AN ABSTRACT OF THE DISSERTATION OF

Alisha M. Aagesen for the degree of Doctor of Philosophy in Microbiology presented on October 30, 2012.
Title: Investigating *Vibrio parahaemolyticus* Interactions with the Pacific Oyster, *Crassostrea gigas*.

Abstract approved: _____
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Vibrio parahaemolyticus is a Gram-negative, halophilic, human pathogenic bacterium ubiquitous in the marine environment. Like many *Vibrio* species, *V. parahaemolyticus* commonly associates with shellfish, particularly oysters. Ingestion of a raw or under cooked oysters contaminated with *V. parahaemolyticus* can cause gastroenteritis, which is typically self-limiting and rarely causes death. Globally, oyster production is highly lucrative, especially on the West Coast of the United States where approximately 60% of oyster production occurs each year. Outbreaks of *V. parahaemolyticus* can result in a significant public health problem as well as an economic burden for the oyster farms implicated in the outbreak. With the increase in overall *V. parahaemolyticus* outbreaks, improved post-harvest processing strategies have been developed to reduce this natural contaminant. Depuration was developed to allow shellfish to purge contaminants from their tissues into the clean, flowing seawater where they are held. This post-harvest processing technique can typically reduce fecal contaminants from the oyster tissues but is relatively ineffective at eliminating *V. parahaemolyticus* and other *Vibrio* species.. Thus, improved methods

for reducing this and other human pathogenic *Vibrio* are needed to effectively produce safer oysters for the consumer. To develop more effective and novel *V. parahaemolyticus* intervention strategies, first we must identify the factors that are involved in *V. parahaemolyticus* colonization of the oyster, allowing them toresist depuration. This study sought to investigate specific factors utilized by *V. parahaemolyticus* and, in the process, determined that various strains of *V. parahaemolyticus* have different alleles of the Type IV pili, mannose-sensitive hemagglutinin (MSHA)and chitin-regulated pilus (PilA). In addition, we expanded our investigations into the allelic diversity of MSHA and PilA from *Vibrio cholerae* and *Vibrio vulnificus* and found that *V. cholerae* strains that possess the Type IV toxin co-regulated pilus (TCP) maintained highly conserved MSHA and PilA sequences while strains of *V. cholerae* without TCP, and all of the *V. vulnificus* and *V. parahaemolyticus* strains examined, had highly divergent sequences with no discernable connection to isolation source or observed phenotype. Following that discovery, we determined that Type I, and Type IV pili, as well as polar and lateral flagellar systems contribute to *V. parahaemolyticus* persistence in the Pacific oyster during depuration, while Type III secretion systems and phase variation do not. Overall, we have identified factors involved in colonization of the Pacific oyster by *V. parahaemolyticus*. Future studies investigating conditions that affect pili and flagella production in *V. parahaemolyticus* may provide novel depuration conditions that could easily and effectively increase the efficiency of oyster depuration, ultimately

reducing the risk of seafood-borne illness by *V. parahaemolyticus* associated with oysters.

Investigating *Vibrio parahaemolyticus* Interactions with the Pacific Oyster, *Crassostrea gigas*

by
Alisha M. Aagesen

A DISSERTATION

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Chair of the Department of Microbiology

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Alisha M. Aagesen, Author

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Dr. Claudia C. Häse and Alisha M. Aagesen were involved in the design, data collection, analysis, and writing of Chapters 2 and 3, and Appendices A and B. Dr. Yi-Cheng Su and Sureerat Phuvasate were involved in the writing and data collection of Chapter 3. Dr. Hiroaki Hasegawa helped in collecting data for Appendix B.

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Dedicated to
the loves of my life,
Bret Aagesen and Sadie Aagesen

Investigating *Vibrio parahaemolyticus* Interactions with the Pacific Oyster, *Crassostrea gigas*

Chapter 1

Introduction and literature review

General introduction

The *Vibrio* genus is comprised of Gram-negative bacilli commonly known for their slightly curved (comma shaped) rods and are naturally found in the aquatic environment. They cover a wide variety of niches ranging from commensal organisms, *Vibrio fischeri* symbiosis with the Hawaiian bobtail squid *Euprymna scolopes* (Yip, Grublesky et al. 2005), to animal pathogens such the oyster pathogen *Vibrio splendidus* (Duperthuy, Schmitt et al. 2011), to human pathogens such as *Vibrio cholerae*, the causative agent of cholera, *Vibrio vulnificus,* and *Vibrio parahaemolyticus*, common causes of food-borne illness (CDC 2011). Unlike many *Vibrio* spp., *V. parahaemolyticus* cells are typically found as small, straight rods, which caused incorrect identification of the species as *Pasteurella parahaemolytica* following a shirasu food poisoning outbreak in Osaka, Japan in 1950 by Fujino (Broberg, Calder et al. 2011; Shinoda 2011). In 1963, *P. parahaemolytica* was correctly identified as a *Vibrio* and the name was subsequently changed to *V. parahaemolyticus*.

V. parahaemolyticus is a human pathogen with hemolytic properties on a special media called Wagatsuma agar and is referred to as the Kanagawa phenomenon (KP) named for the researchers at Kanagawa Prefectural Public Health laboratory that first correlated this hemolysis with human pathogenicity (Shinoda

2011). This medium is used to identify pathogenic *V. parahaemolyticus* isolates by the presence of clearing zones on the agar, indicating that the strain possesses the thermostable direct hemolysin (TDH) virulence factor, named for its stability at 100°C and ability to lyses red blood cells from many animal sources (Shinoda 2011). Interestingly, many years after the original *V. parahaemolyticus* type strain, ATCC17802, was isolated, it was determined that this strain was actually KP^- but possessed a different hemolytic virulence factor named TDH-related hemolysin (TRH). TRH^+ strains exhibit hemolytic clearing on typical blood agar plates but not on Wagatsuma (Broberg, Calder et al. 2011; Shinoda 2011). *V. parahaemolyticus* strains can encode either TDH or TRH or both in their chromosomes and these virulence factors share sequence homology (Broberg, Calder et al. 2011).

In addition to the hemolytic virulence factors, TDH and TRH, *V. parahaemolyticus* can encode two functional Type III secretion systems (T3SS). T3SSs are comprised of a needle and syringe-type apparatus that deliver effector proteins, often virulence factors, into host cells (reviewed in (Galan and Collmer 1999; Dean 2011). To date, all *V. parahaemolyticus* strains encode T3SS-1, the dominant contributor to cell cytotoxicity, and is found on chromosome 1, the larger of the two *V. parahaemolyticus* chromosomes. T3SS-2 is found on a pathogenicity island on chromosome 2 of pathogenic *V. parahaemolyticus* strains, causing enterotoxicity and some cytotoxicity to certain cells lines (Park, Ono et al. 2004; Hiyoshi, Kodama et al. 2010; Dean 2011). Interestingly, it has been shown that many environmental *V. parahaemolyticus* isolates encode T3SS-2 without any of the other

characteristic virulence factors (TDH or TRH) and can cause disease similar to clinical isolates. This suggests that many naturally occurring *V. parahaemolyticus* strains have the potential for human disease with or without TDH or TRH (Caburlotto, Lleo et al. 2010). Thus, *V. parahaemolyticus* encodes for many virulence factors and possessing one or a combination of them can result in human disease. Overall, determination of a strains' capability to cause disease is not nearly as well defined as certain other pathogenic bacterial species.

V. parahaemolyticus have two major surface antigens that can be serologically identified to distinguish strains, the O-antigens of the lipopolysaccharide (LPS) and the K-antigens of the capsular polysaccharide (CPS). Thirteen O-antigens and 71 different K-antigens have been identified (Hsieh, Liang et al. 2003). The current pandemic *V. parahaemolyticus* strain belongs to the O3:K6 serogroup (Chen, Dai et al. 2010). Other polysaccharides, termed exopolysaccharides (EPS), have been found on the surface of *V. parahaemolyticus* strains and changing the amount of EPS produced results in an altered colony morphology and the ability to exhibit phase variation from an opaque (OP) to translucent (TR) colony phenotype (McCarter 1998; Enos-Berlage and McCarter 2000; Guvener and McCarter 2003; Hsieh, Liang et al. 2003). Phase variation results in difference adherence capacity and has been linked to human disease. OP and TR cells of *V. parahaemolyticus* are proficient in forming biofilms, although differences do exist in their biofilm kinetics and loss of EPS results in biofilm defective strains (Enos-Berlage, Guvener et al. 2005). The OP phenotype cells are more adherent to epithelial cells than their TR counterparts (Hsieh, Liang et

al. 2003) and in a related *Vibrio* species, *V. vulnificus*, OP strains were more virulent in mice than TR (Yoshida, Ogawa et al. 1985). Opacity in *V. parahaemolyticus* is under the control of the transcriptional regulator *opaR* gene, which has high similarity and is functionally interchangeable with the *Vibrio harveyi* LuxR protein, responsible for quorum-sensing induced bioluminescence (McCarter 1998). OpaR also responds to quorum sensing and regulates about 5.2% of the genome of *V. parahaemolyticus* directly or indirectly, including the entire surface sensing regulon, which includes lateral flagella, EPS, and T3SS-1 (Gode-Potratz, Kustusch et al. 2011; Gode-Potratz and McCarter 2011). It has been suggested that the TR phenotype primes cells to respond to environmental cues for growth over a surface while the OP phenotype is designed for a sessile, biofilm-type lifestyle (Gode-Potratz and McCarter 2011), illustrating the complex interactions that occur in *V. parahaemolyticus* survival in the diverse environments it may encounter.

Motility is an important aspect of survival in various environments. In general, flagella are helical propellers providing locomotion for the bacterial cell. Bacterial movement in the environment can be the result of chemotaxis towards a food source as well as for colonizing a surface. Flagella are often the first means of adherence to a surface during colonization (reviewed in (McCarter 2001; Kirov 2003; McCarter 2004; McCarter 2006; Merino, Shaw et al. 2006). *V. parahaemolyticus* possesses two flagellar systems, a single, polar flagellum for movement in the liquid environment, and peritrichous (lateral) flagella, for movement over a surface (McCarter 2004) (Fig. 1.1). The polar flagella uses sodium motive force for

locomotion (Atsumi, McCarter et al. 1992), is constitutively produced, and often the first attachment to a surface (Belas and Colwell 1982). The swimming speed of vibrios in the liquid environment have been measured at approximately 60 μm/s but as viscosity increases and polar flagellar rotation is inhibited, this triggers a regulatory cascade signaling the surface sensing regulon to produce lateral flagella and induce the swarmer cell state (Kawagishi, Imagawa et al. 1996; McCarter 2001; Gode-Potratz, Kustusch et al. 2011; Gode-Potratz and McCarter 2011). The *V. parahaemolyticus* swarmer cells are characterized by elongation of the bacterial cells (McCarter and Silverman 1990) and production of lateral flagella, driven by the proton motive (Atsumi, McCarter et al. 1992), for movement over a surface, a phenotype called swarming (McCarter and Silverman 1990). As stated previously, lateral flagella production is controlled by the regulator, OpaR (Jaques and McCarter 2006), which also controls virulence factor production (Gode-Potratz, Kustusch et al. 2011; Gode-Potratz and McCarter 2011), suggesting lateral flagella and virulence factor production are tightly regulated for expression under appropriate circumstances and in different environments. This further demonstrates the vast interplay of gene regulation for pathogenicity in *V. parahaemolyticus*.

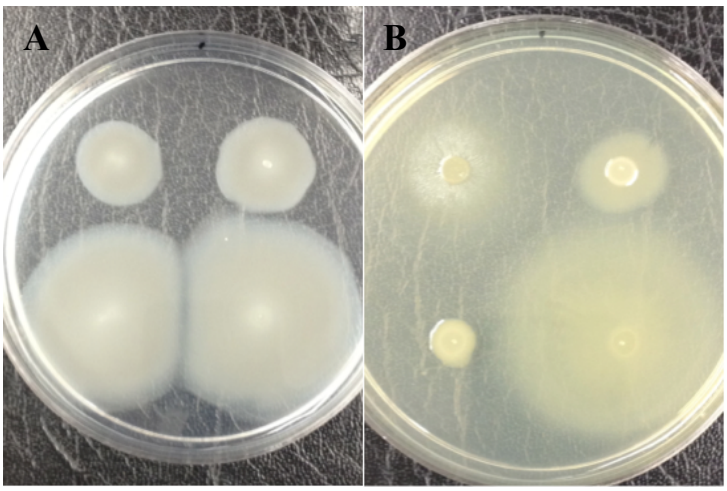

Figure 1.1. *V. parahaemolyticus* polar and lateral flagella. Different strains of *V. parahaemolyticus* in B) semisolid swimming motility agar and C) swarming motility agar (Chapter 3).

Adhesins, such as pili or fimbriae, are bacterial surface structures often important in adhering to multiple surfaces and for pathogenicity in many bacteria (reviewed in (Craig, Pique et al. 2004). The *V. parahaemolyticus* genome (Makino, Oshima et al. 2003) encodes homologues of the Type I pili from human pathogens such as *Acinetobacter baumanii* (Gaddy, Tomaras et al. 2009) and enteropathogenic *Escherichia coli* (Li, Poole et al. 2009), and Type IV pili from various human pathogenic *Vibrio* species including *V. cholerae* (Jonson, Lebens et al. 1994; Marsh and Taylor 1999; Chiavelli, Marsh et al. 2001) and *V. vulnificus* (Paranjpye, Lara et al. 1998; Paranjpye and Strom 2005; Paranjpye, Johnson et al. 2007). The Type I pili from *V. parahaemolyticus* are encoded by the *csu* locus which is most similar to the *csuA/B* operon from *A. baumanii*, which has been shown to be important in biofilm formation of *A. baumanii* on abiotic surfaces (Tomaras, Dorsey et al. 2003; Gaddy, Tomaras et al. 2009). In *E. coli*, Type I pili are known to be mannose-sensitive

hemagglutinins and are used for adherence to mussel hemocytes, which can be inhibited by the addition of D-mannose (Canesi, Pruzzo et al. 2001).

V. parahaemolyticus encodes at least two Type IV pili, the mannose-sensitive hemagglutinin (MSHA) and the chitin-regulated pilus (PilA) (Makino, Oshima et al. 2003). These and other Type IV pili in vibrios are processed by the Type IV pilin peptidase, PilD (Paranjpye, Lara et al. 1998; Fullner and Mekalanos 1999). In *V. cholerae*, MSHA and PilA have not been shown to be directly involved in pathogenicity and are suggested to be environmental factors because of their use for attachment to various surfaces such as zooplankton and mussel hemocytes, as well as chitin utilization (Attridge, Manning et al. 1996; Chiavelli, Marsh et al. 2001; Zampini, Canesi et al. 2003; Meibom, Li et al. 2004; Zampini, Pruzzo et al. 2005; Hsiao, Liu et al. 2006; Hsiao, Toscano et al. 2008). Both MSHA and PilA are used for biofilm formation in many *Vibrio* species (Watnick, Fullner et al. 1999; Watnick and Kolter 1999; Enos-Berlage, Guvener et al. 2005; Paranjpye and Strom 2005; Shime-Hattori, Iida et al. 2006) and expression is induced by the presence of chitin (Meibom, Li et al. 2004; Shime-Hattori, Iida et al. 2006). *V. fischeri*, the bioluminescent squid symbiont, uses both MSHA and PilA to successfully colonize the light organ of the squid (Stabb and Ruby 2003; Ariyakumar and Nishiguchi 2009). Furthermore, loss of *pilA,* the PilA pilin, and *pilD*, the Type IV pilin peptidase, in *V. vulnificus* results in colonization defects of the Eastern oyster, *Crassostrea virginica* (Paranjpye, Johnson et al. 2007; Srivastava, Tucker et al. 2009). Taken together, the Type IV pili MSHA and PilA in *Vibrio* species are

important for surface colonization, biotic or abiotic, in what appears to be natural associations in the aquatic environment and a potential role in human disease has not been established.

Like many vibrios, *V. parahaemolyticus* has been isolated from various locations in the marine environment with seasonal fluctuations in their abundance. Typically in the United States, *V. parahaemolyticus* can be detected in high densities in the water column during the warmer summer months and the bacteria often reside in the sediments during the colder winter months, with sea surface temperatures being strong predictors of *V. parahaemolyticus* abundance (Kaneko and Colwell 1973; Johnson, Bowers et al. 2012). During the summer, *V. parahaemolyticus* is commonly found attached to zooplankton as well as colonizing shellfish, such as oysters (Cook, Bowers et al. 2002; Cook, Oleary et al. 2002; Johnson, Bowers et al. 2012). This natural association between human pathogenic vibrios and shellfish, particularly oysters, presents a potential public health concern for individuals who consume oysters raw or partially cooked.

The Fisheries and Aquaculture Department, division of the Food and Agriculture Organization of the United Nations (FAO), estimates that more than 4.38 million tonnes of oysters are produced globally, more than any other fish or shellfish in aquaculture, and resulting in almost $4 billion in revenues. The West Coast produces approximately 60% of all the oysters consumed in the United States, primarily the Pacific oyster, *Crassostrea gigas* (FAO 2005-2012). Pacific oysters were originally cultivated in Japan and have subsequently been spread globally. They

were first introduced to the Americas in the 1920. Pacific oysters are typically found in estuarine environments and are highly adaptable. The newly sequenced genome of the Pacific oyster has provided insight into the abilities of the sessile animals to survive in turbulent intertidal zones (Zhang, Fang et al. 2012). They can survive in salinities between 10 ppt and 35 ppt and tolerate a broad temperature range, from -1.8 to 35°C (FAO 2005-2012). Since *Vibrio* species, including *V. parahaemolyticus*, naturally inhabit waters surrounding oyster beds, there is a potential for seafood-borne illness associated with the consumption of these animals.

V. parahaemolyticus has been attributed to seafood-borne illness in Japan for decades and has become a leading cause of seafood-borne illness in the world (Broberg, Calder et al. 2011; Shinoda 2011). According to the FoodNet Report 2010 from the CDC, the incidence of *Vibrio* infections has been increasing while other food-borne pathogens are steadily decreasing. In the United States, numerous *V. parahaemolyticus* outbreaks have occurred, particularly from the consumption of oysters, and the incidence of illness is seasonal with highest prevalence during the summer months (CDC 1998; CDC 1998; CDC 1999; CDC 1999; CDC 2006). In particular, many of the outbreaks have been connected with oyster production in the Pacific Northwest (PNW) (CDC 1998; CDC 2006). The largest reported outbreak of laboratory culture-confirmed *V. parahaemolyticus* in North America occurred from May through December of 1997 and resulted in 209 cases of gastroenteritis and one death associated with consumption of raw oysters harvested from California, Oregon, Washington and British Columbia. This resulted in an advisory to cook any

molluscan shellfish harvested from the PNW to reduce the incidence of illness. Oyster beds implicated in the outbreak were closed from mid-August to the end of September. From May-September 1997, the sea surface temperatures were 1-5°C higher than the same period of time the previous year (CDC 1998), possibly contributing to the outbreak as a result of increased abundance of *V. parahaemolyticus* in the environment. *V. parahaemolyticus* was isolated from oysters in the implicated oyster farms and ranged from less than 200 colony-forming units (CFU) to more than 11,000 CFU per gram of oyster. At this point, the infectious dose of *V. parahaemolyticus* was considered to be equal to or greater than 100,000 CFU and regulations allowed the sale of oyster with less than 10,000 CFU per gram of oyster. Prior to this, the largest outbreak of *V. parahaemolyticus* in North America occurred in 1982 with 10 culture-confirmed cases (CDC 1998). In 2006, another large, national outbreak of *V. parahaemolyticus* associated with shellfish occurred, resulting in 177 cases linked consumption of clams and oysters harvested from Washington and British Columbia. Shellfish harvest areas implicated in this outbreak were routinely tested and found to contain acceptable levels of *V. parahaemolyticus* prior to the outbreak, and eight harvest areas in Washington were closed as a result of being associated with outbreak (CDC 2006). Thus, *V. parahaemolyticus* outbreaks are not only a concern from a public health standpoint, they also present a significant economic burden on a lucrative aquaculture industry thriving on the West Coast. With the steady increase in *Vibrio* infections occurring in the US each year, focus

must be directed at tackling this significant health threat to reduce seafood-borne illness and economic loss.

Previous studies have shown a link between increased sea surface temperatures and higher incidence of *V. parahaemolyticus* in the environment, particularly the warmer summer months (Kaneko and Colwell 1973; Johnson, Bowers et al. 2012). This direct correlation has been the focus of modeling strategies to predict *V. parahaemolyticus* incidence in oysters and has been suggested to as a factor to be taken into considering during harvests (Fernandez-Piquer, Bowman et al. 2011) (DePaola, Hopkins et al. 1990) As evident with the outbreak in the PNW in 1997, a 1-5°C increase in sea surface temperatures can result in a *V. parahaemolyticus* outbreak (CDC 1998). A similar incidence occurred on a cruise ship in Alaska in 2004 (McLaughlin, DePaola et al. 2005). Animals harvested from a farm in Alaska that maintained sea surface temperatures greater than 15°C produced the oysters responsible for the *V. parahaemolyticus* outbreak. This further illustrates the seasonality of *V. parahaemolyticus* infections, although it is rather troublesome considering the prediction that sea surface temperatures may increase with climate change. Therefore, the incidence of *V. parahaemolyticus* infections may increase in the future if ocean waters continue to warm as well as bring *V. parahaemolyticus* illness to regions that may not have had issues in the past. Continued monitoring of the *V. parahaemolyticus* in the environment may aid in controlling outbreaks but post-harvest processing strategies may be best option for controlling *V. parahaemolyticus* in oysters.

Multiple strategies have been employed to monitor *V. parahaemolyticus* in seafood and to reduce this naturally occurring bacterium from the tissues of oysters to decrease the risk of seafood-borne illness. The traditional method for enumeration of *V. parahaemolyticus* in oysters is through culture-based methods, such as direct plate counts or the most-probably number method (MPN) (FDA 1998). More sophisticated methods for rapid identification of pathogenic *V. parahaemolyticus* strains have been developed such as the use of microarray technology combined with culture-based techniques to increase the sensitivity of the assay (Chen, Yu et al. 2011) or the use of real-time polymerase chain reaction (PCR) (Rizvi, Panicker et al. 2006; Nordstrom, Vickery et al. 2007). In addition to detection of bacteria in oysters, different post-harvest processing methods have been developed to reduce the amount of bacteria in harvested oysters as well as prevent an increase in the bacterial populations prior to consumption. *V. parahaemolyticus* is capable of multiplying in shellstock oysters if improperly maintained (Johnson, Salinger et al. 1973). Naturally occurring *V. parahaemolyticus* can increase by 50-fold in oysters during storage at 26°C for 10hrs while storage at 3°C for 14 to 17 days can reduce the bacterial amounts by six-fold (Gooch, DePaola et al. 2002). Thus, it has been recommended to rapidly put oysters on ice to prevent increases in bacterial populations post harvest.

Another valid method for reducing bacterial populations in oysters and other shellfish is through depuration, which is a controlled process that allows animals exposure to clean seawater to purge their tissues of contaminating bacteria (Fig. 1.2). This method is successful in reducing the amount of fecal contaminants, such as *E.*

coli, that molluscan shellfish may encounter in the marine environment due to run-off (Croci, Suffredini et al. 2002). Unfortunately, *Vibrio* species are highly resistant to depuration under conditions that significantly reduce the numbers of *E. coli* contaminants (Marino, Crisafi et al. 1999; Croci, Suffredini et al. 2002; Marino, Lombardo et al. 2005), suggesting that *Vibrio* species, including *V. parahaemolyticus*, possess specific factor(s) that allow them to resist the depuration process. Reducing the temperature of the seawater during depuration has been shown to significantly reduce the amount of artificially contaminated *V. parahaemolyticus* present in oysters (Chae, Cheney et al. 2009; Phuvasate, Chen et al. 2012). However, it remains to be seen how effective temperature alone is in reducing naturally occurring *V. parahaemolyticus* because adjusting temperature was ineffective at reducing naturally occurring *V. vulnificus* in oysters (Lewis, Rikard et al. 2010). Thus, identifying *V. parahaemolyticus* factors involved in efficient colonization of the Pacific oyster could ultimately lead to novel intervention strategies, such as new depuration parameters, that would successfully and consistently reduce *V. parahaemolyticus* in the tissues of the Pacific oyster.

Figure 1.2. Example of a small-scale oyster depuration system.
Flow-thru depuration systems at Hatfield Marine Science Center, Newport, OR. Filtered seawater, combined with algae is pumped through the lines and the effluent drained out the bottom where they are treated in accordance with quarantine procedures at HMSC.

Research objectives

Relatively little is known about the *V. parahaemolyticus* factors involved in colonizing the Pacific oyster. This study sought out to identify specific adherence factors hypothesized to be important in *V. parahaemolyticus* resistance to oyster depuration to identify new ways for improving post-harvest processing techniques. To achieve this, we identified genes in the *V. parahaemolyticus* chromosome that could contribute to successful oyster colonization. These gene targets included pili, both Type I and Type IV, flagella, polar and lateral, T3SSs and phase variation. Deletions were constructed in these genes and in the process of deleting *mshA* and *pilA*, which encode MSHA and PilA respectively, striking sequence heterogeneities were observed between the type strain used ATCC17802, the original isolate from

Japan 1950, and the published strain RIMD2210633, a current pandemic isolate O3:K6. Based on these findings, we expanded our initial observations to examine the MSHA and PilA sequences in two other human pathogenic vibrios, *V. cholerae* and *V. vulnificus*, to determine if this allelic diversity was unique to *V. parahaemolyticus*. The results of this investigation are presented in Chapter 2.

Following the successful creation of gene deletions of Type I and Type IV pili in ATCC17802 and acquiring flagella and T3SSs mutants from Dr. Linda McCarter (University of Iowa), depuration studies were conducted to determine if these factors are involved in *V. parahaemolyticus* colonization of the Pacific oyster. During the depuration experiments with the mutant strains, differences in bacterial retention were observed for different parental strains, ATCC17802 and BB22 (OP). This also led us to investigate differences between these two *V. parahaemolyticus* strains and 5 clinical *V. parahaemolyticus* FDA isolates for bacterial retention in the oyster as well as biofilm formation under different laboratory conditions. The results of the investigation are presented in Chapter 3.

Overall, these investigations into the interactions between *V. parahaemolyticus* and the Pacific oyster are providing a starting point with which future studies can expand and ultimately increase the effectiveness of post-harvest processing of oysters to produce safer seafood products for the consumer.

Chapter 2

Sequence analyses of Type IV pili from *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*

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Abstract

Bacterial surface structures called pili have been studied extensively for their role as possible colonization factors. Most sequenced *Vibrio* genomes predict a variety of pili genes in these organisms including several types of Type IV pili. In particular, the mannose-sensitive hemagglutinin (MSHA) and the PilA pili, also known as the chitin-regulated pilus (ChiRP), are Type IVa pili commonly found in *Vibrio* genomes and have been shown to play a role in the colonization of *Vibrio* species in the environment and/or host tissue. Here, we report sequence comparisons of two Type IVa pilin subunit genes, *mshA* and *pilA*, and their corresponding amino acid sequences, for several strains from the three main human pathogenic *Vibrio* species, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. We identified specific groupings of these two genes in *V. cholerae*, whereas *V. parahaemolyticus* and *V. vulnificus* strains had no apparent allelic clusters and these genes were strikingly divergent. These results were compared with other genes from the MSHA and PilA operons as well as another *Vibrio* pili from the Type IVb group, the toxin co-regulated pilus (TCP) from *V. cholerae.* Our data suggests that a selective pressure exists to cause these strains to vary their MSHA and PilA pilin subunits. Interestingly, *V. cholerae* strains possessing TCP have the same allele for both *mshA* and *pilA*. In contrast, *V. cholerae* isolates without TCP have polymorphisms in their *mshA* and *pilA* sequences similar to what was observed for both *V. parahaemolyticus* and *V. vulnificus*. This data suggests a possible linkage between host interactions and maintaining a highly conserved Type IV pili sequence in *V. cholerae*. Although the mechanism underlying

this intriguing diversity has yet to be elucidated, our analyses are an important first step towards gaining insights into the various aspects of *Vibrio* ecology.

