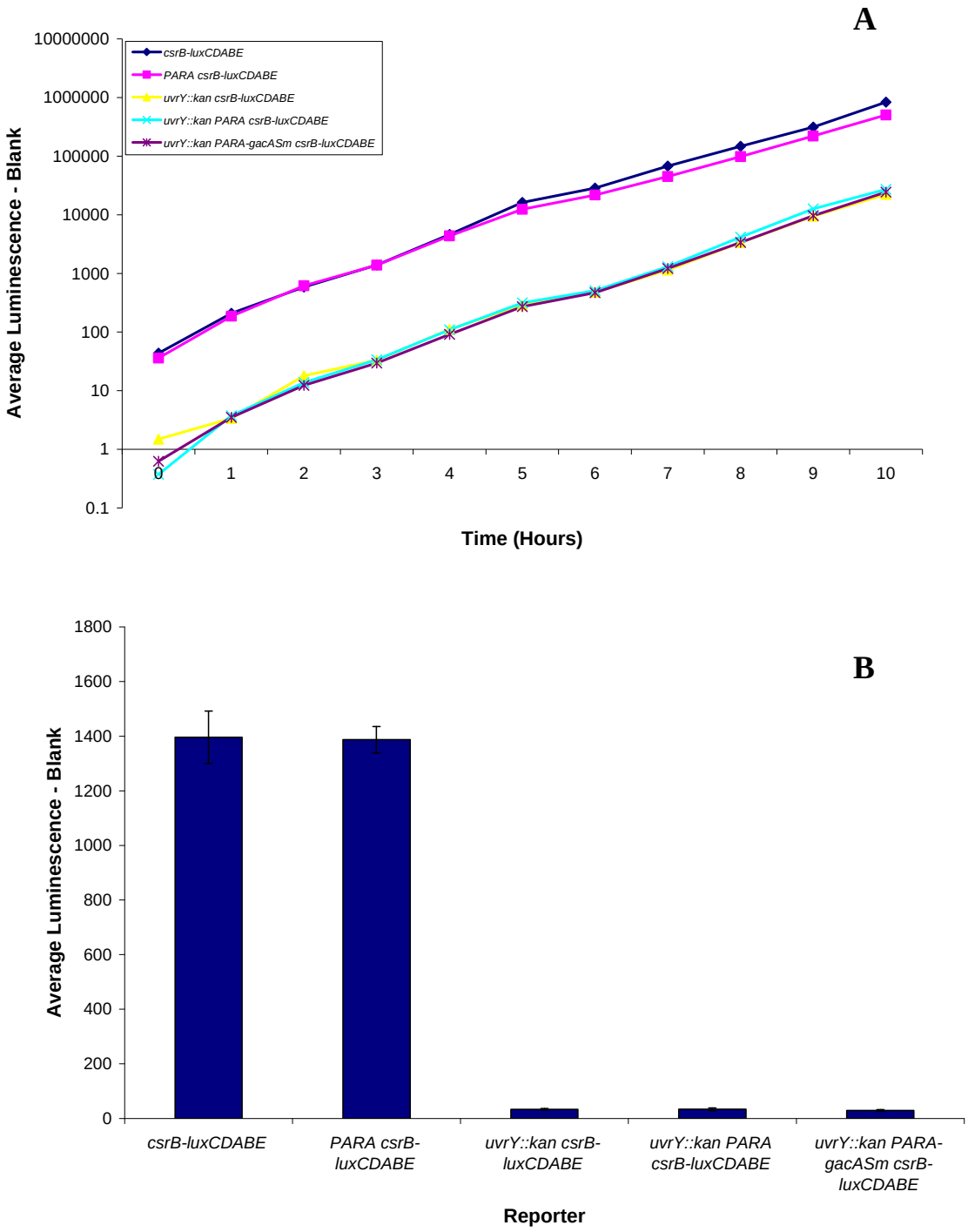

Figure 4-5. Complementation of *uvrY* mutant in *E. coli* by gacA with glucose repression of pCJK3 compared to wild-type luminescence production (A) and average induction at 3 hr (B)

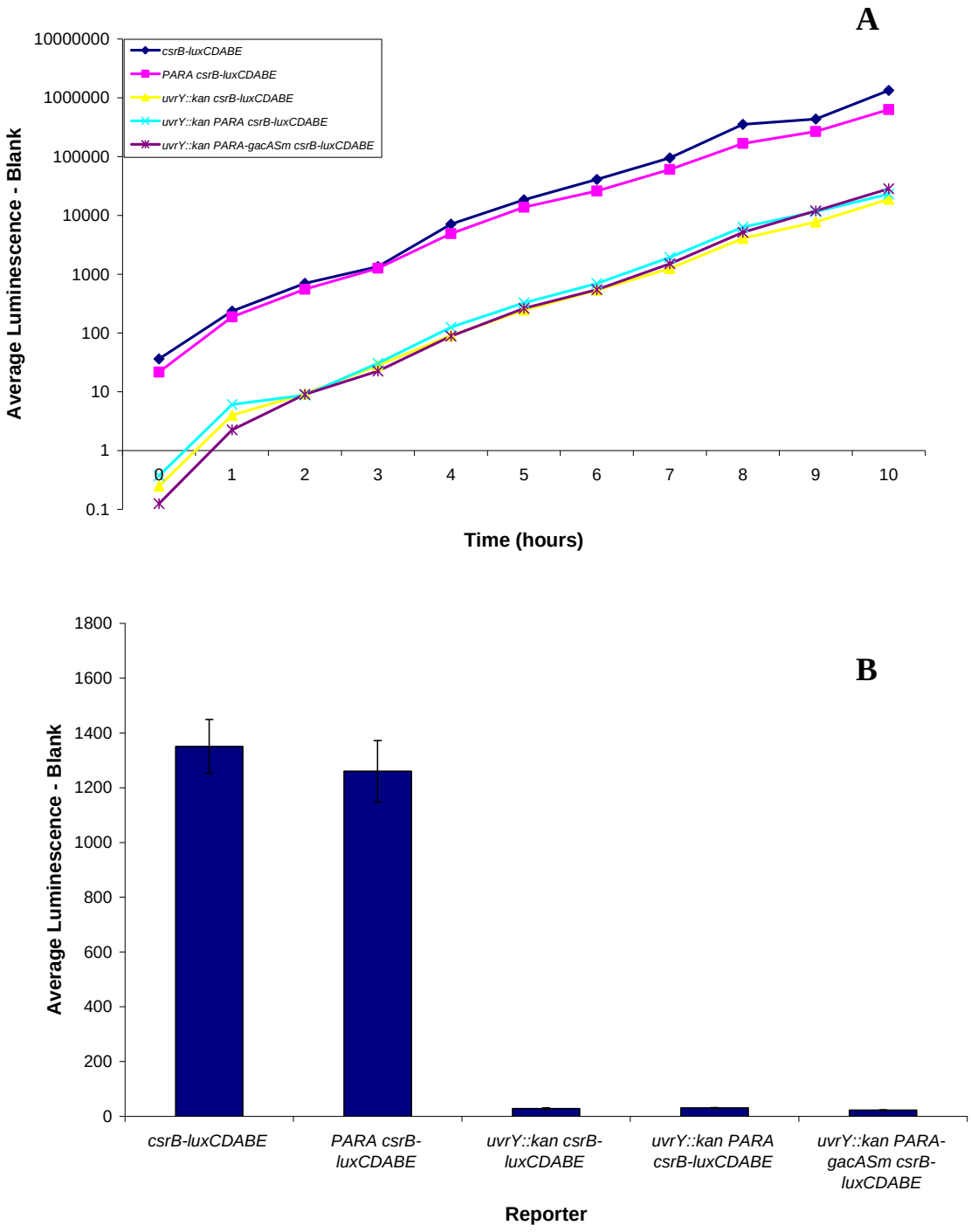

Figure 4-6. Complemtation of *uvrY* mutant in *E. coli* by *gacA* with no sugar induction of pCJK3 compared to wild-type luminescence production (A) and average induction at 3 hr (B)

CHAPTER 5
BACTERIAL QUORUM SENSING SIGNALS AND SETTLEMENT OF CORAL LARVAE

5.1 Introduction

Coral exhibit a range of reproductive strategies, including both sexual and asexual propagation. Some species of coral brood well-developed larvae after internal fertilization throughout the year. Most corals, however, reproduce during annual mass spawning events when gametes are synchronously released into the water column and undergo fertilization outside of the coral polyp (Harrison and Wallace 1990, Ball et al. 2002). Larvae of broadcast spawning scleractinian corals typically become competent to metamorphose into juvenile polyps approximately one week after the spawning and fertilization event (Babcock and Heyward 1986, Negri et al. 2001). Metamorphosis of coral larvae, and other Cnidarians, is naturally triggered by the perception of external cues, both from the abiotic environment and from other organisms on the reef (Morse et al. 1996, Webster et al. 2004, Kitamura et al. 2007).

