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Analysis of Extracellular Proteins Expressed by Host, Crassostrea Virginica and Pathogen, Roseovarius Crassostreae in Roseovarius Oyster Disease

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ANALYSIS OF EXTRACELLULAR PROTEINS EXPRESSED BY HOST, *CRASSOSTREA VIRGINICA* AND PATHOGEN, *ROSEOVARIUS CRASSOSTREAE* IN ROSEOVARIUS OYSTER DISEASE

By

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An Abstract of the Thesis Presented
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Roseovarius Oyster Disease (ROD) involves the colonization of the inner shell of the Eastern Oyster (*Crassostrea virginica*) by the marine alpha-proteobacterium *Roseovarius crassostreae*. The ensuing disease can result in upwards of 90% mortality in hatchery-raised juvenile (< 25 mm shell length) oysters. Symptoms of ROD include heavy, brown ring deposits of conchiolin on the surface of the shell, as well as uneven valve growth and shell curvature. Although the bacterium does not invade oyster tissue or digestive tract, fluorescent localization of an *R. crassostreae* biofilm on the inner shells of ROD-affected oysters, and toxic effects of *R. crassotreae* extracellular products on hemocytes and larval oysters suggest that a chemical warfare takes place between the *R. crassostreae* biofilm and the oyster host.

In this thesis, I cultured *R. crassostreae* under laboratory conditions and analyzed the affects of physical growth condition, temperature, culture volume, and iron limitation

on bacterial growth and bacterial extracellular protein (ECP) production. Culture growth rate increased with increasing temperature. Iron limitation resulted in slower growth but had no affect on maximum culture density. Iron limited cells produced a greater volume of ECPs, especially at low cell density. ECPs from liquid and solid-phase cultures were analyzed by LC-tandem mass spectrometry. The resulting protein sequences had high similarity to proteins in other bacterial species that act as virulence factors in other hosts, including metal transporters (2), proteases (2) and a surface-associated GroEL chaperonin.

I also exposed live oysters to ECPs of live *R. crassostreae* cells by embedding a filter capsule into the oyster shell. I sampled extrapallial fluid from oysters pre-exposure and post-exposure and analyzed the relative expression of the oyster defense protein dominin in addition to the extent of brown shell deposition as a function of bacterial presence. The injury response due to capsule embedment masked any potential response by oysters to *R. crassostreae* ECPs.

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INTRODUCTION

Oyster Culture & Disease

A major fishery along the Atlantic and Gulf coasts of North America has historically been sustained by natural populations of the Eastern oyster, *Crassostrea virginica*. These populations have been in decline over the past century due to the effects of overharvesting, deteriorating coastal water quality, and disease (Mackenzie 2007). An alternative to wild harvesting, oyster aquaculture has existed in some form or another since at least Roman times (Castell 2000). The culture of Eastern oysters has served as a means to offset some of the decline in the wild fishery in the Northeast (Allen et al., 1993). In addition to providing a reliable commercial harvest, oyster aquaculture provides a product that is superior in quality to wild-caught oysters (Allen et al. 1993; Eastern Oyster Biological Review Team 2007).

There is an active and growing oyster culture industry throughout the Northeast. For example, the Maine oyster industry has expanded in recent years with the harvest of the cultured oysters increasing from about 1.9 million to 3 million between 2005 and 2010 (Fig. 1.1). The market value of these oysters likewise increased from $0.85 to $1.75 million dollars over the same time frame. Oyster culture in the state typically takes place in high salinity waters on tidally dominated rivers that experience large phytoplankton blooms and significant seasonal warming so that high-quality marketable oysters can be raised with two to three years of effort. Despite the increasing popularity of Maine cultured oysters in the market, further expansion of the industry is limited by winter temperatures that are too cold to support oyster growth, constraints on the number of lease sites, and crop losses due to disease.

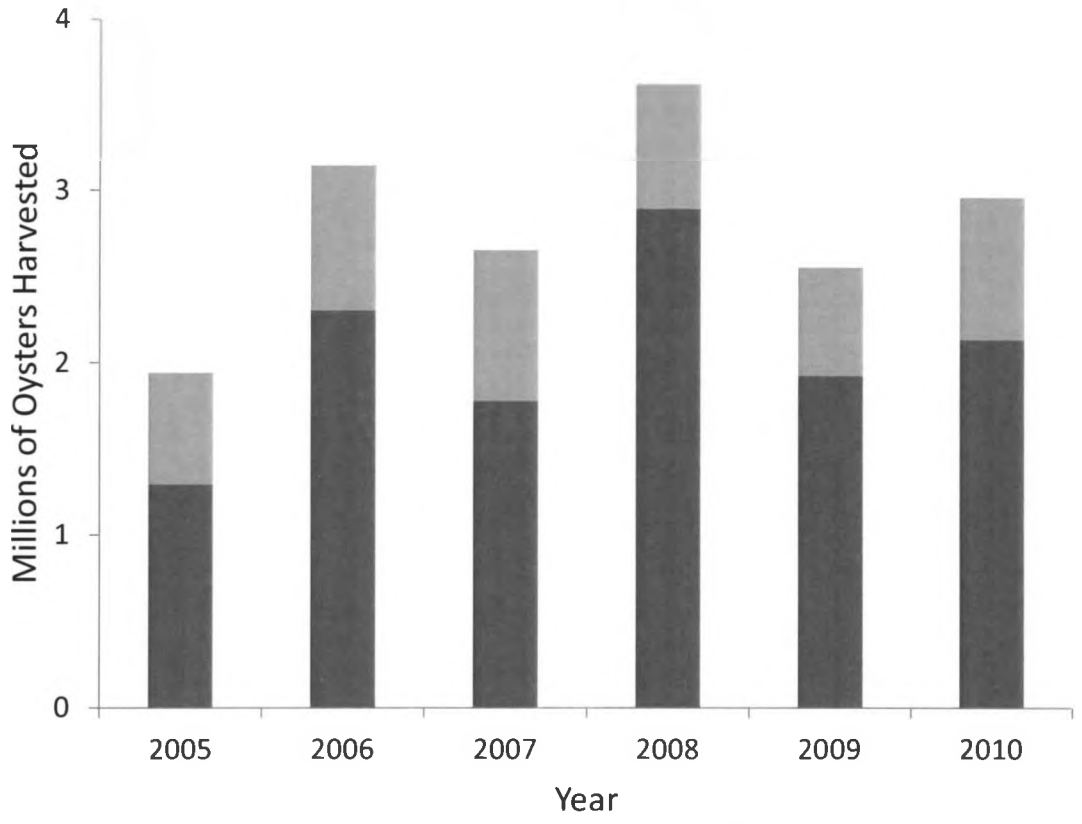

Figure 1.1 Annual Harvest of Maine Farm-Raised Oysters from 2005-2010. The dark portion of each bar represents oysters farmed on the Damariscotta River. Data from Maine Department of Marine Resources (maine.gov/dmr)

There are several diseases that impact oyster culture in the Northeast, including Multinucleated Sphere Species Unknown (MSX), Seaside Organism (SSO), Dermo and Roseovarius Oyster Disease (ROD). MSX is caused by the protist *Haplosporidium nelsoni*. The first known outbreak of this parasite in Eastern oysters was observed in the Delaware Bay in 1957 (VIMS 2012) and outbreaks have since decimated populations in some of the most historically successful oyster grounds in the Chesapeake and Delaware Bays. MSX outbreaks typically occur from May through October with mortalities peaking in August. Infection results in decreased condition (body weight) and upwards of 100% mortality. The effects are particularly acute when the optimal environmental conditions for disease, such as high temperature and high salinity, are met. The parasite

can persist over the winter months in estuaries where the winter salinity remains high and results in increased mortality in the early spring. The range of *H. nelsoni* has expanded in recent years and recent outbreaks have been reported as far north as the Damariscotta River in Maine and farms in Nova Scotia (VIMS 2012). SSO (seaside organism) is caused by *Haplosporidium costale,* which is closely related to the pathogen responsible for MSX. The conditions associated with SSO outbreaks, warm temperatures and high salinity, are similar to those associated with outbreaks of MSX (Carnegie 2009). Fewer large mortality events have been attributed to SSO, but this disease has resulted in considerable losses on several oyster farms in southern New England.

Perkinsus marinus, the causative agent of "Dermo" disease, is another serious pathogen of oysters. The range of *P. marinus* extends along the U.S. Gulf Coast and most of the U.S. East Coast. Although some *P. marinus*-associated mortality may begin as early as June following a warm winter, most mortality occurs during late summer and early fall. Mortality from Dermo increases with increasing salinity as it does with MSX and SSO. However, *P. marinus* persists within a single oyster much longer than *H. nelsoni*, even when it is exposed to salinities much lower than that at which oyster mortality commonly occurs. Thus, *P. marinus* may be present in an oyster for up to three years before it causes mortality and can cause substantial losses among large oysters just prior to harvest (VIMS 2012).

Roseovarius Oyster Disease (ROD) is caused by the alpha proteobacter, *Roseovarius crassostreae*. Previously termed Juvenile Oyster Disease, ROD most commonly affects oysters less than 25 mm in shell length. Davis and Barber (1999)

reported over 90% mortality of hatchery-raised juveniles and considerable financial loss from ROD outbreaks in Maine. In Maine, ROD is endemic on the Damariscotta River, where over 70% of oyster production in the state takes place.

Changes in oyster culture husbandry practices and the implementation of breeding programs have alleviated some of the impact of these diseases. MSX and Dermo have received the most attention in efforts to select for disease resistance. Selective breeding for MSX- and Dermo-resistant Eastern oysters at several institutions in the mid-Atlantic spans nearly four decades (Rawson et al. 2010). Generally, these programs have relied on propagating disease challenge survivors in order to found a particular line. For example, several lines of oysters demonstrating MSX resistance were developed from oysters that survived an MSX outbreak in Delaware Bay in the late 1950s (Ford & Haskin 1982). With each successive generation of selection in the presence of a disease challenge, the offspring produced from the survivors of outbreaks have demonstrated increasing resistance to MSX (Ford & Haskin 1987, Ford et al. 2012). In the late 1980s, researchers in Maine founded a breeding program with oysters obtained from the Frank M. Flowers Co. (NY), a line that had reportedly been selected for resistance to ROD. Even so, the oysters developed in Maine (now designated as the University of Maine Flowers Select Line or UMFS) have improved survival and growth compared to Flowers oysters when grown at sites on the Damariscotta River in Maine where ROD is endemic (Barber et al. 1998, Rawson & Feindel 2012). More recently, a selective breeding program has been established by the State of Connecticut Bureau of Aquaculture in partnership with Connecticut oyster growers. Founders for this program were selected from among the oysters that survived a major MSX-epizootic responsible

for up to 87% mortality in 1997 and 1998 that reduced oyster production in Connecticut from more than 500,000 bushels in 1996 to 80,000 in 2000. This line, called the Clinton line, has also been exposed to ROD and the surviving oysters were spawned in the spring of 2000 to generate a line that has fast growth, and MSX and ROD resistance (Sunila pers. comm.).

Changes in husbandry practices have also been successful in reducing the impact of disease. For example, ROD mortalities on the Damariscotta River typically occur in September and October. To counteract this, the hatchery production of oyster seed in Maine begins during late winter so that growers can deploy the seed in early summer, taking advantage of the full growing season and allowing oysters to grow past the disease threshold size (~25mm shell height) prior to the onset of disease conditions (Rawson & Fiendel 2011).

Although both selective breeding and husbandry measures have been successful in reducing the impact of ROD, the biological basis of this success is unclear. As mentioned above, ROD is endemic in Damariscotta River and problematic in several locations in Rhode Island and Connecticut (Markey et al. 2008, Sunila pers comm.). Thus, some major questions remain, including, what is the genetic and physiological basis of variation in resistance to ROD among lines of oysters? Why are larger-sized oysters more resilient and able to survive ROD under the same conditions under that can kill younger oysters? What virulence factor(s) are expressed by *R. crassostreae* and how do resistant and non-resistant oysters respond? A better understanding of how the physiology of *R. crassostreae* changes as a function of varying environmental conditions may provide answers to some of these questions. Specifically, knowledge of the

mechanisms underlying the colonization of oysters by *R. crassostreae* and how host-parasite interactions lead to onset of disease, the appearance of symptoms and eventually host mortality will be essential to help improve management and breeding programs intended to avoid future outbreaks. The goal of my thesis research has been to use a proteomic approach to help elucidate some of these interactions.

ROD

ROD in Eastern oysters is characterized by a brown ring-shaped deposit of conchiolin on the inner shell and cupping of the left valve caused by retarded and uneven growth of the two shell valves. Conchiolin is the protein matrix excreted by the oyster that sets the stage for aragonite nucleation in successive layers of normal shell growth. Lesions and retraction of the oyster mantle tissue away from the growing edge of the shell are often seen in severe cases (Davis and Barber 1994). If conchiolin is deposited between the shell and the adductor muscle, the oyster gapes and dies (Boardman et al. 2008). However, not all oysters killed by ROD exhibit conchiolin deposition under the adductor muscle, suggesting that many mortalities are likely caused by the stress imparted on the oyster, particularly on smaller oysters, as the host responds to the bacterium. Unfortunately for growers, the symptoms are only apparent a week before mortality occurs (Ford & Borrero 2001) and there are no known treatments for the disease.

ROD is caused by *R. crassostreae*, a marine alpha proteobacteria isolated from oysters showing symptoms of ROD during three separate enzootic cases in 1998 (Boettcher et al. 1999). Although the initial isolates of the bacterium were not identified to species, Maloy et al. (2005) later sequenced a portion of the 16S ribosomal RNA gene

of the pathogen. They found the resulting sequences had high similarity to 16S rRNA sequences from other species in the Roseobacter clade. They subsequently named the species *Roseovarius crassostreae*. Injecting oysters with cultures of *R. crassostreae* resulted in high mortality compared to controls (Boettcher et al. 2000) suggesting that the bacterium is associated with mortality. However, the symptoms typical of ROD during outbreaks were not observed in the oysters artificially "infected" in Boettcher et al.'s experiments. Eventually, Maloy et al. (2007) were able to replicate disease symptoms and mortality in lab treatments, thus fulfilling Koch's postulates linking *R. crassostreae* to ROD. Their experience, however, illustrates the difficulty of creating a challenge model for ROD in the lab, a challenge that is at least partly due to a lack of knowledge of how the host and pathogen interact and of the pathogen's population dynamics in any given estuarine environment during outbreaks of the disease.