Introduction

Vibrio species are marine bacteria that naturally inhabit aquatic environments worldwide and are commonly associated with marine organisms. Some *Vibrio* species are pathogenic bacteria capable of producing life-threatening infections in humans typically following consumption of contaminated food, including seafood. Although the specific factors that contribute to the pathogenicity of vibrios in humans are well studied, little is known about the bacterial factors involved in the association of the bacteria with environmental organisms.

Bacteria display a variety of mechanisms that enable them to specifically interact with target cells. Many bacteria produce hair-like surface structures, called pili or fimbriae, which are often important for survival (Mandlik, Swierczynski et al. 2008; Kline, Falker et al. 2009; Proft and Baker 2009). These adhesins have been clustered into groups based on amino acid sequence similarities among their pilin subunits (Craig, Pique et al. 2004). One type of pili, the Type IV group, are known to be involved in adhesion, immune escape, microcolony formation, transformation, phage transduction, (Craig, Pique et al. 2004) and are commonly found in Gram-negative bacteria, including numerous pathogens (Strom and Lory 1993; Craig, Pique et al. 2004). Type IV pili are known to assist many bacterial species in survival in various environments, ranging from attachment to a variety of surfaces for biofilm

formation (Watnick, Fullner et al. 1999; Enos-Berlage, Guvener et al. 2005; Shime-Hattori, Iida et al. 2006; Barken, Pamp et al. 2008) to colonizing the host (Zhang, Tsui et al. 2000; Hang, John et al. 2003; Stabb and Ruby 2003; Paranjpye and Strom 2005; Wu, Zhang et al. 2005; Paranjpye, Johnson et al. 2007; Forslund, Salomonsson et al. 2010; Mahmoud and Koval 2010). These pili begin as prepilins possessing a hydrophilic leader peptide and are processed by a unique peptidase that cleaves the leader sequence to form a mature pilin protein (Pelicic 2008). After processing, mature pilin subunits assemble together to form pili through interactions between the conserved N-termini in the pilin cores, leaving the variable C-terminal regions to interact with the environment (Craig, Pique et al. 2004). Type IV pili are divided into two subclasses based on differences in amino acid sequence and length. Type IVa pili have both a shorter leader peptide and mature protein sequence while Type IVb pili have considerably longer leader sequences and overall length (Strom and Lory 1993; Craig, Pique et al. 2004; Pelicic 2008). In addition to similarities in their amino acid composition, all Type IV pili appear to have analogous architecture (Craig, Pique et al. 2004).

When examining the genomes of Gram-negative bacteria possessing Type IV pili, Type IVa pili biogenesis genes are scattered throughout the genome but the genes or gene clusters are almost always flanked by the same genes, typically housekeeping genes. In addition, homologous gene sets for Type IVa pili are found in virtually identical locations throughout more than 150 sequenced genomes. Considering these genes have not be found on any identifiable pathogenicity island, it

suggests that these pili are ancient to many of the bacterial phyla possessing these genes (Pelicic 2008). In contrast, Type IVb pili genes are fewer in number than Type IVa genes and are typically found clustered within the genome. Moreover, the gene sequence order does not appear to be conserved amongst different organisms possessing the Type IVb pili except for the universally conserved core proteins. Also, when comparing N-terminal sequence homology, Type IVa pilin subunits are more similar amongst themselves than to Type IVb pilins or within the Type IVb pili group. Furthermore, Type IVa pili occur in bacteria with a broad host range while Type IVb pili have only been identified in colonizers of the human intestinal tract (Craig, Pique et al. 2004).

Vibrio species possess many type IV pili from both Type IVa and b groups but only a select few have been studied for their role in environmental and/or host survival. One thoroughly studied pili from the Type IVb group is the toxin co-regulated pilus (TCP) from *Vibrio cholerae* and it is known for its key role in virulence (Taylor, Miller et al. 1987; Attridge, Manning et al. 1996; Tacket, Taylor et al. 1998). It is expressed by *V. cholerae* classical and El Tor biotypes from the O1 and O139 serogroups (Gaddy, Tomaras et al. 2009). TCP is composed of TcpA subunits and appears as thick bundles on the Electron Microscope (Craig, Pique et al. 2004). TcpA is processed by a TCP specific signal peptidase, TcpJ, to form mature pilin subunits for assembly (Kaufman, Seyer et al. 1991; Manning 1997). The structure of TCP consists of the conserved N-terminal α-helices of TcpA buried in the core of the pilus, maximizing contact between subunits to provide overall strength.

The structurally variable regions of the pilins interact to hold the core units together and coat the surface where interactions take place with the environment, i.e. the intestines (Craig, Pique et al. 2004). In addition to colonization, TCP is the receptor for the CTXΦ phage (Faruque, Kamruzzaman et al. 2003; McQueary and Actis 2011).

An additional well studied *V. cholerae* Type IV pilus is the mannose-sensitive hemagglutinin (MSHA), which belongs to the Type IVa group. When examining operon composition, MSHA in *V. cholerae* consists of two operons where one operon encodes five prepilin subunits, including the major pilus subunit MshA, and the other contains genes involved in assembly and secretion (Marsh and Taylor 1999). In *V. cholerae,* the PilD peptidase has been shown to process the MshA subunits for assembly of the mature pilus structure (Marsh and Taylor 1998; Fullner and Mekalanos 1999). The MSHA pilus hemagglutinates red blood cells (Hanne and Finkelstein 1982; Jonson, Sanchez et al. 1989) and is a receptor for filamentous phage (Jouravleva, McDonald et al. 1998; Faruque, Bin Naser et al. 2005; Campos, Martinez et al. 2010). It has been studied extensively in *V. cholerae* to identify any involvement in host colonization (Attridge, Manning et al. 1996; Thelin and Taylor 1996; Tacket, Taylor et al. 1998). In *V. cholerae*, only the El Tor biotypes produce functional MSHA pili (Hanne and Finkelstein 1982; Jonson, Sanchez et al. 1989) and during human colonization studies, the protein was repressed (Hsiao, Liu et al. 2006). Expression of the MSHA pilus was tightly regulated so that when TCP was expressed, the MSHA protein was repressed; therefore, the MSHA pilus is considered

an anti-colonization factor in human disease (Hsiao, Toscano et al. 2008). When the MSHA pilus was constitutively expressed during colonization, it resulted in immune system recognition (Hsiao, Liu et al. 2006). Thus, the MSHA pilus does not appear to be a virulence factor for *V. cholerae*, suggesting expression of the gene product is for utilization in the environment. Studies have shown that the MSHA pilus is used to adhere to zooplankton exoskeletons as a survival strategy in the aquatic environment (Chiavelli, Marsh et al. 2001; Moorthy and Watnick 2004), presumably by forming biofilms. *V. cholerae* and *Vibrio parahaemolyticus* are known to use the MSHA pilus to form biofilms on various surfaces (Watnick, Fullner et al. 1999; Moorthy and Watnick 2004; Shime-Hattori, Iida et al. 2006), including chitin (Meibom, Li et al. 2004), which provides some supporting evidence for the role of the MSHA pilus in environmental survival.

Another pilus found in *Vibrio* spp. is the Type IVa PilA pilus, also known as the chitin-regulated pilus (ChiRP). The PilA operon is composed of five open reading frames that constitute a single operon, consistent with other Type IVa pili (Fullner and Mekalanos 1999). A mature PilA pilus is composed of PilA subunits that were processed by the PilD peptidase (Fullner and Mekalanos 1999), the same peptidase that processes the MshA pilin subunits (Marsh and Taylor 1998; Fullner and Mekalanos 1999). The PilD peptidase is the fourth open reading frame in the PilA operon (Fullner and Mekalanos 1999). The PilA type IVa pilus is an integral player in the *V. cholerae* chitin utilization program (Meibom, Li et al. 2004). Expression of the PilA protein has been shown to be induced by chitin in both *V. cholerae* (Meibom, Li

et al. 2004) and *V. parahaemolyticus* (Shime-Hattori, Iida et al. 2006). PilA is involved in biofilm formation (Paranjpye and Strom 2005; Shime-Hattori, Iida et al. 2006), adherence to human epithelial cells (Paranjpye and Strom 2005), and colonization of oysters (Paranjpye, Johnson et al. 2007). It has been implicated as a virulence factor for *V. vulnificus* (Paranjpye and Strom 2005), although direct evidence of its role in virulence has not been clearly described in other human pathogenic vibrios.

Taken together, the studies of the Type IVa pili MSHA and PilA in various *Vibrio* spp. suggest that these proteins might be utilized by vibrios for environmental survival by attaching to chitinous substrates such as zooplankton. In contrast, the Type IVb pilus, TCP, from *V. cholerae* is critical for host colonization and has not be implicated in environmental survival, pointing out the possibility of two very distinct roles for the different subclasses of Type IV pili.

During our efforts to investigate the roles of MSHA and PilA in *V. parahaemolyticus* colonization of the Pacific oyster, *Crassostrea gigas*, we noted sequence heterogeneities in these genes. This led us to examine these genes in other human pathogenic *Vibrio* species, such as *V. cholerae* and *V. vulnificus*. Here we present a comparative sequence analysis of the *mshA* and *pilA* pilin genes from several strains of *V. cholerae, V. parahaemolyticus,* and *V. vulnificus*. These sequence analyses suggest that a selective environmental pressure has been applied to these genes, resulting in the observed sequence heterogeneities for all three *Vibrio* species examined.

Materials and Methods

Bacterial strains

Thirteen of the *V. parahaemolyticus* bacterial strains sequenced were kindly provided by Dr. Yi-Cheng Su, Oregon State University Seafood Laboratory, Astoria, OR. Genomic DNA for five tdh/trh negative strains of *V. parahaemolyticus* was obtained from Dr. Narjol-Gonzalez-Escalona, FDA, College Park, MD. Genomic DNA for ten of the *V. vulnificus* strains sequenced were provided by Dr. Paul Gulig, University of Florida, Gainesville, FL. Five of the *V. vulnificus* strains sequenced were provided by Dr. Kathy O'Reilly, Oregon State University, Corvallis, OR. Bacterial strains were grown on LB agar supplemented with sodium chloride to a final concentration of 2%. All strains used in this study are listed in Table 2.1.

Table 2.1. Strains used in this study

Vibrio cholerae

Strain	Serogroup	Biotype	Relevant Genotype	Strain Information	GenBank (PilA,MshA)
MAK757	O1, Ogawa	El Tor	TCP+, CTxA+	pre-7th pandemic patient isolate, mildly toxigenic, from Celebes Islands in 1937	ZP_01954444, ZP_01953747
NCTC 8457	O1, Inaba	El Tor	TCP+,CT-	Saudi Arabia patient 1910, non-pandemic	ZP_01971084, ZP_01970913
B33	O1, Ogawa	hybrid Classical/El Tor	TCP+,CTxA+	clinical isolates Beira, Mozambique 2004	ZP_04401766, ZP_01974939
O395	O1, Ogawa	Classical	TCP+, CT+	clinical isolate, strain of the 6th pandemic in South Asia, isolated in 1965 from India	YP_001217923, YP_001218677
2470-80	O1, Inaba	El Tor	TCP+, CT-	nontoxigenic environmental water isolate from the Gulf Coast, 1980, clonal with TCP+ CT+ isolates	ZP_01677376, ZP_01677345
N16961	O1, Inaba	El Tor	TCP+,CT+	Clinical isolate from Bangladesh, 1971	NP_232053, NP_230063
V51	O141		TCP+,CT-	Clinical Isolate from the United States, 1987	ZP_00748678, ZP_00749816
V52	O37		TCP+, CT+	clinical isolate from Sudan, limited epidemic, not endemic	ZP_00746513, ZP_00747249
MO10	O139		TCP+, CT+	Clinical isolate from India and Bangladesh outbreak 1992, early isolate of O139 emergence	ZP_00758906, ZP_00758992
MZO-2	O14		TCP-, CT-	Clinical isolate from Bangladesh patients with diarrhea in 2001	ZP_01979911, ZP_01978309
1587	O12		TCP-, CT-	Clinical isolate from Lima, Peru, 1994, limited epidemic, not endemic, invasive	ZP_01949212, ZP_01949969
RC385	O135		TCP-, CT-	persistent and luminescent environmental plankton isolate, Chesapeake Bay, 1998	ZP_00751854, ZP_00753463
623-39	non-O1/-O139		TCP-,CT-	environmental water isolate from Bangladesh, 2002	ZP_01983325, ZP_01981981
AM-19226	O39		TCP-, CT-	Clinical isolate from Bangladesh, 2001	ZP_04962566, ZP_04962108
MJ-1236	O1, Inaba	El Tor "Matlab variant"	TCP+,CT+	clinical isolate from patients with acute diarrhea, Matlab, Bangladesh 1994	YP_002877671, YP_002876957
VL426	non-O1/-O139	Albensis	TCP-,CT-	diseased fish from Elbe River, Germany	ZP_04413813, ZP_04414508
TM 11079-80	O1, Ogawa	El Tor	TCP-,CT-	enviromental sewage isolate from Brazil, 1980	ZP_04410672, ZP_04409450
RC9	O1, Ogawa	El Tor	TCP+,CT+	clinical isolate from Kenya 1985	ZP_04408866, ZP_04409227
TMA21	non-O1/-O139		TCP-,CT-	environmental seawater isolate from Brazil, 1982	ZP_04403511, ZP_04402128
BX 330286	O1, Inaba	El Tor	TCP+,CT+	water isolate from Australia, 1986	ZP_04397022, ZP_04396555
CIRS 101	O1, Inaba	El Tor, classical ctxB	TCP+,CTxA+	Clinical isolate from Dhaka, Bangladesh 2002	ZP_05420851, ZP_05417751
M66-2	O1		TCP+,CT-	1937 outbreak Indonesia, pre-7th pandemic isolate	YP_002811095, YP_002809171
CT 5369-93			TCP-,CT-	sewage, Brazil 1993	ZP_06048448, ZP_06049688
RC27	O1	Classical	TCP+,CT-	Indonesia 1991	ZP_06036099, ZP_06035331
INDRE 91/1	O1, Inaba	El Tor	TCP+,CTxA+	Mexico 1991, first case of 7th pandemic in Mexico	ZP_06030661, ZP_06028721

Vibrio parahaemolyticus

Strain	Serotype	Relevant Genotype	Strain Information	GenBank (PilA,MshA)
RIMD 2210633	O3:K6	tdh+/trh-	clinical strain from Osaka, Japan 1996	NP_798902, NP_799077
Peru-466		tdh+/trh+		ZP_05904882, ZP_05905780
K5030		tdh+/trh+		ZP_05776528, ZP_05778018
AN-5034	O4:K68	tdh+/trh+	Bangladesh 1998	ZP_05891366, ZP_05889900
SFL1009	O1:K2	tdh+/trh+	isolated from sediment at Goose Point oyster farm, Willapa Bay, Washington, October 2002	JF923890, JF923914
SFL1027	O5	tdh+/trh+	isolated from sediment at Oregon Oyster farm Yaquina Bay, Oregon, December 2002	JF923885, JF923903
SFL1050	O1:K7	tdh+/trh+	isolated from sediment at Goose Point oyster farm, Willapa Bay, Washington, July 2003	JF923892, JF923904
SFL1079	O1:K4	tdh+/trh+	isolated from seawater at Goose Point oyster farm, Willapa Bay, Washington, July 2003	JF923894, JF923905
SFL1080	O5:K1	tdh+/trh-	isolated from sediment at Goose Point oyster farm, Willapa Bay, Washington, July 2003	JF923891, JF923906
10290	O4:K12	tdh+/trh+	1997 Washington outbreak strain	JF923888, JF923916
10292	O6:K18	tdh+/trh+	1997 Washington outbreak strain	JF923893, JF923915
BE98-2029	O3:K6	tdh+/trh-	1998 Texas outbreak strain	JF923886, JF923901
O27-1C1	O5:K15	tdh+/trh+	1997 Oregon outbreak strain	JF923887, JF923908
M25-0B	O4	tdh-/trh-	Environmental isolate from Washington, 1993	JF923900, JF923913
UCM-V586	O8:K22	tdh-/trh-	Environmental isolate from Spain, 2003	JF923899, JF923912
UCM-V441	O4	tdh-/trh-	Environmental isolate from Spain, 2002	JF923898, JF923911
049-2A3	O4:K29	tdh-/trh-	Environmental isolate from Oregon, 1997	JF923896, JF923909
357-99	O3	tdh-/trh-	Clinical isolate from Peru, 1999	JF923897, JF923910
ATCC 17802		trh+	Shirasu food poisoning, Japan 1965	JF923889, JF923907

Vibrio vulnificus

Strain	Capsule	vcg	Virulence Group	Strain Information	GenBank (PilA,MshA)
98-783 DP-A1	1	E	2	Environmental isolate	JF923941, JF923921
99-520 DP-B8	2	E	3	Environmental isolate	JF923932, JF923920
99-581 DP-C7	2	E	3	Environmental isolate	JF923930, JF923917
99-584 DP-B12	2	E	2	Environmental isolate	JF923931, JF923922
99-736 DP-C7	2	E	2	Environmental isolate	JF923934, JF923924
99-738 DP-B5	2	E	5	Environmental isolate	JF923942, JF923923
S1-13	1	E	4	Environmental isolate	JF923933, JF923919
ATL-9580	1	C	5	Clinical isolate	JF923935, JF923918
CMCP6	1	C	4	Clinical isolate	NP_760518, NP_760356
YJ016	2	C	5	Clinical isolate, Taiwan	NP_935571, NP_935733
CP Mussel 10 PT					JF923939, JF923929
95-10-15 PT				isolated 10/18/05	JF923938, JF923928
960926 -1/4c PT				isolated 10/19/05	JF923937, JF923927
ATCC 27562				clinical isolate, Florida	JF923936, JF923926
OLOL-1				Katrina, isolated 10/18/05	JF923940, JF923925

Sequencing

Genomic DNA from *V. parahaemolyticus* and *V. vulnificus* strains were isolated using the Qiagen DNeasy Blood and Tissue Kit, following the protocol for DNA

isolation included in the kit. Primers for sequencing each gene were designed for the region approximately 100 base pairs upstream from the start codon and 100bp downstream of the stop codon for the gene of interest (Table 2.2). Polymerase chain reaction (PCR) was conducted using Invitrogen Platinum HiFi Supermix, following their standard protocol for PCR. PCR samples were quantified using the NanoDrop Spectrophotometer ND-1000. Sanger sequencing reactions for *V. parahaemolyticus* and *V. vulnificus* PCR products were performed at the Center for Genomic Research and Bioinformatics (CGRB), Oregon State University, Corvallis, OR.

Table 2.2. Primers used in this study

Locus	Gene	Primer name	Primer Sequence
pilA	VP2523	shorter5' VP2523 SpeI P1	5'-GATAATTGGGGGCATATCAACCTCTATAGTTTG-3'
		New3'VP2523 NotI P4	5'-ACCATGGGTGCATTCGTTGCAACCATCTGGATT-3'
		VP2523 sequence	5'-GCAACTTTCTACCAAAGAGTTTTACCTCACTCG-3'
	VV2278	VV pilA seq primer	5'-GTAAGTAACCAGATGTAAATAAAG-3'
		3'VV pilA seq P2	5'-GCCAAAAATCGCGCTTAGCTG-3'
mshA	VP2698	New5'VP2698 SpeI P1	5'-CGTAAACGCATTAAAGCCGCGATGCGCTATCCG-3'
		New3'VP2698 NotI P4	5'-CCATTAAGGTGAAACCACGAGTTTTCATTCAGT-3'
		5'VP2698 seq2	5'-TCGTCATTCTGCTCAAGCGGTAGA-3'
	VV2940	VV mshA seq primer	5'-CAAATGCTAAATGTACTTATATTC-3'
		VV 3' mshA seq P2	5'-CTGCCAGTGCCAATATAGCGACTG-3'

In silico analyses

The *in silico* sequence data for all the *V. cholerae* strains and additional *V. parahaemolyticus* and *V. vulnificus* strains were obtained from the Department of Energy Joint Genome Institute website: http://img.jgi.doe.gov/cgi-bin/pub/main.cgi. The *V. parahaemolyticus* and *V. vulnificus* sequenced DNA was translated into their predicted amino acid sequences using SeqTool and sequence alignments were created in ClustalW at the bioinformatics website for the CGRB:

http://bioinfo.cgrb.oregonstate.edu/. Maximum Likelihood phylogenetic trees were constructed using the Mega 5 program: http://www.megasoftware.net/ using the Tamura-Nei model with nucleotide substitutions. Bootstrap values were calculated with 500 replicates. For the analysis of synonymous and nonsynonymous substitutions, calculations were made using SNAP, the Synonymous Non-synonymous Analysis Program: www.hiv.lanl.gov (Korber 2000). The program is based on the Nei and Gojobori (Nei and Gojobori 1986; Thompson, Neto et al. 2011) method for calculating synonymous and nonsynonymous rates of substitution with the incorporation of Ota and Nei (Ota and Nei 1994) statistics. The package is described by Ganeshan et. al (Ganeshan, Dickover et al. 1997).

Results

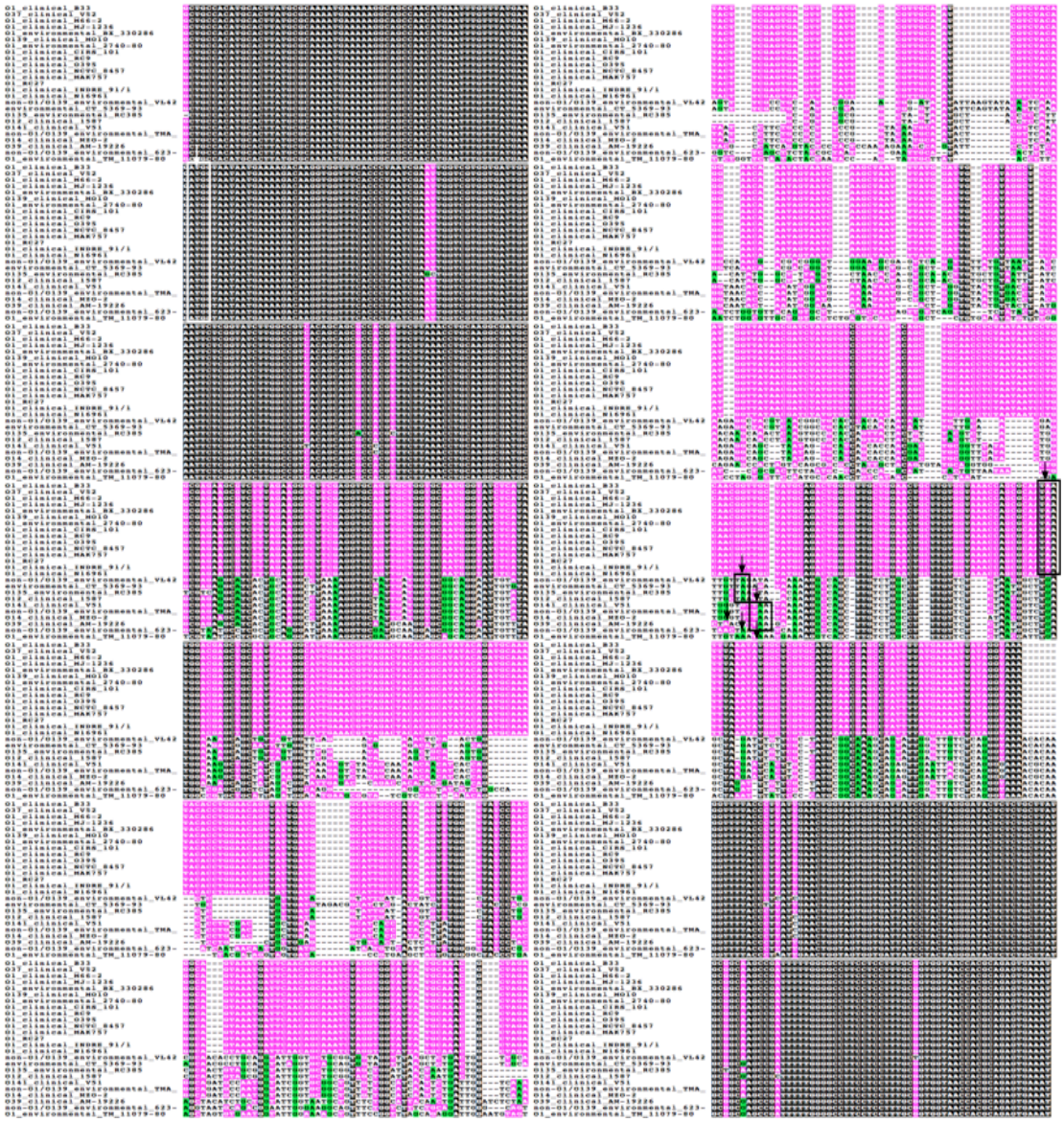
Sequence alignments

Overall, the sequence alignments for the DNA encoding the *mshA* and *pilA* genes from different strains of *V. cholerae, V. parahaemolyticus* and *V. vulnificus* showed considerable sequence heterogeneity (Supplemental Fig. S2.1 and S2.2). Although the immediate 5' regions are highly conserved in both genes, most of the gene sequences varied depending on the strain. Interestingly, *V. cholerae* exhibited distinct groupings for both genes, separating most clinical isolates from environmental isolates. In contrast, *V. parahaemolyticus* and *V. vulnificus* strains did not appear to group based on isolate origin or any other phenotype. Sequence alignments of the predicted amino acid sequences of MSHA and PilA from *V. cholerae, V.*

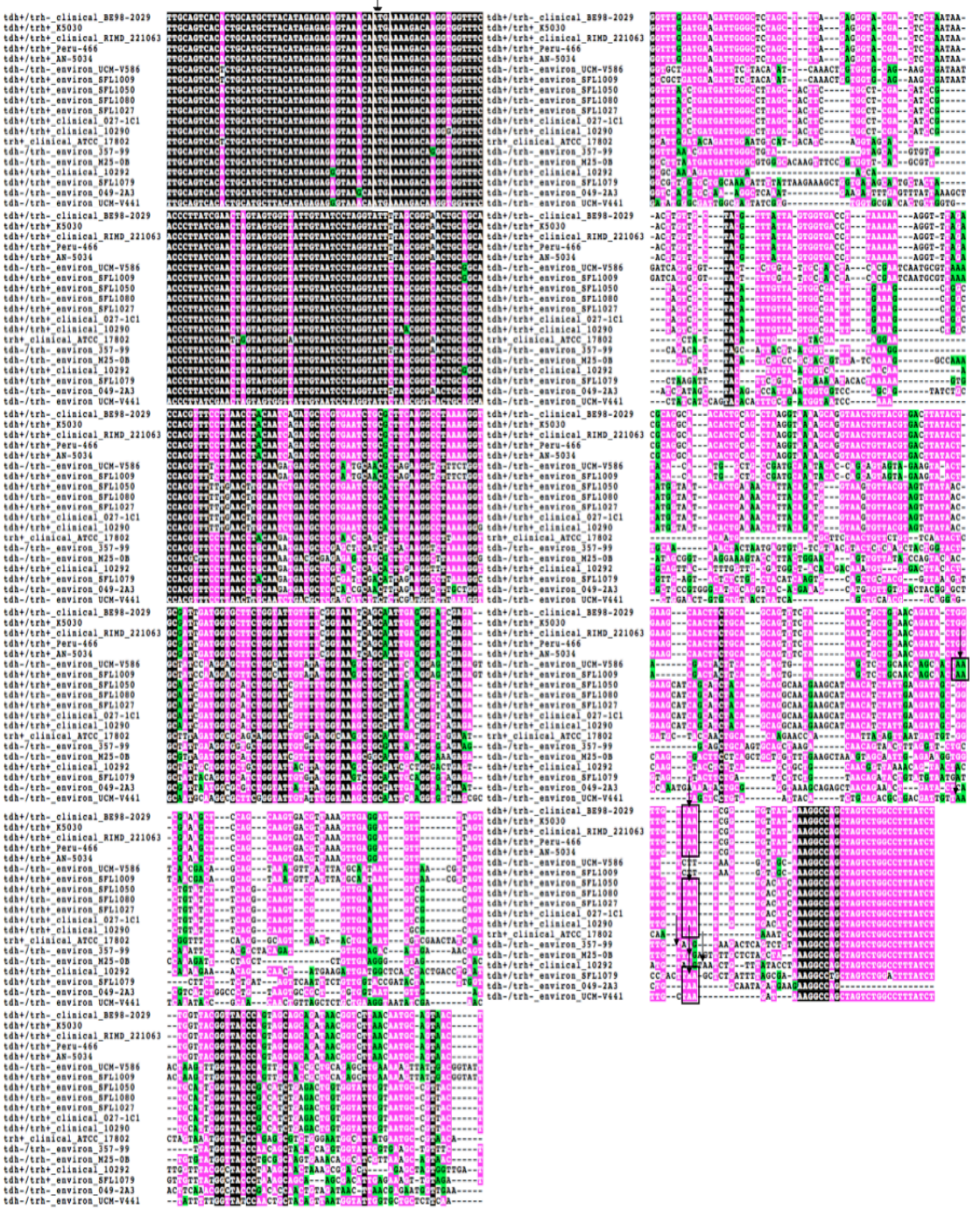
parahaemolyticus, and *V. vulnificus* are shown in Figures 2.1 and 2.2. For *V. parahaemolyticus* and *V. vulnificus,* the predicted amino acids sequences for MSHA and PilA from both environmental and clinical isolates displayed notable sequence heterogeneity. With *V. cholerae* strains, most clinical isolates had conserved sequences for both MSHA and PilA. Most environmental isolates exhibited marked sequence heterogeneity, comparable to what was observed for the *V. parahaemolyticus* and *V. vulnificus* isolates.