Settlement, metamorphosis and recruitment of coral larvae are often used interchangeably, however, each refers to different stages in the development of corals. Settlement describes the physical process, during which larvae become pear-shaped, leave the water column, and casually attach to the substrate at the aboral end. This process is reversible, in that coral larvae "test" available substrates and can potentially leave unsuitable substrata and return to the water column. Larval metamorphosis is a physiological response, during which morphological, physiological and metabolic changes occur that are nearly always non-reversible (Negri et al. 2001, Golbuu and Richmond 2007). Metamorphosis of acroporid corals often occurs within 12 hours of settlement when the larvae have flattened dorsally and developed obvious septal mesenteries radiating out from the central mouth region (Harrison and Wallace 1990, Heyward and Negri 1999). Recruitment is the combination of these two events and the continued survival of the

metamorphosed larvae into a juvenile polyp and to adulthood (Koehl and Hadfield 2004). Both settlement and post-settlement events influence the recruitment rates of corals. Temporal and spatial patterns combined with the innate perception of different substrata also play important roles in the rate and efficiency of larvae recruitment.

The selectivity of coral larvae depends on both the type of larvae and the specificity for certain environmental cues. Brooding coral species tend to be more non-selective when determining where to settle. Often, coral larvae are selective to substrates of dead coral with the same morphologies (i.e. branching larvae settle on dead branching corals) independent of location and substrate availability (Norström et al. 2007). Coral larvae may also specifically settle on algal species, rocks, shells and sand (Morse et al. 1988, Huggett et al. 2006). There, however, are clear exceptions demonstrating that these species can be highly selective, such as the brooding coral, *Stylaraea punctata* (Golbuu and Richmond 2007). Chemosensory cues that induce members of the Agariciidae and Faviidae families function independent of the type of reproduction (Morse et al. 1996), as do those of the genus *Acropora* (Baird and Morse 2004). Often times, larval selectivity is related to coral habitat distribution and can be determined to some degree by surveying adult corals (Abelson et al. 2005, Golbuu and Richmond 2007, Norström et al. 2007). Two models have been used to compare coral recruitment based on larvae selectivity (Morse et al. 1988). The "lottery" model is used to describe those non-specific corals which settle when space becomes available, while the "deterministic" model describes selective corals in which larvae selectivity for appropriate substrata is important in determining spatial patterns in recruitment (Morse et al. 1988, Golbuu and Richmond 2007).

Corals that fit the "lottery" model of recruitment may exhibit general life history strategies relating to their success. *Stlyphora pistillata* and other pocilloporid corals are important early

successional species in coral communities throughout the world. The dominance of these types of species in newly available substrates is attributed to early reproduction, high fecundity, a long breeding season and a fast growth rate. In addition, these corals demonstrate a lack of a strict requirement for surface contact with specific chemical cues, such as crustose coralline algae (CCA) (Baird and Morse 2004). *S. pistillata* is able to colonize substrata as soon as they become available, which allows it to pre-empt some species that may be superior competitors as adults (Baird and Morse 2004).

Many corals, including acroporid corals, do require either direct contact or perception of a chemosensory cue in order to induce metamorphosis. Crustose coralline algae (CCA) have been linked to the induction of metamorphosis in many coral species, including members of the genus *Acropora* (Morse et al. 1994, Morse et al. 1996, Heyward and Negri 1999, Negri et al. 2001) (Golbuu and Richmond 2007). It is thought that the coralline algae produce cell-wall-bound polysaccharides that are recognized by chemoreceptors on the planula (Morse et al. 1996, Kitamura et al. 2007). Biochemical purification of the compound from Pacific and Caribbean congeners of CCA identified it as a member of a unique class of sulfated glycosaminoglycan that is associated with the cell walls of numerous CCA species. Bacteria associated with the surface of algal thallus may also be responsible for the polysaccharides perceived by the larvae (Negri et al. 2001). Mixed and monospecific biofilms of the 50 bacteria isolated from *Lithophyllum* sp. induced settlement and metamorphosis of *Acropora* and *Porites* spp. Both hydrophilic extracts and fragments of CCA are able to induce metamorphosis (Golbuu and Richmond 2007, Kitamura et al. 2007). Besides a chemosensory inducer of coral metamorphosis, CCA may also serve as indicators of environmental conditions to the coral larvae. CCA dominate reef front areas and their presence may indicate favorable conditions for growth and development (Golbuu and

Richmond 2007). While CCA has clearly demonstrated its influence in the settlement and metamorphosis of coral larvae, it is not the only chemosensory cue that coral larvae respond to.

Coral larvae induce settlement in response to both biotic and abiotic cues from the environment. Dead coral rubble and fragments have been shown to induce metamorphosis of the coral *Acropora millepora* (Heyward and Negri 1999) and in both branching and massive morphology of different coral species (Norström et al. 2007). Biofilms, bacteria isolated from CCA and other substrata have also been reported to induce larval metamorphosis (Morse et al. 1988, Negri et al. 2001, Webster et al. 2004). Chemosensory cues produced by active biofilms (e.g. extracellular polysaccharides and water soluble, stable molecules) are critical for the settlement and attachment of larvae of the polychaete, *Hydroides elegans* and the bryozoan, *Bugula neritina* (Dobretsov et al. 2007, Huang et al. 2007) and the acroporid coral *Acropora microphthalma* (Webster et al. 2004).

Bacteria regulate their growth and population densities through the regulatory mechanism named quorum sensing (QS) that consists of excreted chemical signals that either activate or de-activate target bacterial genes involved in cell division and adhesion, thus controlling the formation of biofilms (Waters and Bassler 2005, West et al. 2007). Gram-negative bacteria use signaling molecules, *N*-acetyl homoserine lactones (AHLs), of different lengths for intercellular communication (Miller and Bassler 2001). There are also chemical signals used to communicate between bacterial populations and their eukaryotic hosts. Both riboflavin (vitamin B-12) and its chemical derivative lumichrome have been associated with inter-kingdom signaling, and lumichrome acts to induce settlement and metamorphosis in some marine larvae (Phillips et al. 1999, Tsukamoto 1999, Tsukamoto et al. 1999). The majority of bacteria that exhibit quorum sensing (inclusive of *Alpha-*, *Beta-*, and *Gammaproteobacteria*) are typically dominant in

tropical waters (Webster et al. 2004, Wagner-Dobler et al. 2005, Huang et al. 2007). The activities within a biofilm (whether comprised of one species or a heterogeneous population of bacteria) are critical to induction of larval metamorphosis. A recent study treated biofilms with a protein synthesis inhibitor at two time points. Early treatment greatly disrupted and reduced settlement while late treatment did not influence settlement rates. This suggests that the proteins synthesized and/or regulatory proteins involved in formation of the biofilm are important to induction of larval settlement (Huang et al. 2007).