The causative agent of ROD *(R. crassostreae)* is a gram-negative alpha-proteobacterium that expresses one or two flagella and polar fimbriae. The bacterium does not appear to invade oyster tissues directly. Instead, the bacterium attaches to the inner shell surface using the polar fimbriae and forms a biofilm (Boardman et al. 2008). Biofilms generally behave very differently than do free-living, individual cells of the same bacterial species. The cells in a biofilm communicate with each other so that the physiology of each cell benefits the whole colony rather than the well-being of any individual cell. This behavior is regulated by quorum sensing, a process by which production and recognition of autoinducers helps to regulate gene expression on the population level (Henke & Bassler 2004). In this way, a biofilm behaves more like a multi-cellular organism rather than a group of individuals (Popat et al. 2012). In the case

of ROD, it is likely that the abnormal shell deposition of the oyster host is a response to compounds or toxins excreted by *R. crassostreae* biofilms. Alternatively, oyster hemocytes are involved in pathogen recognition and a recent hypothesis of Mount et al. (2004) is that shell growth is hemocyte-mediated, thus the abnormal shell growth may be due to a hemocyte response to the biofilm.

Little is currently known about how oysters contract ROD. The bacterium has only been obtained from colonized oysters, thus the environmental reservoir or source of the bacterium is unknown. However, aspects of the feeding biology of oysters and functional role of Roseobacters in pelagic communities suggest a role for marine aggregates. In general, marine Roseobacters, of which *R. crassostreae* is a member (Maloy et al. 2005), are the most common group to colonize marine aggregates (Geng & Belas 2010). The Damariscotta River generally experiences a large summer bloom of phytoplankton. Although the bloom may begin in spring, there is a clear pattern of species succession through the summer (Petrie 1975). Exudates from the phytoplankton, as well as increasing abundance of senescent phytoplankton, are not only rich organic sources of nutrients that support bacteria, but also contribute to the increasing abundance and increased size of marine aggregates in late summer and early fall, the same time that ROD mortalities occur in this region. Species of phytoplankton found by Petrie (1975) to reach peak abundance during this time period include *Chaetoceros socialis* and *Asterionella japonica.*

Like many other bivalves, oysters are filter feeders of plankton and algae. The clearance rate of many species is often related to the size of the particles consumed. Ward & Kach (2009) found that only 15 % of particles < 1 micrometer in size

(nanoparticles) are retained by *C. virginica* unless they are incorporated into marine aggregates. Marine aggregates are collections of organic and inorganic particles that cluster in the water column, and are much more accessible as food to the oyster due to their size and stickiness. Kach & Ward (2007) also found that the removal rate of picoplankton – sized (< 1 nanometer) particles by filter feeders was enhanced greatly by their attachment to marine aggregates and that the rate of ingestion of bacteria was significantly enhanced in clams, mussels, oysters, snails, and scallops by feeding on aggregates. Lyons & Ward (2005) observed that the bacterial agent responsible for Quahog Parasite Unknown (QPX), which affects hard clams (*Mercenaria mercenaria)*, is harbored by marine aggregates and suggested that aggregates provide a path for the transmission of this and other diseases to benthic, suspension feeders. Thus, one likely route by which oysters acquire *R. crassostreae* is through marine aggregates colonized by the bacteria in the water column which then enter the mantle cavity during feeding.

Other lines of evidence suggest a role for marine aggregates in the transmission of ROD. Maloy et al. (2005) have shown that there is little variation in the growth of this species across a wide range of salinities, but variation in temperature can dramatically affect bacterial activity and the likelihood of bacteria-related diseases. In the case of ROD, *R. crassostreae* is generally metabolically more active and cell division more rapid at higher temperatures (34-37°C; Maloy et al. 2005). At sites impacted by the disease on the Damariscotta River, ROD mortalities often occur in the early fall. The timing of mortality is thus strongly correlated with the highest temperatures of the year on the Damariscotta River (23-25°C; Perry and Thompson 2005). Gomez-Leon et al. (2008) have shown that the susceptibility of oysters to bacterial toxins, including those

from *R. crassostreae* (see below) is greater for oysters at 25°C compared to oysters held at 20°C, while ROD-associated mortality is highest in water that is 21-26°C and 25-32% salinity (Sunila 2012). These environmental conditions are often found in late summer in the Damariscotta River (Perry & Thompson 2005). However, direct experimental evidence for the role of aggregates in ROD transmission is currently lacking. If they are important to the transmission of ROD, however, then expression of extracellular proteins by *R. crassostreae* during colonization of phytoplankton cells and aggregates may act as virulence factors that negatively affect oysters that feed on such cells and aggregates.

Virulence Factors

The severity of disease outbreaks and impact of pathogens is often related to the expression of virulence factors. Virulence factors are phenotypic characteristics that facilitate the entry, persistence, or disease-causing properties of a pathogen (Brown et al. 2012). In this thesis, I have sought to identify potential virulence factors in *R. crassostreae* and how expression of such factors depends on nutritional composition of the culture media. Several studies have documented potential virulence factors produced by marine bacteria, including *Vibrio* and *Roseovarius*. For example, Gomez-Leon et al. (2008) found that extracellular products generated by of *R. crassostreae* were toxic to hemocytes of Eastern oysters. They isolated the extracellular proteins using a cellophane overlay technique wherein the bacterial cells are not in direct contact with the media but rather sit on a layer of cellophane. The bacteria express extracellular nutrient-scavenging molecules to access resources through the cellophane. After exposing oyster hemocytes to these extracellular molecules, Gomez-Leon et al. (2008) observed substantial

hemocyte mortality. However, they did not further characterize which molecule or molecules were responsible for mortality. Similarly, Labreuche et al. (2006) found that extracellular products produced by *V. aestuarianus* contain virulence factors that cause mortality in *Crassostrea gigas* juveniles.

Recent studies have attempted to narrow down the likely virulence factors involved in pathogen-associated mortality. In some studies, authors have inferred the role of metalloproteases based on the proteolytic activity of and metal incorporation in ECPs associated with pathogens in host mortality. For example, Labreuche et al. (2010) determined that a metalloprotease from the ECPs of *V. proteolyt*icus was found to cause mortality in *C. gigas* adults as well as suppression of hemocyte immune function. Metalloproteases isolated from three *Vibrio* species, *V. tubiashii, V. cholerae,* and *V. splendidus,* have been shown to cause larval mortality in *C. gigas* (Hasegawa et al. 2009). Metalloproteases have also been found associated with disease processes in other non-bivalve aquaculture species. For instance, sepsis related to *V. anguillarum* infection in juvenile Atlantic cod and a number of other fin fish species is related to metalloprotease activity (Norqvist et al. 1990), and a metalloprotease from *A. salmonicida* is a major virulence factor in the infection of a number of fish species (Arnadottir et al. 2009).

Iron is scarce in the marine environment even though it is an essential micronutrient for most organisms. Ocean surface waters enriched from dust plume events may contain nanomolar amounts of dissolved iron, but typically, other ocean waters contain picomolar (< 1 nM) amounts (Sarthou et al. 2007). Dissolved iron in coastal areas can be higher due to enrichment from runoff, but these concentrations have

not been measured in the Damariscotta River. The availability of iron has a significant impact on bacterial growth and cell division, and pathogens generally have limited access to free iron inside the host. To overcome this, bacteria express iron-uptake systems and compete with the host for the host's iron reserves. Many siderophores, which are extracellular proteins capable of chelating iron, have been implicated in bacterial-associated diseases. For instance, Fetherston et al. (2010) found iron uptake to be vital to the pathogenicity of *Yersiniae pestis*, the bacterium that causes bubonic plague and pneumonic plague.

Temperature not only affects growth rates, but also can affect the molecular behavior of cells in a way that can be significant to their ecology and the expression of virulence factors. For example, temperature regulates multiple virulence mechanisms in *V. coralliilyticus*, a pathogen of corals, where the expression of virulence factors is independent of cellular abundance. The pathogen kills corals only in water temperatures above 27°C (Kimes et al. 2012). In the research reported in this thesis, I examined potential virulence factors expressed by *R. crassostreae* in response to iron limitation across a range of temperatures.

Oyster Defense and Host-Parasite Interactions

Oysters can respond to pathogens via innate immunity, but do not appear to possess the more complex, adaptive immune system typical of higher vertebrates (Gosling 2003). The hemolymph of *C. virginica*, like most other invertebrates, contains phagocytic cells that encompass invading pathogens and break them down using reactive oxygen intermediates (Anderson et al. 1997). These immune cells are generally referred to as hemocytes, but they differentiate to combat different types of invaders

(Gosling 2003). In addition to reactive oxygen species, hemocytes produce other non-specific molecules, such as bactericidins, opsonozins, agglutinins, serine proteases, and lysozymes that have adverse affects on invading pathogens (Roch 1999). When the pathogen *V. aestuarianus* infects *C. gigas*, it downregulates superoxide dismutase (an antioxidant) gene expression in the oyster's hemocytes and enhances production of reactive oxygen species, a strategy that may lead to impaired oyster defense (Labreuche 2006). Opsonozins aid in phagocytosis by reversing the slippery effect of the bacterial capsule, while lysozyme breaks the bond between N-acetyl muramic acid and N-acetyl glucosamine, which form the bacterial cell wall. Lysozyme is more destructive to gram positive bacteria, which have the peptidoglycan cell wall as its outermost layer. Agglutinins are glycoproteins that cause the pathogen to cluster in a way that is disadvantageous to its growth and survival inside the host. In the case of ROD, hemolymph protein expression or activity might be affected by compounds produced by *R. crassostreae* that suppress the oyster's immune system.

Another division of defense molecules produced by oysters and other shellfish is the anti-microbial peptides (AMPs). As the name infers, these are small peptides that have specific roles in combating foreign organisms. The DNA and protein sequences of some shrimp and mussel AMPs have been known for a while, but not until recently have they been isolated and sequenced from *C. virginica.* For example, a histone H2B protein was isolated from *C. virginica* and found to be very effective as an antimicrobial against *V. vulnificus* and *V. parahaemolyticus* (Seo et al. 2010). Defensins are another type of AMP that have similar structure and function, three variants of which have been discovered in oysters. These defensins share functional similarities with AMPs of other

species, which is not unexpected, since homology is what aided in the discovery of the proteins in the first place. The oyster defensins have been shown to interfere with steps in the bacterial cell wall synthesis pathway, thus halting binary fission of the pathogen (Schmitt 2010). While this may be effective in preventing the colonization of hosts by gram positive bacteria, defensins are less effective against cell wall-less, gram-negative bacteria like *R. crassostreae*.

Other molecules identified in shellfish play important roles in host defense. Dominin, the most abundant protein in *C. virginica* hemolymph, was isolated and characterized by Itoh and colleagues (2011). This protein is thought to play a role in host defense as well as a number of other metabolic processes, including metal transport, antioxidation, wound repair, and shell mineralization. Dominin's metal transport activity may allow oysters to sequester iron out of reach of pathogens and thus may be part of the defense response of the oyster. Dominin mRNA was detected in oyster hemocytes, which are responsible for defense processes and may also be involved in shell construction, suggesting that the protein plays a role in both processes. If it is related to oyster defense, dominin expression or activity might be affected directly by the presence of a pathogen as a means of downregulating the oyster's immune system. If the function of dominin or another protein involved in shell mineralization function is impaired by *R. crassostreae*, it may explain the abnormal conchiolin deposition and patterns of shell growth characteristic of ROD. While multiple hemolymph proteins may be involved in defense, the sheer abundance of dominin suggests it plays a particularly important role (Itoh et al. 2011).

The onset and severity of disease is often determined by a complex interplay between host and pathogen at the molecular level. In my thesis research, I have sought to identify proteins that are likely to play a role in such molecular interactions when oysters are colonized by the bacteria *R. crassostreae*. The goals of the research I describe have been two-fold, characterize extracellular proteins (ECPs) expressed by *R. crassostreae* under defined nutrient and growth conditions, and characterize the expression of a potential defense protein in the extrapallial fluid of oysters, *C. virginica*, when exposed to *R. crassostreae*.

METHODS AND MATERIALS

Extracellular Protein (ECP) Expression in *Roseovarius crassostreae*

I examined the variation in ECP expression by *R. crassostreae* as a function of iron limitation and environmental temperature. Iron limitation was intended to mimic the reduction in iron availability that *R. crassostreae* may experience in natural environments and within a host (oyster) which may be sequestering iron to limit pathogen growth. I also wanted to explore the effects of increased temperature, particularly those above 25°C, on ECP expression reflecting the late summer conditions under which ROD outbreaks most often occur. In particular, I wanted to test whether growth at high temperature triggers the expression of one or more proeteins that may function as virulence factors, as was observed for *V. coraliilyticus* (Kimes et al. 2012).

Sea water tryptone (SWT) media was used as the control; it is the standard media for culturing *R. crassostreae* (Maloy et al. 2005) and provides abundant carbon substrates and nutrients. Liquid SWT media was prepared by combining 70% filtered (0.2μm) artificial sea water, 5% tryptone (Bacto), 3% yeast extract (Bacto), and 3% glycerol (Fisher Scientific), adjusted to pH 7.0. Solid SWT media for plates was prepared similarly, but with the addition of 1.5% agar (Amresco). All media, solid and liquid, were autoclaved immediately after preparation to ensure sterility prior to my experiments. Iron-limited media was prepared to determine protein expression patterns of *R. crassostreae* under iron-depleted conditions. Iron-limited SWT media (Fe^{-}SWT) was prepared by adding 2, 2-dipyridyl (Sigma-Aldrich) at a final concentration of 100 μM to the standard recipe for SWT media. This chemical is often used for chelating iron

in bacterial culture media (e.g., Bakopoulos et al. 1997). To test whether SWT itself was limiting with respect to iron, I also prepared an iron-replete media (Fe^{+}SWT) by adding $FeCl_3$ (Sigma-Aldrich) to a final concentration of 100 µM. An algae-based media was prepared in which the general nutrient source, yeast extract, was replaced with an equal mass of concentrated microalgae (Shellfish diet 1800, Reed Mariculture). This algae-based media was meant to provide the bacteria with a nutritional source more like marine aggregates, the source it may exploit in its native habitat when ROD outbreaks are common.