Supplemental Figure S2.1. DNA sequence alignments of *mshA* from *Vibrio cholerae* (A), *Vibrio parahaemolyticus* (B), and *Vibrio vulnificus* (C). The DNA sequences for *mshA* from *V. cholerae* (A), *V. parahaemolyticus* (B), and *V. vulnificus* (C) were aligned using the ClustalW program. White indicates normal residues. Green are similar residues. Pink are identical residues. Black indicate globally conserved residues. The white arrow indicates the start codon of the gene and the white box encases all the start codons of the strains in the alignment. The black arrows and boxes indicate the stop codons for the strains in the alignment.

Supplemental Figure. S2.1. A



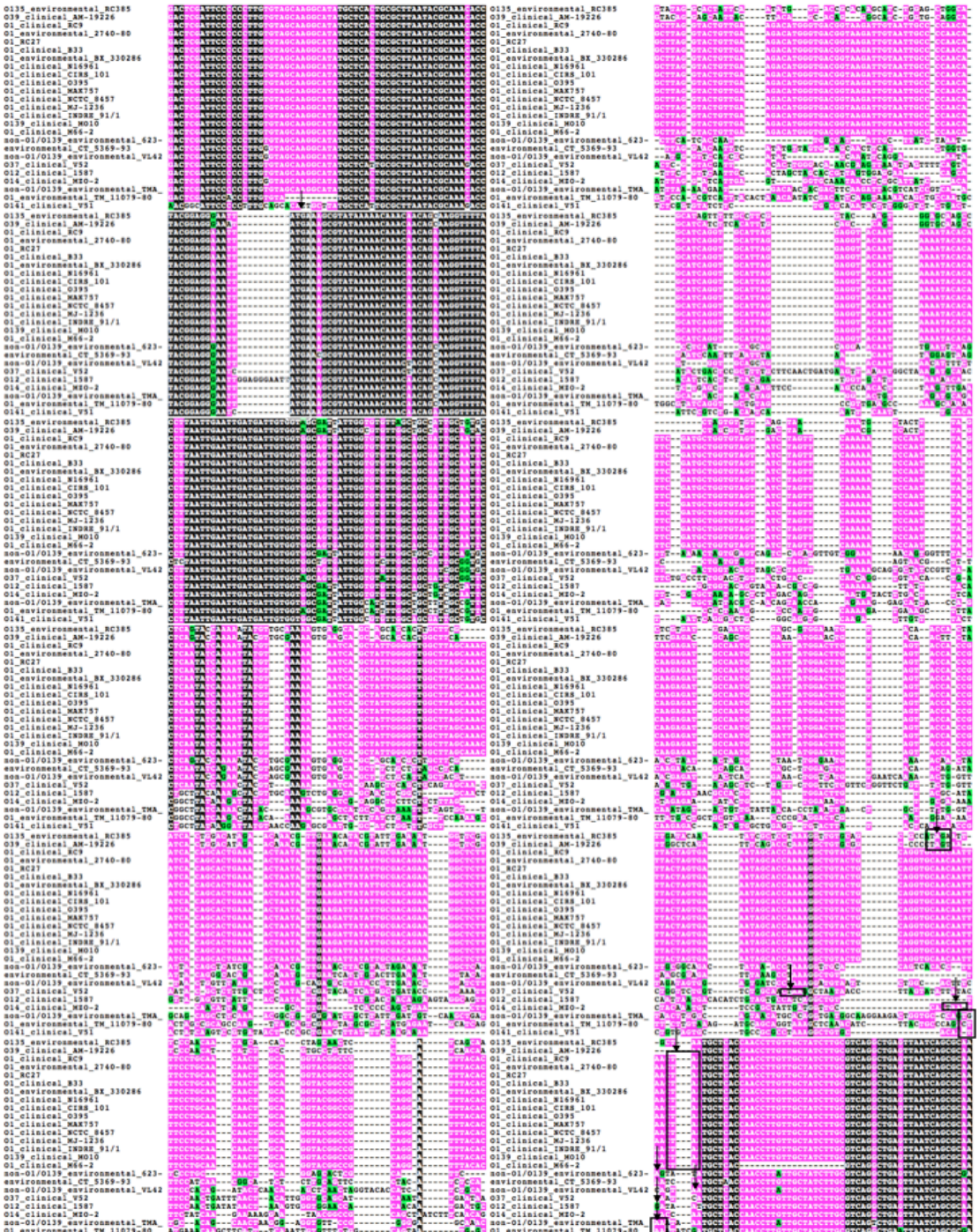
B



Supplemental Figure S2.1. DNA sequence alignments of *mshA* from *Vibrio cholerae* (A), *Vibrio parahaemolyticus* (B), and *Vibrio vulnificus* (C).

Supplemental Figure S2.2. DNA sequence alignments of *pilA* from *Vibrio cholerae* (A), *Vibrio parahaemolyticus* (B), and *Vibrio vulnificus* (C). The DNA sequences for *pilA* from *V. cholerae* (A), *V. parahaemolyticus* (B), and *V. vulnificus* (C) were aligned using the ClustalW program. White indicates normal residues. Green are similar residues. Pink are identical residues. Black indicate globally conserved residues. The white arrow and box indicates the start codon of the genes in the alignment. The black arrows and boxes indicate the stop codons for the strains in the alignment.

Supplemental Figure. S2.2. A



B

Supplemental Figure S2.2. DNA sequence alignments of *pilA* from *Vibrio cholerae* (A), *Vibrio parahaemolyticus* (B), and *Vibrio vulnificus* (C).

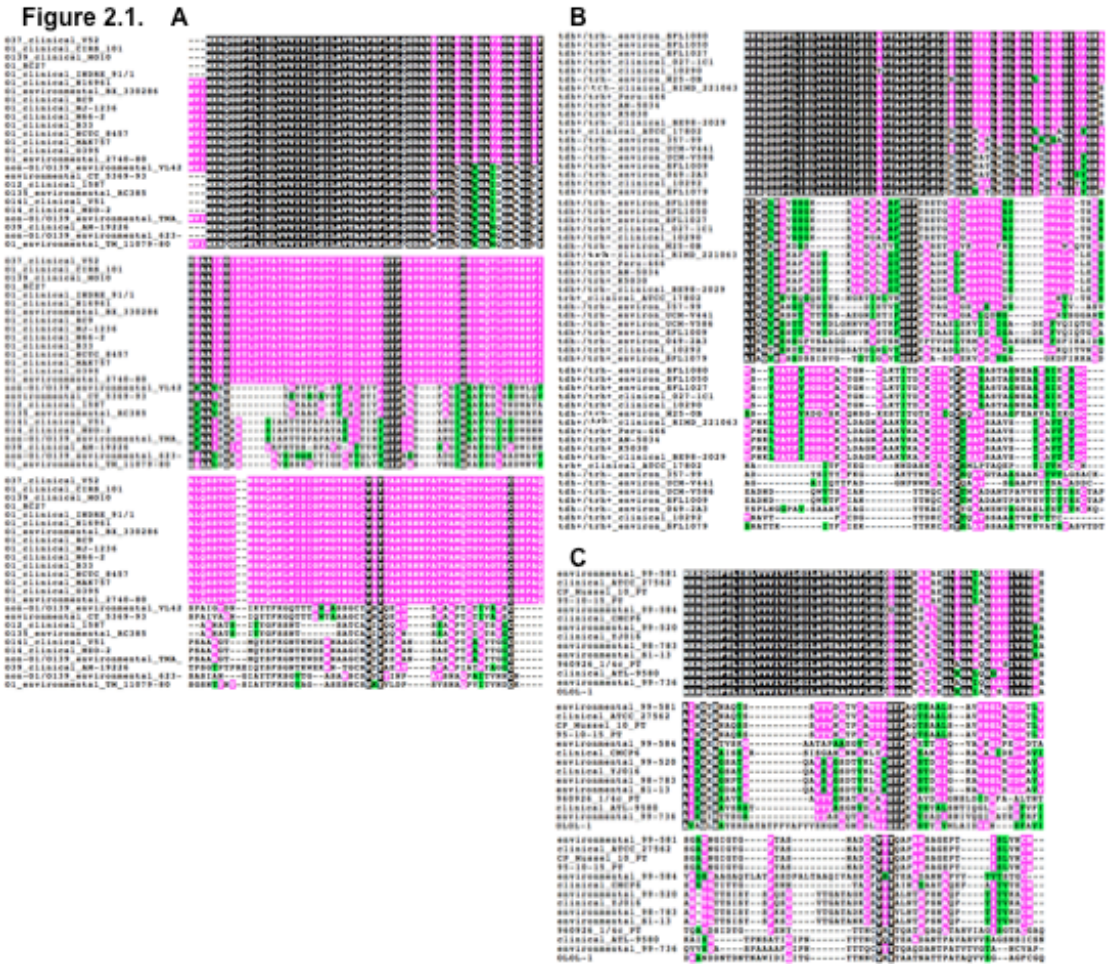


Figure 2.1. Amino acid sequence alignment of MSHA from *Vibrio cholerae* (A), *Vibrio parahaemolyticus* (B), and *V. vulnificus* (C). The predicted amino acid sequence alignments of MshA for *V. cholerae* (A), *V. parahaemolyticus* (B), and *V. vulnificus* (C) were constructed using the ClustalW program. White indicates normal residues. Green are similar residues. Pink are identical residues. Black indicates globally conserved residues.

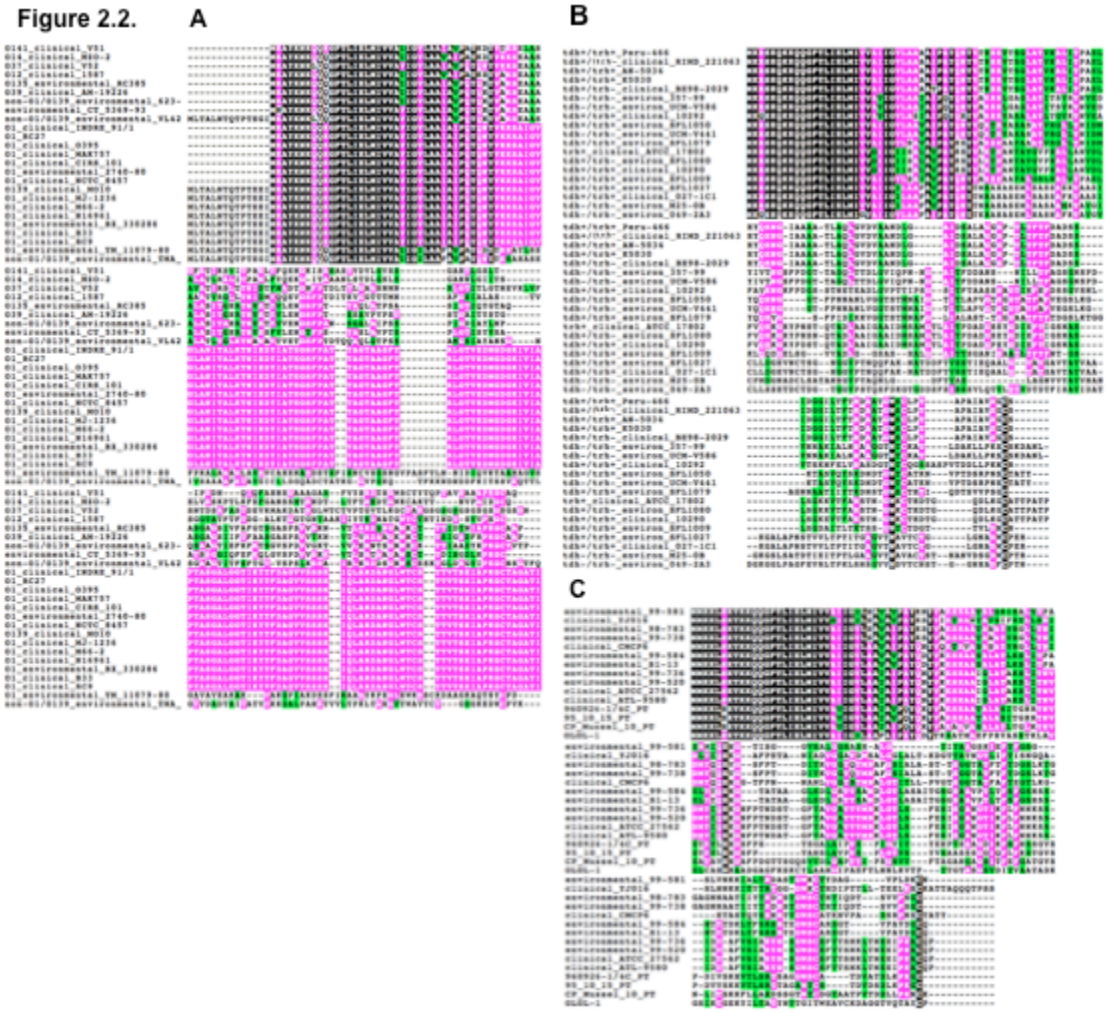


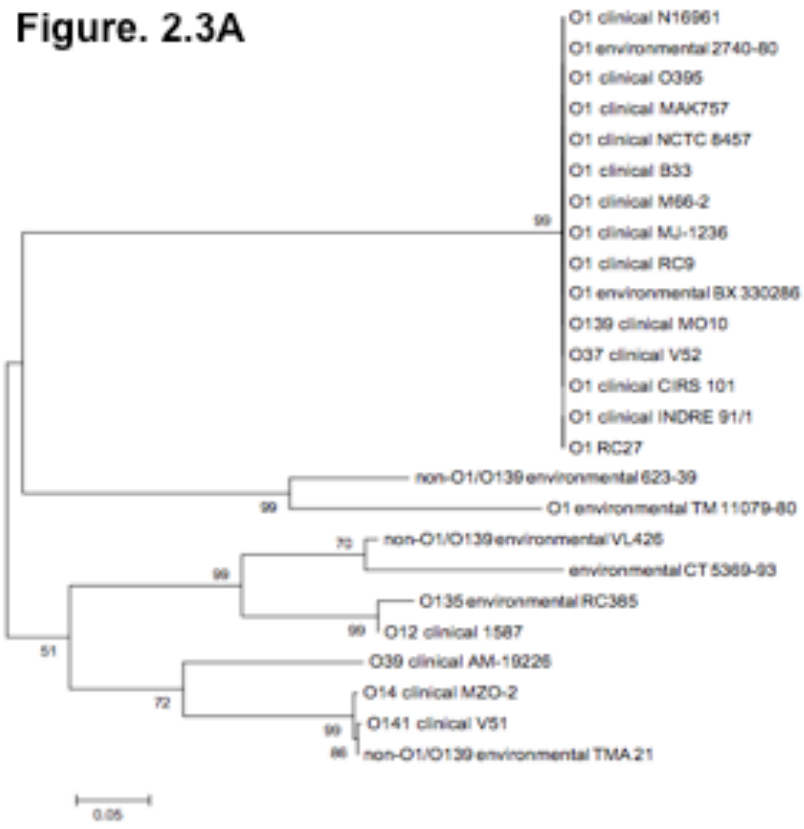
Figure 2.2. Amino acid sequence alignment of PilA from *Vibrio cholerae* (A), *Vibrio parahaemolyticus* (B), and *Vibrio vulnificus* (C). The predicted amino acid sequences of PilA for *V. cholerae* (A), *V. parahaemolyticus* (B), and *V. vulnificus* (C) were aligned using the ClustalW program. White indicates normal residues. Green are similar residues. Pink are identical residues. Black indicates globally conserved residues.

Phylogenetic trees

Maximum Likelihood (ML) phylogenetic trees were constructed from the *mshA* (Fig. 2.3) and *pilA* (Fig. 2.4) sequences for the *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* isolates. Similar to the DNA and amino acid alignments, the *mshA* (Fig.

2.3A) and *pilA* (Fig. 2.4A) ML phylogenetic trees for *V. cholerae* clustered most clinical isolates into one branch while environmental isolates exhibited various branching patterns. When ML phylogenetic trees were constructed for these two gene sequences from *V. parahaemolyticus* (Fig. 2.3B and 2.4B) and *V. vulnificus* (Fig. 2.3C and 2.4C), no discernable grouping patterns appeared for either species, unlike the *V. cholerae* phylogenetic trees.

Figure 2.3. Bootstrap Maximum Likelihood phylogenetic trees for *mshA* from *Vibrio cholerae* (A), *Vibrio parahaemolyticus* (B), and *Vibrio vulnificus* (C). The bootstrap Maximum Likelihood phylogenetic trees *for mshA* from *V. cholerae* (A), *V. parahaemolyticus* (B), and *V. vulnificus* (C) were constructed using the gene sequences for *mshA* in the Molecular Evolutionary Genetics Analysis (MEGA) 5 software. All bootstrap values are listed.



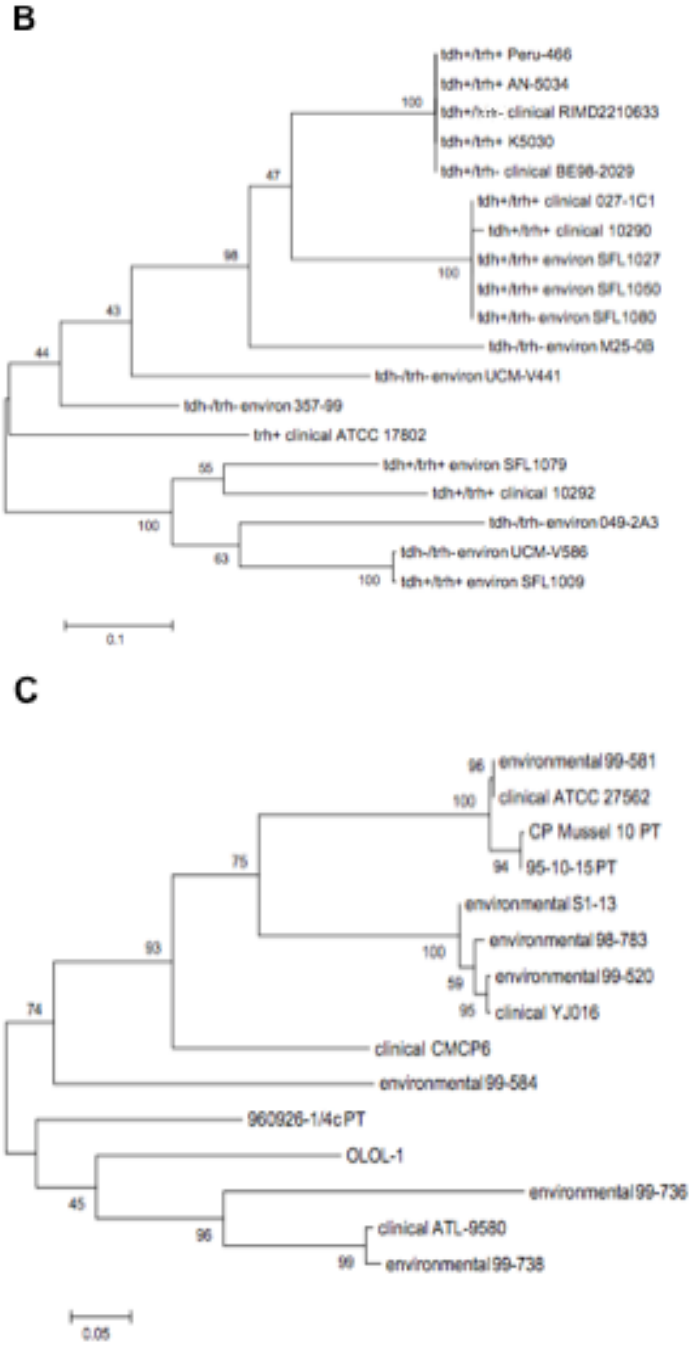
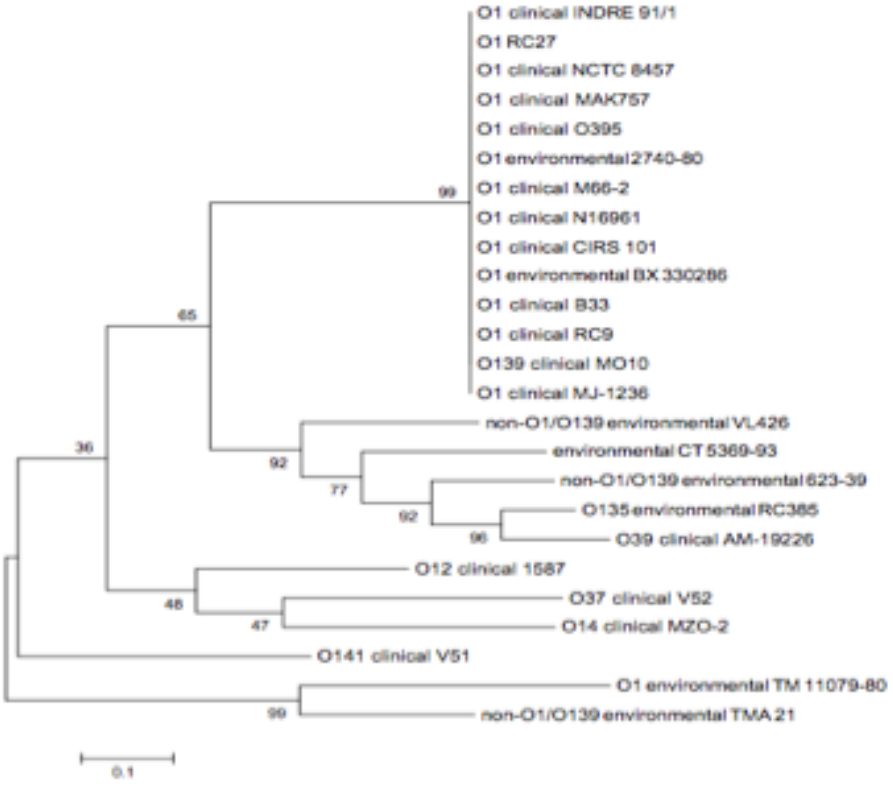


Figure 2.3. Bootstrap Maximum Likelihood phylogenetic trees for *mshA* from *Vibrio cholerae* (A), *Vibrio parahaemolyticus* (B), and *Vibrio vulnificus* (C).

Figure 2.4. Bootstrap Maximum Likelihood phylogenetic trees for *pilA* from *Vibrio cholerae* (A), *Vibrio parahaemolyticus* (B), and *Vibrio vulnificus* (C). The bootstrap Maximum Likelihood trees for *pilA* from *V. cholerae* (A), *V. parahaemolyticus* (B), and *V. vulnificus* (C) were constructed using the gene sequences for *pilA* in the Molecular Evolutionary Genetics Analysis (MEGA) 5 software. All bootstrap values are listed.

Figure. 2.4A



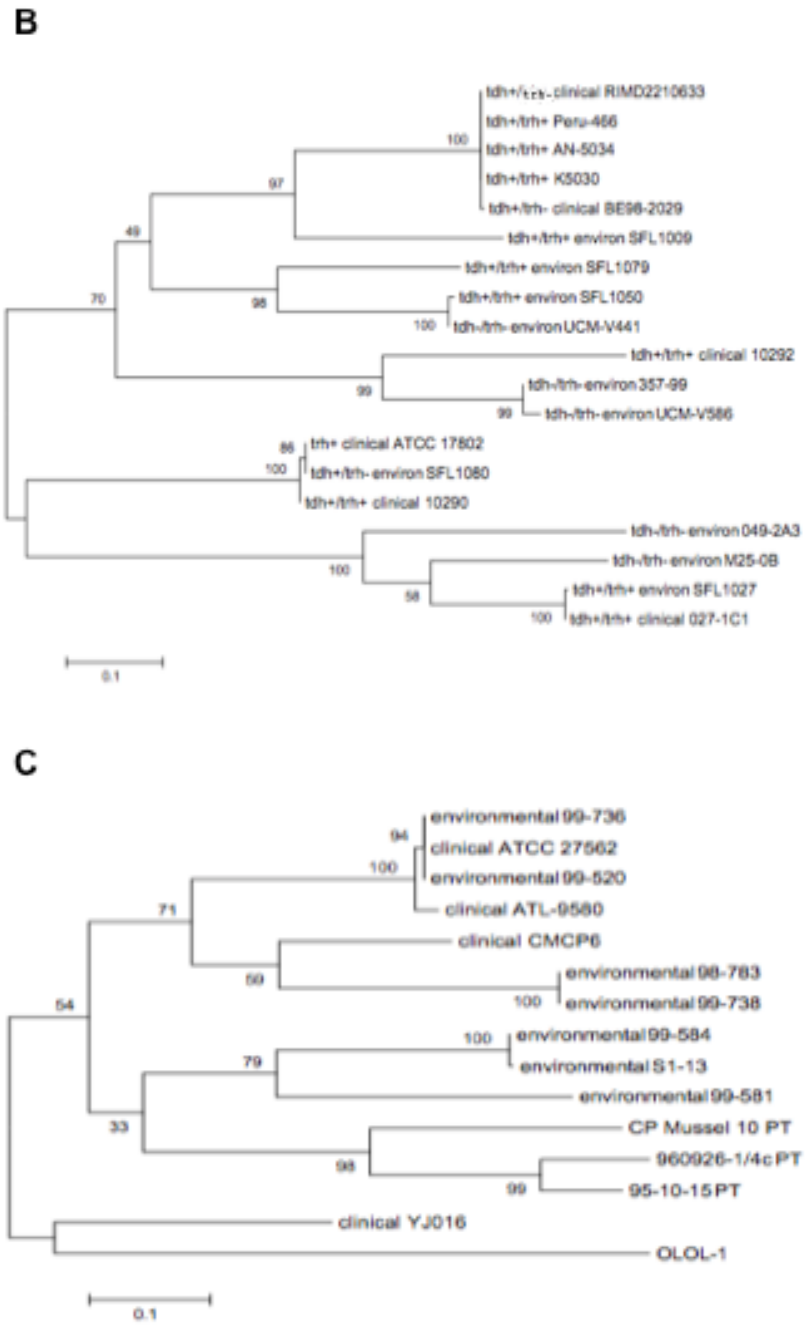


Figure 2.4. Bootstrap Maximum Likelihood phylogenetic trees for *pilA* from *Vibrio cholerae* (A), *Vibrio parahaemolyticus* (B), and *Vibrio vulnificus* (C).