Just as chemical cues from the environment stimulate and enhance coral larvae settlement, chemical signals present in the environment serve to inhibit coral metamorphosis and recruitment. The red algae, *Delisea pulchra* produces furanones, which directly interfere with QS signals mediated by AHL production (Rasmussen et al. 2000). This interference disrupts biofilm formation and ultimately leads to decreased larval settlement observed in *Hydroides elegans* and *Bugula neritina* (Dobretsov et al. 2007). Furanones are also produced by marine bacteria, green, red and brown algae, sponges, fungi, and ascidians (Kjelleberg et al. 1997). Triclosan (TRI) is a chlorinated aromatic compound also found in marine systems that directly disrupts bacterial biofilms and thus decreases settlement of some pelagic larvae (Zhang and Dong 2004, Dobretsov et al. 2007). These compounds function as anti-fouling agents against bacteria, fungi, and other marine invertebrates.

Algae are in direct competition for space on coral reefs and any form of degradation or disturbance of coral reefs generally results in an increased dominance by benthic algae (Birrell et al. 2005). Algal production of anti-fouling chemicals leads to a diminished rate of coral larval recruitment that is enhanced by anthropogenic inputs to the system in the form of terrestrial run-off and sedimentation (Abelson et al. 2005, Birrell et al. 2005). Increased turf algae,

cyanobacteria and sedimentation greatly decreased the success of coral larvae metamorphosis and also led to decreased survival of juvenile recruits (Birrell et al. 2005, Kuffner et al. 2006). Many species of coral are dominant as adults but are inferior to algae as larvae or recruits. This is predominately due to their slow growth rates as compared to algae (Kuffner et al. 2006). *Dictyota* spp. are now the dominant algae in the Caribbean and reefs show upwards of 50% *Dictyota* cover in the Florida Keys. Direct and indirect contact between the algae and coral recruits resulted in increased mortality as compared to algal mimics (plastic aquarium plants), suggesting that something more than just shading and abrasion on the part of the algae influenced settlement and survivability of coral larvae (Kuffner et al. 2006).

In this study, the role of two signaling cues of bacterial origin in the induction of settlement and metamorphosis of *Acropora palmata* and *Montastrea faveolata* larvae was investigated. I hypothesized that known signals commonly associated with microbial biofilms and intercellular communication may function as settlement cues for these species of scleractinian corals, which are the primary reef building corals found in the Florida Keys National Marine Sanctuary. These signaling cues were chosen based on their involvement in the induction of settlement and metamorphosis of other marine invertebrates, in addition to coral larvae.

5.2 Materials and Methods

5.2.1 Extraction of AHLs from Coral-Associated Bacteria

Transgenic microbial biofilms were constructed using bacteria isolated from *A. palmata*. Two isolates of *Agrobacterium tumefaciens* and one isolate of *Vibrio harveyi* were selected due to their production of typical AHL-like compounds as identified by thin layer chromatography (TLC). Overnight cultures were grown on GASW broth and extracted with equal volumes ethyl acetate. The organic phase was dried and resuspended in 10 µl methanol. Extracts were spotted

on a TLC plate (Whatman KC18 Silica Gel 60 with fluorescent indicator, 10 x 10 cm, 200 μm thick) as well as standard AHLs as controls. The plate was developed with a mobile phase of methanol:water (3:2) for approximately 30 minutes. The presence of AHLs was detected by an *Agrobacterium tumefaciens* reporter strain carrying the plasmid pZLR4, which contains the *traCDG* operon with its promoter region (Table 2-1). *traG* is transcriptionally fused to *lacZ* (Cha et al. 1998). The reporter construct is stimulated during interaction with AHLs. The reaction requires 60 μg/ml of the substrate 5-bromo-4-chloro-3-indolyl β-galactopyranoside (X-gal) and results in blue color production. 2 ml of overnight culture of the reporter was subcultured into 50 ml ABM medium (per liter: 3.0 g K_2HPO_4, 1.0 g NaH_2PO_4, 1.0 g NH_4Cl, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.15 g KCl, 0.01 g $CaCl_2$, 2.5 mg $FeSO_4 \cdot 7H_2O$, 5% mannitol) and incubated at 30˚C for five hours (Hwang et al. 1994, Shaw et al. 1997). The reporter strain was then mixed with 100 ml of cooled ABM agar supplemented with Gm 30, 60 μg/ml X-gal. The agar mixture was slowly poured over the tiles to cover them, allowed to solidify and incubated at 30˚C overnight. After overnight growth of the reporter strain, blue color development over the test lanes were compared to the AHL control lane.

5.2.2 Biofilm Formation

Transgenic biofilms to account for consequences of loss of AHL function were constructed by mating the plasmid pE7-R3 into each other three coral isolated bacteria (Table 2-1). This plasmid is an IncP broad range host cosmid vector (pLAFR3) carrying the *aiiA* gene from *Bacillus* sp. 240B1 which encodes for an enzyme that cleaves the lactone ring of AHLs, rendering them functionless (Dong et al. 2000). The resulting biofilms served as the "transgenic" biofilms. As a vector control, pLAFR3 vector alone was mated into each bacterial isolate (Staskawicz et al. 1987). The resulting biofilms served as the "wild-type" biofilms for the following settlement experiments.

Settlement induction experiments were set-up in plastic containers (approximately 300 ml) to measure the consequences of AHL hydrolysis in coral larval settlement. Tiles (porous ceramic, 1 x 1 inch with 0.5 cm x 0.5 cm grid pattern) were bathed in either sterile GASW supplemented with 5% CFA media inoculated with the wild-type (vector control) bacteria or sterile GASW supplemented with 5% CFA inoculated with the transgenic (AHL-lactonase) bacteria and biofilms were allowed to form on the tiles. As a control, tiles were also bathed in sterile GASW supplemented with 5% CFA. 150 ml of filter sterilized seawater was added to each plastic container. Before tiles were added to the container, they were washed twice with filter sterilized seawater to remove any residual media. Settlement induced in each treatment (tiles + wild-type, tiles + transgenic, and tiles + media alone) was measured with and without the addition of crustose coralline algae (CCA), giving a total of eight treatments. Each treatment was performed in triplicate. CCA was collected from a rubble zone on the west side of the Bahia Honda Bridge and washed with running filter sterilized sea water at least 5 times or until water ran clear. *Acropora palmata* gametes were collected during a mass spawning event at Looe Key Reef, FL in August 2006. Gametes from different colonies were crossed-fertilized and larvae were maintained in flowing seawater for eight days until they reached competency. Fertilization took place at the Mote Marine Laboratory Tropical Research Laboratory in Summerland Key, FL. Twenty competent *A. palmata* larvae were added to each container, the lids were caped and containers were placed in a randomized pattern (to ensure blind sampling) in running seawater raceway table to maintain temperature. Larval counts and water changes were performed daily for a total of three days.

The effect of the presence of AHL and crustose coralline algae (CCA) on the induction of acroporid coral larval settlement was also tested. Settlement experiments were set-up in plastic

containers as above. Sterile tiles were added to either 150 ml filter sterilized seawater or 150 ml filter sterilized seawater supplemented with 100 nM 3-oxo-C6-Homoserine Lactone (3-o-C6-HSL). A fragment of CCA was added to each treatment and negative controls without the addition of CCA were included. Each treatment was performed in triplicate. Twenty competent *A. palmata* larvae were added to each container, lids were capped and containers were placed in a randomized pattern (to ensure blind sampling) in a running seawater raceway table to maintain temperature. Larval counts and appropriate water changes were performed daily for a total of three days.

5.2.3 Extraction of Coralline Algae Compounds

In order to determine the conditions necessary for pure lumichrome and riboflavin to sufficiently migrate on the TLC plate (Whatman KC18 Silica Gel 60 with fluorescent indicator, 10 x 10 cm, 200 μm thick), saturated solutions of lumichrome and riboflavin in methanol:HCl (49:1) and in pure methanol were prepared (Phillips et al. 1999). Samples were pelleted to eliminate any particulate matter in solution as lumichrome and riboflavin have low solubility in many solvents. A total of 3 μL of each mix and the solvent (methanl:HCl) were spotted onto the TLC plate. The plate was developed with a mobile phase of chloroform:methanol:water (17.5:12.5:1.5) (Phillips et al. 1999). A total time of approximately 40 minutes was required for the mobile phase to migrate to the top of the plate.