In my first experiment, I estimated the growth rate of *R. crassostreae* in SWT and Fe-SWT media at small volumes to establish ECP sampling protocols. I inoculated cultures of SWT and Fe-SWT and recorded the increase in cell density as a function of incubation time. The density of all cultures was estimated spectrophotometrically using a previously established relationship between the optical density of cultures at 600 nm in wavelength and te number of colony forming units (CFUs) determined by standard dilution, plating, and counting. Two 15-mL replicate culture tubes containing 10 mL each of these two liquid media types were inoculated with 50 uL of *R. crassostreae* cells at approximately $5.0x10^5$ CFU/mL. Cells were mixed in the room temperature liquid media and incubated at room temperature (~25°C) with gentle rotation. At approximately 11, 17, 34, 41, 59, 77, 123, 143, and 153 hours, I sampled 500 µl from each replicate to estimate cell density. Because liquid media types varied in color, I centrifuged and resuspended all samples in standard SWT before measuring cell density. Unfortunately, due to carryover of algal debris after centrifugation and resuspension, ABM culture densities could not be determined spectrophotometrically.

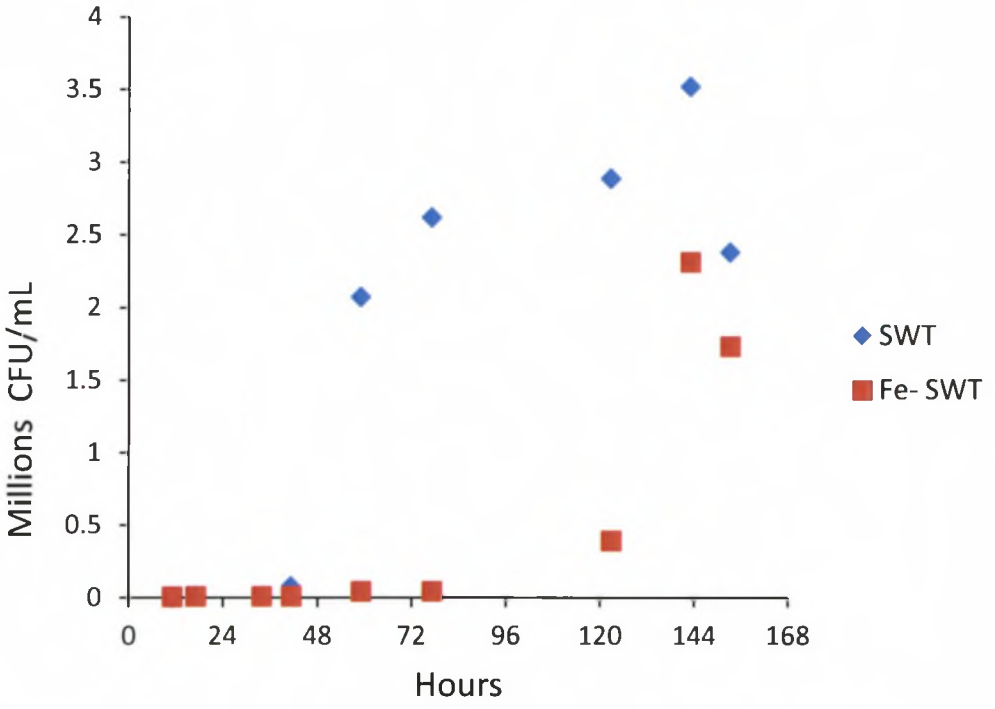

Figure 2.1 Media-dependent Variation in Growth of *R. crassostreae*. The relationship between cell density and time was used to estimate culture growth phases for *R. crassostreae* grown in liquid SWT and Fe-SWT at room temperature (~25°C).

The variation in media-specific *R. crassostreae* growth was analyzed graphically (Fig 2.1). Cell density in both media types peaked by ~143 hours post inoculation. However, cell density in the Fe-SWT media increased more slowly than cell density in the control media (SWT). While SWT cultures showed a rapid increase in cell density around 50 h, a similar increase in Fe-SWT cultures did not occur until after 120 h. Growth phase of a given culture through time was determined by dividing the cell density (CFU/mL) of samples taken at defined time points by the maximum density reached in that culture. The resulting ratios were used to define growth stage according to the standards shown in Table 2.1. I had intended to use these definitions to sample ECPs in subsequent experiments. However, densities in experiment 2 did not correspond

Proportion of Maximum Density	Growth Phase Determination	Hours post inoculation (SWT)	Hours post inoculation (Fe-SWT)
< 0.1	Pre-log	< 64	< 82
.10 - .30	Early log	64-84	82-103
.30 - .70	Mid-log	84-109	103-129
.70 - .90	Late log	109-129	129-150
> .90	Stationary	>129	>150

Table 2.1 Growth Phase Definitions for *R. crassostreae* Grown in Liquid Media

to those in experiment 1. This was mostly likely due to the use of a smaller culture volume in the second experiment. Thus, in subsequent experiments (#3-5), samples were taken more frequently and growth curves were established for each experiment, individually. In experiments 2-5, Fe-SWT and ABM cultures were always sampled at the same time points. Although I could not directly measure cell density for ABM cultures, I assumed growth in the ABM cultures was similar to that in the Fe-SWT so that I was sampling the two media types at similar densities.

I ran five separate experiments to investigate how temperature, culture volume, and media type affected the growth of *R. crassostreae* and ECP expression (Table. 2.2). For estimating the variation in ECP expression, I sampled 500 uL of cells from each culture at various time points, centrifuged the sample at 8,000 rpm/ 5,900 xg to pellet cells, and removed supernatant containing ECPs. These supernatants were combined

Exp. #	Temp (°C)[±]	Vol (mL)	ECP Isolation	Media type/No. Replicate Cultures			
				SWT	Fe- SWT	Fe+ SWT	ABM
1	25	10	No	2	2	0	0
2	25	2	Yes	2	2	2	2
3	28	150*	Yes	1	1	1	1
4	30	10	Yes	2	1[†]	2	2
5	23.5	10	Yes	2	2	2	2

Table 2.2 Experiments Investigating ECP Expression in *R. crassostreae*. I conducted five separate experiments investigating the effects of temperature (Temp), volume (Vol), and media type on *R. crassostreae* growth and ECP expression. An asterisk indicates cultures grown in shaking incubator due to the size of the cultures. All others were mixed using small rotator. A cross indicates a treatment in which the last ECP sample is missing. [±]Experiment #1-4 temperatures represent average temperatures with +/- 2°C, while Experiment 5 was maintained at a more constant temperature (+/- 0.5°C).

with an ECP buffer (Roche protease inhibitor cocktail, TRIS (Amresco) to 50mM, and EDTA (Sigma-Aldrich) to 1 M and stored at -20°C.

To examine the effect of temperature on growth and ECP expression by *R. crassostreae*, I compared samples from experiments 1, 4, and 5, wherein culture volume was held constant. Given temperature-dependent growth rate variation, ECPs from all media types held at 23.5°C were sampled at 30, 60, 100, 120, 145, 167, 190, and 217 h post-inoculation so that all growth phases (pre-log, log, etc) would be represented in all

media types. Similarly, the cultures held at 30.0°C were sampled at 25, 44, 70, 96 and 122 h post-inoculation. I examined the effect of culture volume on ECP expression by inoculating 150 mL of each liquid media type with 50 uL of *R. crassostreae* cells at $1x10^6$ CFU/mL in 250 ml Erlenmeyer flasks with gentle agitation (100rpm) at room temperature (27-29°C). All four of these larger cultures (one replicate per media type) were sampled at 21, 45, 68, 115, 139, and 188 h post inoculation. The samples from the replicate cultures used to test for temperature and volume effects were centrifuged and the supernatants, containing ECPs, processed as described above.

Finally, I also analyzed how ECP expression differed for free-living versus solid phase *R. crassostreae*. For this comparison, a 75 uL suspension of *R. crassostreae* cells at approximately $1x10^6$ CFU/mL was spread on a 100 mm SWT-agar plate and incubated for approximately 5 d at room temperature until the bacterial growth covered the surface of the media thoroughly. The biofilm was scraped, resuspended in 2 mL of sterile, full strength sea water, and then centrifuged at 8,000 rpm/ 5,900 xg to pellet the cells. The resulting supernatants containing solid phase ECPs were stored in ECP buffer with protease inhibitor, as described above. In addition, *R. crassostreae* cells were grown on plates with a cellophane barrier between the cells and the media. Cellophane was cut into 100mm diameter circles and autoclaved before being placed onto SWT agar plates. *R. crassostreae* inoculant (75 uL at $1x10^6$ CFU/mL) was spread over the top of the cellophane, avoiding direct contact with the edge of the plate and the underlying agar. This method of cell growth promotes extracellular expression of nutrient-scavenging molecules and was utilized by Gomez-Leon et al. (2008) to collect extracellular products of *R. crassostreae*. Cells were scraped off the top of the

cellophane, resuspended, and centrifuged to collect ECPs. The agar surface was also scraped and treated as a collection of extracellular products.

SDS-PAGE Analysis of *R. crassostreae* ECPs

I attempted to quantify protein concentrations in the culture supernatants to ensure equal protein loading on SDS-PAGE gels. Due to small sample volumes, I used the Coomassie Plus Standard Microplate Assay (Pierce; effective range 100-1500 ug/mL protein). In most cases, protein concentrations in culture supernatants were below the range of detection, even after they were concentrated using Nanosep 10 kD filter spin columns (Pall). Thus, I loaded the maximum volume of prepared supernatant on the gel for each sample and protein volume was estimated from bands resolved on each gel (see below).

ECPs were separated using Novex precast 12% 1 mm acrylamide gels (NuPAGE; Invitrogen). Twenty-three microliters of each *R. crassostreae* supernatant in ECP buffer were combined with 2 uL of reducing agent and 5 uL of loading buffer according to manufacturer's protocols. Ten microliters of Color Sieve prestained protein color marker (Invitrogen) were used as the size marker in the first lane on each gel. Electrophoresis was carried out in a Sure-Lock mini-gel apparatus with MOPS (Invitrogen) running buffer and 500 uL of antioxidant added to the upper chamber. Initial gels were run for 45 minutes. However, this did not provide adequate resolution of bands greater than 60 kDa in size, and because I observed few to no bands lesser than 30 kDa in size on initial gels, subsequent gels were run between 60 and 90 minutes. Typically, I ran the gel until the 30 kD marker on the protein ladder reached the bottom

edge, removing all proteins < 30kD in size providing more clarity and spacing in the larger size ranges. All gels were stained using Silver Snap Stain (Pierce), due its low (nanogram to subnanogram) detection limits and significant contrast between stained bands and background.

Hemolymph Protein Expression

I attempted to investigate variation in *R. crassostreae* growth and ECP expression when the cells were exposed to hemolymph and extrapallial fluid of adult oysters. This experiment was intended to investigate the molecular interactions between *R. crassostreae* and *C. virginica* that may lead to the onset of ROD. Bacteria were directly exposed to oyster hosts through the use of a microcapsule embedded into the oyster shell (Fig 2.2). The microcapsule included a 0.2 μm barrier filter that blocked movement of bacteria but did not impede movement of host or pathogen extracellular proteins. My goal was to observe the variation of host protein expression in the extrapallial fluid that was concurrent with any changes in ECP expression by *R. crassostreae*.

Adult oysters were provided by the Oyster Broodstock Program at the Darling Marine Center in Walpole, ME. Oysters were transported to Orono, ME on May 9, 2012. They were initially held at 12°C for 24 h in artificial sea water at a salinity of 25 ppt, then acclimated to 23°C over the next 48 h, and held at 23°C for another two weeks prior to the experiment. They were fed $1.25*10^9$ cells per day per oyster of Shellfish Diet (Reed Mariculture) during holding, but were not fed the day before or during the experiment. On May 28, 32 oysters were split into four experimental groups. A ~1 cm

Figure 2.2 Microcapsule Embedded in Oyster Shell. Nanosep 10 kDa microcapsules with filters were affixed in the left valve of experimental oysters allowing the passage of liquids and molecules < 10 kDa in size.

diameter hole was drilled in the left valve of each oyster with a Dremmel tool into which a 0.2 uM filter microcapsule was secured using cyanoacrylate (Krazy) glue (Fig. 2.2). Those oysters in Groups A and C were only drilled half-way through the shell before affixing the capsule (exposure control), while for those in groups B and D, holes were drilled all the way through, exposing the mantle cavity.

Oyster capsules in groups A and B were filled with culture of *R. crassostreae* in SWT at $3x10^5$ CFU/mL and the capsules in groups C and D were filled with SWT media only. Oysters were returned to holding tanks and held on top small platforms so that the oysters were submerged but the top of the capsules were exposed above the water surface to avoid contaminating the culture with sea water and other bacteria. Capsules and drilled opening were sealed using Krazy Glue. The water temperature was kept at 23°C (+/- 1°C) and ambient air temperature was approximately 25°C. After 6 d, all *R.*

crassostreae cultures and media were removed from the microcapsules and pelleted to collect the supernatant. Supernatants were stored similarly to culture samples from media experiments described above.

I attempted to obtain hemolymph and extrapallial fluid from all oysters pre- and post- exposure. Hemolymph was extracted via shell notching for Groups A and C or from the drilled holes,for Groups B and D. Hemolymph was collected from the same holes post-experiment. At the end of the experiment, if I had difficulty obtaining adequate volumes of fluid, the oyster was shucked and hemolymph was removed from the pallial cavity. All hemolymph samples were spun at 8,000 rpm/ 5,900 xg to pellet hemocytes and the supernatant was stored in a manner similar to that for ECP supernatants. The protein concentration in most hemolymph supernatants was quantified using the Coomassie Plus Standard Microplate Assay (working range, 100-1500 ug/mL).

Conchiolin deposition patterns were scored from 0 to 3 based on the localization and/or expanse of conchiolin on the left valve of each oyster. A zero represents little or no deposition, a score of one represents deposition around the injury (hole) or from the injury to the growing edge of the shell only, a score of two represents either a deposition pattern extending more than an inch from the injury or conchiolin on the entire outer edge of the shell, and a score of three indicates the occurrence more than one deposition event on a single oyster shell. Examples of a pattern of deposition corresponding with each score can be seen in Figure 3.11.