Substitution analyses

We analyzed *mshA* and *pilA* for the rate of synonymous (silent) (d_S) and nonsynonymous (structural) (d_N) changes for the *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* isolates. For *mshA* from *V. cholerae*, the rate of synonymous (d_S) was 0.759 while the rate of nonsynonymous (d_N) was 0.471, with a d_N/d_S ratio of 0.621 (Table 2.3). The rate of synonymous changes for *V. parahaemolyticus* was 0.746 and for *V. vulnificus* was 0.662. The rate of nonsynonymous changes for *V. parahaemolyticus* and *V. vulnificus* was 0.431 and 0.384, respectively. This resulted in a d_N/d_S of 0.577 for *V. parahaemolyticus* and 0.580 for *V. vulnificus* (Table 2.3). For *pilA*, the rate of synonymous changes was 1.109 for *V. cholerae*, 1.691 for *V. parahaemolyticus*, and 1.186 for *V. vulnificus*. The rate of nonsynonymous changes was 0.629 for *V. cholerae*, 0.642 for *V. parahaemolyticus* and 0.503 for *V. vulnificus*. This resulted in a d_N/d_S of 0.567, 0.380, and 0.424 for *V. cholerae, V. parahaemolyticus* and *V. vulnificus*, respectively (Table 2.3).

Table 2.3. Analysis of synonymous and nonsynonymous nucleotide substitutions for genes involved in Type IV pili function from *V. cholerae, V. parahaemolyticus,* and *V. vulnificus*

Gene Locus	Organism	Sequence Length (bp)	No. of Strains	d_S	d_N	d_N/d_S
mshA	*V. cholerae*	438-537	25	0.759	0.471	0.621
	V. parahaemolyticus	456-504	19	0.746	0.431	0.577
	V. vulnificus	447-510	15	0.662	0.384	0.580
mshC	*V. cholerae*	489-513	25	0.135	0.039	0.290
	V. parahaemolyticus	131 (1-131 5'end)	15	0.229	0.017	0.072
	V. vulnificus	94 (1-94 5'end)	12	0.042	0.015	0.356
pilA	*V. cholerae*	420-504	25	1.109	0.629	0.567
	V. parahaemolyticus	405-486	19	1.691	0.642	0.380
	V. vulnificus	402-453	15	1.186	0.503	0.424
pilB	*V. cholerae*	1689	24	0.176	0.008	0.047
	V. parahaemolyticus	248 (1-248 5'end)	19	0.288	0.037	0.127
	V. vulnificus	122 (1-122 5' end)	14	0.208	0.016	0.074
pilD	*V. cholerae*	876	24	0.122	0.005	0.039
	V. parahaemolyticus	870	3	0.000	0.000	-
tcpA	*V. cholerae*	675	13	0.486	0.052	0.106
tcpJ	*V. cholerae*	762	13	0.003	0.000	0.000
mshA	*V. cholerae* with TCP		13	0.000	0.000	-
	V. cholerae without TCP		12	0.663	0.377	0.568
pilA	*V. cholerae* with TCP		13	0.000	0.000	-
	V. cholerae without TCP		12	1.270	0.615	0.484

Region analyses

To compare the diversity of *mshA* and *pilA*, we examined neighboring genes from their respective operons, *mshC* and *pilB*, as well as the Type IV pilin peptidase, *pilD*. The rate of synonymous and nonsynonymous changes for *mshC* was 0.135 and 0.039 for *V. cholerae*, 0.229 and .017 for *V. parahaemolyticus*, and 0.042 and 0.015 for *V. vulnificus*. This resulted in a d_N/d_S ratio of 0.290 for *V. cholerae*, 0.072 for *V. parahaemolyticus*, and 0.356 and *V. vulnificus* (Table 2.3). For *pilB*, the rates of synonymous and nonsynonymous for *V. cholerae, V. parahaemolyticus*, and *V. vulnificus* was 0.176 and 0.008, 0.288 and 0.037, and 0.208 and 0.016 respectively. The d_N/d_S ratio for *pilB* was 0.047 for *V. cholerae,* 0.127 for *V. parahaemolyticus*, and

0.074 for *V. vulnificus*. For *pilD*, the synonymous and nonsynonymous rates calculated for *V. cholerae* were 0.122 and 0.005 with a d_N/d_S of 0.039. The *V. parahaemolyticus* strains used to calculate the synonymous and nonsynonymous rates of substitution for *pilD* had identical sequences; thus the synonymous and nonsynonymous rates of substitution were zero and the d_N/d_S ratio cannot be calculated. These rates are comparable with data from Chattopadhyay et. al (2009), which calculated the rates of synonymous and nonsynonymous substitutions for *pilD* from *V. vulnificus* as 0.092 and 0.007 with a d_N/d_S ratio of 0.076.

TcpA and TcpJ

To compare the findings for *mshA* and *pilA* with another Type IV pilin and its corresponding peptidase, we calculated the rates of synonymous and nonsynonymous substitutions for the toxin co-regulated pilus pilin subunit *tcpA* from *V. cholerae* and its processing leader peptidase *tcpJ* (Table 2.3). Only 13 *V. cholerae* strains out of the available 25 possess *tcpA* and *tcpJ*. The d_S and d_N for *tcpA* was 0.486 and 0.052 with a d_N/d_S ratio of 0.106. For *tcpJ*, the d_S and d_N was 0.003 and 0.000 with a d_N/d_S ratio of 0.000.

Discussion

The results from our sequence analyses of the *mshA* and *pilA* genes from several strains of three human pathogenic *Vibrio* species, *V. cholerae, V. parahaemolyticus*, and *V. vulnificus*, suggested the various alleles observed were the result of selective pressure. When examining the *V. cholerae* predicted amino acid alignment (Fig.

2.1A) and phylogenetic tree (Fig. 2.3A) for the *mshA* gene, one distinct grouping emerged with highly conserved sequences for the MSHA pilin subunit. In fact, the isolates in this group, identifiable as one branch of the phylogenetic tree (Fig. 2.3A), were primarily from the O1 serogroup (13 out of 15) and clinical isolates (11 out of 15). This differs considerably from the remaining *V. cholerae* isolates examined, which were predominately environmental, non-O1/O139 strains (9 out of 10) with no apparent grouping pattern in the phylogenetic tree (Fig. 2.3A). When comparing the predicted amino acid alignments and phylogenetic trees for the *V. parahaemolyticus* (Fig. 2.1B and 2.3B) and *V. vulnificus* (Fig. 2.1C and 2.3C) strains sequenced, no grouping could be established based on either isolation source or phenotype, in contrast to what was observed for *V. cholerae*.

Reviewing the sequence data for the PilA pilin subunit from *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, the *pilA* sequences exhibited a trend similar to what was observed for the MSHA pilin subunit. For *V. cholerae* strains, a group of highly conserved PilA sequences emerged and were primarily from the O1 serogroup (13 out of 14) and of clinical origin (11 out of 14). The remaining isolates were predominately non-O1/O139 (10 out of 11) and from an environmental source (6 out of 11). They did not have any clear pattern to their alignment (Fig. 2.2A) or tree branching (Fig. 2.4A). Consistent with the *mshA* findings, no apparent grouping pattern was observed for either the amino acid alignment or branching on the phylogenetic tree for any of the *V. parahaemolyticus* (Fig. 2.2B and 2.4B) and *V. vulnificus* (Fig. 2.2C and 2.4C) *pilA* genes sequenced. Taken together, our hypothesis

is that a selective pressure has caused the differences observed in these two Type IVa pili in *V. cholerae, V. parahaemolyticus* and *V. vulnificus*.

To test for selective pressure, the synonymous and nonsynonymous nucleotide substitution rates were calculated to determine a d_N/d_S ratio (Nei and Gojobori 1986). In protein-coding sequences, synonymous substitutions (d_S) are structurally silent while nonsynonymous substitutions (d_N) result in a change to the amino acid sequence. When a d_N/d_S ratio is calculated, typically the value suggests whether the substitutions are largely neutral ($d_N/d_S = 1$), under a negative selection ($d_N/d_S < 1$), or a positive selection ($d_N/d_S > 1$) (Yang and Bielawski 2000). Table 2.3 shows the calculations for d_S, d_N, and d_N/d_S for the *mshA* and *pilA* genes from the different *Vibrio* strains analyzed and the data suggest a selective pressure has been applied to these two genes for all three *Vibrio* species. To further analyze the selective pressure applied to the Type IV pili examined, we compared *mshA* and *pilA* with another gene in their corresponding operon, the neighboring genes *mshC* gene and *pilB* respectively, to determine if a selective pressure has been applied strictly to the gene encoding the pilin subunit or to the entire operon. When comparing the d_N/d_S value for *mshA* with *mshC* and *pilA* with *pilB* for all three vibrios, the d_N/d_S values for the pilin subunits (*mshA* and *pilA*) are considerably larger than the neighboring gene in the operon (*mshC* and *pilB*) (Table 2.3). These results suggest that the neighboring genes (*mshC* and *pilB*) in both the MSHA and PilA operons are more conserved than their corresponding pilin subunits (*mshA* and *pilA*). Thus, it is possible that the pilin subunits are not under the same selective pressure as their neighboring genes.

Both *mshA* and *pilA* encoded pilins are processed by the same Type IV prepilin peptidase*, pilD* (Marsh and Taylor 1998; Paranjpye, Lara et al. 1998; Fullner and Mekalanos 1999; Kim and McCarter 2004). When examining the d_N/d_S value for *pilD*, it was evident that the *pilD* gene maintained a highly conserved sequence. We calculated the *pilD* d_N/d_S for *V. cholerae* (0.039) but were unable to calculate it for *V. parahaemolyticus* because the sequences were identical for d_S (0.000) and d_N (0.000) so the d_N/d_S was 0/0 (Table 2.3). Despite the inability to calculate the d_N/d_S for *V. parahaemolyticus*, the results for *V. cholerae pilD* (0.039) were congruent with what was found for *V. vulnificus* (0.076) by Chattopadhyay et. al (2009). This suggests that a strong purifying selection has maintained the highly conserved *pilD* sequence in contrast to the general observation for the *mshA* and *pilA* sequences. When examining the predicted amino acid sequences for both *mshA* (Fig. 2.1) and *pilA* (Fig. 2.2) for all three vibrios, it was clear that the N-termini remain highly conserved while the C-termini varied considerably. The N-termini region is recognized by the PilD peptidase for processing the protein into a mature pilin subunit (Craig, Pique et al. 2004). If the N-terminal region of the Type IVa pili proteins MSHA and PilA varied, it is possible that PilD would no longer process these proteins into mature subunits while variations in the C-termini should still result in a mature pilin subunit. Thus, it appears that PilD has maintained a highly conserved sequence unlike the MSHA and PilA proteins it processes.

To further understand the variations observed in the MSHA and PilA pilins, the *V. cholerae mshA* and *pilA* sequences were compared to the Type IVb pilin TCP from

V. cholerae. The *tcpA* gene encodes the major pilin subunit of TCP and is processed by its own Type IV pili peptidase TcpJ, encoded by *tcpJ* (Kaufman, Seyer et al. 1991). Contrary to *tcpA* that exhibit some variability in its sequences with mostly synonymous substitutions (d_S of 0.486) and few nonsynonymous substitutions (d_N of 0.052), *tcpJ* has relatively few substations overall (d_S of 0.003 and d_N of 0.000). The d_N/d_S for *tcpA* is 0.106 and *tcpJ* is 0.000, suggesting that these genes are under strong negative selection to maintain their sequences and structures. When examining the *V. cholerae* phylogenetic trees constructed for the *mshA* and *pilA* genes, the strains that possess TCP are all from the O1 serogroup and on a single branch (Fig. 2.3A and 2.4A). Looking at the amino acid alignment data, it was evident that the *V. cholerae* isolates containing all three Type IV pili were highly conserved (Fig. 2.1A and 2.2A). To break it down further, the d_N/d_S ratio for *mshA* and *pilA* from the *V. cholerae* strains possessing TCP were also calculated and the d_S and d_N for both genes were 0.000, resulting in an undefined d_N/d_S ratio (Table 2.3). Therefore, *V. cholerae* strains possessing all three Type IV pili appear to be under a strong purifying selection. Even though some O1 *V. cholerae* isolates in this conserved branch were from environmental or unknown sources (3 out of 13), the fact that they possess TCP implies they could cause cholera. Taken together, the evidence suggests a connection between host interactions and highly conserved Type IV pili in *V. cholerae*.

A previous study by Chattopadhyay et. al (2009) analyzed *pilA* from 55 *V. vulnificus* strains of various origins and also determined that *pilA* is highly divergent. A total of 25 unique alleles were identified from the 55 analyzed strains and the

authors did not determine any relationship between the various alleles and pathogenicity of *V. vulnificus* (Chattopadhyay, Paranjpye et al. 2009). They concluded that the genetic diversity of *pilA* in *V. vulnificus* was higher than neighboring genes (*pilBCD*) and thus was under strong positive, diversifying selection (Chattopadhyay, Paranjpye et al. 2009). This conclusion was made despite the fact that the d_N/d_S ratio calculated for *pilA* was less than 1. The usefulness of the d_N/d_S ratio to detect positive selection is reduced when comparing gene polymorphisms within a single population compared to divergent populations (Kryazhimskiy and Plotkin 2008). Our results are consistent with their findings and also demonstrate that MSHA and PilA from *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* exhibit higher genetic diversity than other genes in their corresponding operon (*mshC* and *pilB* and *pilD*).

Chattopadhyay et. al (2009) suggested various ideas to explain their observation, including that the allelic variability in PilA for *V. vulnificus* could be the result of oyster innate immune system (Chattopadhyay, Paranjpye et al. 2009). It was noted that since *V. vulnificus* commonly associate with shellfish in the environment and infections in humans are typically opportunistic, the selective pressure applied to this gene was probably not in response to an adaptive immune system (Chattopadhyay, Paranjpye et al. 2009). Shellfish have an innate immune system that recognizes highly conserved motifs while lacking a well-developed adaptive immunity (Zampini, Canesi et al. 2003; Pruzzo, Gallo et al. 2005). Thus, the driving force behind the variations observed in the PilA protein could be the result of the

innate immunity of shellfish, such as oysters, in part based on a previous study showing that PilA was involved in oyster colonization by *V. vulnificus* (Paranjpye, Johnson et al. 2007; Chattopadhyay, Paranjpye et al. 2009). Data from our laboratory also indicated that PilA and MSHA play a role in *V. parahaemolyticus* colonization of the Pacific oyster, *Crassostrea gigas* (Aagesen, A.M., and C.C. Häse, unpublished results), further supporting the idea that the shellfish immune system might be involved in applying pressure to these pili proteins, thus causing variability. Studies using different strains expressing the various alleles for MSHA and PilA from *V. cholerae, V. parahaemolyticus*, and *V. vulnificus* in shellfish interaction experiments are required to fully address this issue.

In addition to the shellfish immune system, other selective pressures in the environment could exist to cause the observed allelic diversity in MSHA and PilA, such as protozoan grazing, bacteriophages and DNA uptake (Chattopadhyay, Paranjpye et al. 2009). Ideally, various alleles for MSHA and PilA from *V. cholerae, V. parahaemolyticus* and *V. vulnificus* would need to be examined to better understand the role of bacteriophages as a selective pressure causing the variations observed for these proteins. However, future studies using various alleles for MSHA and PilA are required to support these hypotheses.

In summary, this study illustrates significant diversity of the MSHA and PilA pilin subunits from *V. cholerae, V. parahaemolyticus,* and *V. vulnificus*. For all three vibrios examined in this study, *mshA* and *pilA* had considerably higher d_N/d_S ratios than any of the other genes examined, suggesting these genes are under a possible

positive selection while the other genes examined are not. Another interesting finding was that *V. cholerae* strains that possess TCP also maintain highly conserved MSHA and PilA sequences, suggesting a connection with the host. Even though a selective pressure appears to exist causing the allelic variations observed for *mshA* and *pilA*, the mechanism(s) driving this diversification have yet to be determined. Several suggestions can be made, yet evidence to support these ideas awaits further experimental analyses. In addition, our observations raise an important point about the use of these genes in detection methods for these important human pathogens. In particular, some PCR-based detection methods utilize certain pathogen-associated genes as targets, including Type IV pili genes (Gubala 2006; Jagadeeshan, Kumar et al. 2009). Realizing that the *Vibrio mshA* and *pilA* genes can be extremely variable at the 3' ends of the genes is important to consider when designing primers to target these genes. Therefore, it is possible that a PCR protocol designed to amplify *mshA* and *pilA* from various *V. cholerae, V. parahaemolyticus*, and *V. vulnificus* strains may not detect these genes simply due to the variations observed in this study. This is certainly something to consider when utilizing these genes in a PCR protocol.

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Chapter 3

Vibrio parahaemolyticus persistence in the Pacific oyster, *Crassostrea gigas*, is a multifactorial process involving pili and flagella but not Type III secretion systems or phase variation

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Unpublished

Abstract

Vibrio parahaemolyticus is a human pathogen and a leading cause of seafood-borne illness in the United States, typically resulting from eating raw or undercooked oysters. *V. parahaemolyticus* has been shown to be highly resistant to oyster depuration, suggesting that the bacterium possesses specific mechanisms or factors for sufficiently colonizing oysters and subsequently persisting during depuration. Here we show that pili, in particular Type I and Type IV, and both flagellar systems, polar and lateral, positively contribute to *V. parahaemolyticus* persistence in the Pacific oyster, *Crassostrea gigas* during depuration; however, known virulence factors for human disease, such as Type III Secretion Systems 1 and 2, or phase variation, do not. Furthermore, we characterized eight different *V. parahaemolyticus* strains for differences in depuration, biofilm formation, and motility and determined that the factors commonly involved in biofilm formation on abiotic surfaces, glass or plastic, do not directly correlate with bacterial colonization of oysters. Further studies examining the molecular mechanisms underlying the observed differences in colonization by these *V. parahaemolyticus* strains may provide beneficial insights into what critical factors are required for proficient colonization of the Pacific oyster, possibly leading to novel post-harvest processing techniques to sufficiently reduce the numbers of *V. parahaemolyticus* in market oysters.

Introduction

The genus *Vibrio* consists of a group of bacteria that naturally inhabitant aquatic environments worldwide. Among this diverse group of microorganisms are a few human pathogens, namely *Vibrio cholerae, Vibrio vulnificus,* and *Vibrio parahaemolyticus*. *V. parahaemolyticus* is a ubiquitous, halophilic, marine organism commonly found associated with shellfish, particularly oysters (Vieira, Costa et al. 2011; Johnson, Bowers et al. 2012). Depuration is a controlled process where shellfish, such as oysters, are placed into clean seawater with the intent to reduce the bacterial contaminants in their edible tissues. Studies have shown that depuration is not very effective at reducing the numbers of *V. parahaemolyticus* in oysters, which are part of the natural microflora of these marine invertebrates (Croci, Suffredini et al. 2002). If ingested by humans, *V. parahaemolyticus* can cause gastroenteritis, thereby posing the most significant threat to the health of shellfish consumers who eat their oysters raw or under-cooked. Virulence factors from *V. parahaemolyticus* that are involved in causing human disease have been studied extensively, whereas relatively little is known about the components involved in the bacterial association with oysters.

When colonizing a surface, including host tissue, bacteria can employ a range of factors or surface structures to aid in successful adherence and attachment (Kline, Falker et al. 2009). Many of these factors have been identified, such as pili and flagella, and studied for their roles in biofilm formation and host colonization (Yildiz and Visick 2009). Pili (or fimbriae) are one of the many components involved in

bacterial attachment to a variety of surfaces. Type I pili are known to be crucial for attachment of uropathogenic *Escherichia coli* to urinary tract cells and invasion (Crepin, Houle et al. 2012). Similarly, Salmonella also use Type I pili for host colonization, an essential component of pathogenesis (Althouse, Patterson et al. 2003). *V. parahaemolyticus* encodes a homologue of these Type I pili, the CsuA/B operon (http://img.jgi.doe.gov/). This *V. parahaemolyticus* operon has the highest similarity to genes in *Acinetobacter baumanii* that are used for attachment to various surfaces (Tomaras, Dorsey et al. 2003). Another group of pili, the Type IV group, have been examined for their involvement in surface contact and attachment. Two well-studied Type IV pili from vibrios are the mannose-sensitive hemagglutinin (MSHA) and the chitin-regulated pilus (PilA). These two pili have been shown to aid in the adherence of *V. cholerae* to form biofilms on various surfaces, such as chitin (Tarsi and Pruzzo 1999; Meibom, Li et al. 2004), but are not known to aid in human disease (Attridge, Manning et al. 1996; Hsiao, Liu et al. 2006). *V. vulnificus* uses PilA for biofilm formation and adherence to epithelial cells (Paranjpye and Strom 2005) and bacterial retention in Eastern oysters, *Crassostrea virginica* (Paranjpye, Johnson et al. 2007; Srivastava, Tucker et al. 2009). In addition, the squid symbiont *Vibrio fischeri* use both MSHA and PilA for colonizing the light organ of the juvenile squid, *Euprymna scolopes* (McFall-Ngai, Brennan et al. 1998; Stabb and Ruby 2003), further suggesting that these two Type IV pili are environmental factors that vibrios possess for their survival in the aquatic environment, possibly for associating with mollusks. These two Type IV pili have been shown to be involved in biofilm

formation in *V. parahaemolyticus* (Shime-Hattori, Iida et al. 2006).

Flagella are helical surface structures that provide locomotion for many bacterial species and are critical during early stages of colonization of a surface, biotic or abiotic (McCarter 2001; Yildiz and Visick 2009). *V. parahaemolyticus* possess a single, polar flagellum that is constitutively produced and used for movement in a liquid (reviewed in (McCarter 2001)). In addition, *V. parahaemolyticus* also produce peritrichous (lateral) flagella upon contact with a surface or under iron-limiting conditions ((McCarter and Silverman 1989; McCarter and Silverman 1990; Kirov 2003) reviewed in (Stewart and McCarter 2003)). These bacterial structures are known to be involved with surface adherence during biofilm formation and have been linked with virulence (McCarter 2004; Gode-Potratz, Kustusch et al. 2011). When *V. parahaemolyticus* encounters a surface, the rotation of the polar flagellum slows down or ceases, creating a signal cascade that induces lateral flagella gene expression and represses capsular polysaccharide production (Kawagishi, Imagawa et al. 1996). This change in gene regulation from swimmer cell to swarmer cell has many phenotypic changes such as cell elongation and an increase in virulence (McCarter 2004). The increase in virulence after growth on a surface has been connected with an increase in toxin delivery via Type III Secretion Systems (T3SSs) (Hueck 1998; Gode-Potratz, Kustusch et al. 2011). Many bacterial pathogens use T3SSs for survival in the host by injecting virulence factors or effectors directly into the host, thereby altering host defenses (reviewed in (Galan and Collmer 1999)). *V. parahaemolyticus* encodes two T3SSs, found on different

chromosomes and exhibiting different phenotypes during disease. All *V. parahaemolyticus* strains possess T3SS1, mainly responsible for cytotoxicity, while T3SS2 is predominately found on pathogenic *V. parahaemolyticus* isolates and causes enterotoxicity (Hiyoshi, Kodama et al. 2010; Matlawska-Wasowska, Finn et al. 2010; Noriea, Johnson et al. 2010).

Some bacteria exhibit "phase variation" and switch from displaying an opaque (OP) phenotype, the result of production large quantities of CPS, to a translucent (TR) phenotype where CPS production is dramatically reduced (Hsieh, Liang et al. 2003). Phase variation in *V. parahaemolyticus* is controlled by the LuxR homologue, OpaR (McCarter 1998). Deletions in the *opaR* gene result in a fixed, translucent phenotype (McCarter 1998). Phase variation and lateral flagella expression are co-regulated where an OP strain produces much less lateral flagella than a TR strain (Jaques and McCarter 2006).

Clearly, host colonization is a complex process and determining the contributions of each factor is a complicated task. In this study, we examined the role of *V. parahaemolyticus* pili, flagella, phase variation and T3SSs in colonization of the Pacific oyster, *Crassostrea gigas*, and conclude that bacterial colonization of oysters is a multifactorial process.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 3.1. The *V. parahaemolyticus* strains were grown using Tryptic Soy medium supplemented with sodium chloride for a final concentration of 2%. The *E. coli* strains were grown using Luria-Bertani medium. All strains were cultured at 37ºC unless otherwise indicated. Antibiotics in this study were used at the following concentrations: streptomycin, 100ug/ml; chloramphenicol, 20ug/ml for *E. coli* and 10ug/ml for *V. parahaemolyticus*; kanamycin, 50ug/ml; and ampicillin, 100ug/ml.

Table 3.1. Strains used in this study

V. parahemolyticus	Description	Origin
ATCC 17802	ATCC17802 spontaneous streptomycin resistant isolate. Parent to all pili mutants	This study
VPAA8	ATCC 17802 ΔVPA1506 *csuB*-	This study
VPAA9	ATCC 17802 ΔVP2698 *mshA*-	This study
VPAA26	ATCC 17802 ΔVP2526 *pilD*-	This study
VPAA27	ATCC 17802 ΔVP2523 *pilA*-	This study
VPAA28	ATCC 17802 ΔVP2698 *mshA*-, ΔVP2523 *pilA*-	This study
LM5432	spontaneous phosphomycin resistant isolate of BE22 (OP)	Linda McCarter
LM5431	spontaneous phosphomycin resistant isolate of Δ*opaR1* (TR)	Linda McCarter
LM5392	Δ*opaR1 flaM*::Tn5 Kanamycin fla-	Linda McCarter
LM7789	Δ*opaR1 lafK*::Chloramphenical laf-	Linda McCarter
LM7901	Δ*opaR1 flaK*::Chloramphenical *lafK*::Kanamycin fla- laf-	Linda McCarter
LM7341	Δ*opaR1* VPA1342::Chloramphenical Type III Secretion System 1 VP1672::Kanamycin Type III Secretion System 2	Linda McCarter
O27-1C1	FDA clinical isolate	Yi-Cheng Su
BE98-2029	FDA clinical isolate	Yi-Cheng Su
10290	FDA clinical isolate	Yi-Cheng Su
10292	FDA clinical isolate	Yi-Cheng Su
10293	FDA clinical isolate	Yi-Cheng Su
Escherichia coli		
TOP10	cloning strain	Invitrogen
DH5αλpir	cloning strain	
SM10λpir	cloning strain	
Plasmids		
pCR2.1TOPO	cloning vector	Invitrogen
pWM91λpir	cloning suicide vector	Metcalf, 1996

Construction of V. parahaemolyticus deletion mutants

All primers used in the PCR are listed in Table 3.2. To construct the pili deletion mutants in *V. parahaemolyticus*, PCR was used to amplify regions 1.5kb upstream

and downstream of the desired gene to essentially create an in-frame gene deletion fusing the start and stop codons of the gene. Briefly, primers 1 and 2 were designed to amplify the region 1.5kb upstream of the desired gene, including the start codon. Primers 3 and 4 were designed to amplify the region 1.5kb downstream of the desired gene, including the stop codon. Primers 2 and 3 are designed to have complementary regions that allow the two PCR fragments to combine, fusing the start to the stop with the complementary regions built into the primers in between. The PCR products from reactions using primers 1 and 2, and 3 and 4 were mixed. Primers 1 and 4 were added to combine and amplify the two fragments into one PCR product of approximately 3kb. The fragment was cloned into the plasmid pCR2.1TOPO TA (Invitrogen) and transformed into *E. coli* TOP10 (Invitrogen) cells. Using restriction sites designed into the primers, the fragment was isolated from pCR2.1TOPO using restriction digests and cloned into the suicide vector pWM91 using the complementary restriction sites, followed by transformation into DH5αλpir. Once a pWM91 construct containing the deletion allele was identified, a chloramphenicol cassette was added to the construct via restriction digests. The resulting construct contained the gene deletion with 1.5kb upstream and downstream of the gene of interest (for allelic exchange), a cat cassette (for selection) in the pWM91 suicide vector background. This final construct was then transformed into SM10λpir for conjugation in *V. parahaemolyticus*. SM10λpir conjugations with *V. parahaemolyticus* occurred by streaking each strain onto an LB plate and the mating occurred overnight at 37°C. Conjugations were harvested the followed day by adding 2mls of Tryptic soy broth

(TSB) 2% NaCl to the bacterial mass and plating dilutions on Tryptic soy agar (TSA) 2% NaCl plates containing chloramphenicol (Sigma) and streptomycin (Sigma) and incubated at 37°C. The resulting ex-conjugates were screened by PCR to confirm suicide plasmid integration into the chromosome and single colonies were plated onto counter selection plates containing 1% tryptone, 0.5% yeast extract, 0.25% NaCl and 10% sucrose, and incubated at room temperature for 24hrs. Single colonies from the counter selection plates were patched onto TSA 2% NaCl plates containing either streptomycin or chloramphenicol to verify counter selection was successful. Chloramphenicol sensitive strains were screened by PCR to determine if the wild type or mutant deletion allele was present.