Dilution series of the pure samples was performed in order to optimize the concentration for visualization on TLC plates. Using stock solutions of 2800 g/L lumichrome and riboflavin, 1, 10 and 50 μL were spotted onto the TLC plate in addition to 50 μL of the solvent (methanol:HCl). The TLC was developed using the same mobile phase as above.

The presence of lumichrome and riboflavin in coralline algae was tested by methanol extraction (Phillips et al. 1999, Kitamura et al. 2007). Briefly, approximately 10 g of coralline

algae, frozen in liquid nitrogen, were ground into a paste to which 100% HPLC-grade methanol was added and transferred to a 15 ml plastic tube. The suspension was vortexed vigorously, and allowed to settle on ice. The contents were filtered using a Whatman 0.45 μL filter. This extraction process was performed three times. Methanol was rotary-evaporated at 45ºC at a pressure of 337 mbar, and then at 80 mbar for five hours on a Büchi Rotavapor R-200 (Büchi Labortechnik AG, Flawil, Switzerland). The final dried sample was reconstituted in 400 μL of methanol:HCl (49:1) to be used for TLC.

5.2.4 Thin Layer Chromatography of Coralline Algae Extracts

The methanol-extracted coralline algae samples were spotted onto the TLC plate in volumes of 1, 5, 10, and 25 μL. Five microliters of the pure lumichrome and riboflavin stock solutions were spotted as well as 25 μL of the methanol:HCl solvent. The plate was developed with chloroform:methanol:water (17.5:12.5:1.5) for 40 minutes, allowed to dry and visualized using a UV transluminator.

Due to the suspicion that chlorophyll is also extracted with methanol from the coralline algae, solvent portioning was attempted to separate lumichrome from chlorophyll. As a chlorophyll control, chlorophyll was extracted from grass blades with methanol. The starting solvent was methanol, which was then mixed with either ethyl acetate, isopropanol, chloroform, or tetrahydrofuran. If the two solvents were miscible then a 1:1 chloroform:water step was added. The solution was vortexed and then centrifuged to separate phases. Since lumichrome and riboflavin are yellow/orange in solution and chlorophyll is green, simple observation on the phase color indicated the presence of each chemical. Acid (0.05 M HCl) and base (0.05 M NaOH) were added to each solvent mix to test the effect of pH on the partitioning.

Solvent partitioning was applied to the coralline algae extracts in order to separate chlorophyll from lumichrome and riboflavin and therefore result in a cleaner run on the TLC.

The extracts were treated with methanol and ethyl acetate solvents mixed with chloroform and water and treated with 0.05 M NaOH. This resulted in the yellow lumichrome in the top phase and the green chlorophyll in the bottom phase. The top phase was transferred to a new Eppendorf 1.5 ml tube and stored until used for TLC.

Solvent partitioned coralline algae extracts were separated by TLC with both chloroform:methanol:water (17.5:12.5:1.5) and also methanol:water (3:2) mobile phases. The samples were running quickly with the mobile front so a more hydrophobic mobile phase of chloroform:methanol:water (35:12.5:1.5) was used.

5.2.5 Induction of Coral Larvae Settlement and Metamorphosis

Elkhorn coral, *Acropora palmata*, gametes were collected from Looe Key Reef, FL in August 2007 during a mass spawning event. Fertilization and rearing of larvae were conducted at Mote Marine Laboratory Tropical Research Laboratory (Summerland Key, FL). Settlement experiments were set up in six well Petri plates to test the effects of pure lumichrome, riboflavin, and *N*-acyl-homoserine lactones (AHLs) have on the settlement and metamorphosis of coral larvae. Lumichrome and riboflavin were used due to the observation that lumichrome induces settlement in ascidian larvae (Tsukamoto 1999). Larval settlement was scored positive if the larvae was attached at the aboral end to any part of the polystyrene well and did not detach with gentle agitation with water. Differences among treatments were compared using ANOVA and student's t-test with STATISTICA software, version 6.0.

N-acyl-homoserine lactones are signaling molecules and are critical components of the communication system, quorum sensing (Waters and Bassler 2005, West et al. 2007). For this experiment, 3-oxo-C6 homoserine lactone (a short-chain AHL commonly produced by marine vibrios (Taylor et al. 2004)) and C14 homoserine lactone (a long-chain AHL) were used. 3-oxo-C6 HSL is a common AHL produced by bacteria involved in quorum-sensing systems . C14

HSL was selected for these experiments based on the observation that many marine associated alpha- and gamma-proteobacteria produce long-chain AHLs (Wagner-Dobler et al. 2005, Mohamed et al. 2008).

Two reporter strains were utilized in order to detect the presence of lumichrome and/or AHLs in the methanol extracted coralline algae samples. *Agrobacterium tumefaciens* pZLR4 contains the *traCDG* operon with its promoter region. *traG* is transcriptionally fused to *lacZ* (Cha et al. 1998). The reporter construct is stimulated during interaction with AHLs. MG32-dapA is a strain of *Sinorhizobium meliloti* construct with the dapA promoter fused with a *gus* reporter gene, which uses 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-gluc) as a substrate (25 μg/ml). The *dapA* promoter activity was induced on M9 agar in response to 200 nM lumichrome in a promoter probe screen of *Sinorhizobium meliloti* (Gao et al., unpublished data). Therefore, the construct was used as a potential lumichrome reporter in this experiment.

20 μl of the lumichrome and riboflavin solutions (4.55 mM and 1.46 mM, respectfully) and of each AHL tested (3.21 mM C14-HSL and 1 mM 3-o-C6-HSL) was impregnated onto 0.002 g C18 resin. The mixtures were allowed to dry overnight in a flow hood as the chemicals adhered to the resin. A small amount of aquarium-grade silicone adhesive was applied to the center of a 1 x 1 inch porous ceramic tile and spread evenly with a metal spatula. The impregnated C18 resin was then spread onto the adhesive as evenly as possible for each of the four chemicals tested. As controls, two tiles with silicone adhesive only were prepared. Tiles were allowed to dry completely in the flow hood overnight (to effectively release acetic acid during the curing process). Prior to the set-up of the bioassay to test for the presence of the chemicals on the tiles, the tiles were washed in filter sterilized seawater for 3 hours and then placed in a thin layer of

distilled water. The distilled water was to allow the salt to diffuse out of the tiles so as to not interfere with the reporter strains.

2 ml of overnight culture of each reporter (*Agrobacterium tumefaciens* pZLR4 and *Sinorhizobium meliloti* MG32-dapA) were subcultured into 50 ml ABM medium and incubated at 30˚C for five hours (Hwang et al. 1994, Shaw et al. 1997). Each of the washed tiles were placed into large Petri plate (lumichrome, riboflavin and control in one plate; AHLs and control in the other). The reporter strains were mixed with 100 ml of cooled ABM agar supplemented with Gm 30, 60 μg/ml X-gal for the *Agrobacterium* reporter and 60 μg/ml X-gluc for the MG32 reporter. The agar mixture was slowly poured over the tiles to cover them, allowed to solidify and incubated at 30˚C overnight.

5.3 Results

5.3.1 Consequences of AHL Hydrolysis on Coral Settlement

Settlement induction experiments carried out with competent *Acropora palmata* coral larvae approximately one week after fertilization to investigate the involvement in common bacterial signaling molecules in the induction of coral larvae. Settlement in response to biofilms of wild-type bacteria (vector control) were compared to biofilms of the same bacterial strains that carried a plasmid-borne gene encoding for an AHL-lactonase enzyme that had developed on ceramic tiles. Each type of biofilm tested with and without the addition of a small piece of crustose coralline algae (CCA) and a negative control of tiles bathed in the bacterial media +/- CCA was included.