Mass Spectrometry

Ten protein bands of interest, all resolved using the Silver Snap Stain (Pierce), were processed and sequenced by mass spectrometry at the Lerner Research Institute's Mass Spectrometry Core Facility (http://www.lerner.ccf.org/services/cs/ms2/) in Cleveland, Ohio. Nine bands were chosen based on preliminary gel analysis suggesting they were expressed by R. *crassostreae* in response to particular culture conditions. For example, band Rc37b (37kDa band from *R. crassostreae*) was initially detected in protein preps from cultures in log or stationary phase growth in Fe-SWT and ABM media. The conditions under which the other 8 bands were identified are given in Table 2.3. The tenth band was an extremely prominent 26 kDa band observed in oyster extrapallial fluid (lane 10, Fig 2.3). Each band was excised from the gel, rinsed with water, dehydrated in acetonitrile, washed in 0.1M ammonium bicarbonate, dehydrated a second time in acetonitrile and then dried. The protein in each band was digested overnight in 50 mM ammonium bicarbonate with 10-15 µl 20 ng/µl trypsin. The resulting peptides were extracted and analyzed using standard protocols on a Finnigan LCQ-Deca ion mass spectrometer system.

ID	Treatment	Growth Phase	Presence
Rc38	SWT plate	N/A	Absent from SWT liquid or ABM Plate
Rc60a	ABM liquid	prelog	Only band in pre-log ABM
Rc110	ABM liquid	log/stat	ABM and Fe-SWT, not in Fe+SWT or SWT
Rc37a	ABM liquid	log/stat	ABM and Fe-SWT, not in Fe+SWT or SWT
Rc37b	Fe-SWT	log/stat	Fe-SWT and ABM, not in Fe+SWT or SWT
Rc32	Fe+SWT	log/stat	SWT and Fe+SWT, not in ABM or Fe-SWT
Rc60b	SWT plate	N/A	Strongest/only band from SWT plate
Rc36	SWT on cellophane	N/A	Not on SWT plates w/o cellophane
Rc49	Fe-SWT liquid	log/stat	Fe-SWT and ABM, not Fe+SWT or SWT
Cv26	Contact w/ hemolymph	N/A	Largest band from capsule contact

Table 2.3 Bands Chosen for Mass Spectrometry Analysis. Band designations indicate species of origin and protein size. For instance, Rc38 represents a *Roseovarius crassostreae* band, estimated to be about 38 kDa in size.

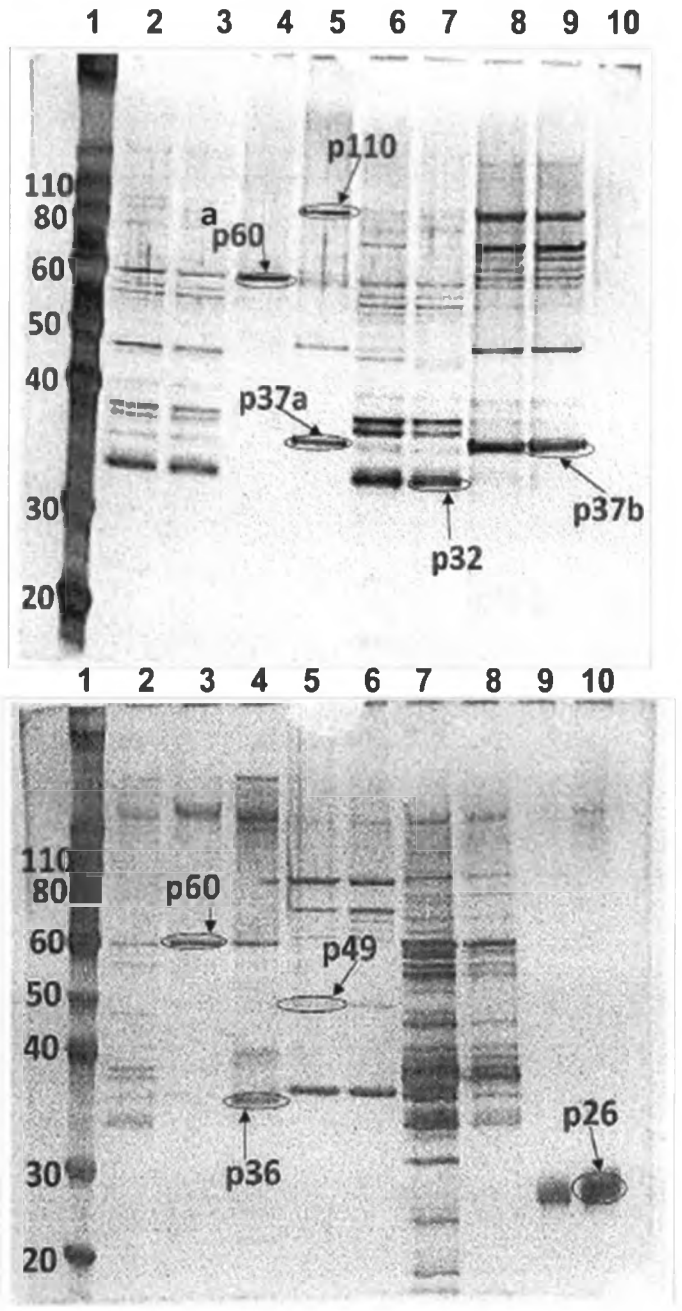

Figure 2.3 Mass Spectrometry Sample Preparation. Samples for mass spectrometry sequencing were identified on preliminary gels and rerun on preparatory gels pictured, above. Top Gel includes ECPs sampled from *R. crassosteae* at stationary phase in SWT media (lanes 2 and 3), ABM at 184 hours (lanes 4 and 5), Fe+SWT at stationary phase (lanes 6 and 7), and Fe-SWT at stationary phase (lanes 8 and 9). Bottom gel samples include SWT in stationary phase (lane 2), SWT solid phase (lane 3), SWT cellophane plate (lane 4), Fe-SWT stationary phase (lanes 5 and 6), Fe-SWT solid phase (lanes 7 and 8), and oyster capsule fluid (lanes 9 and 10). Lane 1 on both gels contains Proseive size standards. Size of standard bands in kDa are given at left.

RESULTS

The growth of *R. crassostreae* was highly dependent on culture temperature. The cultures incubated at 30°C during Experiment 3 had the fastest growth regardless of media type. In contrast, the 23.5°C cultures in Experiment 4 had the slowest growth. These differences were most apparent when quantified as the time it took for a given culture to reach 90% of its maximum cell density (Fig. 3.1). An ANOVA with temperature and media as main effects, along with their interaction term, explained much of the variation in growth ($R^2 = 0.93$). The effect of temperature on the time it took to reach the end of late log phase (90% max) was highly significant ($F_{1,7} = 73.593$, $p < 0.0001$). On average, Fe-SWT cultures took ~ 23 hours longer to reach late log phase, although the effect was not statistically different between the cultures grown ($F_{1,7} = 2.621$, $p=0.149$). Although the magnitude of difference in growth in Fe-SWT and SWT media appeared to vary with temperature, the interaction between the two effects was not statistically significant ($F_{1,7} = 1.793$, $p=0.222$).

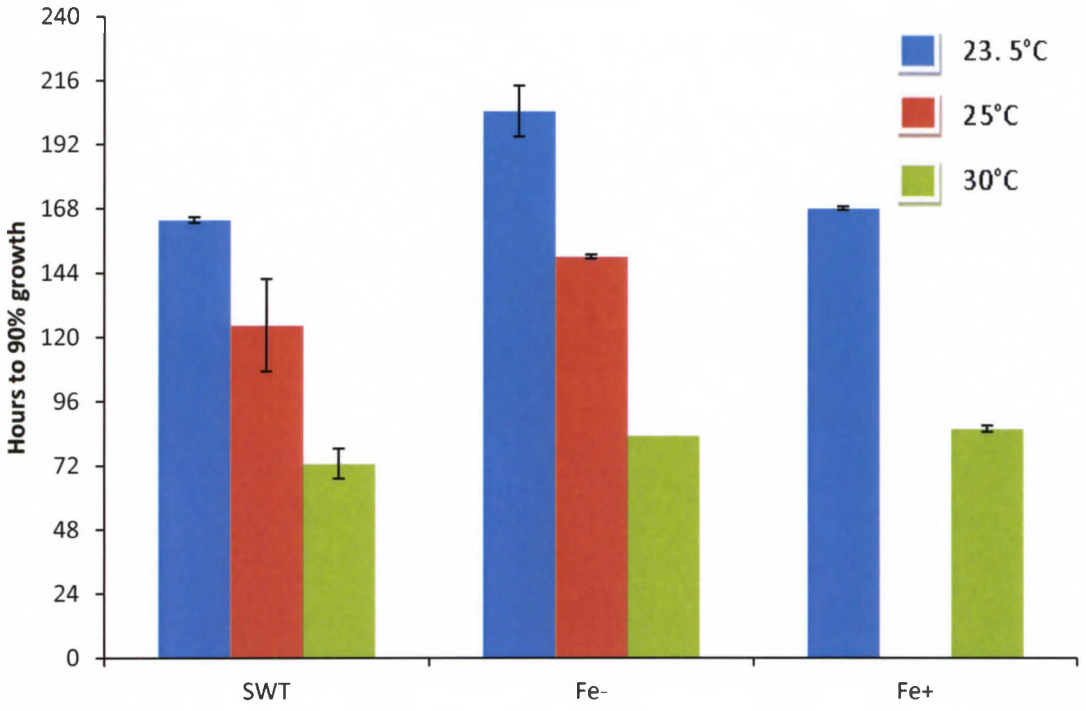

Figure 3.1 Temperature Effect on *R. crassostreae* Growth. The growth of *R. crassostreae* to late log phase (90% of max growth) was measured at 3 experimental temperatures. Bars represent means of two replicates (except Fe-SWT at 30°C) and error bars represent standard deviation. No Fe+SWT sample was taken at 25°C.

Analysis of Variance

Source	Type III SS	df	Mean Squares	F-ratio	p-value
TEMP	17001.23	1	17001.23	73.593	0
MEDIA	605.58	1	605.58	2.621	0.149
MEDIA*TEMP	414.255	1	414.255	1.793	0.222
Error	1617.112	7	231.016		

Table 3.1 ANOVA for the Effect of Temperature and Media on the Growth of *R. crassostreae* (N=11).

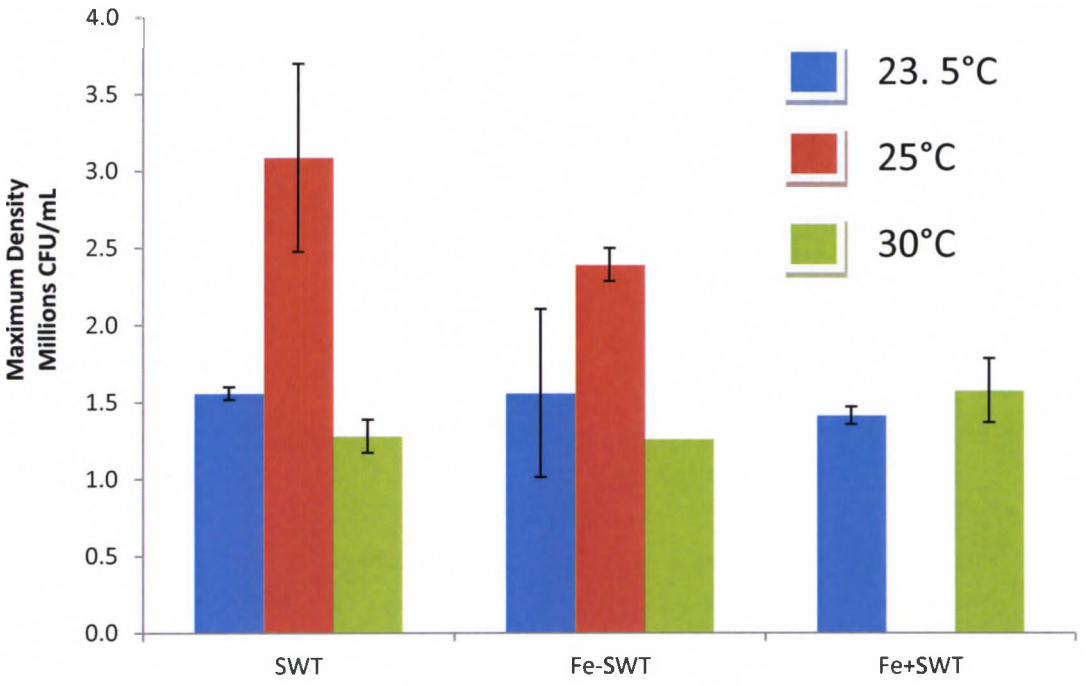

Figure 3.2 Temperature Effect on Maximum Density of *R. crassostreae*. The maximum cell density of cultures measured spectrophotometrically (OD_{600}).

Analysis of Variance

Source	Type III SS	df	Mean Squares	F-ratio	p-value
TEMP	6.66E+11	1	6.66E+11	0.985	0.354
MEDIA	6.58E+10	1	6.58E+10	0.097	0.764
MEDIA*TEMP	4.90E+10	1	4.90E+10	0.072	0.796
Error	4.74E+12	7	6.77E+11		

Table 3.2 ANOVA for the Effect of Temperature and Media on the Maximum Density of *R. crassostreae* (N=11).

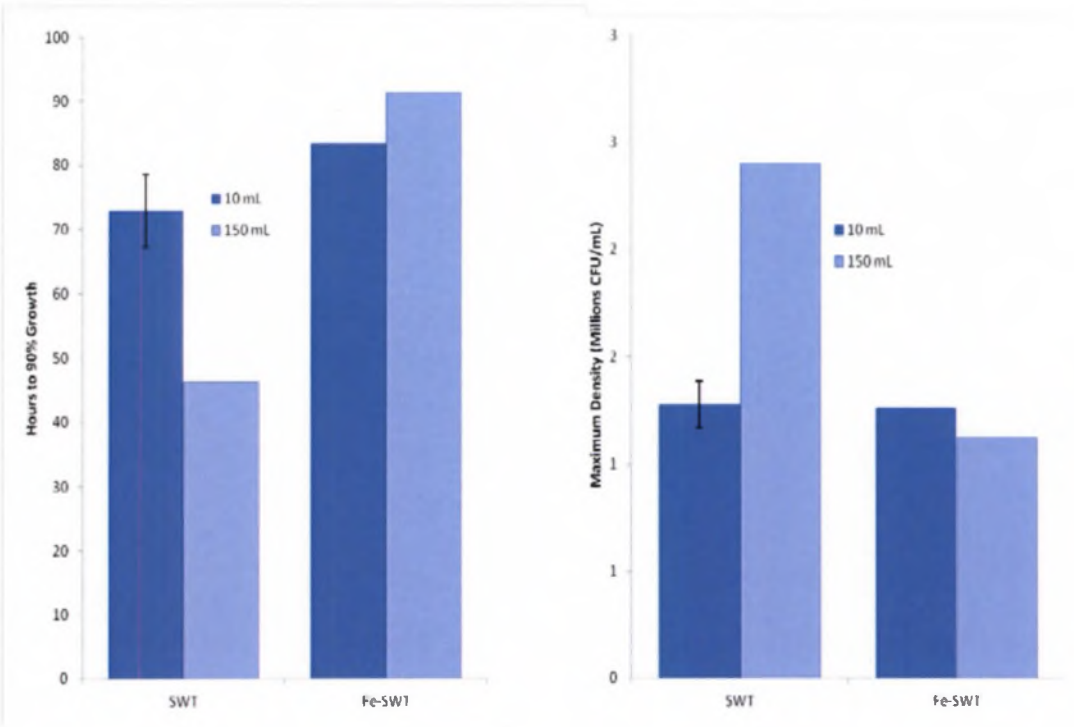

Figure 3.3 Effect of Volume on Growth Rate and Maximum Density of *R. crassostreae.* Growth rate (time required to reach 90% maximum density) is shown as a function of media type and volume in the left panel, while the maximum density in CFU/mL of these cultures is in the right panel. Error bars represent standard deviation when N=2; all other treatments N=1.