Table 3.2. Primers used in this study

Gene Deletion	
*ΔcsuB*1-	ggggactagtTCTTTCGACCTTGTGGGTGAAGACGTGCTG
*ΔcsuB*2-	gactgactgactgactgactCGTCACGGTTTACCAGCTTAATGTCACACT
*ΔcsuB*3-	agtcagtcagtcagtcagtcTGGTAAGTGATGTTTCGGAGGGATGAAATG
*ΔcsuB*4-	gggggcggccgcGACTCCAAGACAAAGGACGGACGATGTAGT
ΔpilA 1-	gggggactagtGATAATTGGGGGCATATCAACCTCTATAGTTTG
ΔpilA 2-	gactgactgactgactgactTTTCATTTTCTTTCCTTCTTAATGAAATAA
ΔpilA3-	agtcagtcagtcagtcagtcTAATCACACGACATAATGCACTCC
ΔpilA4-	gggggcggccgcGCATTCGTTGCAACCATCTGGATTGGC
*ΔmshA*1-	gggggactagtCGTAAACGCATTAAAGCCGCGATGCGCTATCCG
ΔmshA2-	gactgactgactgactgactTTTCATTGTTTACTCTCTCTATGTAAGCAT
*ΔmshA*3-	agtcagtcagtcagtcagtcTGTTAATCGGTGTTATAAAGGCCAGCTAGT
ΔmshA4-	gggggcggccgcCCATTAAGGTGAAACCACGAGTTTTCATTCAGT
*ΔpilD*1-	gggggactagtACCCATGGTTATTCTGGCCGAACAGGTATC
*ΔpilD*2-	ggaaccttccaaggccaattggccCATATTTATCTCTTGAGCGATAAGTCAAAT
*ΔpilD*3-	ggccaattggccttggaaggttccTAAATGGCTTTGGTAATAGGCTTAACG
*ΔpilD*4-	gggggcggccgcCTCCCCATTCAACGTCGGTACCACATTGAG

Oyster collection and preparation

Diploid Pacific oysters were provided by the Oregon Oyster Farms (Yaquina Bay, Newport, OR) and the animals were transported to the laboratory on the day of harvest. Experimental animals were cleaned to remove epibiota and housed in a holding facility at Hatfield Marine Science Center (Newport, OR) with filtered, aerated seawater containing cultured algae and allowed to acclimate to 18ºC for a minimum of four days.

Oyster exposure to V. parahaemolyticus

All *V. parahaemolyticus* strains were streaked onto TSA 2% from frozen stock stored at -80ºC and incubated at 37ºC for 18 hours. A single colony from each strain was inoculated into 10ml of TSB 2% for 5 hours at 37 ºC under static conditions. The cultures were placed into sterile 30ml centrifuge tubes and collected by centrifugation at 5000x*g* for 15min at 4ºC. The supernatant was decanted and the pelleted cells were washed with 10ml of sterile 1% NaCl. The cells were centrifuged a second time and the pellet was resuspended in 1% NaCl to an optical density of 3.0 at 600nm, which is approximately 10^9 cfu/ml. For each experiment, 12 animals were placed into five 30L tanks for exposure and five animals were placed into one, 30L control tank. Each tank was filled with 20L of filtered seawater with cultured algae, heated to 18ºC, and aerated to maintain optimal dissolved oxygen levels. Each exposure tank received 2ml of the appropriate *V. parahaemolyticus* strain for a final concentration of ~10^5 cfu/ml and the control tank received 2ml of 1% NaCl. Oysters were exposed to *V. parahaemolyticus* for 18hrs then placed into separate tanks with

flowing, 18ºC filtered seawater with algae and aeration. Animals were sampled at various time points throughout depuration. At each time point, three to five animals from each *V. parahaemolyticus* exposure tank and one animal from the control tank were removed from the depuration system. Each animal was shucked with a sterile knife, weighed, and placed into a sterile blender jar. Then animals were blended with three volumes of sterile artificial seawater and blended at high speed for 30 seconds in a laboratory blender (Waring Laboratory, Torrington, CT) to create an oyster slurry for serial dilutions. Each slurry dilution was plated on TSB 2% NaCl with the appropriate antibiotic and incubated at 37ºC for 18 hours. Control animals that did not receive *V. parahaemolyticus* were included in the experimental design to determine levels of indigenous bacteria.

Biofilm assay

All strains were cultured in filtered Zobell Marine broth at 30ºC in a roller drum overnight. Cultures were diluted 1:100 (OD600nm ~0.1) in fresh Zobell Marine broth and incubated at appropriate temperatures and times in either 12 x75 mm borosilicate glass culture tubes or in Nunc 96 well culture plates.

Motility assays

All strains were assayed for motility using swimming agar (1% Bacto tryptone, 2% NaCl, 0.32% Bacto agar) and swarming agar (2% Bacto Heart Infusion Broth, 2% NaCl, 1.5% Bacto agar) (Jaques and McCarter 2006). Each plate was inoculated with 2µl of an overnight culture normalized to approximately OD 600nm of 2.0.

Swimming motility plates were incubated at room temperature (~20°C) for 22hrs. Swarming motility plates were incubated at 30°C for 22hrs.

Statistical analyses

Biological data for oyster depuration experiments were $\log_{10}$ transformed before statistically analyzed. For the depuration experiments, three to five animals from strain were averaged and the averages from four independent experiments were compared using the Student's t test in Excel. P value ≤ 0.05 was considered statistically significant and marked by an asterisk. All assays for the individual *V. parahaemolyticus* strains were analyzed by ANOVA with post-hoc pairwise analysis with Tukey's test using XLSTAT Pro 2012 (Addinsoft, New York, New York). Different letters indicate statistically significant differences in the individual strains for the various assays tested.

Results

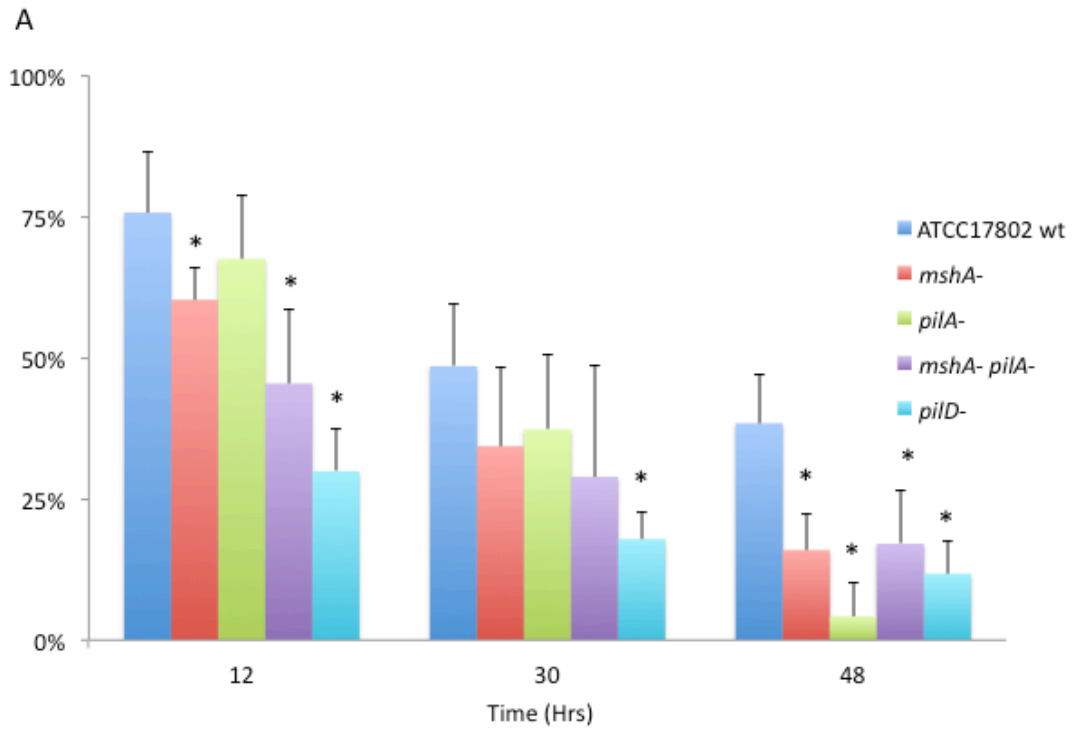
Role of pili, flagella, phase variation and Type III Secretion systems in persistence in the Pacific oyster infection assay

Bacterial species use multiple factors to colonize biotic and abiotic surfaces. In particular, the contribution of pili (Watnick, Fullner et al. 1999; Paranjpye and Strom 2005; Shime-Hattori, Iida et al. 2006) and flagella (Mueller, McDougald et al. 2007) in colonizing surfaces have been well established. To test the role of pili in colonizing the Pacific oyster and resisting depuration, *V. parahaemolyticus* ATCC17802 defined gene deletions were created in the Type I pilin gene, *csuB,* and

Type IV pilin genes, *mshA* and *pilA*. A double gene deletion in *mshA* and *pilA* was also created in ATCC17802, as well as a single gene deletion in the Type IV pilin peptidase, *pilD*. Animals were infected with the *V. parahaemolyticus* ATCC17802 wild type and pili mutants (Table 3.1) and assayed for percent retention of bacterial colony-forming units (CFU) per gram of tissue over time during depuration. After 12hrs of depuration, the Type IV pili mutants Δ*mshA* (*P* value = 0.04), Δ*mshA*/Δ*pilA* (*P* value = 0.01), and Δ*pilD* (*P* value = 0.0004) (Fig. 3.1A) showed a significant defect in percent retention compared with the wild type. The Δ*mshA* and Δ*mshA*/Δ*pilA* strains had slight defects in percent retention while the Δ*pilD* strain had a much more dramatic reduction than either the Δ*mshA* and Δ*mshA*/Δ*pilA* strains. After 30hrs of depuration, all strains had a reduction in overall bacterial loads and the Δ*pilD* strain had a significant reduction in percent retention compared to wild type ATCC17802 (Fig. 3.1A) (*P* value = 0.002). By 48hrs of depuration, Δ*mshA*, Δ*pilA*, Δ*mshA*/Δ*pilA*, and Δ*pilD* strains all exhibited significantly less percent retention of bacterial loads in oyster tissues compared with wild type ATCC17802 (Fig. 3.1A) (*P* values = 0.03, 0.008, 0.05, 0.02, respectively). When examining the Type I pili mutant Δ*csuB* (Fig. 3.1B), at 4hrs of depuration the percent retention of bacterial counts was not significantly different than the wild type ATCC17802 (*P* value = 0.28); in contrast, at 24 and 48hrs of depuration, the Δ*csuB* strain had a significantly less percent retention of bacterial CFU/g of tissue compared with wild type ATCC17802 (Fig. 3.1B) (*P* values = 0.001 and 0.05 respectively). From these data,

it appears that *V. parahaemolyticus* uses both Type I and IV pili to persist in the Pacific oyster during the depuration process.

Fig. 3.1. Percent retention of *V. parahaemolyticus* pili mutants over forty-eight hours of depuration. The percent retention of *V. parahaemolyticus* A) Type IV pili mutants Δ*mshA*, Δ*pilA*, Δ*mshA*/Δ*pilA*, Δ*pilD* and B) Type I pili mutant Δ*csuB* compared to wild type. Asterisk indicates retention of pili mutants are significantly different than the wild type strain. * *P* value of ≤ 0.05. Error bars indicate standard deviation.



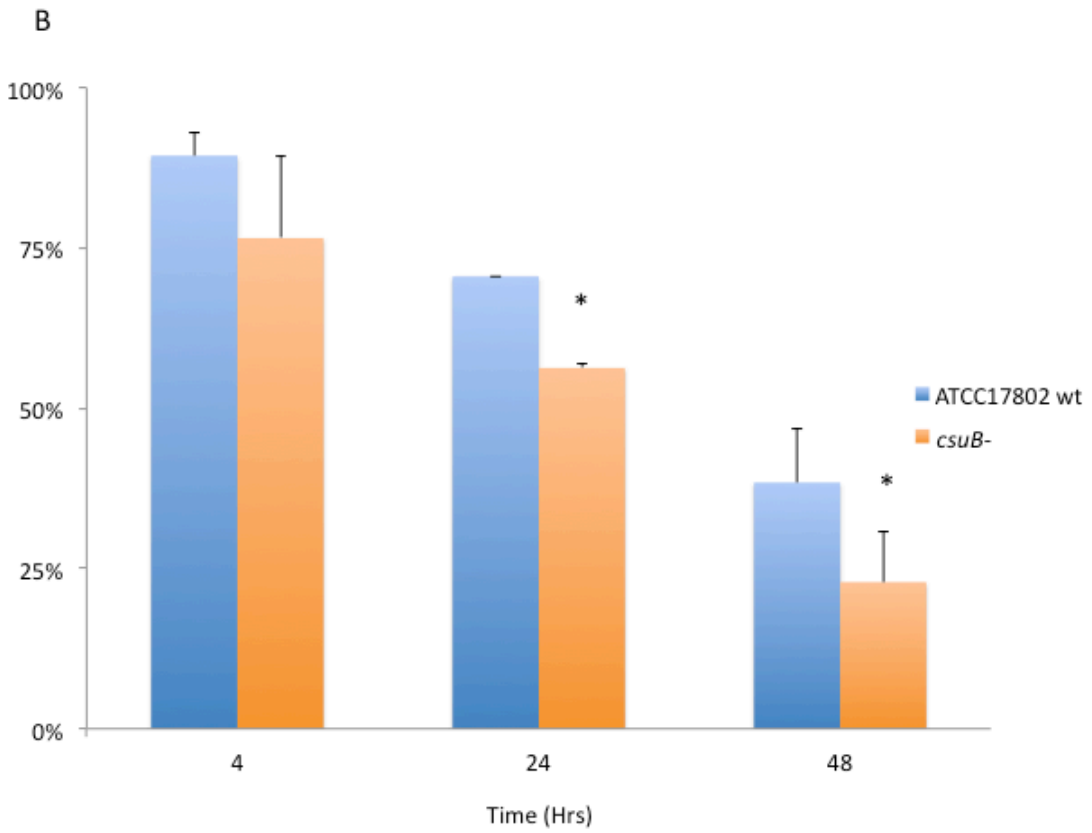


Fig. 3.1. Percent retention of *V. parahaemolyticus* pili mutants over forty-eight hours of depuration.

To examine the possible role of flagellar systems in *V. parahaemolyticus* colonization of the oyster, polar (Δ*flaM*) and lateral flagella (Δ*lafK*) mutants in *V. parahaemolyticus* strain TR, as well as a completely non-motile strain (Δ*flaK*/Δ*lafK*) (Table 3.1) were tested for persistence in the Pacific oyster during depuration. The percent retention of Δ*lafK* and *Δ*flaK*/Δ*lafK* strains after 24hrs of depuration was significantly different than wild type TR strain (*P* value = 0.022 and 0.017, respectively) (Fig. 3.2). By 48hrs, the Δ*flaM* and *Δ*flaK*/Δ*lafK* strains was retained significantly less than the wild type TR strain (Fig. 3.2) (*P* value = 0.0076 and 0.029,

respectively). These data suggest that both flagellar systems play an important role in persistent colonization of the Pacific oyster.

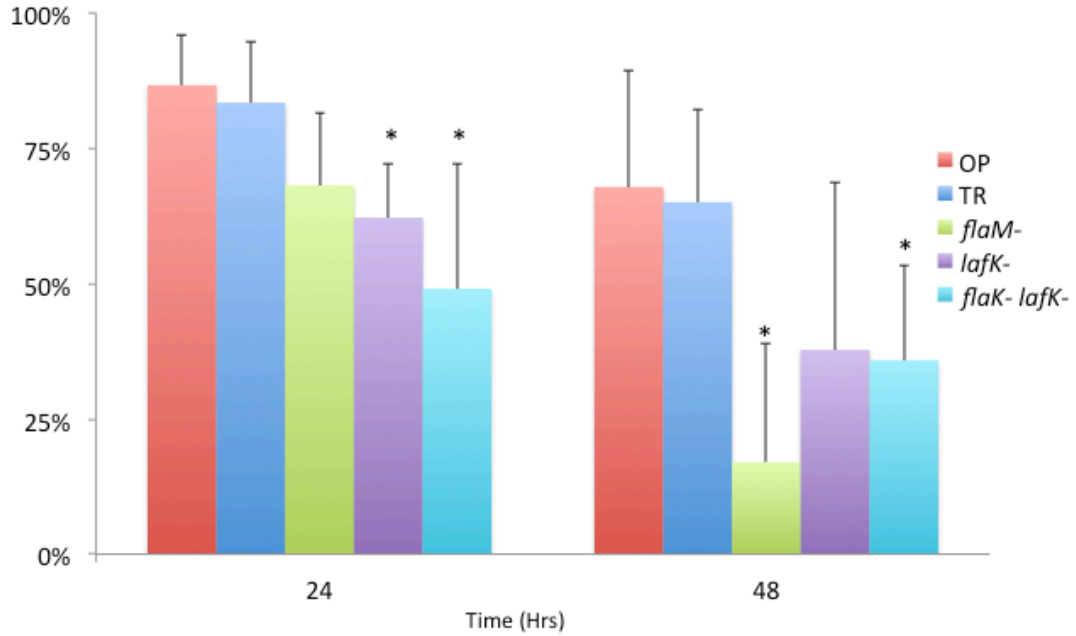


Fig. 3.2. Percent retention of *V. parahaemolyticus* flagella mutants over forty-eight hours of depuration. The percent retention of *V. parahaemolyticus* Δ*flaM*, Δ*lafK*, Δ*flaK*/Δ*lafK* compared with wild type. Asterisk indicates retention of flagella mutants are significantly different than the wild type strain. * *P* value of ≤ 0.05. Error bars indicate standard deviation.

V. parahaemolyticus exhibits phase variation from the opaque (OP) to translucent (TR) cell type due to the amount of CPS produced by the cell (Enos-Berlage and McCarter 2000). OP strains produce larger quantities of CPS than TR strains and have increased adherence to epithelial cells (Hsieh, Liang et al. 2003). The OP and TR strains were not statistically different in their percent retention at either 24hrs (*P* value = 0.66) or 48hrs of depuration (*P* value = 0.83) (Fig. 3.2), suggesting phase variation is not involved in colonizing the Pacific oyster.

Type III secretion systems (T3SS) are important virulence factors in many bacterial species. *V. parahaemolyticus* possesses two distinct T3SSs, T3SS1 and T3SS2. T3SS1 is located on chromosome 1 and is used for cytotoxicity (Park, Ono et al. 2004; Zhou, Konkel et al. 2009; Matlawska-Wasowska, Finn et al. 2010) while T3SS2 is located on chromosome 2 and causes enterotoxicity (Park, Ono et al. 2004). When the Type III secretion system double mutant was tested, there was no significant different in percent retention of the ΔT3SS1/ΔT3SS2 double mutant strain compared to the wild type TR strain over 24hrs (*P* value = 0.61), 48hrs (*P* value = 0.83), 72hrs (*P* value = 0.27) and 96hrs (*P* value = 0.19) of depuration (Fig. 3.3), suggesting that *V. parahaemolyticus* does not utilize either T3SSs for persisting in the Pacific oyster.

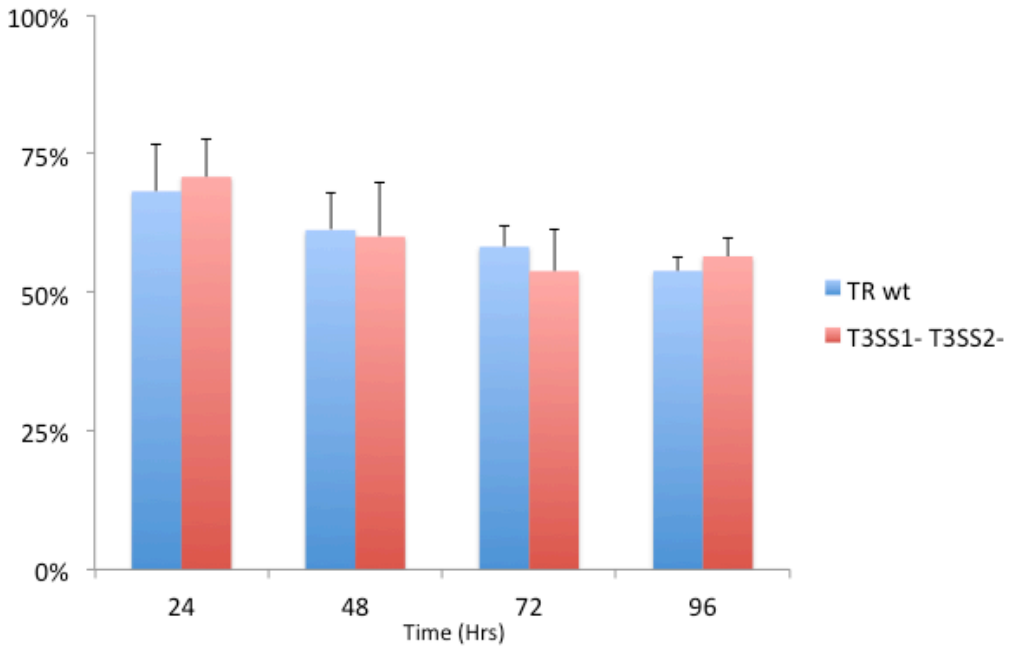


Fig. 3.3. Percent retention of *V. parahaemolyticus* Type III secretion system mutant. The percent retention of *V. parahaemolyticus* ΔT3SS1-/ΔT3SS2- compared with wild type. Asterick indicates retention Type III secretion system mutant was significantly different than the wild type strain. * *P* value of ≤ 0.05. Error bars indicate standard deviation.

Differences in depuration amongst different V. parahaemolyticus strains

In the process of identifying that pili and flagella but not phase variation or T3SSs are involved in persisting in the Pacific oyster, we noted that the two different wild type background strains used for mutant creation exhibited different depuration rates. The OP/TR strains were highly resistant to depuration while the ATCC17802 strain appeared more susceptible to depuration (Figs 3.1, 3.2, and 3.3). By 48hrs, the OP and TR strains remained at a much higher percent bacterial retention (65-68%) than the ATCC17802 strain (38%). Studies using ATCC17802 were concluded by 48hrs due to the significant reduction of the wild type strain, making it difficult to determine differences in colonization by the pili mutants past that point. In contrast,

the OP and TR strains remained at the relatively high percent bacterial retention (57%) even at 96hrs of depuration (Fig. 3.3). Based on these observations, various *V. parahaemolyticus* strains isolated from different sources were tested individually to determine if depuration rates were significantly different. Table 3.3 shows the percent retention of 8 strains of *V. parahaemolyticus* after 24 and 48hrs of depuration. Even though the percent bacterial retention of OP and TR were not significantly different (*P* value = 0.66 for 24hrs and 0.83 for 48hrs), both strains were included and used for comparison. After 24hrs of depuration, the 5432 (OP) and 5431 (TR) strains had the highest percent retention of bacteria of all the strains tested with 86-87% recovered (Table 3.3). FDA isolate O27-1C1 and ATCC17802 had a slightly lower percent retention at this time point retaining 70-75% of the initial input, but overall were not significantly different than either OP or TR. In the remaining strains, 10290, 10292, 10293 and BE98-2029, only 56-59% of the bacteria were retained in the animal after 24hrs of depuration, with BE98-2029 persisting at the lowest level (56.4±14.8%) (Table 3.3).

Table 3.3. Percent retention of eight *V. parahaemolyticus* strains in the Pacific oyster over 48hrs of depuration

	V. parahaemolyticus strain							
Time (hrs)	ATCC 17802	10292	10290	BE98-2029	10293	O27-1C1	5431	5432
24	70.5%±0.01a ABC	59.5%±6.1a BC	58.3%±9.5a C	56.4%±14.8a C	57.2%±2.9a C	74.9%±0.5a AB	87.3%±8.5a A	86.7%±9.4a A
48	38.4%±8.5b C	41.7%±10.3b BC	40.2%±4.5b C	43.9%±13.7a BC	57.2%±14.8a ABC	72.1%±3.4a A	66.1%±19.7a AB	67.9%±21.7a AB

At 24hrs, 5431 > 5432 > O27-1C1 > ATCC17802 > 10292 >10290 > 10293 > BE98-2029
After 48hrs, O27-1C1 > 5432 > 5431 > 10293 > BE98-2029 > 10292 > 10290 >ATCC 17802

Capital letter indicates signifcant differences amongst strains at either 24 or 48hrs of depuration.
Lower case letters indicate significant differences in percent retention within a strain over time.

By 48hrs of depuration, O27-1C1 maintained the highest percent retention in the oysters with 72.1±3.4% (Table 3.3), followed by 5432, 5431, and 10293 although the differences were not statistically different. Strains BE98-2029, 10292 and 10290

were significantly reduced, compared to O27-1C1, in their bacterial retentions with only approximately 40-44% of their starting bacterial inputs recovered. Lastly, strain ATCC17802 had significantly less bacterial retention than O27-1C1, 5431, and 5432 after 48hrs of depuration with only 38.4±8.5% recovered and only ATCC17802, 10292 and 10290 had significant reductions in their retention in the oyster from 24hrs to 48hrs of depuration (Table 3.3). These data show that different *V. parahaemolyticus* strains exhibit different oyster colonization abilities, suggesting that possible genetic differences exist between these strains that confer an ability of certain strains to better persist in the Pacific oyster.