On each of the three days that settlement was measured, the media control with CCA resulted in the highest settlement events (Fig. 5-1A). In both the media control and the wild-type biofilm treatments, the addition of CCA enhanced larval settlement. The transgenic biofilms and CCA together, however, did not show enhanced settlement (Fig. 5-1A). Over the three day

settlement experiment the media control with CCA led approximately 47% of the larvae to settle (Fig. 5-1B), which was significantly higher than all other treatments ($F_{5, 21} = 32.6746$; $p < 0.0001$). Exposure to wild-type biofilms with CCA resulted in settlement of approximately 13% of the larvae added (Fig. 5-1B). Post hoc comparisons using t-tests indicated that media alone with CCA significantly induced settlement more than wild-type biofilms with or without CCA ($t = 2.079$; $p = 0.0005$). The transgenic biofilms did not show significant differences in settlement in the presence or absence of CCA ($p = 0.878$).

Settlement induction in response to exposure to known concentrations of AHL (3-o-C6-HSL, synthetic AHL) was also tested with *Acropora palmata* larvae. The hypothesis that the AHL signal in the water would induce settlement at a higher rate than seawater alone was tested. The addition of CCA was also predicted to enhance coral settlement. Filter sterilized seawater supplemented with 100 nM 3-o-C6-HSL did not induce coral larvae to settle more than filter sterilized seawater alone (Fig. 5-2; $F_{1,16} = 0.4337$; $p = 0.5195$). The addition of CCA, however, to both treatments did increase settlement, although the total percentage of settlement only reached approximately 15% (Fig. 5-2B).

5.3.2 Isolation of Coralline Algae Compounds

Thin layer chromatography (TLC) was used to first separate pure samples of lumichrome and riboflavin. The hypothesis that lumichrome and riboflavin were present in crustose coralline algae (CCA) was tested. Compounds from CCA were extracted with methanol and crude extracts were separated by TLC. The crude extracts did not separate as cleanly as the pure compounds with chloroform:methanol:water (17.5:12.5:1.5) or methanol:water (3:2). They ran as a long smear with no distinct separation, however when viewed under UV light, regions did fluoresce similar to the pure compounds. To eliminate potential chlorophyll contamination in the extraction process, samples were solvent partitioned to remove chlorophyll from the

lumichrome. The extracts were treated with methanol and ethyl acetate solvents mixed with chloroform and water, and finally treated with 0.05 M NaOH. This combination resulted in successful separation of the lumichrome and chlorophyll standards. The solvent partitioned samples, however, still did not show full separation as compared to the pure compounds. The samples migrated up the plate very quickly so a more hydrophobic mobile phase was used (chloroform:methanol:water; 35:12.5:1.5). This mobile fraction resulted in a shorter migration by the pure compounds but the extracts still migrated as a smear.

5.3.3 Roles of Signaling Molecules in Coral Larvae Settlement

Due to limited number of *Acropora palmata* collected following the spawning event, only a preliminary pilot study investigating the effects of lumichrome on settlement could be performed. Eight larvae were placed in each treatment well and monitored for three days. No larvae settled in that time, although larvae in the lumichrome treatments appeared to undergo more of a morphological change than the larvae in the other treatments. The aboral end of the larvae was noticeably more swollen than in other treatments suggesting that larvae were responding to lumichrome more so than seawater alone (Fig. 5-4).

Based on the limited observations of *Acropora palmata* larvae in response to exposure to lumichrome, the influence on settlement of *Montastrea faviolata* was examined. Settlement of *Montastrea faviolata* larvae in response to lumichrome, riboflavin and AHLs with acyl side chains of different lengths was extremely low. Lumichrome, riboflavin and C14-HSL appeared to slightly induce settlement (Fig. 5-3); however, some settlement was also observed in the negative controls. No significant differences were observed between the treatments ($F_{10,61}$ = 0.9564; p = 0.4899). No larvae settled in response to 3-o-C6-HSL regardless of concentration applied. This contradicts the results observed for *Acropora palmata* larvae in response to this

AHL (Fig. 5-2). While lumichrome, riboflavin, and C14-HSL appear to induce settlement, the high standard error limits potential conclusions.

5.4 Discussion

This study begins to shed light on the environmental and biological cues that *Acropora palmata* and *Montastrea faviolata* larvae perceive and respond to during their transition from pelagic to benthic organisms. While the conclusions that can be made from these settlement experiments are limited, there are specific trends that are both consistent with previously conducted studies and indicative of some general cues that induced larval settlement and metamorphosis.

Settlement in response to wild-type and transgenic biofilms of a consortia of bacteria primarily comprised of isolates of *Agrobacterium tumefaciens* and *Vibrio harveyi* demonstrated that larvae of *A. palmata* show slightly different degrees of settlement as compared to seawater alone. This is primarily due to the production of AHLs by the wild-type biofilms and the lack of AHLs in the transgenic biofilms due to the *aiiA* gene encoding for an AHL-lactonase enzyme that cleaves the ring of the AHL molecule, resulting in loss of function (Dong et al. 2000, Dong et al. 2001, Gao et al. 2007a). The presence of CCA led to an increased level of settlement in both the wild-type biofilm treatment and the negative control (> 40% settlement), while no such increased was observed in the transgenic biofilm treatment. This increase was greatest in the seawater negative control, suggesting that settlement may be induced by CCA more than the presence of AHL signaling compounds produced by biofilms. This result was further supported through the experiment using a known concentration of a synthetic AHL (3-o-C6-HSL). Induction of settlement with and without the presence of CCA was not significantly different from the seawater negative control with and without CCA. In the treatments with CCA, induction of settlement was higher but similar between treatments.

Larvae used in these experiments required approximately eight days to become fully competent. Once settled, larvae tended to begin to metamorphosize after 12-24 hours, which supports previous studies (Negri et al. 2001). The induction of settlement in response to CCA has been demonstrated in Pacific corals from the families Acroporidae and Faviidae (Morse et al. 1996, Baird and Morse 2004) and the results presented here suggest that acroporid corals in the Atlantic are induced to settle after exposure to CCA cues in the environment.

Riboflavin and its derivative lumichrome are chemicals involved in the cell-to-cell communication between bacteria and their eukaryotic host (Phillips et al. 1999). Lumichrome is involved in the settlement and metamorphosis of sessile marine organisms such as the asicidin, *Hhalocynthia roretz*i (Tsukamoto 1999, Tsukamoto et al. 1999). Therefore, it is reasonable to hypothesize that riboflavin and lumichrome may be produced by either CCA or the bacteria associated with it. Methanol extractions of CCA and subsequent analysis with thin layer chromatography failed to successfully isolate both riboflavin and lumichrome from extracts of CCA. This is not to say, however, that neither compound is present in coralline algae. CCA does contain compounds that fluoresce similarly to pure samples of riboflavin and lumichrome (green and blue respectfully). While the potential for numerous natural compounds to fluoresce blue and green these results should not be discounted. Alternative separation methods may be employed, such as high pressure liquid chromatography (HPLC) to better separate compounds extracted from CCA. These compounds can then be screened for their involvement in the induction of coral larvae settlement and metamorphosis. A recent study isolated a novel compound from CCA by HPLC shown to induce metamorphosis. The natural inducer was identified as 11-deoxyfistularin-3, a bromotyrosine derivative (Kitamura et al. 2007). This chemical was also isolated from marine sponges and related compounds have a wide range of

biological activities, such as antiviral, antibiotic, cytotoxic, Na^+/K^+ ATPase inhibitory, and anticancer (Kitamura et al. 2007).

Settlement experiments investigating the roles of lumichrome, riboflavin and short- and long-chain AHLs in settlement induction of *Montastrea faviolata* larvae were inconclusive. While optimization of these settlement techniques are ongoing, sub-optimal larval and settlement conditions may have affected the outcome of the experiments. The spawning event at Looe Key Reef, FL in August 2007 resulted in nearly non-existent acroporid coral spawning and very few colonies of *Montastrea faviolata* that spawned. With limited competent coral larvae and adverse environmental conditions, representing natural conditions for settlement becomes challenging. From the limited results of this study, it appears that the presence of multiple cues enhance the effect of each other. In order to determine if one cue is able to induce settlement alone, larvae must be presented with a wide range of treatments and sufficient yields of competent larvae from spawning events are required.