There was no clear effect of temperature on maximum culture density (Fig. 3.2). The maximum density of most cultures plateaued at ~1.4-1.5*10^6 cfu/mL except those grown at 25°C, which peaked at ~2-3*10^6 cfu/mL. An analysis of variance examining the effect of temperature, media, and their interaction on maximum density explained little of the variation in density (R^2 = 0.14). There was no evidence for an effect of temperature, media, nor their interaction on maximum density (Table 3.2).

I also investigated the effect of culture volume on both the growth rate and maximum density of *R. crassostreae* (Fig 3.3). Culture volume had litte effect on either growth rate (time to reach 90% maximum density) or maximum density in Fe-SWT cultures. In contrast, growth rate was substantially faster in SWT cultures at the larger volume and they reached a greater maximum density. Because of limited numbers of replicates, particularly at 150 mL volume, I could not conduct an ANOVA to test for the statistical significance of this apparent interaction between media type and culture volume.

There was a high degree of variation in the expression of ECP bands for *R. crassostreae* from different experiments. Therefore, I compared the variation in ECP patterns among treatments within experients. In Experiment 1 cultures grown at 25°C, I detected from one to as many as eight porminent bands in SDS-PAGE gels from cells sampled at stationary phase (Fig 3.4). Cultures grown in ABM produced fewer bands, but because I could not determine culture density, I cannot directly ascertain whether expression of these bands is dependent on culuture growth phase. However, assuming growth rate of cultures in ABM was similar to that in iron limited media, the ECPs from ABM cultures at 184 hours (Fig 3.4) are likely those sampled from "stationary phase" cells. This suggests that, overall, there is greatly reduced ECP expression both in density and number in ABM cultures. In contrast, upwards of 7-8 prominent and several other less abundant proteins were recovered from cells grown in other media types. Banding patterns were highly repeatable within replicates with the exception of ABM cultures. ABM stationary cultures (lane 5) express what appear to be three of the same large, dense protein bands expressed in the Fe-SWT cultures (lanes 8 and 9).The lack of ECPs in ABM cultures may also reflect slow growth, compared to Fe-SWT cultures.

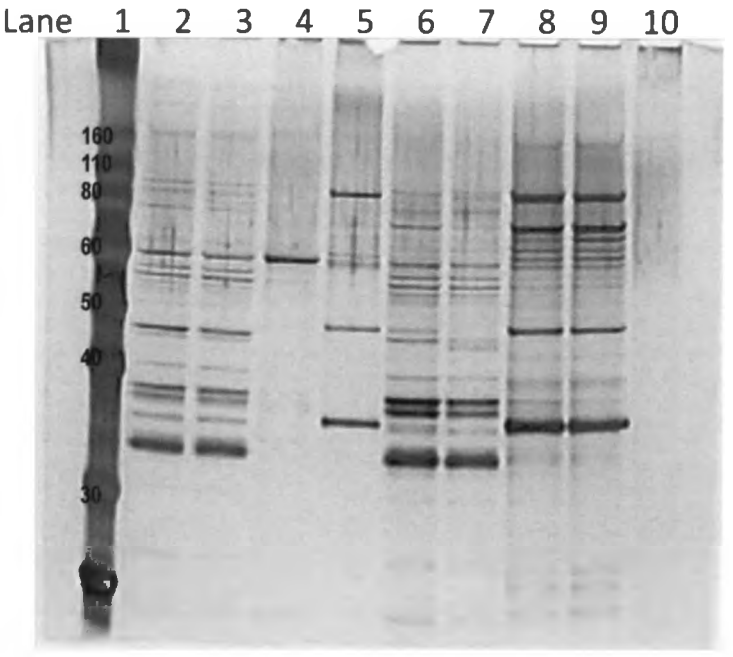

Figure 3.4 SDS-PAGE Analysis of Media-specific Extracellular Protein Expression in *R. crassostreae*. ECPs were sampled from 10 mL, 25°C *R. crassostreae* cultures at stationary phase in SWT media (lanes 2 and 3), ABM at 184 hours (lanes 4 and 5), Fe+SWT at stationary phase (lanes 6 and 7), and Fe-SWT at stationary phase (lanes 8 and 9). Lane 1 is Proseive size standards. Size of standard bands in kDa is given on left.

In all of the ECP experiments, the banding patterns associated with Fe+SWT cultures were highly similar to those for the SWT cultures. This pattern was illustrated in Experiment 2, wherein SWT (lanes 2 and 3) and Fe+SWT (lanes 6 and 7) patterns are extremely similar (Fig 3.4), suggesting that the addition of iron had no effect on ECP expression. This similarity in protein expression in SWT and Fe+SWT media was also observed in subsequent experiments. On the other hand, in three out of the four ECP experiments, band intensities and molecular weights changed dramatically for cells grown in Fe-SWT (Fig. 3.4). I also compared the relative

protein expression as a function of culture volume and media type for samples taken at mid-log phase in Experiments 3 and 4 (10 mL at 30°C, 150 mL at 28°C; Fig 3.7).

Cultures grown in Fe-SWT media expressed ECPs at earlier time points (corresponding to earlier culture growth stages) than was observed in other media types (Fig 3.5). To illustrate this difference in expression, I compared the estimates of relative protein volume (protein volume/cell density) for ECP samples from Fe-SWT and SWT cultures which were run on the same gel. For example, the samples from Experiment 3 were run on three separate gels. On gel 1, I observed a 15-fold increase in the relative band volume for cells grown in Fe-SWT cultures over those grown in SWT. Similarly relative protein volumes for Fe-SWT were 3-6-fold higher on gels three and two, respectively. Samples on gels 2 and three were taken from stationary phase cells, while samples on gel 1 were from mid-log and late log cultures. On average, however, the relative volume in Fe-SWT was about 8-fold higher, regardless of growth stage (Fig 3.6).

Overall, there was a consistent difference between SWT and Fe-SWT samples in relative ECP expression (Fig 3.7). This difference in ECP expression was even greater when culture volume was increased to 150 mL (Fig 3.6). The relative expression as a function of cell density decreased nearly 75% for cells grown in SWT control media. In contrast, relative band volume increased < 120% in Fe-SWT cultures. Due to space limitations, Experiment 3 only included 1 replicate of each 150 mL culture, so formal statistical analysis is not possible.

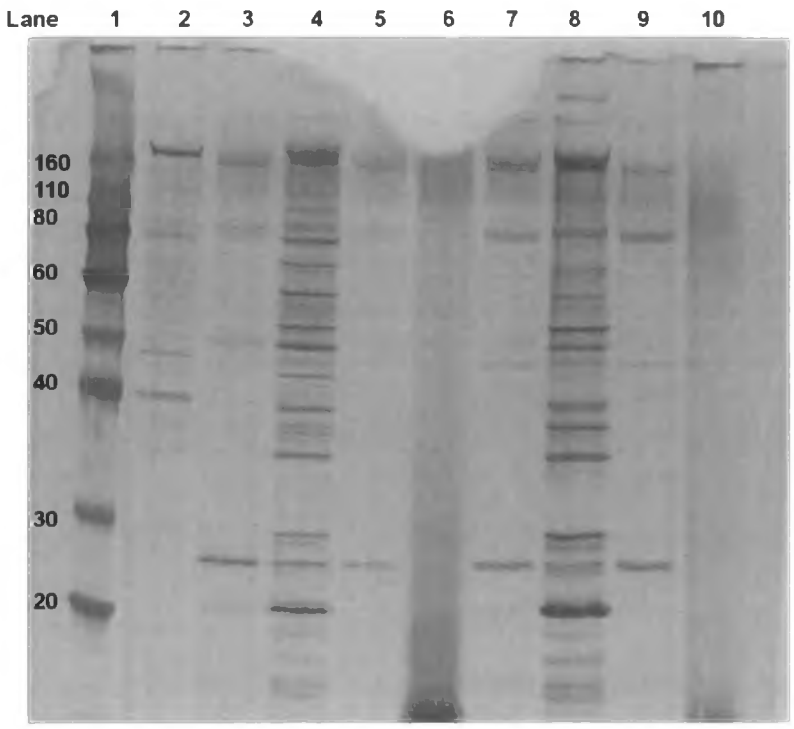

Figure 3.5 SDS-PAGE Analysis of Extracellular Proteins expressed by *R. crassostreae* in Large Culture Volume. ECPs were sampled from 28°C, 150 mL cultures of *R. crassostreae* at solid phase on SWT plates (lane 2), SWT at mid-log phase (lane 3) Fe-SWT at mid-log phase (lane 4), Fe+SWT at mid-log phase (lane 5), ABM at 21 hours (lane 6), SWT at late log phase (lane 7), Fe-SWT at late log phase (lane 8), Fe+SWT at late log phase (lane 9), and ABM at 43 hours (lane 10). Lane 1 is Proseive size standards. Size of standard bands in kDa is given on left.

ECPs from 150 mL Fe-SWT cultures contained bands at similar size to the major bands expressed in 10 mL Fe-SWT cultures from Experiment 2 (Fig 3.4) in addition to the expression of <10 more significant bands (Fig 3.5).

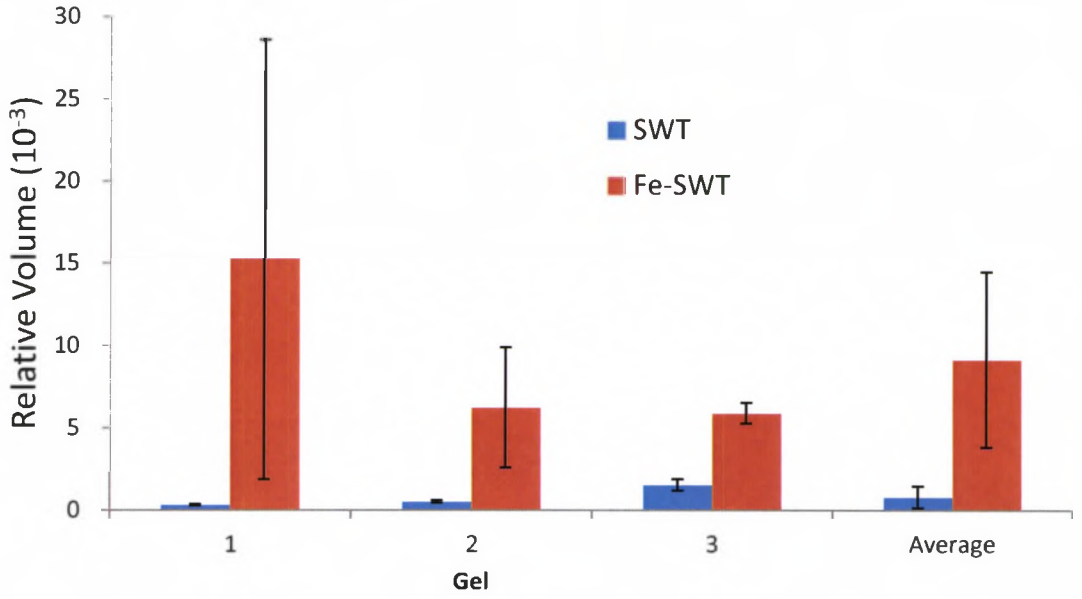

Figure 3.6 Relative Protein Expression in *R. crassostreae* as a Function of Iron Availability. The average protein volume of ECPs, as estimated using Gel Analyzer was graphed as a function of culture cell density. I compared ECP preps for SWT and Fe-SWT cultures and analyzed averages from each gel (N=2) as well as an average of all gels combined (N=6). Gel background values were subtracted and raw volume was standardized to the 50 kDa band in the protein ladder. Error bars represent standard deviation. Relative volume (Rawvol/(CFU/mL)) was considered a proxy for (proteins exported/cell).

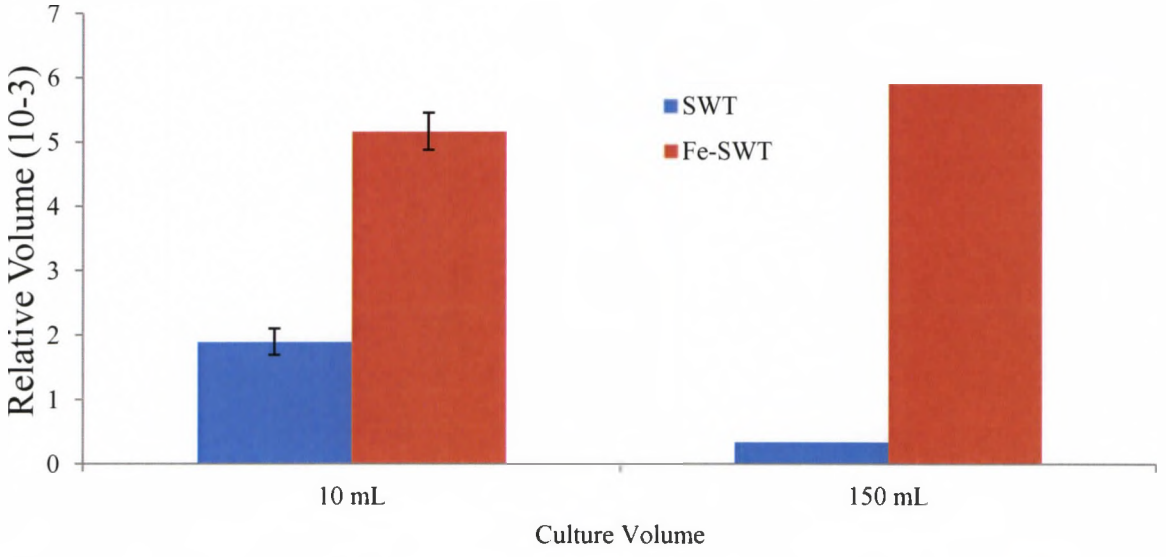

Figure 3.7 Relative Protein Volume as a Function of Culture Volume in *R. crassostreae*. I compared mid-log culture ECPs from media types SWT and Fe-SWT at 10 mL(N=2) and 150 mL(N=1) culture volumes. Relative protein volume estimates as in Fig 3.6.