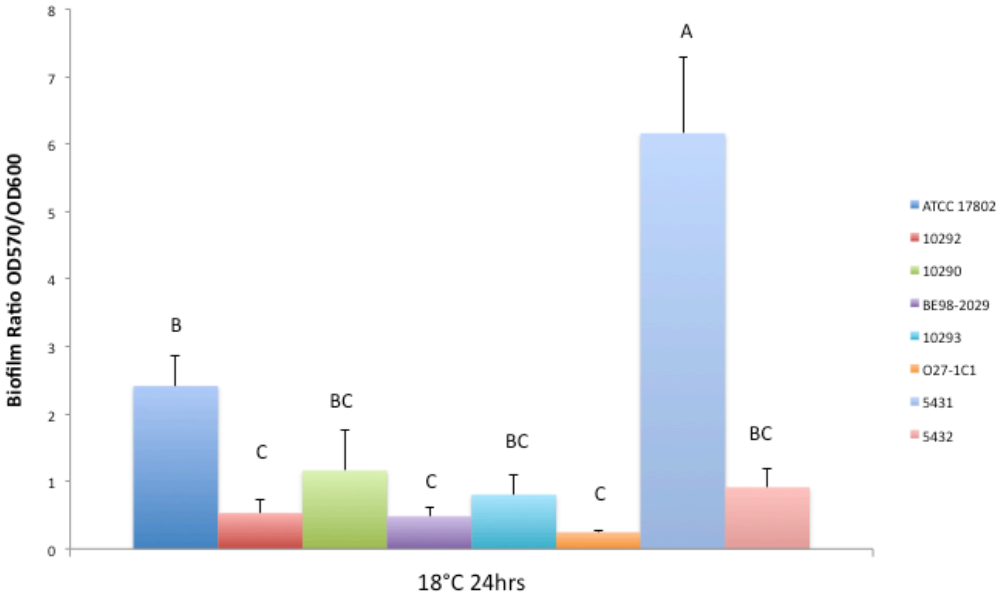
Biofilm formation in various V. parahaemolyticus strains

To identify if colonizing an abiotic surface, typically resulting in biofilm formation, correlated with the depuration data, each *V. parahaemolyticus* strain was tested for biofilm formation on borosilicate glass tubes (Fig. 3.4A) or plastic microtitre plates (Fig. 3.4B) at 18°C for 24hrs, 30°C for 4hrs or 24hrs, and 37°C for 4hrs or 24hrs. To account for any variability in growth rates that may exist between these strains, a growth curve was conducted and the biofilm data was normalized to the OD600nm for the strain at that time point at the indicated temperature. A normalized biofilm ratio > 1 indicated that a large quantity of cells adhered to the surface at low cell densities (OD600nm), whereas values near 1 had a similar cell attachment compared to overall growth. Biofilm ratios < 1 indicate that while cell growth increased, little biomass was accumulating on the surface. Biofilms grown at 18°C for 24hrs were examined because in the oyster infection assays, animals were exposed to *V*

parahaemolyticus at approximately 18°C for 16-18hrs, thus making this biofilm condition relevant to the oyster depuration data. Fig. 3.4A shows the biofilm data at 18°C for 24hrs on glass tubes for strains ATCC17802, 10290, 10292, 10293, BE98-2029, O27-1C1, 5431 and 5432, and shows significant differences between these strains. Strain 5431 had a significantly higher biofilm ratio than all the strains tested, followed by ATCC17802, 10290, 5432, and 10293. 10292, BE98-2029 and O27-1C1 had very low biofilm ratios, with O27-1C1 exhibiting the lowest biofilm ratio overall. The biofilm data for growth on plastic at 18°C for 24hrs (Fig. 3.4B) were similar to what was observed on glass, 5431 had the highest biofilm ratio amongst the strains tested, followed by ATCC17802. The remaining strains (10292, 10290, BE98-2029, 10293, O27-1C1 and 5432) varied slightly amongst each other and strain O27-1C1 had the lowest overall biofilm ratio. When comparing these biofilm forming abilities on glass or plastic with depuration experiments (both conducted at 18°C), these rankings did not correlate with each other. For instance, a strain that had the highest animal retention (O27-1C1) (Table 3), presumably by adhering well to the oyster surface, did not adhere as well to either glass or plastic (Fig. 3.4).

Fig. 3.4. Quantification of biofilm formation of different *V. parahaemolyticus* strains. Strains were grown in A) glass tubes and B) polystyrene microtiter plates at 18C for 24hrs, 30C for 4hrs, 30C for 24hrs, 37C 4hrs, and 37C 24hrs. Attached cells were stained with 0.1% crystal violet, solubilized with DMSO, and measurements were made on a spectrophotometer at OD 570nm. The values with different letters are significantly different with a *P* value of ≤ 0.05.

A



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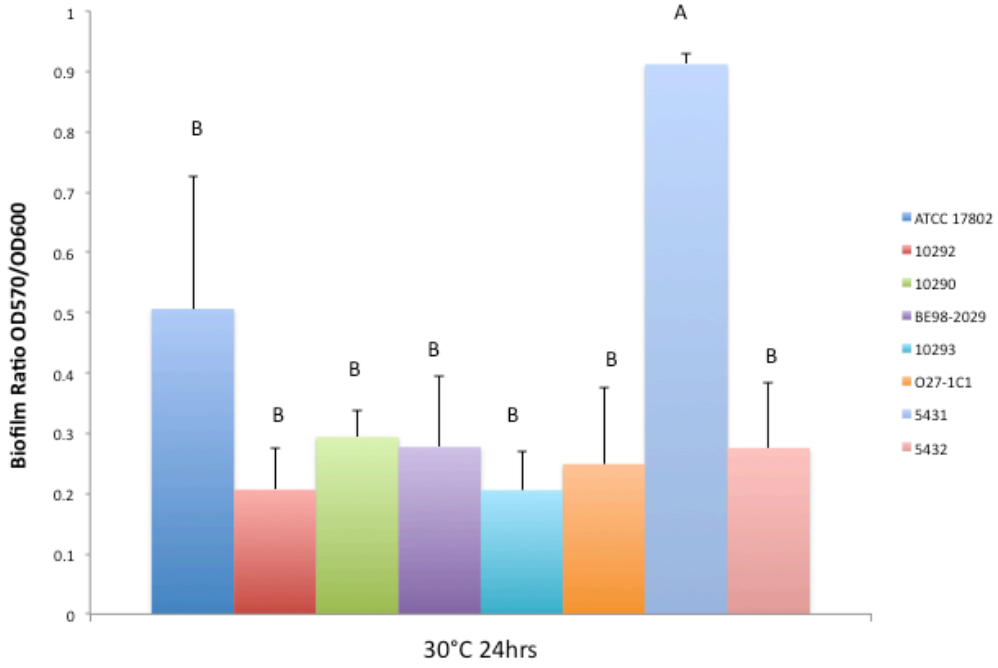
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0.7
0.6
0.5
0.4
0.3
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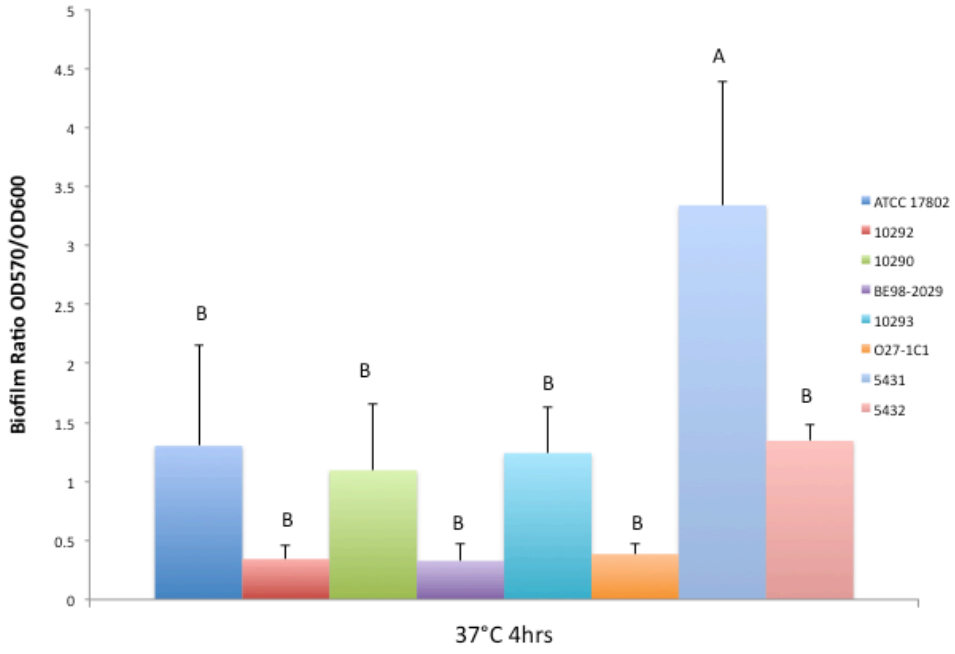
0.1
0
B
B
B
B
B
B
A
B
ATCC 17802
10292
10290
BE98-2029
10293
O27-1C1
5431
5432
30°C 24hrs

A


Biofilm Ratio OD570/OD600
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4
3.5
3
2.5
2
1.5
1
0.5
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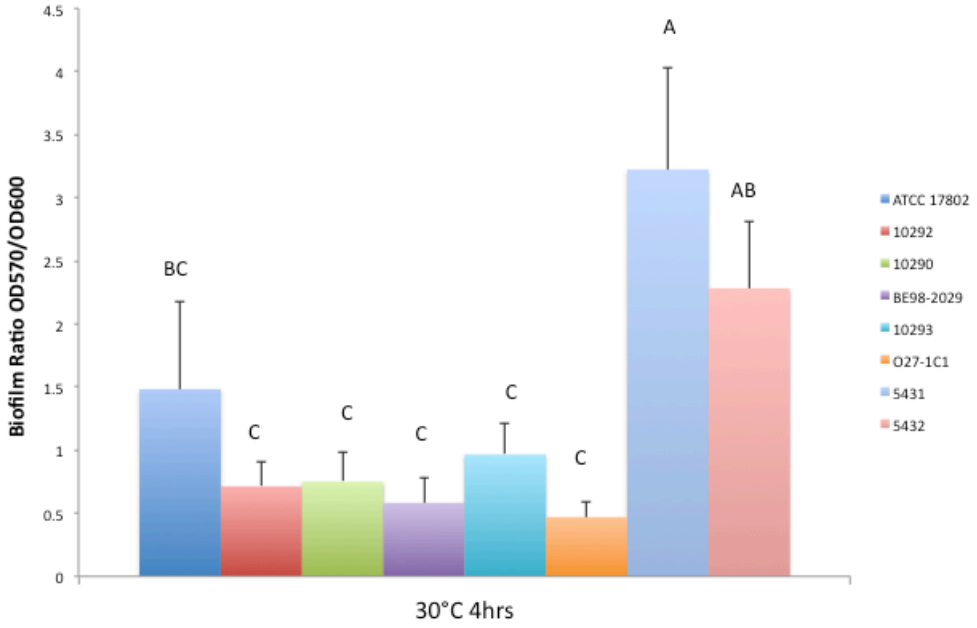
B
B
B
B
B
B
A
B
ATCC 17802
10292
10290
BE98-2029
10293
O27-1C1
5431
5432
37°C 4hrs

A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30°C 4hrs
ATCC 17802
10292
10290
BE98-2029
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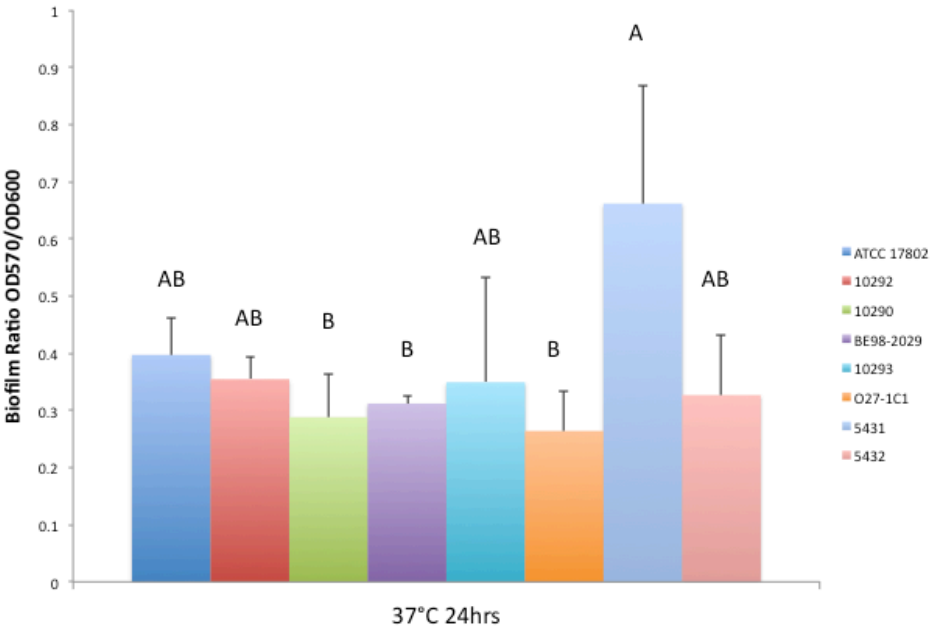
5431
5432

A


Biofilm Ratio OD570/OD600
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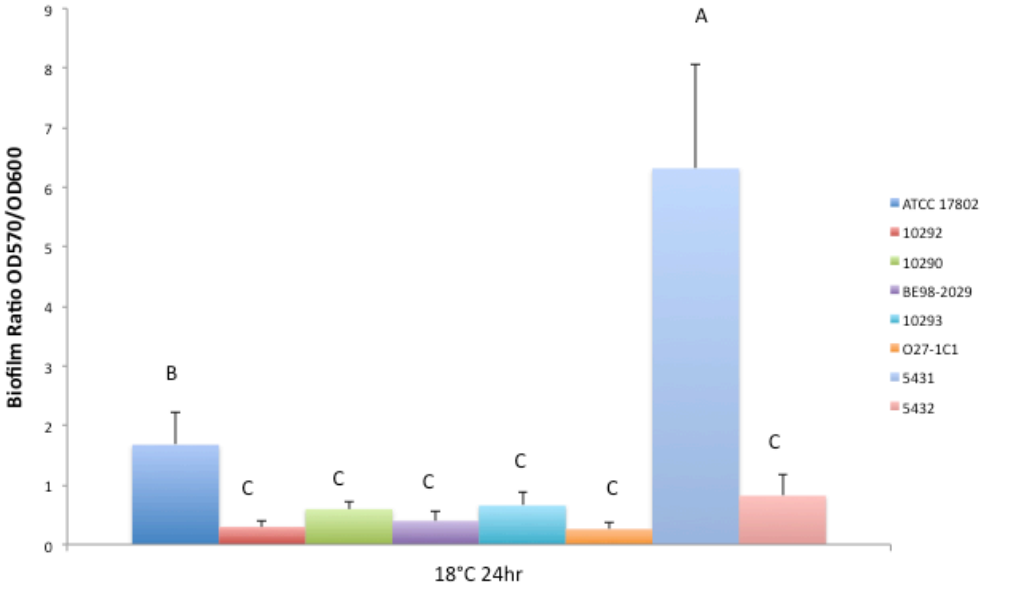
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
AB
AB
B
B
AB
B
A
AB
37°C 24hrs
ATCC 17802
10292
10290
BE98-2029
10293
O27-1C1
5431
5432

B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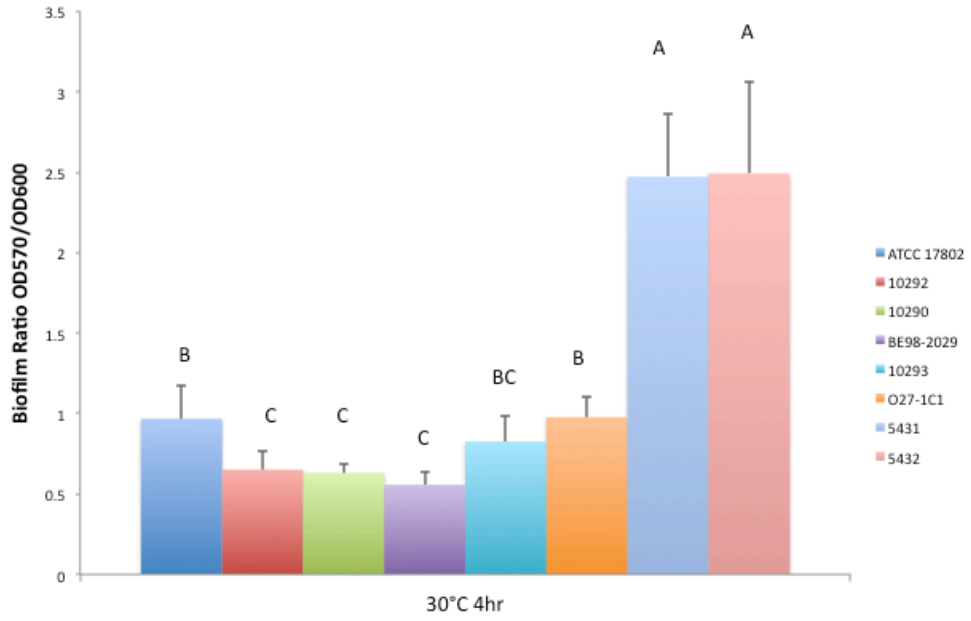
9
8
7
6
5
4
3
2
1
0
B
C
C
C
C
C
A
C
18°C 24hr
ATCC 17802
10292
10290
BE98-2029
10293
O27-1C1
5431
5432

B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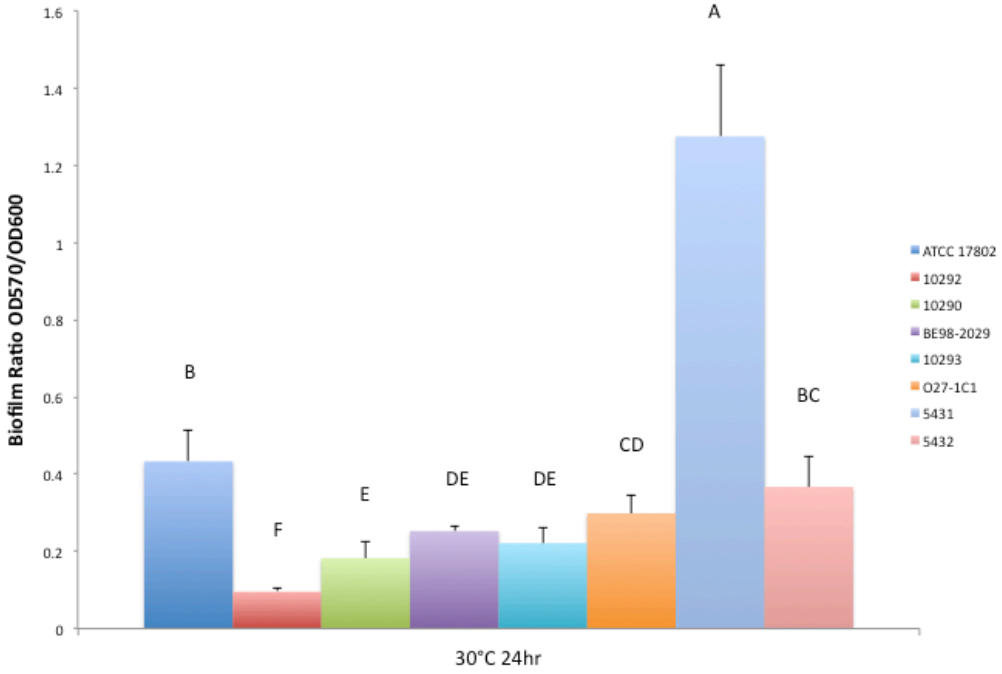
3
2.5
2
1.5
1
0.5
0
B
C
C
C
BC
B
A
A
30°C 4hr
ATCC 17802
10292
10290
BE98-2029
10293
O27-1C1
5431
5432

B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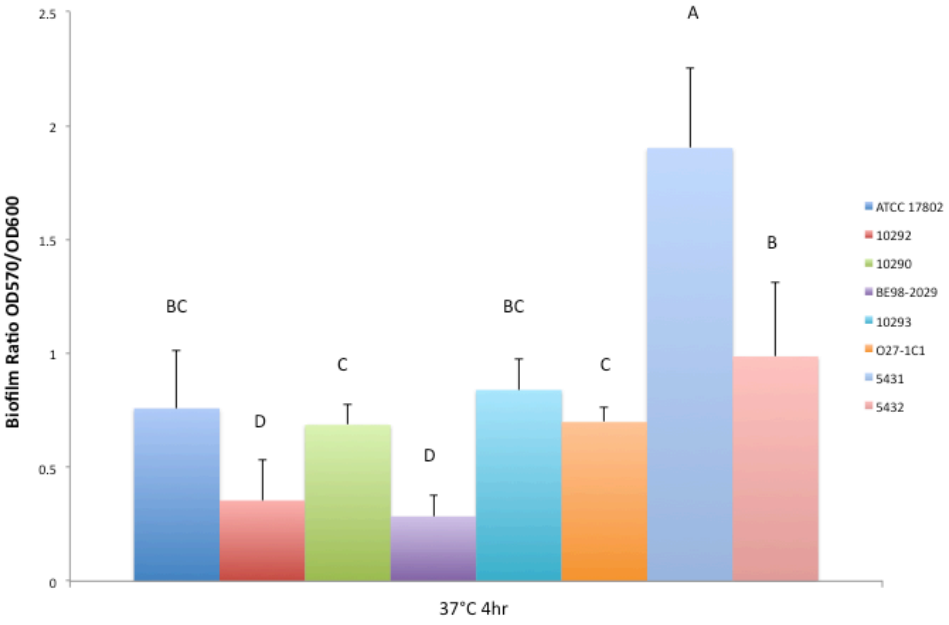
1.4
1.2
1
0.8
0.6
0.4
0.2
0
B
F
E
DE
DE
CD
A
BC
30°C 24hr
ATCC 17802
10292
10290
BE98-2029
10293
O27-1C1
5431
5432

B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2
1.5
1
0.5
0
BC
D
C
D
BC
C
A
B
37°C 4hr
ATCC 17802
10292
10290
BE98-2029
10293
O27-1C1
5431
5432

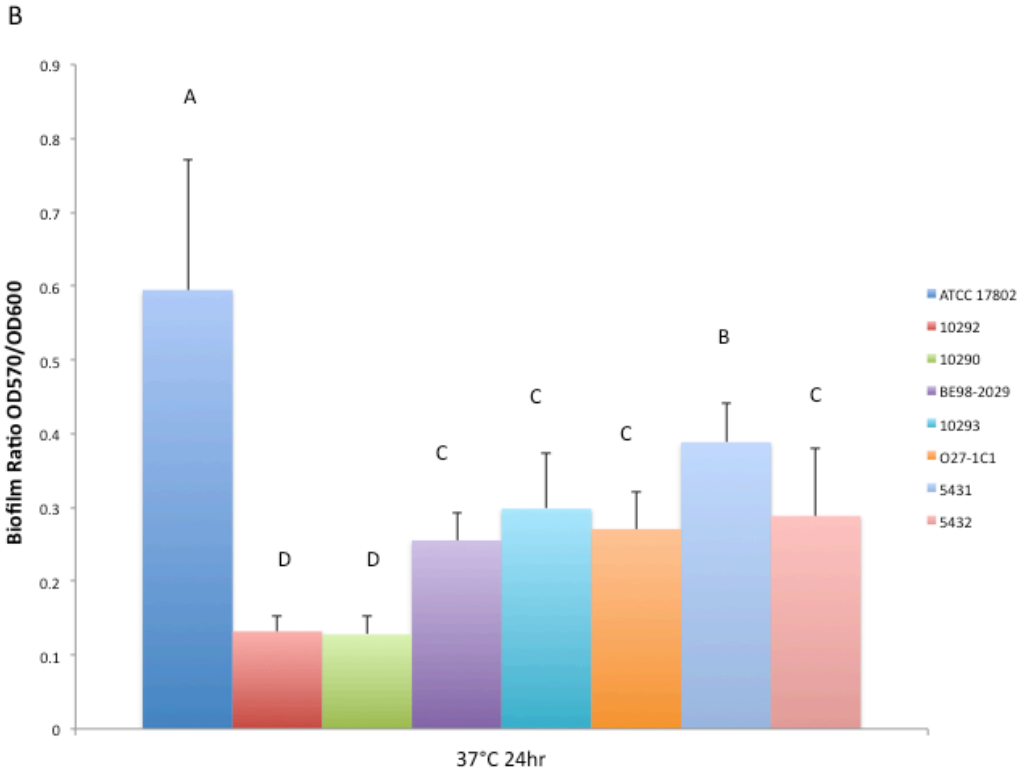

Fig. 3.4. Quantification of biofilm formation of different *V. parahaemolyticus* strains.

For oyster depuration experiments, *V. parahaemolyticus* strains were cultured under static conditions for 4 to 5 hours at 37°C, which would correspond to an early biofilm time point. Therefore, strains were tested at an early biofilm time point to determine if any correlation existed between biofilm formation and oyster attachment after 4 hours of growth at 37°C. Again, biofilm formation on glass (Fig. 3.4A) was highest for strain 5431, followed by 5432, ATCC17802, 10293 and 10290. Strains O27-1C1 and 10292 had a very low biofilm ratio on glass after 4hrs at 37°C. Biofilms on plastic after 4hrs of growth at 37°C is shown in Fig. 3.4B. Similar to the findings for glass, strain 5431 had the highest biofilm ratio of all the strains, followed by 5432, 10293, ATCC17802, O27-1C1, 10290, 10292 and BE98-2029. Differences

did exist between these strains and their ability to form biofilms on glass versus plastic. This was evident for strain O27-1C1, which had the lowest biofilm ratio on glass but was in the middle for plastic and interesting because it is one of the highly retained bacterial strains in the depuration experiments (Table 3.3). Overall, early biofilm formation (4hrs) at 37°C did not correlate well with the depuration data for these strains at either 24hrs or 48hrs (Table 3.3) and does not adequately represent the adherence abilities of these strains immediately prior to their interactions with oysters.

Since the biofilm experiments performed under conditions most similar to our oyster infection assay (18°C for 24hrs and 37°C for 4hrs) did not correspond to the depuration data for these strains (Table 3.3), other conditions were also examined. Although unrelated to the conditions used during the oyster depuration assays, an early biofilm time point (4hrs) at 30°C and late biofilm time points (24hrs) at 30°C and 37°C were examined in an attempt to identify any connection between biofilm adherence and oyster colonization,. At 30°C for 4hrs on glass (Fig. 3.4A), the results were very similar to what was observed at 37°C after 4hrs of growth. Strain 5431 had the highest overall biofilm ratio, followed by 5432 and ATCC17802. The five FDA clinical isolates, 10290, 10292, 10293, BE98-2029, and O27-1C1 had very low biofilm ratios, significantly less than 5431 and 5432. The results for biofilms on plastic (Fig. 3.4B) at 30°C after 4hrs did not show dramatic differences from what was observed for glass. Strains 5431 and 5432 had significantly higher biofilm ratios than all the other strains, followed by ATCC17802, O27-1C1 and 10293. 10290,

10292 and BE98-2029 had the lowest biofilm ratios of all the strains tested, significantly less than 5431, 5432, ATCC17802 and O27-1C1. The results for 30°C at 4hrs on glass versus plastic were very similar with no dramatic differences observed and under these conditions the strains did not appear to exhibit similar trends in their biofilm forming abilities and adherence to oysters.

Biofilm ratios on glass after 24hrs of incubation at 30°C are shown in Figure 4A. It is important to note that by 24hrs, all strains had achieved stationary phase (data not shown). The highest biofilm ratios were observed for strain 5431 and ATCC17802, whereas the remaining strains10292, 10290, BE98-2029, 10293, O27-1C1, and 5432 had very low biofilm ratios. Comparing these data with the biofilms grown on plastic for 24hrs at 30°C (Fig. 3.4B), a similar trend was observed; 5431 and ATCC17802 had the highest biofilm ratios, followed by 5432. Moreover, strains 10290, 10292, 10293, BE98-2029, and O27-1C1 exhibited low biofilm ratios (Fig. 3.4B), resembling what was observed for these strains growing on glass (Fig. 3.4A). While the biofilm ratios of glass and plastic are similar for these strains, the pattern of biofilm adherence by these strains at 30°C with 24hrs of growth did not relate to the data obtained during the depuration experiments (Table 3.3).

When these strains are grown on glass for 24hrs at 37°C, all strains had similar biofilm ratios except 5431, which had the highest biofilm ratio. Comparing glass biofilm ratios of these strains with those from growth on plastic, a definite contrast was observed. Strain ATCC17802 had the highest biofilm ratio, followed by strain 5431. This is in direct contrast to the data for all the other biofilm conditions

tested (18°C 24hrs glass and plastic, 30°C 4 and 24hrs glass and plastic, 37°C 4hrs glass and plastic and 37°C 24hrs glass) where 5431 maintained the highest biofilm ratio of all the strains. The next highest biofilm ratios were from 10293, 5432, O27-1C1, BE98-2029, 10292 and 10290, respectively. Previous comparisons between glass and plastic biofilm conditions showed a somewhat consistent trend for all the strains tested. Comparing glass and plastic biofilms at 37°C for 24hrs actually shows a switch in the highest biofilm ratio from strain 5431 to ATCC17802. It is interesting to point out that strain ATCC17802 had the lowest percent bacterial retention (38%) after 48hrs of depuration while strain 5431 had one of the highest (66%) (Table 3.3), again showing that biofilm forming abilities did not reflect the depuration data observed for these strains. Overall, biofilm data under any of the conditions tested (Fig. 3.4), whether they were relevant or non-relevant to the oyster depuration experimental conditions, did not correspond consistently with depuration data. This suggests that bacterial interactions with biotic and abiotic surfaces are a complex interplay between multiple factors and colonization of one type of surface does not accurately reflect colonization of another.