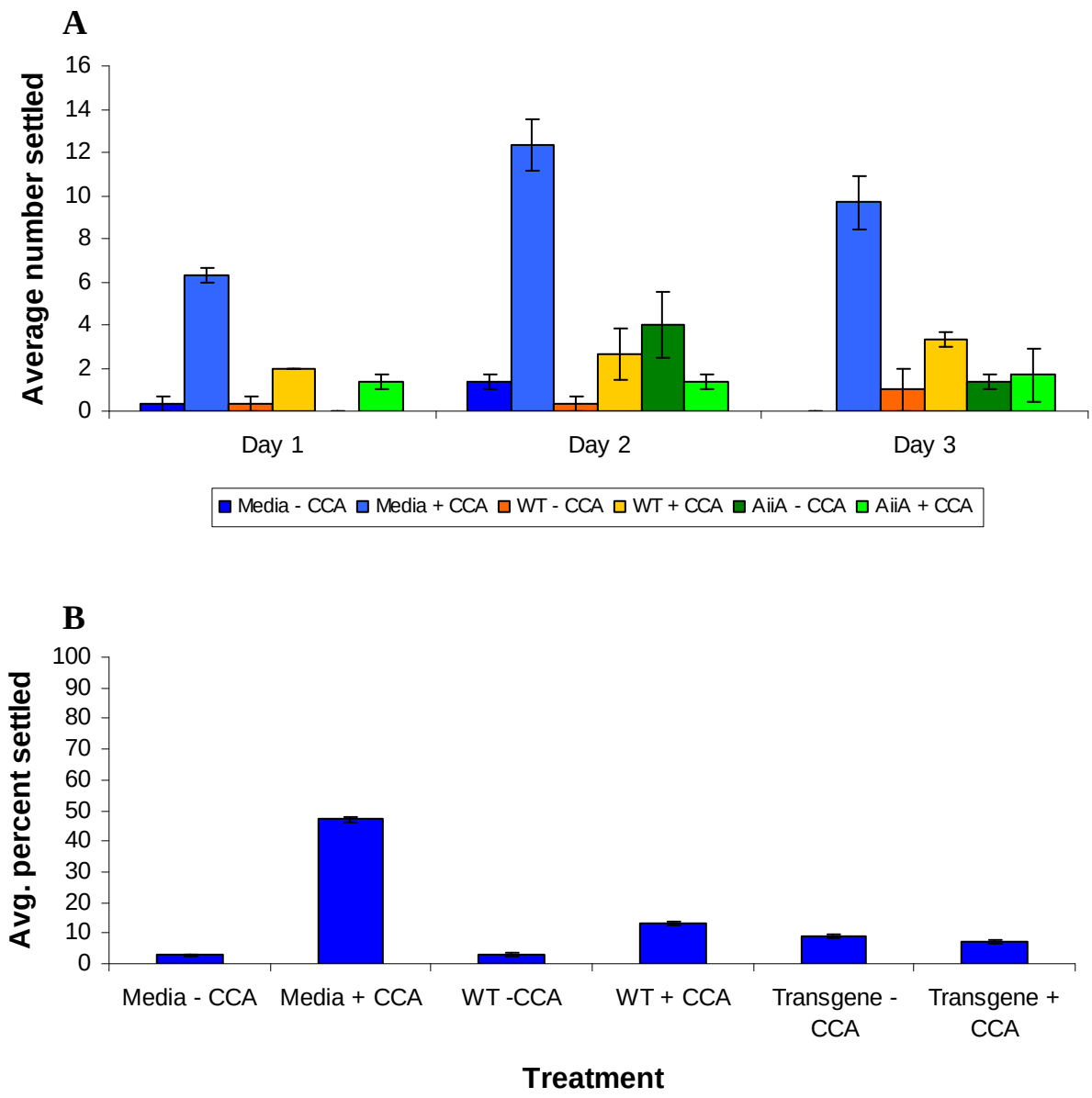

Figure 5-1. Coral larvae settlement in response to tiles bathed in GASW media supplemented with 5% CFA +/- either wild-type biofilm formation or transgenic biofilm formation with strains carrying the pAiiA (AHL-lactonase gene). Panel A shows average larval settlement measured each day. Each treatment was tested for both the ability to induce settlement and also if settlement was enhanced with the addition of a piece of crustose coralline algae (CCA). Panel B shows average percent of coral larvae settlement after 3 day exposure to each treatment. A total of 20 larvae were added to each treatment at the start of the experiment. Combined averages among treatments were used to calculate the total percentage of larvae that settled. Settlement was defined as adherence to any surface in the container with the aboral end of the pear-shaped larvae.