Dominin Expression in Oyster Hemolymph

I examined the differential expression of the presumed hemolymph defense protein dominin under physical stress (shell damage) and physical stress plus exposure to *R. crassostreae*. In order to embed the capsules, I exposed oysters to injury in addition to the extracellular products of live *R. crassostreae*. To investigate the effects of injury, I sampled hemolymph from control and injured oysters pre- and post-experiment and examined the difference in dominin expression. Among the oysters that were injured by capsule placement, I compared those that had control capsules with media only and those with *R. crassostreae* in SWT. Five protein bands (A-E) consistently dominated the banding signature for pre-exposure oyster hemolymph. These proteins are labeled in Fig 3.8 (right); their sizes were estimated using Gel Analyzer as 58, 43, 33, 31 and 28 kDa, respectively.

Obtaining hemolymph from oysters proved more difficult post-exposure. Apparently, the mantle pulled away from the shell surface for those oysters where the capsule hole was drilled all the way through the shell. The fluid I removed through the capsule hole contained little protein, and I suspect it was mostly seawater. I obtained measureable amounts of hemolymph protein from only 7 oysters post-exposure. Of these, only 5 showed protein bands consistent with the pre-exposure samples, as determined by dominin presence on SDS-PAGE gels (Fig. 3.9).

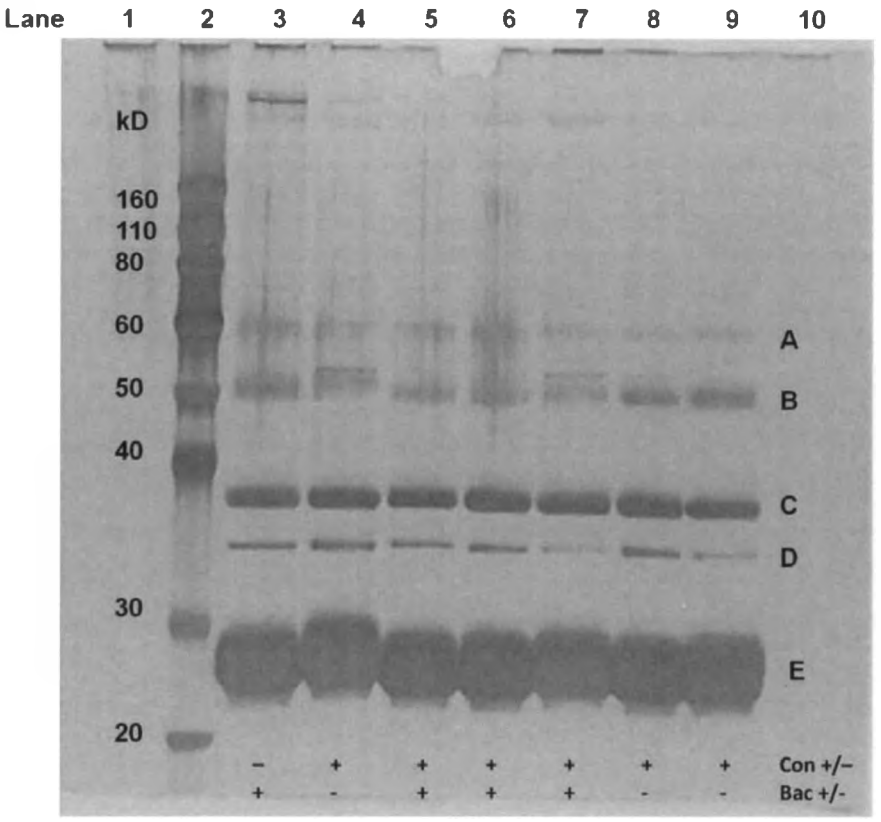

Figure 3.8 SDS-PAGE Analysis of Oyster Hemolymph Proteins Sampled Pre-Exposure. Oysters were sampled for hemolymph prior to the capsule experiment. Shown is an SDS-PAGE gel with 5 ug of hemolymph protein from seven oysters (lanes 3 – 7). Lane 2 contains the Prosieve protein marker, sizes in kDa at left. Letters at right represent five major proteins. Letter E represents dominin.

Post-exposure hemolymph samples displayed very different protein banding patterns compared to those in pre-exposure samples. Of the five samples for which dominin could be clearly resolved, only two express the 31 kDa band D, all express the 33 kDa band C, two express the 44 kDa band B, and only one expressed band A (58 kDa). The only sample that expressed all five bands typically observed in pre-exposure hemolymph was a sample from an oyster not exposed to *R. crassostreae* (Fig 3.9, lane 8).

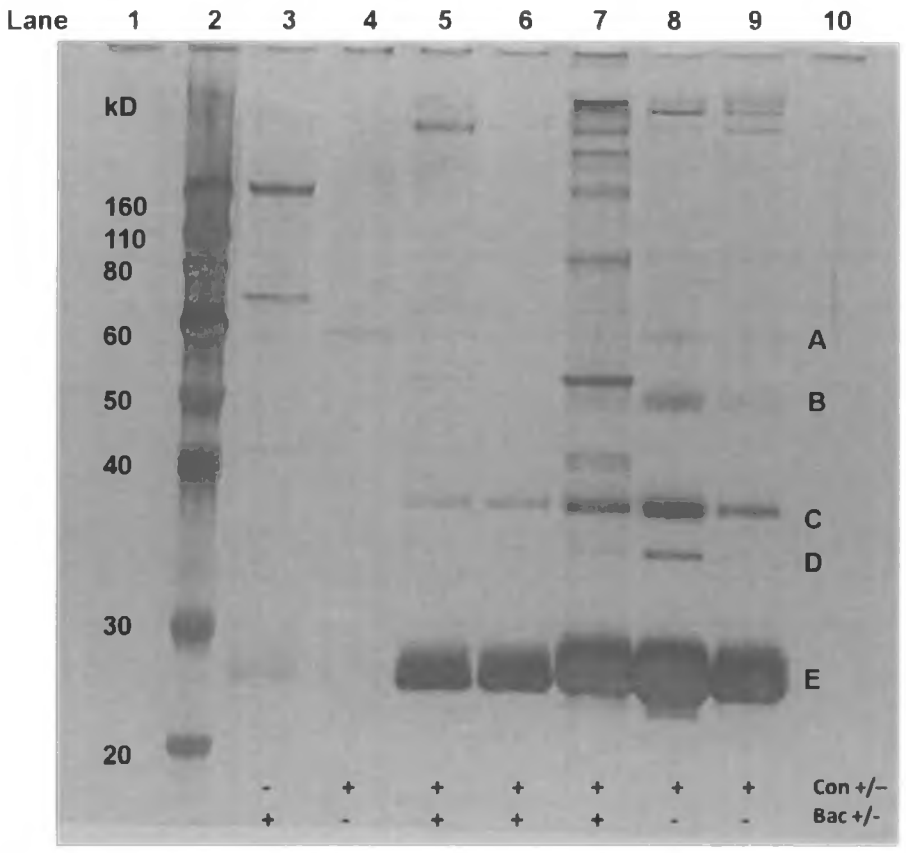

Figure 3.9 SDS-PAGE Image of Oyster Hemolymph Proteins Sampled Post-Exposure. Hemolymph from post-exposure oysters including Control-no injury (lane 3), Injured-Bac- oysters (lane 4, 8, 9) and Injured-Bac+ oysters (lane 5, 6, 7). Lane 2 is Proseive size marker, kDa values at left.

To quantify changes in hemolymph expression, I calculated the proportion of the total protein volume of each hemolymph sample comprised by dominin (Fig. 3.10). The relative expression of dominin in post-exposure oysters was approximately 17% lower when compared to pre-exposure oysters. However, the between sample variance was high and so the difference was not statistically significant. The proportion of dominin was slightly higher post-exposure for oysters exposed to the bacteria (Bac+) versus the exposure control (Bac-) oysters. There was no clear statistical difference in the proportion of dominin in response to bacterial presence.

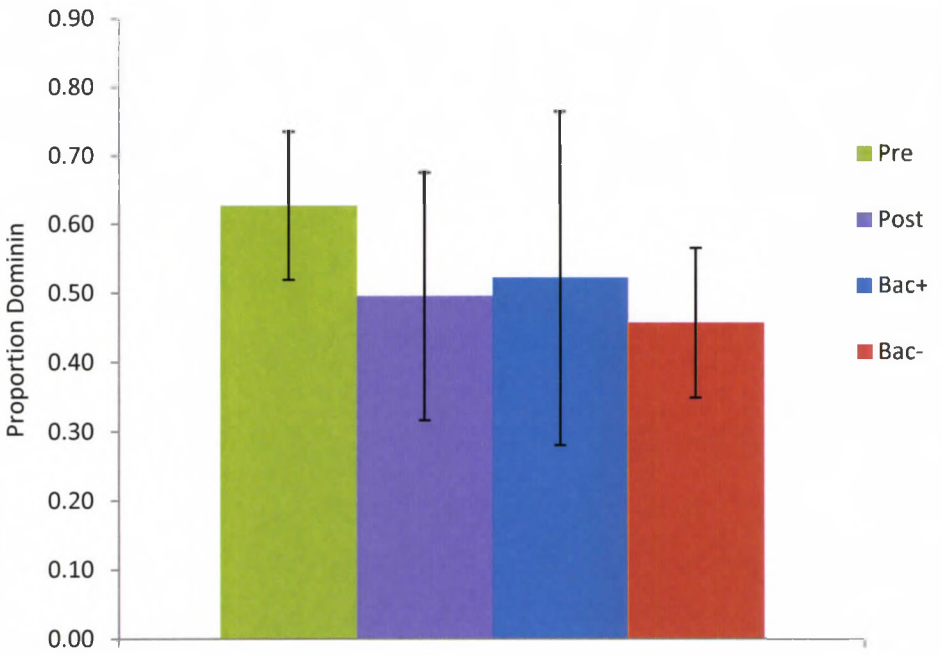

Figure 3.10 Relative Expression of Dominin in Response to Injury. The proportion of dominin (volume dominin/total volume) in oyster hemolymph between pre-experimental oysters with no additional injury (N=5) and post-experimental oysters which were injured(N=5) is represented. I also examined capsule-treated oysters (Bac+, N=3) and control-capsule oysters (Bac-, N=2). Error bars represent standard deviation.

Conchiolin deposition on the inner surface of oyster valves is a hallmark of ROD. Conchiolin deposition patterns were scored from 0 to 3 based on the location and extent of deposition on the inner shell. I examined the effects of capsule placement and presence or absence of *R. crassostreae* in capsule on conchiolin deposition. Oysters for which the hole did not extend through the shell (contact-) all had scores of 0 or 1. For those that had a score of I, deposition appeared to be due to the hole I drilled at the edge of the shell for sampling hemolymph pre-exposure. In contrast, when the hole was drilled all the way through, oysters had at least mild and, in 33% of cases, a severe deposition of conchiolin.

Shell Appearance

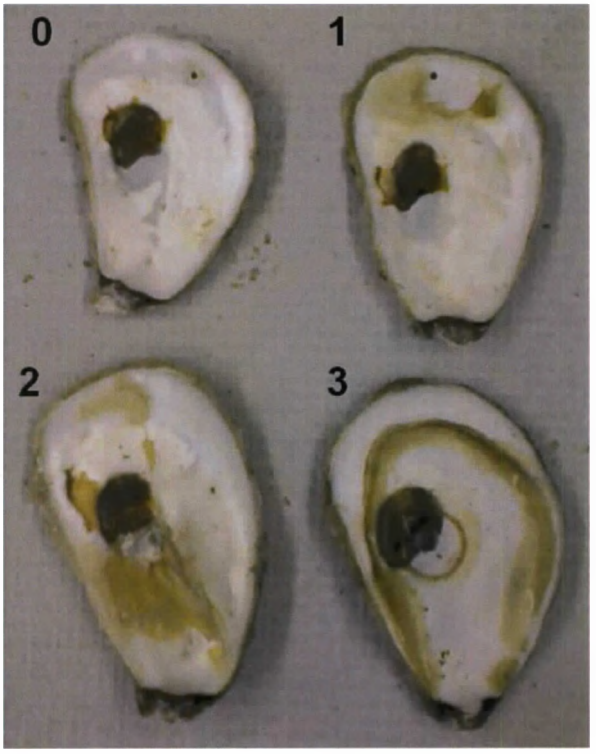

Figure 3.11 Conchiolin Deposition Analysis. The extent of conchiolin deposition was scored on oysters post-experiment. The score for each of four oysters, based on criteria outlined in the methods is shown above, left of each shell. The heavy brown ring of conchiolin exhibited by the oyster with a score of 3 is typically observed in severe cases of ROD.

Statistical analysis (RxC contingency test) indicated that there was significantly more conchiolin deposited on the inside of the shells when the capsule extended through the shell (x^2=8.75, df=3, p=0.033). Of oysters with capsules fully embedded, those with bacteria in the capsule had lower conchiolin deposition scores than those without. This difference, however, was not statistically significant (x^2=11.6, df=6, p=0.072), indicating that conchiolin deposition in experimental (contact+) oysters was more likely a response to injury than to the presence of *R. crassostreae*.