Motility of the different V. parahaemolyticus strains

Figure 3.2 demonstrated that both flagellar systems, polar and lateral, are important for persistence of *V. parahaemolyticus* in the oyster infection assay. Therefore each *V. parahaemolyticus* strain was tested for use of flagella in swarming and swimming assays to identify possibly differences in motility between these strains. Swarming is

a surface-contact dependent motility that utilizes peritrichous flagella to move across the surface when the constitutively produced polar flagella becomes restricted in rotation by increased viscosity or is no longer capable of locomotion (McCarter 2001). Figure 3.5A shows swarming abilities of the various strains. Strain 5431 is a fixed translucent (TR) phase variant and the TR phenotype has been published as an excellent swarmer, while strain 5432 is an opaque (OP) phenotype that has been published as a weak swarmer (Jaques and McCarter 2006), making these two strains excellent comparisons to characterize the various *V. parahaemolyticus* isolates. Strains O27-1C1, 10292, 5431 and BE98-2029 were swarm positive (++), meaning these strains likely produce lateral flagella to move across surfaces. Strain 5432 was a weak swarmer (+), confirming what has been previously published, and strains 10290, 10293 and ATCC17802 appear to be non-swarming (-). Comparing swarming abilities with the depuration data, there is a loose correlation between swarm$^+$ strains and increased bacterial retention in the oyster. Swarm$^+$ strains O27-1C1, 5431 and 5432 were maintained in the oyster more than the other strains, such as the swarm$^-$ strains ATCC17802 and 10290 (Table 3.3). However, swarm$^-$ strain 10293 was maintained at relatively high amounts in the oyster, comparable to two swarm$^+$ strains, O27-1C1 and BE98-2029. Swarming did not appear to correlate with the biofilm data obtained in this study. O27-1C1 was a proficient swarmer (Fig. 3.5), was highly retained in the oyster (Table 3.3) but a poor biofilm former (Fig. 3.4), comparatively speaking, under all conditions tested. Although possessing lateral flagella is important for persistence (Fig. 3.2), strains unable to swarm proficiently

are still capable of efficiently colonizing the animal. Overall, these data further demonstrate how complex and multifaceted the interactions are between *V. parahaemolyticus* and the Pacific oyster.

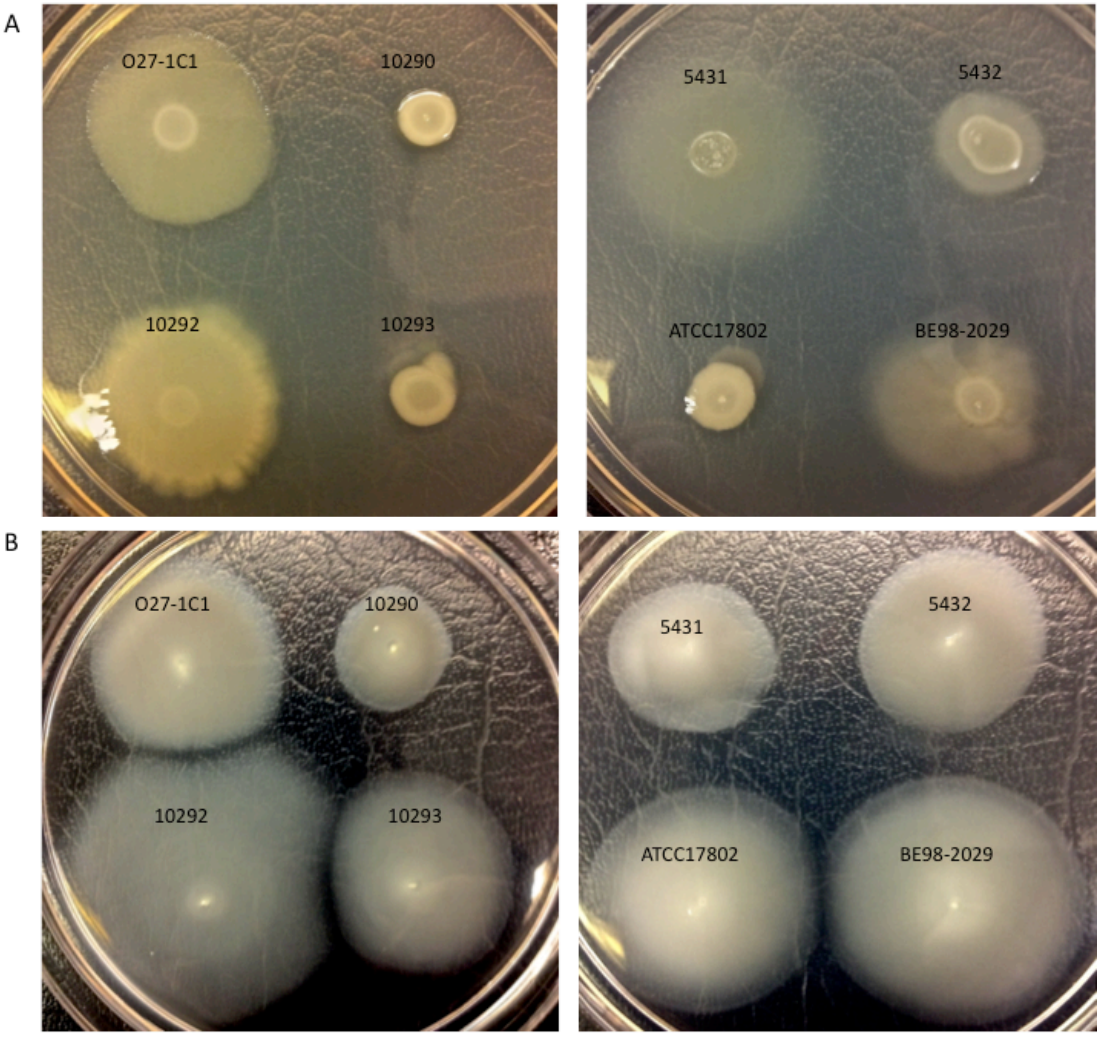

Fig. 3.5. Motility assays of different *V. parahaemolyticus* strains. 2ul of overnight cultures were inoculated in A) swarming motility (30°C for 22hrs) and B) swimming motility plates (20°C for 22hrs).

Figure 3.5B shows a swimming motility assay of the various strains and it appears that all strains are capable of swimming with similar swimming diameters, although 10290 appears to have a slightly smaller swimming diameter than the other

strains. It is important to point that out that swimming motility agar is not completely conclusive for measuring the sole contribution of the polar flagellum because both polar and lateral flagella are utilized to swim in the semi-solid agar motility assays (McCarter and Silverman 1990). Comparisons between swimming and depuration showed that there is not a direct correlation between swimming abilities and persistence in the oyster, although it is attractive to suggest that the smaller swimming diameter of 10290 may correlate with its lower retention in the animal. Further studies investigating the individual roles of polar and lateral flagella in these strains are required to conclusively answer these questions. Overall, motility does not appear to directly correlate with a *V. parahaemolyticus* strain's ability to persist in the oyster.

Discussion

Mollusks are natural reservoirs for various *Vibrio* sp., ranging from *V. fischeri* colonizing the light organs of the Hawaiian squid (McFall-Ngai, Brennan et al. 1998; Stabb and Ruby 2003), *Euprymna scolopes*, to the human pathogen *V. parahaemolyticus* associating with shellfish (Croci, Suffredini et al. 2002; Vieira, Costa et al. 2011; Johnson, Bowers et al. 2012). These inherent associations with human pathogens such as *V. parahaemolyticus* illustrate a potential health hazard from the consumption of edible mollusks, such as oysters. Since oysters naturally encounter various bacterial species in the marine environment, including vibrios, a process called depuration was developed to place harvested oysters into clean seawater in a controlled system, allowing them to purge contaminates from their

tissues. Vibrios have been shown to be resistant to depuration, suggesting they possess specific adherence mechanisms to persist in the animal under these conditions (Marino, Crisafi et al. 1999; Croci, Suffredini et al. 2002; Marino, Lombardo et al. 2005). Several studies have investigated the relationship between *Vibrio* sp. and shellfish (Marino, Crisafi et al. 1999; Chiavelli, Marsh et al. 2001; Marino, Lombardo et al. 2005) and more recently, genetic analyses have begun to identify specific factors contributing to *V. vulnificus* colonization of *C. virginica* (Paranjpye, Johnson et al. 2007; Srivastava, Tucker et al. 2009). In this study, we examined the role of various bacterial traits predicted to be involved in *V. parahaemolyticus* persistence in the Pacific oyster, *C. gigas*. Bacteria are known to use a variety of structures to adhere to different surfaces, including biotic surfaces such as eukaryotic cells (Stabb and Ruby 2003; Paranjpye and Strom 2005; Dalisay, Webb et al. 2006) and abiotic surfaces such as borosilicate glass (Shime-Hattori, Iida et al. 2006; Abdallah, Chaieb et al. 2009) or plastic microtitre plates (Watnick, Fullner et al. 1999; Enos-Berlage, Guvener et al. 2005; Snoussi, Noumi et al. 2008); some of these structures include pili (McFall-Ngai, Brennan et al. 1998; Chiavelli, Marsh et al. 2001; Enos-Berlage, Guvener et al. 2005; Dalisay, Webb et al. 2006) and flagella (Graf, Dunlap et al. 1994; Mobley and Belas 1995; Ottemann and Miller 1997). To determine if *V. parahaemolyticus* uses pili and/or flagella for resisting depuration in the Pacific oyster, mutants in the Type I pilin gene *csuB*, the Type IV pilin genes *mshA*, *pilA*, and *mshA/pilA*, the Type IV pilin peptidase *pilD*, and the flagellar regulatory genes *flaM*

(polar) *lafK* (lateral) and *flaK/lafK* (non-motile) were tested in a Pacific oyster infection assay.

Loss of Type I and Type IV pili in *V. parahaemolyticus* resulted in a decrease in overall percent of bacterial retention in oyster tissue over the 48hrs of depuration. The most dramatic reduction in bacterial retention was seen by mutation in *pilD,* encoding the Type IV pilin peptidase. Deletion of the *pilD* gene results in loss of all Type IV pili processed by the peptidase from the bacterial surface (Marsh and Taylor 1998; Paranjpye, Lara et al. 1998), including MSHA and PilA pili and potentially any others that *V. parahaemolyticus* may possess that were not targeted in this study. The Δ*mshA*/Δ*pilA* double gene deletion strain exhibited slightly higher bacterial retention compared to the Δ*pilD* strain and considerably less than either single deletion (Δ*mshA* or Δ*pilA*), suggesting that *V. parahaemolyticus* may use both of these pili (and possibly other, unidentified Type IV pili) for sufficient adherence to the oyster, resulting in the resistance to depuration. These results are consistent with what was observed in *V. vulnificus* where loss of *pilA* and *pilD* resulted in a decrease in bacterial loads in the tissues of *C. virginica* compared with wild type (Paranjpye, Johnson et al. 2007). Loss of the Type I pilin gene *csuB* resulted in significantly less bacterial retention than the wild type strain after 24hrs and 48hrs of depuration. Type I pili have been studied for their roles in attachment to various surfaces and biofilm formation and are known for their role in attachment to the bladder by uropathogenic *Escherichia coli* (UPEC) (Crepin, Houle et al. 2012) and host colonization by Salmonella (Ewen, Naughton et al. 1997; Althouse, Patterson et al. 2003), as well as

biofilm formation (Li, Hao et al. 2007). Type IV pili are also known for their attachment to various surfaces and involvement in biofilm formation of many bacterial species (Zhang, Tsui et al. 2000; Li, Hao et al. 2007), including vibrios (McFall-Ngai, Brennan et al. 1998; Stabb and Ruby 2003; Paranjpye and Strom 2005; Shime-Hattori, Iida et al. 2006; Paranjpye, Johnson et al. 2007; Barken, Pamp et al. 2008; Srivastava, Tucker et al. 2009). Based on our data, it appears that *V. parahaemolyticus* use both Type I and Type IV pili to adhere to the oyster. That could explain why *V. parahaemolyticus* is quite resistant to depuration. However, complete loss of attachment to the oyster was not observed for any of the pili mutants tested. The data suggests that attachment by Type I and Type IV pili may have an additive effect and examination of a combination mutant in future studies would be interesting. A study examining the role of MSHA and PilA in *V. parahaemolyticus* adherence to abiotic surfaces also showed that loss of individual pili had a slight reduction, while loss of both had an additive effect (Shime-Hattori, Iida et al. 2006). Therefore, it appears that attachment to a surface by *V. parahaemolyticus*, such as an oyster, utilizes many factors, including Type I and Type IV pili, possibly in conjunction.

Flagella are known to be one of the first steps in colonizing a surface ((Barken, Pamp et al. 2008), reviewed in (Ottemann and Miller 1997)). In *V. parahaemolyticus*, polar flagella are constitutively produced and utilized for locomotion in liquid environments, while lateral flagella are induced when the cell is in contact with a surface or encounters a viscous environment and are used to

"swarm" over a surface ((Jaques and McCarter 2006), reviewed in (McCarter 2001; McCarter 2004)). Polar flagella act as a tactile sensor for lateral flagella production (McCarter and Silverman 1990; Kawagishi, Imagawa et al. 1996) and if the polar flagellum is unable to rotate by either mutation or surface-associated blockage that signals the cell to produce lateral flagella. We showed that loss of flagella results in a decrease in *V. parahaemolyticus* retention in the oyster during depuration. After 24hrs of depuration, all flagellar system mutants had reductions in their overall bacterial retentions, while only the lateral flagella mutant (Δ*lafK*) and the non-motile double flagella mutant (Δ*flaK*/Δ*lafK*) were significantly reduced compared to the wild type (TR) strain. By 48hrs of depuration, all strains had a reduction in the percent of retention but only the polar flagella mutant (Δ*flaM*) and the double flagella mutant (Δ*flaK*/Δ*lafK*) were significantly less than TR. Based on these findings, it appears that both flagellar systems positively contribute to *V. parahaemolyticus* persistence in the oyster. Extensive work has been done to characterize the polar and lateral flagella of *V. parahaemolyticus* ((Ulitzur 1975; Shinoda and Okamoto 1977; Kimura, Tateiri et al. 1979; Belas and Colwell 1982; Belas, Simon et al. 1986; Kawagishi, Imagawa et al. 1996; Stewart and McCarter 2003; Kim and McCarter 2004; Park, Arita et al. 2005) reviewed in (McCarter 2001; McCarter 2004; Merino, Shaw et al. 2006)). Mutations in the polar flagella impair the early stages of *V. parahaemolyticus* cellular attachment in a biofilm (Enos-Berlage, Guvener et al. 2005), whereas loss of lateral flagella in *V. parahaemolyticus* results in a defect in biofilm formation as well as adherence to HeLa cells (Park, Arita et al. 2005). It seems plausible that attachment

to the surface of an oyster is similar to forming a biofilm on other biotic surfaces where flagella are the used as an initial attachment. If colonization of an oyster resembles biofilm formation, loss of factors required for efficient biofilm formation, such as flagella and pili are expected to reduce oyster colonization abilities. Therefore, it might not be surprising that loss of flagella resulted in a significant decrease in bacterial retention during oyster depuration. It appears that while flagella are important for persisting in an oyster, other factors are also involved, such as pili, supporting the notion that persistent colonization of an oyster by *V. parahaemolyticus* is a multifactorial process.

Type III secretion systems (T3SS) are an important virulence factor for many bacterial pathogens (Hueck 1998; Galan and Collmer 1999; Hiyoshi, Kodama et al. 2010; Matlawska-Wasowska, Finn et al. 2010; Zhao, Chen et al. 2010). The genome of *V. parahaemolyticus* encodes two T3SS systems (Park, Ono et al. 2004); T3SS1 is located on chromosome one and it contributes to *V. parahaemolyticus* cytotoxicity while T3SS2 is located on chromosome two and causes enterotoxicity (Park, Ono et al. 2004; Hiyoshi, Kodama et al. 2010; Noriea, Johnson et al. 2010). It has been shown that all *V. parahaemolyticus* strains possess T3SS1 while T3SS2 is predominately found in pathogenic (Kanagawa-positive) strains (Park, Ono et al. 2004). Oysters possess an innate immune system and have macrophage-like hemocytes that are responsible to bacterial killing (Philipp, Lipinski et al. 2012). Therefore, to persist in the oyster, bacteria likely have to overcome their immune defenses (Duperthuy, Schmitt et al. 2011). To test if T3SSs are required for persistent

colonization of the Pacific oyster, a ΔT3SS1/ΔT3SS2 mutant of *V. parahaemolyticus* was tested in the oyster infection assay. Interestingly, no significant difference was observed between the wild type and ΔT3SS1/ΔT3SS2 double mutant strains, suggesting that *V. parahaemolyticus* does not use either T3SSs to persist in the oyster host. Possibly, *V. parahaemolyticus* might use other unidentified factors or tactics to circumvent the oyster immune system for survival in the oyster host.

One possibility for oyster immune system evasion is phase variation in colony morphology from the opaque to the translucent phenotype, which is a result of altering CPS production. In *V. vulnificus*, translucent colonies were associated more with *C. virginica* hemocytes that the opaque colonies (Harris-Young, Tamplin et al. 1993) and have been shown to induce high-frequency variation in the oyster (Srivastava, Tucker et al. 2009). In addition, the *V. vulnificus* opaque phenotype is considered to be more virulent than the translucent phenotype (Amako, Okada et al. 1984; Yoshida, Ogawa et al. 1985). The *V. parahaemolyticus* strain 5432 exhibits an opaque (OP) phenotype that can shift between the opaque (large quantities of CPS) and translucent (little to no CPS production) phenotypes (Hsieh, Liang et al. 2003). In contrast, strain 5431 is a fixed translucent derivative of 5432 due to the deletion of the *opaR1* gene, which is the master regulator of this phenotype (Jaques and McCarter 2006). *V. parahaemolyticus* OP strains are known to adhere to epithelial cells better than TR strains (Hsieh, Liang et al. 2003). Therefore, we compared strains 5431 (TR) and 5432 (OP) to determine if CPS production affected colonization of the oyster. However, there was no significant difference observed

between strains 5431 and 5432, suggesting that phase variation may not play a large role *V. parahaemolyticus* colonization of the Pacific oyster. This finding is similar to what was observed in *V. vulnificus* where a translucent variant, capable of reverting back to the opaque phenotype, showed no difference in recoverability compared with the opaque wild type in whole oyster homogenates of *C. virginica,* but an acapsular deletion mutant and the rugose phenotype had significant reductions in recoverability (Srivastava, Tucker et al. 2009). Interestingly, the authors concluded that *V. vulnificus* translucent and rugose colonies capable of phase variation reverted back to the opaque phenotype after oyster passage (Srivastava, Tucker et al. 2009). This was not observed in our assay because the translucent phenotype (TR) is fixed by a deletion of *opaR1* so reversion to the opaque phenotype is not possible. Further studies are required to identify if phase variation to the opaque phenotype after oyster passage also occurs in *V. parahaemolyticus*.

The mutants tested in this study were created in two different *V. parahaemolyticus* background strains. Strain ATCC17802 was used to create the pili mutants while strain 5431 was the parent to the T3SS and flagellar systems mutants. Upon investigation of these different mutants in our Pacific oyster infection assay, it was noted that while the ATCC17802 wild type strain was rapidly reduced to approximately 40% of the starting bacterial load by 48hrs of depuration (Fig. 3.1), strain 5431 maintained approximately 60% of the starting input (Fig. 3.2 and 3.3). These data suggested that different *V. parahaemolyticus* strains can vary in their abilities to persist in the oyster host. To further investigate this, an additional five *V.*

parahaemolyticus strains were tested in the oyster infection assay to determine natural differences in bacterial retention in different bacterial isolates. The additional *V. parahaemolyticus* strains were all clinical isolates and were obtained from the Food and Drug Administration (FDA) Pacific Regional Laboratory Northwest (Bothell, WA) (Chiu, Duan et al. 2007) (Table 3.1). These FDA clinical strains have been used in previous studies as a combination strain mixture to compare different depuration conditions and post-harvest processing strategies (Su, Yang et al. 2010; Phuvasate, Chen et al. 2012). Here, we examined the strains individually and compared them with strains ATCC17802, 5432 and 5431 to determine any differences in their depuration abilities. Interestingly, bacterial retention in the oyster varied substantially amongst some of these different strains during different time points of depuration. Animals retained some strains (i.e. 5431 and ATCC17802) at considerably higher amounts than other strains after 24hrs of depuration but then lost up to 20% of their starting inputs by 48hrs. In fact, ATCC17802 was one of the highly retained strains after 24hrs only to drop to the least retained strain by 48hrs. In contrast some strains, O27-1C1 for example, maintained a high level of bacterial retention after 24hrs and did not exhibit a significant reduction in bacterial retention in the animals even after 48hrs of depuration. This shows that although some strains may have increased recoverability at early time points, increased depuration time can be successful in reducing bacterial retention for some strains, but not for others, while some strains are retained at similar amounts over the entire depuration experiment. Taken together, these data show that vast differences in oyster persistence exist

between these *V. parahaemolyticus* isolates. We have previously published that *V. parahaemolyticus* strains exhibit allelic diversity in their MSHA and PilA pilin subunit genes (Aagesen and Häse, 2012) and this may contribute to the differences in persistence during oyster depuration observed for the *V. parahaemolyticus* strains examined in this study. Future studies describing this observation, including examining more strains and defined mutants, may provide insights into what constitutes a "good" and "bad" oyster colonizer.

As mentioned previously, vibrios use pili and flagella for sufficient adherence to a surface, as well as capsular polysaccharides, during biofilm formation (Yildiz and Visick 2009). Since we determined that *V. parahaemolyticus* uses pili and flagella to persist in the oyster, the various *V. parahaemolyticus* strains were also characterized for differences in biofilm formation. The strains were tested under different conditions to examine if there was a correlation between biofilm formation and oyster depuration. Initially, we examined conditions representing our oyster infection assay such as growth at 18°C for 24hrs, relevant to oyster depuration conditions, and an early biofilm time point (4hr at 37°C) that most relates to the initial stages of oyster colonization where pili and flagella would be used for initial surface interactions and it may also reflect the bacterial adherence capabilities just prior to oyster exposure. We also examined other conditions, less relevant to the oyster infection condition; however, there did not appear to be any direct correlation between biofilm time, temperature, or surface to what was observed for these strains in depuration. In fact, no biofilm data, relevant or not relevant to the oyster infection assay conditions,

correlated with the oyster depuration data. Interestingly though, a similar trend was observed for all biofilm conditions tested where a "good" colonizer (5431) and "bad" colonizer (ATCC17802) had the two highest biofilm ratios, respectively, except for biofilms on plastic at 37° for 24hrs where the findings were reversed and ATCC17802 actually had the highest biofilm ratio. It is also important to note that all of the FDA clinical isolates are relatively poor biofilm formers under all of these conditions. Therefore, biofilm formation on an abiotic surface does not appear to directly relate to colonization on a biotic surface, consistent for what was concluded for *V. vulnificus* strains colonizing *C. virginica* (Srivastava, Tucker et al. 2009).

Other studies have compared biofilm formation amongst different strains within a species and have found dramatic differences in the biofilms produced by the various isolates (Mueller, McDougald et al. 2007; Snoussi, Noumi et al. 2008; Abdallah, Chaieb et al. 2009; McQueary and Actis 2011). One study examining *V. cholerae* environmental and clinical isolates using genetic analyses determined that 'there appears to be no set of "universal" biofilm genes for all strains (Mueller, McDougald et al. 2007)'. While genetically different strains may use the same mechanisms for biofilm formation, variations in the factors used to colonize the surface may differ. It is important to note that while vibrios are known to use Type IV pili, including MSHA and PilA, for colonizing surfaces (Watnick, Fullner et al. 1999; Chiavelli, Marsh et al. 2001; Paranjpye and Strom 2005; Shime-Hattori, Iida et al. 2006; Mueller, McDougald et al. 2007), striking sequence heterogeneities exist in these pilin subunits from *V. parahaemolyticus* (Aagesen and Hase 2012) that could

contribute to the variations observed in the adherence to different surfaces. Future studies detailing any differences in adherence by the various MSHA and PilA pilin alleles are required to fully understand the role of the sequence variations in these pili for surface attachment to biotic and abiotic substrates observed in this study.

Since flagella are an important structure for the initial adherence to a surface for colonization (Stewart and McCarter 2003) and this study identified that both polar and lateral flagellar systems are important for persisting in the oyster, the various *V. parahaemolyticus* strains were also examined for their motility in agar assays. Typically, expansion across a surface is the result of lateral flagella (McCarter and Silverman 1990) and can be observed on swarming motility agar. The results from the swarming motility assay for the various strains of *V. parahaemolyticus* show that some strains are proficient swarmers (O27-1C1, 10292, 5431 and BE98-2029), while one is a poor swarmer (5432), and three appear to be non-swarmers (10290, 10293, and ATCC17802). Comparing these data with the depuration data for these strains, there is a slight correlation between persistence in the oyster and the ability to swarm, although this did not hold true for strain 10292, which is a proficient swarmer and poor colonizer. In contrast to the swarming assay, all strains were capable of swimming in the semisolid swimming agar. However, some of these strains exhibit slight differences in swimming circle diameter, where strain 10290 had a smaller diameter than the other strains tested and strain 10292 appeared to have the largest swimming diameter of all the strains. In *V. parahaemolyticus,* swimming in semisolid motility (0.3%) agar utilizes both polar and lateral flagella (Kim and

McCarter 2004). Thus, loss of swimming abilities of a polar flagellum mutant could be masked by the use of lateral flagella to radially expand in semisolid agar. Comparing the swimming and swarming assays, one could argue that the smaller swimming diameter produced by 10290 may be the result of lacking lateral flagella to aid in swimming through the semisolid agar, although this may not be the case because a swarm^{+} (BE98-2029) and a swarm^{-} (ATCC17802) appear to have a similar swimming diameter. Swimming diameter in this assay has many factors involved ranging from growth rates to chemotaxis to swimming speed. Therefore, it is difficult to quantitatively compare these different strains based on their swimming diameter and conclusively say there is a difference in swimming motility between these strains. Moreover, we are unable to identify the specific contributions of the polar versus lateral flagella in radial expansion of the swimming diameter in semisolid motility agar by these strains, although swimming circle diameter clearly did not relate to the depuration data for these strains. Despite the findings from the mutant studies showing that both polar and lateral flagellar systems contribute to oyster colonization, it appears that variations in swimming and swarming abilities do not directly correlate with *V. parahaemolyticus* colonization of the Pacific oyster. Taken together, our data show that *V. parahaemolyticus* likely use multiple factors for proficiently colonizing the oyster and some overlap may exist, similar to what has been observed for biofilm formation where loss of one factor does not completely abolish attachment to the surface (Shime-Hattori, Iida et al. 2006).

A summary of the different characteristics for the eight *V. parahaemolyticus* strains tested can be found in Table 3.4. While we were able to conclude from the mutant studies that Type I and Type IV pili, along with polar and lateral flagellar systems aid the bacteria in persistently colonizing the Pacific oyster, there appears to be no direct correlation between biofilm or motility phenotypes and colonization of the oyster. Overall, these findings are consistent with what has been previously shown in *V. vulnificus* studies with *C. virginica* where PilA and flagella mutants had significantly reduced recovery than the wild type strain (Paranjpye, Johnson et al. 2007), while the translucent phenotype showed no significant difference (Srivastava, Tucker et al. 2009). In addition, we showed that T3SSs or phase variation do not play a role in persistence in oysters. Clearly, bacteria utilize a variety of molecular mechanisms to successfully colonize and persist in the oyster host, and further genetic comparisons between "good" and "bad" colonizers may provide important insights into the specific factors required for oyster colonization by *V. parahaemolyticus* that could be targets for novel depuration intervention strategies for the efficient clearance of *V. parahaemolyticus*, thereby reducing seafood-associated illness and creating a safer shellfish product any time of the year.