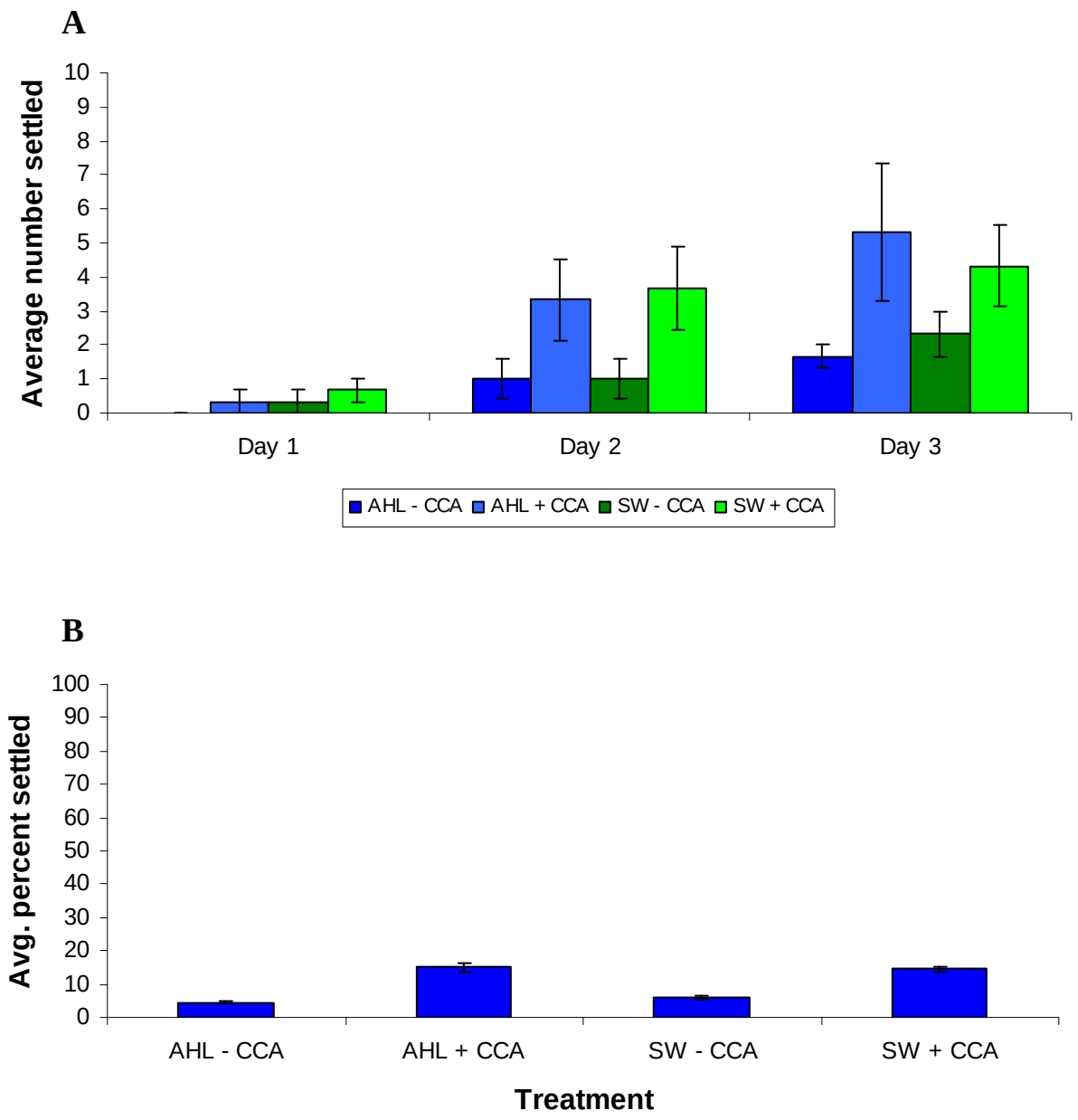

Figure 5-2. Coral larvae settlement in response to tiles bathed in filter sterilized sea water supplemented with 100 nM 3-o-C6-HSL (AHL treatment) and filter sterilized sea water as a control. Panel A shows average larval settlement measured each day. Each treatment was tested for both the ability to induce settlement and also if settlement was enhanced with the addition of a piece of crustose coralline algae (CCA). Panel B shows average percent of coral larvae settlement after 3 day exposure to each treatment. A total of 20 larvae were added to each treatment at the start of the experiment. Combined averages within treatments were used to calculate the total percentage of larvae that settled. Settlement was defined as adherence to any surface in the container with the aboral end of the pear-shaped larvae.

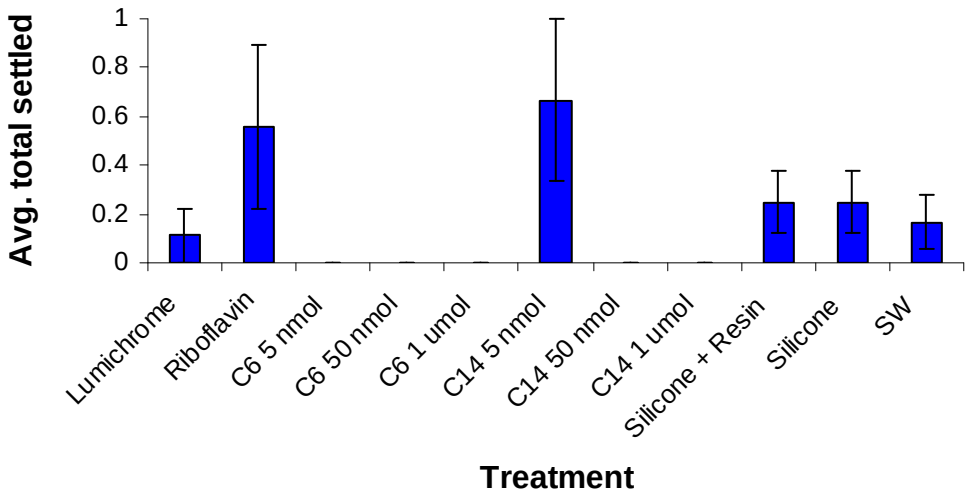

Figure 5-3. Average total coral larvae (*Montastrea faveolata*) over six days in response to potential inducers. Settlement was conducted in 6-well polystyrene Petri plates with 20 larvae added to each well with filter sterilized seawater. Larval counts and water changes were performed daily. Combined averages within treatments were used to calculate the total percentage of larvae that settled. Settlement was defined as adherence to any surface in the well with the aboral end of the pear-shaped larvae.

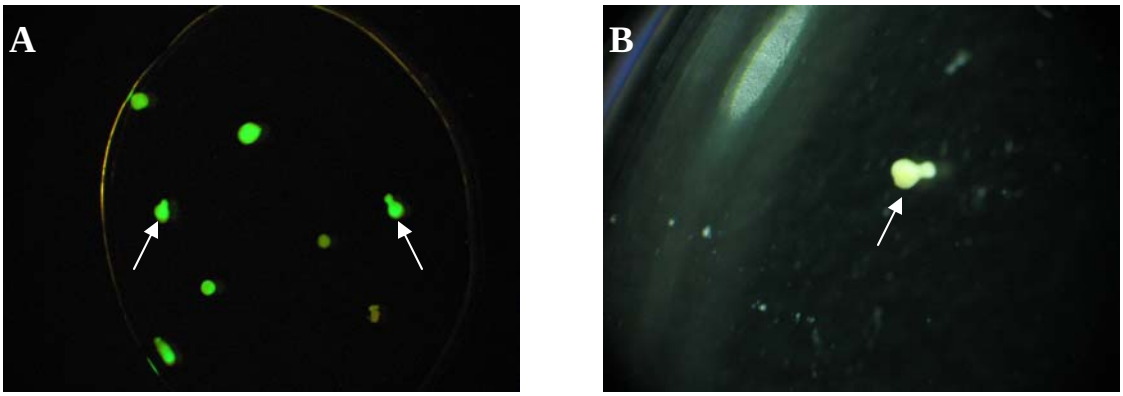

Figure 5-4. Swollen aboral ends of *A. palmata* larvae in response to exposure to lumichrome. Panel A shows swimming larvae under a Leica dissection microscope with an autofluorescence filter. Comparatively more larvae in the lumichrome treatment exhibited swollen aboral ends than in the seawater control, potentially signaling their readiness to settle (white arrows). Panel B depicts a close up of a larvae under dark field microscopy with a swollen aboral end in response to lumichrome (white arrow).

CHAPTER 6
SUMMARY AND CONCLUSIONS

6.1 Value and Decline of Corals

Coral reefs are valuable ecosystems and are vital to the overall health and sustainability of near-shore marine systems. The goods and services originating from these ecosystems support local economies and represent a wide array of benefits to society (Johns et al. 2001). Coral reefs, however, are facing ever increasing environmental stressors, limiting their productivity and ultimately leading to their demise. These studies investigated how the biology of the corals, interactions with other organisms, and environmental cues contribute to the complexity of coral reef ecology and specifically to the study and management of coral diseases.

The environmental stressors confronting corals continues to increase as the world's population and global demands increase (Harvell et al. 1999, Nystrom et al. 2000). Increased nitrification and pollution run-off due to amplified farming practices and pollution from miss-treated wastewater allow for the introduction of opportunistic pathogens into novel environments (Lipp et al. 2002, Griffin et al. 2003). Besides the fact that anthropogenic stressors provide opportunities for pathogens, they alter the normal ecology of coral reef systems and subject the corals to conditions often well beyond their tolerance. Such conditions generally led to coral bleaching, which may be exacerbated by the presence of pathogenic bacteria (Douglas 2003, Hughes et al. 2003, Rosenberg and Falkovitz 2004, Ainsworth et al. 2008).

6.2 Characterization of a Coral White Pox Pathogen

The increase of these types of anthropogenic inputs in the Florida Keys is what led researchers to suggest that the presence of *Serratia marcescens* PDL100, a coral white pox pathogen, was due to introduction via un-treated sewage effluence (Patterson et al. 2002). *Serratia marcescens* is a known enterobacterium capable of causing disease in plant, vertebrate

and invertebrate animals and humans but had not been isolated from a marine invertebrate before. The suggestion that *S. marcescens* PDL100 originated from human sources is reasonably supported by this study through the comparison of the carbon utilization profiles and the enzymatic induction of *S. marcescens* isolates in response to growth on coral mucus. PDL100 correlated the highest with the pathogenic *Serratia marcescens* isolates.

Carbohydrate utilization patterns (CUPs) are not only used to identify bacterial isolates. These classification tools have also been applied to microbial source tracking. Identifying the CUP of a bacterial isolate that can be compared to a database allows for source identification with relative ease. Hagedorn and colleagues used BIOLOG GP2 plates to source track *Enterococcus* fecal pollution in water and found that CUP analysis led to an average rate of correct classification by source to be approximately 95%, well in the upper range of other methods (Hagedorn et al. 2003). CUP mapping has also been shown effective for *E. coli* and fecal streptococci resulting in a 73 and 93% average rate of correct classification (ARCC), respectfully (Seurinck et al. 2005). Nutrient utilization profiling has proven to be effective in source tracking *E. coli* in surface waters, yielding an ARCC of 89.5% using BIOLOG GN2 plates (Uzoigwe et al. 2007).