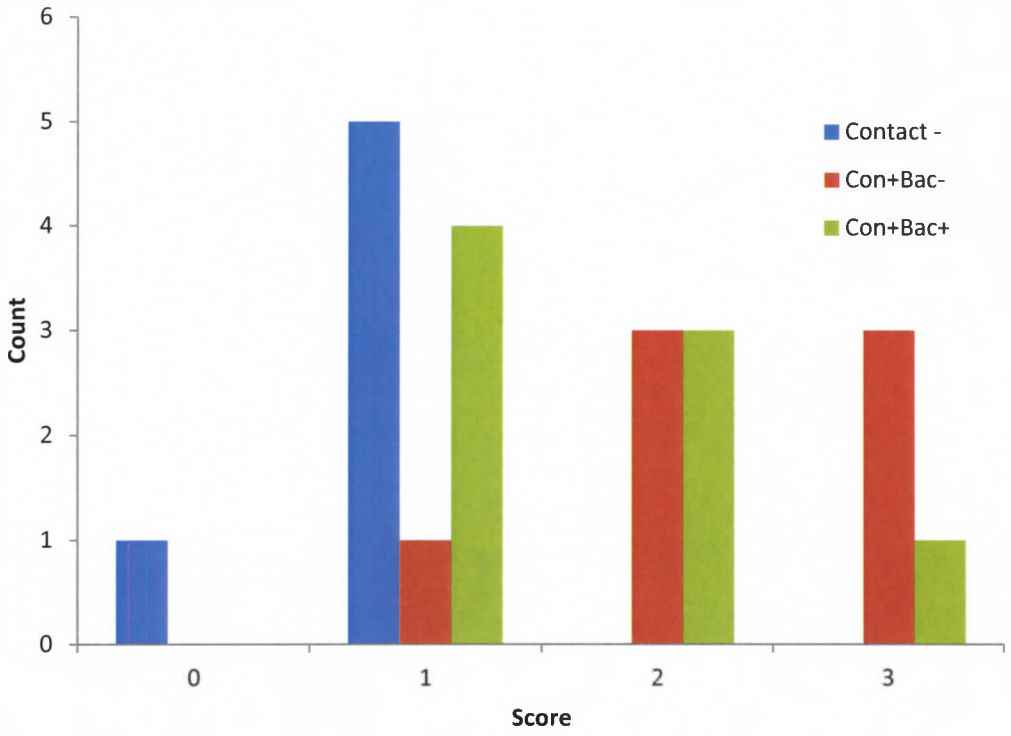

Figure 3.12 Oyster Conchiolin Deposition in Response to Treatment. Along the x-axis are the scores given each shell deposition event (Fig 3.11). The number (count) of oysters representing each score are represented on the y-axis. Blue bars represent oysters that were not injured by capsule embedment. Red bars represent control capsule oysters, and green bars represent oysters embedded with capsules containing *R. crassostreae* cultures.

Protein bands of interest were processed and sequenced by mass spectrometry at the Lerner Research Institute's Mass Spectrometry Core Facility. The information in Table 3.3 summarizes the results of this sequencing effort. In order for the MS-sequencing to find a match between a sample of interest and a protein database, there must be two or more unique peptides shared between the sequenced protein and the database.

Band	Database ID	Species of protein ID	Unique Peptides	Coverage
Rc38	ABC Fe+ siderophore transporter, periplasmic substrate-binding protein	*Maritimibacter alkaliphilus*	3	13%
Rc60	Chaperonin GroEL	*Roseovarius* sp. 217	7, 11	11%, 22%
Rc110	PA domain protein	*Micrococcus luteus*	10	18%
Rc37a,b	Tat (twin-arginine translocation) pathway signal sequence	*Micrococcus luteus*	3,8	8%, 15%
Rc32	TRAP transporter soluble receptor TAXI family protein	*Ruegeria sp.* TW15	6	22%
Rc36	Manganese ABC transporter substrate-binding lipoprotein	*Staphylococcus epidermis*	12	32%
Rc49	PA domain protein	*Micrococcus luteus*	5	12%
Cv26	Dominin precursor	*Crassostrea virginica*	4	31%

Table 3.3 Protein Identification by Mass Spectrometry Sequencing.

The bands submitted all shared three or more unique peptides with specific proteins from other bacterial species listed in Table 3.3. (Or with *C. virginica*, in the case of Cv26.). The bands sequenced from *R. crassostreae* included a variety of transport proteins, proteases, and other surface-associated proteins. The treatments from which these proteins were observed and isolated are given in Figure 3.13.

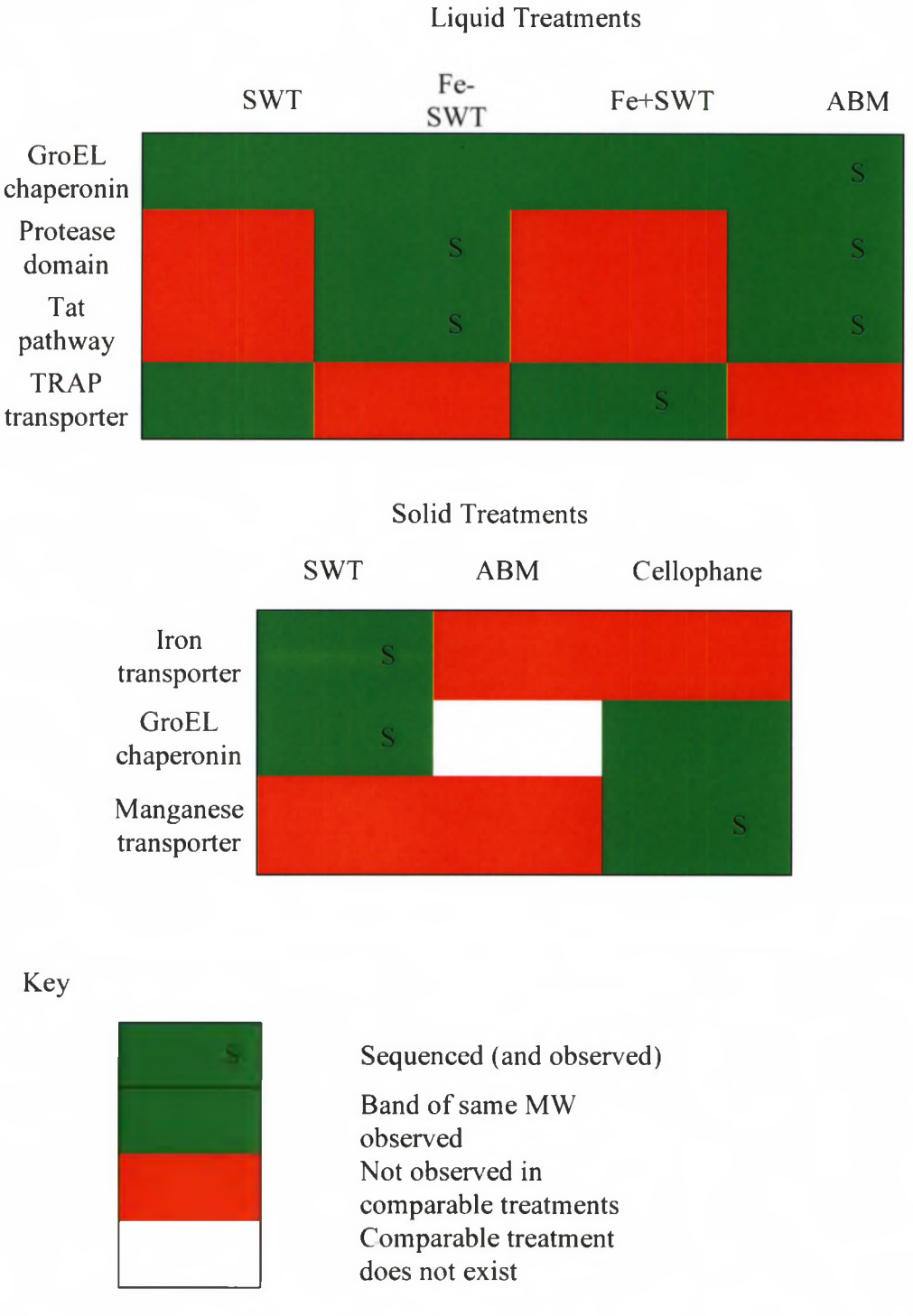

Figure 3.13 Treatment-specific Expression of ECPs by *R. crassostreae*. Sequenced Extracellular proteins were grouped by identity and diagrammed to show the treatment(s) from which the protein type was sequenced.

DISCUSSION

Understanding the mechanisms that induce virulence in pathogenic organisms, such as bacteria, is essential to establishing measures for disease prevention. On the most basic level, we seek to elucidate the underlying biochemical battle that exists between host and parasite in pathogenic systems. Improved knowledge of such interactions can provide improved molecular methods for detecting harmful pathogens. For example, genetic markers can be used to detect the presence of *Vibrio parahaemolyticus*, a marine bacterium that can be pathogenic in humans who have consumed marine shellfish contaminated by this species. Not all *V. parahaemolyticus* strains, however, are pathogenic and genetic markers are only 70% effective at detecting pathogenic strains (Kidham et al. 2012). In contrast, proteomic analysis reflects the actual cell physiology *in situ* and how the expression of virulence factors changes with environmental conditions, including temperature, pH, salinity and nutrient availability. Thus, proteomic analysis facilitates the development of molecular assays based on parasite physiology that can be used to identify and track "pathogenic" strains of *V. parahaemolyticus* and prevent the consumption of unsafe shellfish.

In the case of *Roseovarius crassostreae*, we currently lack detailed knowledge of the environmental conditions that are associated with breakouts of ROD. While *R. crassostreae* is not pathogenic to humans, the disease associated with this species has caused substantial losses for oyster farms in Maine (e.g., Davis & Barber 1994). An improved knowledge of the environmental conditions and host-parasite interactions leading to virulence in this species will facilitate the design of improved treatments and husbandry practices to reduce the impact of ROD throughout the northeastern U.S.

Knowing the aggressins utilized by *R. crassostreae* when it colonizes oysters and that lead to ROD outbreaks will allow us to investigate which innate oyster defenses are most important to resistance and that could be enhanced through selective breeding.

Evidence indicates that *R crassostreae* does not invade the tissues of the oyster host (Boardman et al. 2008), suggesting that the extracellular activity of both host and pathogen are central to the development of the disease. Bacterial virulence is often associated with extracellular proteins normally expressed by the bacteria, or expressed when the cell is stressed, such as during periods of iron limitation. The analysis of extracellular products produced by bacteria, including their effects on host cells, is an approach that has provided substantial information on the expression of virulence factors in a number of marine bacterial species. For example, Gomez-Leon et al. (2008) conducted toxicity assays in which they exposed larval oysters to intact, viable *R. crassostreae* cells grown on standard media (SWT plates) and other toxicity tests wherein oyster hemocytes were exposed to extracellular products collected from cells grown using the cellophane overlay technique. However, the choice of assay used to identify and characterize extracellular virulence factors can impact the relevance of the results. Oftentimes, as in Gomez-Leon et al. (2008), host cells are exposed to extracellular isolates to determine the extent of their role in disease state, but since both host and pathogen are removed from the natural system, the results from these experiments can be extremely variable. In addition, the culture methods used to prepare the pathogen for the experiment may not match the natural, dynamic environment and there is the risk that the isolated extracellular products do not contain the virulence factors present under field conditions. In the Gomez-Leon et al. (2008) study, in

particular, the two approaches used to isolate virulence factors, whole cell and ECP toxicity, are not necessarily comparable. In my research, I have found that some ECPs, such as GroEL chaperonin appear to be expressed constitutively by *R. crassostreae* while other proteins like Tat pathway proteins and metalloproteases are differentially expressed when cells are faced with iron limitation, as they might experience when colonizing a host oyster.

The production of ECPs and virulence factors by pathogenic bacteria depends on a wide variety of environmental conditions. My thesis has sought to identify how iron limitation, temperature, culture volume and exposure to host cells affect the expression of extracellular proteins by *R. crassostreae.* In the five culture experiments reported in this thesis, I found that the growth rate of *R. crassostreae* increased as culture temperatures increased from 23.5°C to 30°C. My findings are consistent with those of Maloy et al. (2005), who also found that growth rate increased with increasing temperatures, up to 34°C. In addition to affecting growth rate, temperature was associated with the extent of the response by *R. crassostreae* to iron deficiency. At 30°C, growth of this species in Fe-SWT media was only slightly slower than growth in control media (SWT) even though growth was very different in two media at 23°C. Thus, iron deficiency has a larger impact on the growth of *R. crassostreae* at temperatures that reflect those that are common on oyster farms in Maine when disease outbreaks typically occur.

Temperature regulation of the expression of virulence factors has been observed in other host-parasite interactions. For example, Kimes et al. (2012) found that temperature regulates multiple virulence mechanisms, independent of abundance, in the

case of coral infection by the bacterium *V. coralliilyticus*. Given such findings, it is important to consider whether virulence varies with temperature for *R. crassostreae* as well. Such temperature dependence is a potential explanation for ROD breakouts occuring during the warmest times of the year. On the other hand, Gomez-Leon et al. (2008) cultured all bacteria at "room temperature" prior to isolating ECPs. Based on my research, I support that much more care needs to be taken when isolating and analyzing the effects of potential virulence factors on host mortality. In particular, experiments must clearly define the environmental conditions, including temperature, under which ECPs and their role as virulence factors are examined. The term "room temperature" does not suffice to repeat bacterial ECPs. At the very least, growth conditions for *R. crassostreae* in ECP exposure trials must be consistent.

The major variable I manipulated in my study of *R. crassostreae* ECPs was iron limitation. I used a variety of media types designed to iron limitation (e.g., Fe-SWT), as well as nutritional sources (ABM) typical of *R. crassostreae's* natural environment. Although the typical location of the bacteria in costal habitats is unknown, the ecology of Roseobacters suggests some possibilities. Roseobacters often express dominance over other bacteria in the colonization of the highly valued surface area of marine aggregates; this dominance may be due to their ability to produce antibacterial compounds that inhibit colonization by other strains of bacteria (Bruhn et al. 2007). Tropodithietic acid, an antibacterial agent produced by Roseobacters, is only synthesized in significant amounts when cultures are grown in a standing broth, allowing for biofilm formation. Many more bioactive molecules are likely differentially produced based on environmental conditions and, in some cases, depending upon specific algal-bacterial

interactions (Bruhn et al. 2007). Of particular importance, however, are the factors that are synthesized in a biofilm, the life-history stage during which Roseobacters are likely to successfully colonize both marine aggregates and perhaps the inner surface of the oyster shell. For example, members of the Roseobacter clade express the quorum sensing molecule N-acylhomoserine lactone (AHL). Zan et al. (2011) suggested that AHL is involved in the transition between mobile and sessile phases in *Ruegeria* sp. KLH11, a sponge-associated Roseobacter, and may be associated with the ability to adapt to new environments.

Extracellular proteins are also differentially expressed by bacteria and, as discussed above, are often involved in pathogenicity. I isolated and characterized several proteins that were differentially expressed by *R. crassostreae*. One protein band, Rc38, was a band that I first observed in ECP samples from R. *crassostreae*. MS-sequencing identified this protein as an ABC Fe+ transporter (siderophore). ABC siderophore transporters are extracellular proteins capable of chelating iron and function in maintaining transition metal homeostasis within the cell (Klein & Lewinson 2011). Many siderophores have been implicated in bacterial-associated diseases. For instance, Fetherston et al. (2010) found iron uptake to be vital to the pathogenicity of *Yersiniae pestis*, the bacterium that causes bubonic plague and pneumonic plague. In human diseases, high free iron availability in the host's blood is a major catalyst of bacterial virulence (Bullen et al. 2005). Innate host defenses often include mechanisms that limit pathogen growth by reconfiguring the host's iron acquisition, transport, and localization during an infection. If iron is stored in isolated tissues to limit pathogen growth, this is

thought to reduce the availability of iron for other host cells, especially macrophages (Ganz 2009).

Given this function, it is not surprising that I originally isolated Rc38 from stationary cells grown on solid media. In such an environment, if cells use up iron in their immediate proximity, then expression of siderophores may follow to scavenge iron from the environment. On the other hand, I also expected to observe the expression of this protein in iron-limited media (Fe-SWT). This latter observation does not mean that Rc38 is not involved in iron scavenging or virulence, but suggests that regulation of its expression is much more complex.

A second protein of interest, Rc36, was isolated from *R. crassostreae* cells grown on a cellophane overlay. There were twelve peptides from this protein that had high similarity to peptides from a manganese ABC transporter protein from *Staphylococcus epidermis*. These proteins have a similar function to the ABC Fe+ transporters in that they allow the cell to maintain physiologically appropriate manganese levels. Like iron, manganese is an important cofactor for enzymes including superoxide dismutases (SODs) and peroxidases that defend against reactive oxygen species. Mn(II) supplementation has antioxidant effects on *Bacillus subtilis* and *Escherichia coli* (Inaoka et al. 1999, Al-Maghrebi et al. 2002). Like iron transporters, manganese transporters have been implicated in bacterial virulence. For example, Paton et al. (1998) and Marra et al. (2002) found that a manganese permease system is virulent in *Streptococcus pneumonia.* Mutation of the gene encoding for the lipoprotein contained within the ABC Mn(II) transport complex has been shown to result in reduced virulence in *Staphylococcus aureus*, and the regulation of Mn homeostasis appears to

overlap with peroxide defense and iron homeostasis in *Staphylococcus aureus*, *B. subtilis* and *E. coli* (Horsburgh et al. 2002).

This relationship between manganese transport and virulence is perhaps in part because of manganese's effect on the management of oxidative stress by *S. pneumonia* cells as observed by Tseng et al. (2002), or perhaps due to the important apparent role of manganese in activating general carbon metabolism (Ogunniyi et al. 2010). Isolation of this protein from cellophane plates suggests that manganese incorporation is important to a growing biofilm. As with the iron transporter, however, we do not know if Rc36 is expressed in response to excess or limiting concentrations of manganese and what its role (if any) may be in the development of ROD.

A protein similar to the GroEL chaperonin was observed in all pre-log cultures at 25°C and directly sequenced from the supernatants of SWT plates (Rc60a) and liquid ABM pre-log cultures (Rc60b). GroEL proteins typically function as intracellular proteins. However, they have been detected at the surface of several mucosal pathogens, such as *Mycobacterium smegmatis,* (Rao & Lund 2010), *Lactobacillus johnsonii, Mycobacterium leprae, Salmonella enterica* serovar typhimurium, *Clostridium difficile, Helicobacter pylori, Legionella pneumophila,* and *Haemophilus ducreyi,* and have been implicated in cell attachment and modulation of the host's immune system (Bergonzelli et al. 2006). The presence of this protein at the bacterial surface suggests that it may play a role in bacterial adherence to surfaces. This role, in addition to the upregulation of GroEL during apoptosis, was confirmed in *L. pneumophila* by Tsugawa et al. (2007). Other probiotic properties (colonization factors) of surface-associated GroEL were suggested by Bergonzelli et al. (2006), including attachment to mucus and epithelial

cells, as well as stimulation of cytokine secretion in macrophages and epithelial cells of hosts. Affek & Jagusztyn-Krynicka (2007) found that a 60 kDa surface-associated chaperonin is a virulence factor in the gingival pathogen *Actinobacillus actinomycetemcomitans.* It is integral the formation of a "tenacious" biofilm by interacting with the host's immune system (inflammatory response). Surface-associated GroEL chaperonins produced by *R. crassostreae* may contribute to ROD by aiding in shell attachment as well as suppressing the oyster's immune system by stimulating cytokine production in hemocytes or oyster tissues.

I observed a band similar in size to GroEL (60 kDa) in all 25°C pre-log liquid cultures (Fig 4.1). I also observed this as the singular band expressed by SWT solid phase (SWT plate) cultures and the sole band expressed by low density ABM cultures. If this protein serves a similar function in *R. crassostreae* as in other bacteria, it could be playing a role in attachment and biofilm formation under a wide variety of environmental conditions.

Two protein bands (Rc110, Rc49) that were sequenced by MS contained protease-domains associated with peptidases. These bands may represent the products of different peptidase genes, or may be two proteins from the same gene that result from differential splicing. In either case, I observed these peptides in both Fe-SWT and ABM cultures but did not detect them in corresponding SWT or Fe+SWT cultures. This suggests that iron limitation and algae sources of nutrition result in increased expression of this potential virulence factor. As mentioned earlier, proteases are often involved in virulence. Labreuche et al. (2006) found that a zinc-dependent metalloprotease was associated with *C. gigas* mortality from *Vibrio aestuarianus*. A potential mechanism of

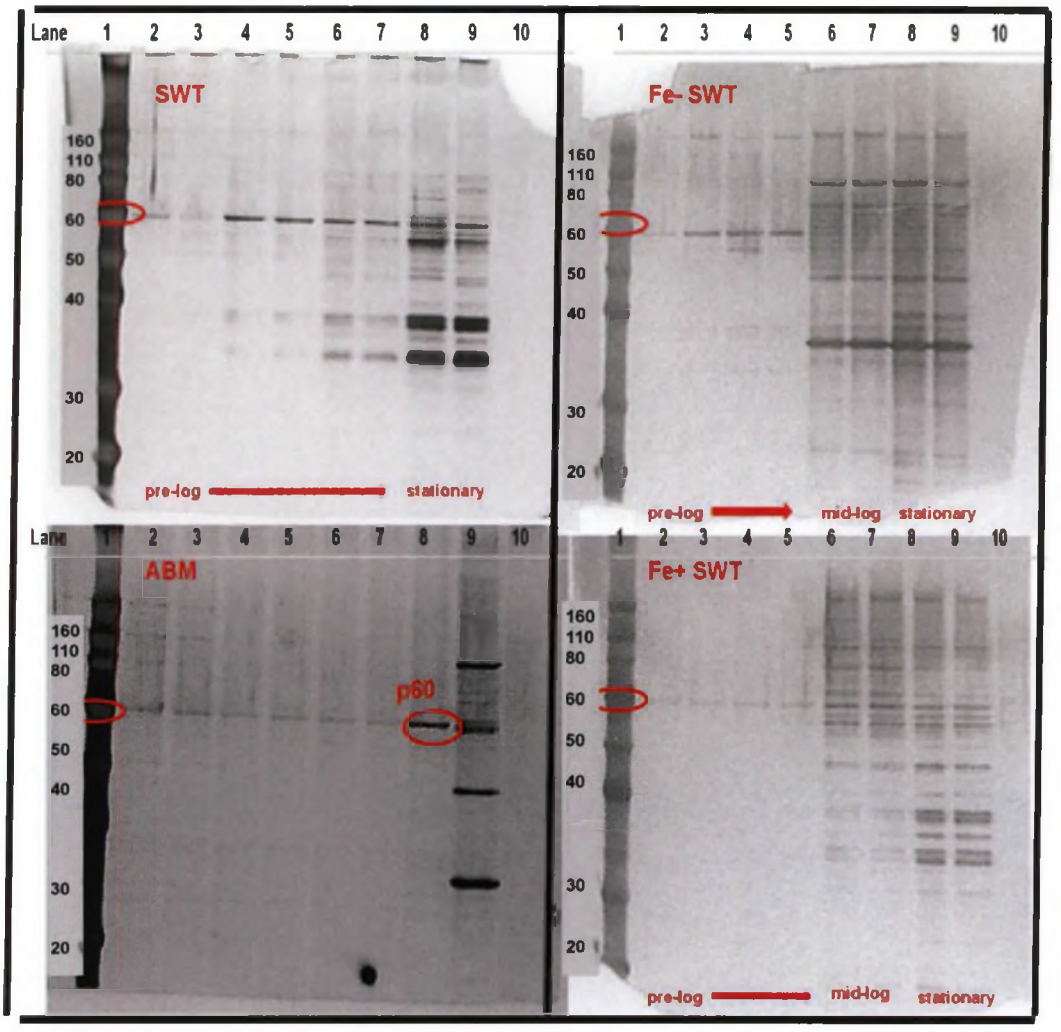

Figure 4.1 Expression of a 60 kDa Band by *R. crassostreae*. Pre-log *R.crassostreae* cultures of every liquid media type at 10 mL and 25°C express a band at 60 kDa. The 60 kDa standard protein is circled on each ladder (lane 1). Band Rc60b on the ABM gel (circled) was one of the two sequenced and matched to the GroEL chaperonin. Growth phases are noted at the bottom of each gel.

virulence for zinc metalloproteases was reported by Oggioni et al. (2004), who observed that a pneumococcal zinc metalloproteinase cleaved a human matrix metalloproteinase.

The expression of band Rc37 also increased under iron-limited conditions and when *R. crassostreae* was cultured with algae-based media (ABM). This band was identified as the twin-arginine translocation (Tat) protein export system, which is present

in the cytoplasmic membranes of most bacteria and archaea and has the highly unusual property of transporting fully folded proteins (Palmer and Berks 2012). As such, this transporter may play a role in the export of proteins more directly involved with virulence. This combination of proteases and a transporter comprises three of the four major extracellular products identified in the Fe-SWT and ABM cultures from cells sampled at stationary phase. Because fewer proteins were isolated from control cultures, I cannot say that iron-limiting conditions contain a greater proportion of proteases; however, other methods may be used to determine protease content of ECPs. Hasegawa et al. (2009) used gel zymography to identify proteolytic activity in supernatants of pathogens. To identify the importance of metalloproteases to virulence, metal chelators can mixed with bacterial supernatants prior to exposing host cells to isolated ECPs. By chelating important cofactors, this approach suppresses the activity of metalloproteases, which in turn should reduce the effects of ECPs on host cells. Another common way of determining the role of a specific protein in bacterial virulence is by performing gene knockouts. This genetic method removes a single variable: the expression of a singular protein.

The final protein band from *R. crassostreae* that I sequenced (Rc32) had high similarity to the TRAP (Tripartite ATP-independent periplasmic) transporter soluble receptor TAXI family protein, a high-affinity, Na+-dependent unidirectional secondary transporter. This protein is common in prokaryotes but is absent from eukaryotes (Mulligan et al. 2011). This protein was isolated from control (SWT) cultures and there is no evidence in the literature implicating it in bacterial virulence.

Dominin Expression

The most abundant protein in hemolymph that I sampled from oysters both pre- and post-exposure to *R. crassostreae* was identified by MS-sequencing as dominin. The actual function of this protein is not well-documented, but Itoh et al. (2011) suggest that it may be involved in metal transport, antioxidation, wound repair, and shell mineralization. Interestingly, this protein also migrated across the capsule membrane and I observed it in protein preparations from the embedded capsules, themselves. It would be tempting to conclude, then, that dominin is active in host defense and was moving in response to the presence of *R. crassostreae* in the capsules. However, as the most abundant protein in the extrapallial fluid of the oyster, the movement of dominin across the filter membrane may simply have been a result of diffusion down a gradient.

Scientists have been unable to reliably initiate an ROD-like colonization of oysters by *R. crassostreae* in the laboratory. Even reliable hemolymph sampling requires injury to the shell, causing a wound response reaction that is similar to the physical characteristics of ROD, such as excessive conchiolin deposition. In my experiment, I sampled hemolymph by creating a 1 mm diameter hole in the shell of some oysters and embedding capsules throught a 1 cm diameter hole into the shell of other oysters. The heavy conchiolin deposition in response to the shell damage imparted by my experimental methods makes it difficult to separate the effects of exposure to *R. crassostreae* from the effects of shell damage. Thus, future experiments will need to be conducted in a manner that separates these effects, and can thus clearly identify the factors influencing the expression of dominin.

As discussed in the introduction, the mechanism(s) of resistance to ROD in oysters has not been elucidated. In studying this disease, it would be beneficial to determine whether oyster resistance is associated with a heightened response to the pathogen, or conversely with a muted response. The correlation of oyster size with mortality suggests that a heightened response, consistent with oyster size and strength, helps to overcome ROD. If dominin is in fact involved in pathogen response, an increase in dominin expression might be a key defense strategy for the oyster. Conversely, successful oysters that have resistance to ROD may have significantly lower expression of defense proteins like dominin and are less likely to continue to grow shell incorrectly. When analyzed on reducing polyacrylamide protein gels, dominin formed one thick band, but under native conditions two distinct bands. This suggests that there are three isoforms of dominin (Itoh et al. 2011) and they may have different functions in the oyster. Comparison of expression of these isoforms or other proteins might reflect different general immune responses.

A potentially fruitful avenue of inquiry may involve the qualitative and quantitative study of protein expression in oysters through healthy growth. For example, the isolation and characterization of American Oyster Defensin (Seo et al. 2010) was done on a group of healthy adults. If a similar study was performed on juvenile oysters, we may find host size-specific variation in the expression of certain antimicrobial proteins in the oyster. Perhaps certain defensins are not expressed or developed until the oyster reaches about 25 millimeters in length. Differential expression of defense proteins through oyster growth might explain the correlation of oyster size with mortality.

In addition to heightened understanding constitutive defense mechanisms, our research would benefit greatly from the successful induction of an *R. crassostreae* colonization on oyster shells in the laboratory. ROD outbreak information to date has relied on post-outbreak observations. Once a challenge model is available, one can compare the production of dominin and other potential defensins in response to an *R. crassostreae* biofilm. One way we could elucidate the expression these proteins is by reverse designing PCR primers (from amino acid sequence to nucleotide sequence) to look at the expression of the corresponding genes using qPCR.

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