The high degree of correlation between the coral white pox pathogen and other pathogenic isolates of *Serratia marcescens* tends to suggest that metabolic potentials and perhaps virulence factors are conserved among pathogenic isolates of this species. This notion provides reason why *S. marcescens* is such a successful opportunistic pathogen, and able to infect vastly different hosts. The fundamental mechanisms that *Serratia marcescens* PDL100 employs during colonization and growth on the coral host are similar to those observed in other pathogenic *S. marcescens* isolates. It appears that this coral white pox pathogen may have the necessary

machinery in place to overcome its host defenses and mount an attack leading to an infection, provided there is an open niche or the coral is vulnerable due to other stressors. This is suggested based on the induction of enzymatic activities and proteases during growth on coral mucus. Both cell-associated and extracellular proteases were shown to be induced during growth of the pathogen on coral mucus. Extracellular proteases are often associated with virulence and a mechanism for pathogenicty (Travis et al. 1995, Young et al. 1999). *Serratia marcescens* PDL100 also possesses the metabolic and regulatory pathways that may be needed to colonize and grow on coral mucus. These pathways, however, may not have specifically evolved during the interaction between PDL100 and *Acropora palmata*. In fact, many of the carbon substrates utilized by PDL100 were common to those utilized by other isolates of *S. marcescens*. Similarly, the enzymes induced during growth on coral mucus were consistent with other pathogenic isolates of *S. marcescens*.

6.3 Potential Regulation of Virulence Factors and Disease Management

The results of this study also indicate that *Serratia marcescens* PDL100 possesses the two-component regulatory system, GacS/GacA. The complementation assay demonstrated that the GacA protein in PDL100 is functional and therefore suggests that this pathogen may regulate its virulence through this response regulator as do many other pathogenic γ-protoebacteria including *E. coli*, *Salmonella enterica*, *Pseudomonas* spp. and *Vibrio* spp. (Lapouge et al. 2008). Potential targeting of the gacA gene for disruption or mutation may lead to a strategy for the management of this pathogen. As discussed earlier, conventional disease treatments, such as antibiotics are not feasible on coral reefs. By disrupting the function of gacA or another component of the regulatory system, the pathogen will still be able to grow and proliferate, however, virulence factors controlled by the regulator protein will not be expressed (Lapouge et al. 2008). Another target within the regulatory system is the inhibition of the

autophosphorylatable GacS in response to an environmental stimulatory signal. Potentially, an environmental signal or microbial isolate could be used as a biocontrol agent to inhibit GacS and therefore, the downstream virulence gene expression. The use of probiotic bacteria to colonize a host and provide a barrier against and/or actually inhibit pathogenic infection is routinely applied in agriculture and commercial aquaculture to control disease in trout (Brunt et al. 2007), shrimp (Chythanya et al. 2002, Farzanfar 2006) and other species (Balcazar et al. 2006). The exploitation of the natural abilities of native coral-associated bacteria to combat invading pathogens, may be a future means to manage opportunistic pathogens capable of causing coral diseases.

6.4 Coral Mucus

Coral mucus has been shown to serve many purposes for the coral host and the surrounding reef ecosystem. Such functions include ciliary-mucoid feeding by the copepod Acartia negligens (Richman et al. 1975) and mucus is hypothesized to protect against fouling, smothering by sediment, physical damage, desiccation during air exposure at extreme low tides, space invasion by other corals, and ultraviolet radiation damage (rev. (Wild et al. 2004, Brown and Bythell 2005)). Mucus also provides rich organic nutrients to bacteria and other microorganisms living on the corals and in the surrounding waters (Wild et al. 2004). While the composition of coral mucus varies among species, season and depth (Crossland 1987), certain types of molecules are routinely present. In many cases, protein and carbohydrate polymers are the major components, where as lipids are less abundant. Generally, fucose, arabinose, galactose and N-acetyl glucosamine are present in high concentrations (Ducklow and Mitchell 1979, Meikle et al. 1988). The enzyme induction assay presented here begins to elucidate some of the carbon sources found in *Acropora palmata* mucus secreted during the summer months. While

these conclusions were deduced indirectly, the induction of the specific enzymes indicates that these molecules were present in coral mucus.

6.5 Settlement and Metamorphosis of Coral Larvae

Settlement and metamorphosis induction experiments demonstrate that Acropora palmata larvae appear to respond to both AHLs and cues from coralline algae. Experimental results also demonstrate that larvae of *A. palmata* induce settlement in response to microbial biofilms comprised of *Agrobacterium* and *Vibrio* isolates from coral mucus. The settlement cues from CCA may either be produced by the algae or by bacteria associated with the algae (Negri et al. 2001). Lumichrome showed potential for induction of settlement in *A. palmata* in a pilot study that led planktonic larvae to swell and become pear shape before those in other treatments (Fig. 5-4). However, due to limited larvae stocks, the influence of lumichrome and riboflavin in settlement and metamorphosis in *A. palmata* larvae was not tested. *Montastrea faviolata* larvae is another important reef-building coral species in the Caribbean. Settlement experiments testing the role of lumichrome, riboflavin and short- and long-chain AHLs were conducted, but results and conclusions were limited due to low larvae counts and unfavorable spawning conditions.

6.6 Future Directions

The mechanisms and virulence factors utilized by opportunistic pathogens remain unclear for many pathogens, including *Serratia marcescens* (Patterson et al. 2002). Although well-studied the full mechanism of *Vibrio harveyi* has yet to be fully elucidated (Austin and Zhang 2006). In *Pseudomonas aeruginosa*, few host-specific virulence mechanisms were identified in virulence mutant screens in insects, mice, and nematodes, suggesting extensive conservation of virulence factors used by this pathogen (Mahajan-Miklos et al. 2000). This study begins to draw some conclusions as to the types of capabilities and potential virulent pathways exhibited by the coral white pox pathogen, *Serratia marcescens* PDL100. These conclusions were drawn from

metabolic characterization experiments in comparison to other environmental and pathogenic isolates of *S. marcescens* as well as native coral bacterial isolates. The results of the metabolic assays suggest potential genes involved in the colonization and growth of the pathogen when it encounters coral mucus. The natural progression of research is the genetic and molecular characterization of white pox disease. Tools to identify specific genes induced or repressed during the colonization and infection process such as a promoter library screen will be employed. Once these genes are clearly identified as being involved in the colonization and infection process, they may serve as targets for disruption in order to reduce virulence in the pathogen. Similar tools can then be applied to other coral diseases in order to better understand the mechanism of infection. Once coral diseases are understood at the microbial level, sustainable management practices to reduce disease and preserve coral reefs may be achievable.

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BIOGRAPHICAL SKETCH

Cory Jon Krediet was born in 1984, in Oklahoma City, Oklahoma. The older of two children, he grew up in Chicago, Illinois, graduating from Glenbard North High School in 2002. He earned his B.A. in biology and German from Drew University in 2006, graduating s*umma cum laude* and with specialized honors in biology.

During his undergraduate career, Cory conducted original research as part of a Research Experience for Undergraduates (REU) program sponsored by the National Science Foundation. Working at Shoals Marine Laboratory in the Gulf of Maine, he investigated growth and mortality trade-offs along a depth gradient in the Jonah crab, *Cancer borealis*. That project developed into an honor's thesis project at Drew Univeristy. Cory's undergraduate experiences also led him to study coral ecology in the Egyptian Red Sea and Belize.

Upon completion of his M.S. program, Cory will continue at the University of Florida for his Ph.D., working with Dr. Max Teplitski to further elucidate the genetic and regulatory pathways that allow *Serratia marcescens* to colonize and infect elkhorn coral, *Acropora palmata*.