Table 3.4. Summary of eight *V. parahaemolyticus* strains

	Depuration		Glass Biofilm Ratios OD570nm/OD600nm				
Strain	24hrs	48hrs	18°C 24hr	30°C 4hr	30°C 24hr	37°C 4hr	37°C 24hr
ATCC17802	70.5%±0.01 ABC	38.4%±8.5 C	2.41±0.46 B	1.48±0.70 BC	0.51±0.22 B	1.30±0.85 B	0.40±0.07 AB
10290	58.3%±9.5 C	40.2%±4.5 C	1.16±0.60 BC	0.75±0.24 C	0.29±0.04 B	1.10±0.56 B	0.29±0.07 B
10292	59.5%±6.1 BC	41.7%±10.3 BC	0.53±0.19 C	0.71±0.20 C	0.21±0.07 B	0.34±0.11 B	0.35±0.04 AB
10293	57.2%±2.9 C	57.2%±14.8 ABC	0.80±0.29 BC	0.97±0.24 C	0.21±0.06 B	1.24±0.38 B	0.35±0.18 AB
BE98-2029	56.4%±14.8 C	43.9%±13.7 BC	0.48±0.13 C	0.58±0.20 C	0.28±0.12 B	0.33±0.13 B	0.31±0.01 B
O27-1C1	74.9%±0.5 AB	72.1%±3.4 A	0.25±0.02 C	0.47±0.12 C	0.25±0.13 B	0.38±0.09 B	0.26±0.07 B
5431	87.3%±8.5 A	66.1%±19.7 AB	6.16±1.13 A	3.22±0.81 A	0.91±0.02 A	3.34±1.05 A	0.66±0.21 A
5432	86.7%±9.4 A	67.9%±21.7 AB	0.91±0.28 BC	2.28±0.52 AB	0.28±0.11 B	1.34±0.14 B	0.33±0.11 AB

Plate Biofilm Ratios OD570nm/OD600nm					Motility			
18°C 24hr	30°C 4hr	30°C 24hr	37°C 4hr	37°C 24hr	Swimming	Diameter (mm)	Swarming	Diameter (mm)
1.67±0.56 B	0.97±0.20 B	0.43±0.08 B	0.76±0.25 BC	0.59±0.18 A	+++	27.5±4.0	-	8.2±0.3
0.59±0.14 C	0.63±0.05 C	0.18±0.04 E	0.69±0.09 C	0.13±0.03 D	+	14.2±5.9	-	6.3±1.1
0.29±0.11 C	0.65±0.12 C	0.10±0.01 F	0.35±0.18 D	0.13±0.02 D	+++	50.7±5.5	++	37.0±17.7
0.65±0.22 C	0.83±0.16 BC	0.22±0.04 DE	0.84±0.13 BC	0.30±0.08 C	++	32.3±7.1	-	12.5±5.0
0.40±0.16 C	0.56±0.08 C	0.25±0.01 DE	0.28±0.10 D	0.26±0.04 C	+++	25.8±7.6	++	20.7±3.8
0.26±0.11 C	0.98±0.12 B	0.30±0.05 CD	0.70±0.07 C	0.27±0.05 C	++	28.5±7.6	++	23.8±1.8
6.31±1.76 A	2.47±0.39 A	1.27±0.19 A	1.90±0.35 A	0.39±0.05 B	++	16.0±4.8	++	25.3±5.8
0.82±0.35 C	2.49±0.57 A	0.37±0.08 BC	0.97±0.33 B	0.29±0.09 C	++	20.5±2.0	+	11.2±1.6

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Chapter 4

Conclusions

Vibrio associated food-borne illness is a continued threat in the United States, as well as the world and is steadily rising year after year (Potasman, Paz et al. 2002; Baker-Austin, Stockley et al. 2010; CDC 2011). Many of these infections result from ingestion of *V. parahaemolyticus* contaminated shellfish, particularly oysters, and many of these infections originate from shellfish sources on the West Coast, mainly the Pacific Northwest (PNW) (CDC 1998; CDC 1999; CDC 2006; CDC 2011). Interestingly, *V. parahaemolyticus* isolates from the PNW generally are not of the pandemic O3:K6 serotype (DePaola, Kaysner et al. 2000) and exhibit genetic profiles distinctly different from other locations in the United States and the world (DePaola, Ulaszek et al. 2003). Clinical isolates from the PNW generally encode for both of the hemolysins, *tdh* and *trh*, and the T3SS-2 genes. However, many environmental isolates are *tdh*+ *trh*- and genetically similar to clinical isolates from different geographic locations and the O3:K6 pandemic strain RIMD2210633 (Paranjpye, Hamel et al. 2012). Despite the continued efforts to understand virulence factors of *V. parahaemolyticus* and the increased monitoring *V. parahaemolyticus* in the environment, including shellfish, it appears that current standards of what differentiates a pathogenic strains from a non-pathogenic have yet to bet determined considering many environmental isolates appear to be capable of causing disease if they were ingested. Therefore, efforts should also focus on understanding the natural

relationship between *V. parahaemolyticus* and oysters to improve shellfish sanitation practices so that in combination with environmental monitoring it can reduce the overall incidence of seafood-borne illness associated with *V. parahaemolyticus* in the United States.

Overall, this study has made progress into understanding the *Vibrio*-oyster interaction(s), and researchers can build from this data and investigate novel ways of increasing the effectiveness of oyster depuration. Currently, depuration is effective against fecal contaminants, such as *E. coli* and *Salmonella*; whereas vibrios are highly resistant to the process under the same conditions (Marino, Crisafi et al. 1999; Croci, Suffredini et al. 2002; Marino, Lombardo et al. 2005). New research has identified that changes in depuration temperature can effectively reduce *V. parahaemolyticus* from oysters that were artificially contaminated in the laboratory (Chae, Cheney et al. 2009; Phuvasate, Chen et al. 2012), although this method was shown to be ineffective for reducing naturally occurring *V. vulnificus* (Lewis, Rikard et al. 2010) and the effectiveness on naturally acquired *V. parahaemolyticus* in oysters has yet to be determined. Based on our findings that pili and flagella are involved in the multifactorial process of colonizing the oyster and resisting depuration, future studies can address whether changes in the depuration conditions result in increased effectiveness in depuration of *V. parahaemolyticus*. The polar flagellum of *V. parahaemolyticus* is powered by Na+ and is constitutively produced (McCarter 2001; McCarter 2004; McCarter 2006), although studies have shown that swimming motility is affected by the concentration of NaCl (Belas, Simon et al. 1986),

suggesting that altering NaCl concentrations might effect depuration of *V. parahaemolyticus*, and it has been shown that salinity does affect depuration (Phuvasate and Su, manuscript in preparation). Unfortunately, lowering salinity to 10 ppt had a negative effect on depuration. Lateral flagella production is regulated (McCarter 2006) by the hindrance of the polar flagella often as a result of contact with a surface (McCarter, Hilmen et al. 1988; McCarter and Silverman 1990), iron limitations (McCarter and Silverman 1989), Ca+ (Gode-Potratz, Chodur et al. 2010), pH and Na+ (Kimura, Tateiri et al. 1979). Some of the factors affecting lateral flagella production are possible conditions that could be changed during depuration, such as pH and salinity. As previously stated, changing salinity alone did not improve depuration (Phuvasate and Su, manuscript in preparation), although the combination of low salinity and increased pH may be beneficial in improving depuration. Lateral flagella are proton driven and increasing the pH of the medium requires increasing amounts of NaCl for lateral flagella production (Ulitzur 1975; Kimura, Tateiri et al. 1979). Based on this information, future studies are required to identify the effects of pH alone, pH plus salinity, and pH plus salinity plus temperature to determine the most effective depuration conditions for elimination of *V. parahaemolyticus* from oysters.

This study also determined that pili, both Type I and Type IV, contributed to *V. parahaemolyticus* persistence in the Pacific oyster. Little is known about the conditions that regulate the production of these particular pili in *Vibrio* species. MSHA is coordinately regulated in *V. cholerae* so that it is repressed during human

colonization (Hsiao, Toscano et al. 2008; Hsiao, Xu et al. 2009). PilA and MSHA expressing are induced by chitin (Meibom, Li et al. 2004; Shime-Hattori, Iida et al. 2006). Thus, better understanding pili regulation in *V. parahaemolyticus* could aid in depuration studies by providing new conditions or additives that could repress pili and possibly freeing the bacterium from the tissues of oysters and naturally this is the next area of investigation to follow up on the studies presented here.

Identifying that MSHA and PilA from three human pathogenic *Vibrio* species, *V. cholerae, V. vulnificus* and *V. parahaemolyticus*, exhibit allelic diversity presents an interesting finding requiring further studies to fully understand the role these gene alleles play in attachment to various surfaces and/or survival in various environments. As presented in Chapter 2, *V. cholerae* strains possessing TCP all maintain an identical MSHA and PilA allele while all other *V. cholerae* strains and all of the *V. vulnificus* and *V. parahaemolyticus* exhibit striking sequence heterogeneities without an apparent connection to a particular phenotype. Many suggestions were made as to why these gene sequences varied but future studies are required to identifying if any of our speculations are true. It was proposed that phage avoidance could be a driving factor for varying these sequences since Type IV pili are known to be receptors for filamentous phage (Jouravleva, McDonald et al. 1998; Karaolis, Somara et al. 1999; Campos, Martinez et al. 2003; Campos, Martinez et al. 2010). Alternatively, maintaining a highly conserved MSHA or PilA could result in acquisition of environmental DNA as a result of phage infection, possibly explaining the observation that O1 *V. cholerae* with TCP maintain highly conserved MSHA and

PilA sequences. It is tempting to speculate that acquisition of TCP by these O1 *V. cholerae* strains could be a result of possessing a particular MSHA and/or PilA sequence. MSHA and PilA are both induced by chitin and part of the *Vibrio* chitin utilization program (Meibom, Li et al. 2004) and chitin is known to induce natural competence in vibrios (Meibom, Blokesch et al. 2005) and has been used as tool for DNA manipulation in many *Vibrio* species (Marvig and Blokesch 2010; Pollack-Berti, Wollenberg et al. 2010; Gode-Potratz and McCarter 2011). These data suggest it may be possible that MSHA and PilA allelic variation may impact acquisition of environmental DNA, possibly explaining the observance in *V. cholerae.* Further investigations are required to address this fully.

Interestingly, Chapter 3 demonstrated that MSHA and PilA are involved in persistence in the Pacific oyster. Additionally, various strains of *V. parahaemolyticus* exhibited differences in retention in the oyster as well as biofilm formation abilities under different conditions with no apparent connection between the two. MSHA and PilA are known to be used in biofilm formation in *V. parahaemolyticus* (Shime-Hattori, Iida et al. 2006). When comparing the MSHA and PilA alleles from the strains examined in Chapter 3, some strains do cluster in a phylogenetic analysis of concatenated MSHA and PilA DNA sequences (Fig. 4.1). Unfortunately, we were unable to PCR amplify the *pilA* sequence from strain 10293, similar to the troubles that originally inspired Chapter 2. Therefore, strain 10293 was left out of our phylogenetic analysis presented in Fig. 4.1. More extreme methods, such as primer walking or whole genome sequencing, should be more successful in obtaining the

pilA sequence from 10293 than the basic PCR primer design used for the other strains. Additionally, TR was not included in this comparison because OP is the parent to this strain and they presumably possess identical alleles since neither one of these genes were targeted for creation of the TR strain.

As can be see in Fig. 4.1, some strains are clustered in the concatenated MSHA-PilA phylogenetic tree, although this apparent grouping does not correspond with either the depuration or biofilm data for these strains. Interestingly, the two strains with the highest bootstrap values, OP and 10292, are considered "good" and "bad" colonizers of oysters and are on the high and low end, respectively, of the biofilm ratio under the conditions tested. As has been shown in Chapter 3 and in numerous biofilm studies, multiple factors are involved in colonizing a surface. Therefore, future work investigation these alleles and their roles in adherence to multiple surfaces will be interesting. It is possible that possessing these various alleles results in differences in attachment to various aquatic surfaces, such as zooplankton, or aiding in DNA acquisition from the environment, or the difference between "good" invertebrate colonizers and "bad". A much more in depth look using gene deletions, allele swapping, and expression studies are required to fully understand the importance of allelic diversity in the MSHA and PilA genes from *V. parahaemolyticus*.

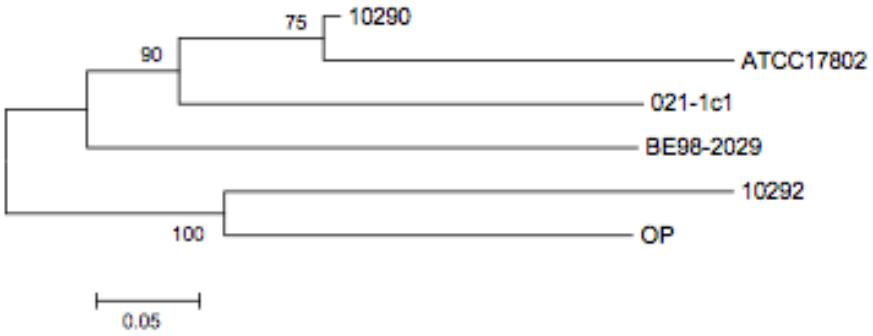

Figure 4.1. Maximum Likelihood tree of concatenated MSHA and PilA sequences from 6 different *V. parahaemolyticus* strains studied in Chapter 3. The bootstrap consensus values are indicated.

Overall, this study has provided insight into *V. parahaemolyticus* interactions with the Pacific oyster and has identified important factors required for persistence in the animal during depuration. Additionally, identifying allelic diversity in the MSHA and PilA genes from *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* has presented an interesting evolutionary finding, possibly providing some roles for these pili in the ecology of *Vibrio* species in the environment. These data presented here are the groundwork for additional investigations into *V. parahaemolyticus* colonization of the Pacific oyster to improve post-harvest strategies and subsequently provide a safer shellfish product for the consumer.

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APPENDICES

Seasonal effects of heat shock on bacterial populations, including artificial *Vibrio parahaemolyticus* infections, in the Pacific oyster, *Crassostrea gigas*

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Unpublished

Abstract

Pacific oysters, *Crassostrea gigas*, experience distinct seasonal differences in their physiological status based on reproduction. During the warmer summer months, oysters are conditioned to spawn, resulting in massive physiological efforts for gamete production. In addition, warmer conditions can induce significant loss of animals as a result of the phenomenon known as summer mortality. Moreover, the higher temperatures during the summer typically result in increased bacteria populations in the oysters, including a rise in bacterial *Vibrio* populations. We hypothesized that these animals are under multiple stresses that lead to possible immune system impairments during the summer months that can possibly lead to death. Therefore, naturally or artificially occurring bacteria may take advantage of an oyster summer stress situation, resulting in bacteria better surviving the immune system and residing in the tissue and hemolymph of animals that would otherwise successfully remove the bacterial contaminants. Here we show that in the summer and the fall animals exposed to a short heat stress respond similarly, resulting in a general trend of more bacteria being found in heat shocked animals than their non-heat shocked counterparts. We also show that naturally occurring bacterial populations fluctuate with time during depuration and actually enrich for vibrios. In addition, oysters artificially contaminated with *V. parahaemolyticus* responded similarly, where *V. parahaemolyticus* was found at higher concentrations in the tissue and hemolymph from heat shocked animals and this was consistent for animals examined during summer and fall. Finally, oyster hemocyte interactions with *V.*

parahaemolyticus differed based on the time of the year. The most dramatic difference in hemocyte interactions occurred in the spring, possibly coinciding with the physiological state of the animals being in early gonad maturation phase. Overall, these findings demonstrate that seasonal changes and/or a short heat shock is sufficient to impact bacterial retention, particularly *V. parahaemolyticus*, in oysters and this line of research might lead to important considerations for animal harvesting procedures.

Introduction

The Pacific oyster, *Crassostrea gigas*, is native to Japan but has been introduced to numerous places around the world with the largest producers being located in China, Japan, Korea and France, followed by the United States of America and Taiwan, with an estimated $3.69 billion in global revenues in 2003 (FAO 2005-2012), demonstrating that Pacific oyster aquaculture has a significant impact on the economy of many countries. The Food and Agriculture Organization (FAO) of the United Nations estimates that the USA consumes almost 60% of all *C. gigas* produced in the world and that 60-70% of Pacific oyster production occurs on the West Coast. Thus, it is important to the West Coast, as well as all of the USA, to maintain a safe and healthy Pacific oyster aquaculture system to meet the demands of this highly consumed shellfish.

During the warmer summer months, oysters are often exposed to increased sea surface temperatures and undergo a change in their physiology in order to spawn,

which is the release of gametes into the water column for reproduction. Spawning in Pacific oysters is generally triggered by an increase in water temperatures as well as an abundance of food (Langdon, Evans et al. 2003). Also during the summer months, mass mortality events have occurred worldwide for these oysters in a phenomenon known as summer mortality and these episodes have been associated with gametogenesis, increased temperatures, and bacteria (Soletchnik, Lambert et al. 2005; Degremont, Ernande et al. 2007; Garnier, Labreuche et al. 2007; Degremont, Bedier et al. 2010). Vibrios were the most commonly isolated bacteria from moribund animals during a massive mortality episode in France (Garnier, Labreuche et al. 2007). While oyster pathogens, such as *Vibrio splendidus* and *Vibrio aestuarianus*, were commonly isolated from animals with septicemia, other *Vibrio* spp., such as *Vibrio parahaemolyticus*, were also found. When sampling the environment, vibrios tend be in higher numbers in the water column during the warmer, summer months (Kaneko and Colwell 1973; Wright, Hill et al. 1996). Thus, in addition to gametogenesis and increased sea surface temperatures, oysters also encounter an increase in the concentration of naturally occurring vibrios, ranging from oyster pathogens to human pathogens, such as *V. parahaemolyticus*. Overall, summer presents a potentially stressful time of the year for oysters.

V. parahaemolyticus, a human pathogen capable of producing gastroenteritis upon ingestion, is the most common seafood-borne illness in the USA and is typically associated with the consumption of raw or undercooked shellfish, in particular oysters (CDC 2008). *V. parahaemolyticus* bacteria are found at higher concentrations in the

environment and more commonly found associated with oysters during the summer months (Kaneko and Colwell 1973; DePaola, Nordstrom et al. 2003; Kaufman, Bej et al. 2003; Johnson, Bowers et al. 2012). Overall, colonization of marine animals is an important aspect of the ecology of some *Vibrio* species, such as the colonization of the Hawaiian squid, *Euprymna scolopes*, by the commensal *Vibrio fischeri* (Chun, Troll et al. 2008). Moreover, vibrios, including *V. parahaemolyticus*, have been shown to be more resistant to shellfish depuration than other bacterial species not common to the marine environment (Marino, Crisafi et al. 1999; Croci, Suffredini et al. 2002; Marino, Lombardo et al. 2005; Su, Yang et al. 2010; Wang, Yu et al. 2010; Phuvasate, Chen et al. 2012).

Clearly, there is a connection between the increased abundance of *V. parahaemolyticus* in the water during the warmer summer months, their natural association with oysters in the environment, and an increase in the incidence of seafood-borne illness associated with consumption of oysters contaminated with *V. parahaemolyticus*. In addition to oysters being exposed to larger numbers of bacteria during warmer temperatures, gametogenesis might play a role. Therefore, we examined the effects of a brief heat shock on the natural bacterial populations found in oyster tissues and hemolymph during the summer and fall, as well as on oysters artificially contaminated with *V. parahaemolyticus* and found that a brief heat shock of 2hrs at 37°C was enough to impair the oyster's ability to successfully clear the artificially contaminated *V. parahaemolyticus* compared with the non-heat shocked animals regardless of the time of year. We also found that hemocytes had different

killing abilities during the spring, summer and fall, suggesting seasonality, corresponding to physiological status of the animal, may affect hemocyte interactions with *V. parahaemolyticus*. In addition, total bacterial populations in the oyster tissue and hemolymph changed with time, where low populations of vibrios were isolated at the start of the experiment and high concentrations of vibrios were found at the end of the experiment, suggesting our depuration conditions enriched for vibrios in the tissues and hemolymph of the oysters and this was consistent for the summer and fall. This study has begun to tease out this natural and complex *Vibrio*-oyster interaction in regards to seasonality of the animal as well as the natural environmental conditions encountered by both oyster and bacterium, possibly explaining the increased occurrence of *V. parahaemolyticus* infections associated with oysters during the summer months.

Materials and Methods

Strains used in this study and culture conditions

The strains used in this study were a mixture of FDA clinical isolates: 10290, 10292, 10293, O27-1C1 and BE98-2029 (Chiu, Duan et al. 2007; Su, Yang et al. 2010; Phuvasate, Chen et al. 2012). Strains were stored in 20% glycerol at -80°C until use. Spontaneous streptomycin resistant isolates of these strains were used for all assays at a streptomycin concentration of 100µg/ml. These isolates were cultured onto Tryptic Soy Agar (Difco) supplemented with NaCl for a final concentration of 2%. From these plates, single colonies were inoculated into 10ml of Tryptic Soy Broth (Difco)

supplemented with NaCl for a final concentration of 2% and incubated at 37°C for 5hrs under static conditions. Then each strain was pelleted individually by centrifugation at 3000 x *g* for 15min at 4°C, washed once with 10ml of 1% NaCl solution and re-pelleted. Then each pellet was resuspended in 1ml 1% NaCl and all strains were combined equally to an OD 600nm of ~3.0, which corresponds to approximately $1x10^9$ colony-forming units (CFU) per ml.

Oyster preparation

Diploid, extra small oysters were collected from Oregon Oyster Farm (Newport, OR) and thoroughly rinsed and scrubbed to remove any dirt or epibiota attached to the shells. Cleaned animals were placed into holding tanks at 18°C for a minimum of seven days, allowing them to acclimate to the change in temperature. During the summer months, animals were housed at Hatfield Marine Science Center, Oregon State University (Newport, OR) and maintained in flow-thru quarantine facilities with filtered seawater containing cultured algae and bubblers were added to each tank for proper aeration. The animal experiments during the winter were housed at Oregon State University main campus (Corvallis, OR) and held in a UV treated, recirculating depuration system where salinity was maintained at 30ppt and a pH of 8.2-8.4. Animals were fed a diet of Phytoplex (Kent Marine, Franklin, WI) for the duration of the experiments.

Oyster infection and heat shock treatment.

Larval culture tanks were filled with 20L of filtered 18°C seawater plus algal cultures and submergible circulating pumps (Petco) were added. Animals were divided into two groups, natural microflora and artificial *V. parahaemolyticus* exposure, and placed into the tanks. Oysters in the *V. parahaemolyticus* group were placed into tanks containing approximately 10^5 CFU/ml of *V. parahaemolyticus*, while the natural microflora group received 1% NaCl, and were held in these tanks for 18hrs. Following the 18hrs exposure, animals were placed into either 18°C seawater or 37°C seawater for 2hrs, then placed into a depuration system containing clean 18°C seawater and algae for 48hrs.

Bacterial enumeration during depuration

Oysters were sampled at various time points throughout the experiment. The exposure isolates correspond to sampling animals immediately after the 18hr exposure. The post heat shock isolates correspond to animals coming out of either the 18°C (non heat shock) or 37°C (heat shock) exposure. Then animals were sampled periodically during depuration. At each time point, four oysters were sampled from each treatment and were sprayed with 70% ethanol and aseptically shucked. Using a 20g needle and 1ml syringe, oyster hemolymph was collected from the pericardial cavity thru the adductor muscle and serially diluted for bacterial enumeration. Following hemolymph collection, oyster bodies plus liquor were weighed and diluted 1:3 with sterile seawater. The mixtures were homogenized in a

laboratory blender (Waring Laboratory, Torrington, CT) for 1 min, serially diluted. Both hemolymph and whole oyster homogenates were plated on TSA 2% with streptomycin for the *V parahaemolyticus* exposed animals or TSA 2% with no antibiotic and TCBS plates for the natural microflora and incubated at 37°C for 18hrs. The amount of *V. parahaemolyticus* in the oyster tissues is presented as CFU/g of oyster tissue while in the hemolymph, it is presented as CFU/ml of hemolymph.

Hemocyte assays

Oysters were aseptically shucked and hemolymph was extracted from the pericardial cavity via the adductor muscle with a 1ml syringe and 20gauge needle. Hemolymph was put directly on ice to prevent aggregation and pooled from each animal. To enumerate hemocytes, hemolymph was mixed 1:1 with 0.4% Trypan Blue and the concentration was determined using the Neubauer hemocytometer. Approximately 10^6 cells were added to each well of a 48 well tissue culture plate (Falcon) and set for 30mins at 18°C to form an adherent monolayer. Wells containing settled hemocytes were washed three times with sterile artificial seawater (ASW). Extra hemolymph was collected and spun for 300 x g for 15mins at 4°C to pellet hemocytes. The cell-free hemolymph was filter sterilized with a .2um filter. Approximately 10^6 CFU of *V. parahaemolyticus* was added per well to the monolayer and incubated at 18°C (non heat shock) or 37°C (heat shock) for 2hrs, followed by 2hrs at 18°C. Hemocyte monolayers were sampled at the 2hr and 4hr time points to enumerate the amount of *V. parahaemolyticus* present. At each time point, the monolayer supernatants were

collected and cold DI water was added to the well with slight agitation for 10mins. The DI water was collected and combined with the supernatants for serial dilution and plating on TSA 2% with and without streptomycin. The concentration of *V. parahaemolyticus* recovered in the hemocyte assays are presented as CFU/ml. Less than 10^2 CFU/ml naturally occurring bacteria were present in the uninfected hemocytes at each experiment.

Statistical analyses

All oyster and hemocyte data were $\log_{10}$ transformed and then averaged for statistical analyses using the Student's t test in Excel (Microsoft Office). A P value ≤ 0.05 was considered statistically significant and indicated by the use of an asterisk.

Results

Effects of heat shock on naturally occurring bacteria in the Pacific oyster during the summer

The marine environment is composed of many different types of bacteria, ranging from commensals (McFall-Ngai, Brennan et al. 1998) to pathogens, both human (Chiu, Duan et al. 2007) and animal (Duperthuy, Schmitt et al. 2011), and various bacterial genera are commonly isolated from the edible tissues of oysters, including vibrios (Olafsen, Mikkelsen et al. 1993; Blackstone, Nordstrom et al. 2003; DePaola, Nordstrom et al. 2003). To observe the effects of a heat shock on bacteria naturally occurring in oysters, market oysters were exposed to a heat shock or no heat shock

treatment and allowed to depurate for 48hrs following the treatment. Animals were sampled at various time points throughout the experiment and the bacterial populations were isolated on non-selective medium (TSA) for the total aerobic, heterotrophic bacterial population, and on selective medium (TCBS) for the subpopulation were likely from the *Vibrio* genus (Table A.1). When examining the total bacterial populations in animals during the month of June, there was a considerably high average of total bacteria present on TSA at the start of the experiment (~3.5 log CFU/g). After the heat shock, there were significantly more overall bacteria present in the heat shocked animals than the non-heat shocked (*P* value = 0.004) and by 48hrs of depuration, both treatment groups had large amounts of bacteria present in their tissues (Table A.1). Animals sampled in June contained low concentrations of bacteria recovered on TCBS at the start of the experiment. Following the heat shock, there was an apparent increase in the amount of TCBS isolates present in the heat shocked animals while the non-heat shocked animals maintained relatively low levels of bacteria on this selective medium. By 48hrs, both treatment groups had an increase in the average amount of bacteria isolated on TCBS (Table A.1).

Table A.1. Natural Flora (Log CFU/g of Tissue) of Whole Oyster Homogenates of Heat Shocked (37°C) and Not Heat Shocked Animals (18°C), Summer

June

	Starting Amount	Post Heat Shock	48hrs
Heat Shocked Animals TSA	3.53 ± 0.49	3.88 ± 0.01*	3.88 ± 0.01
Not Heat Shocked Animals TSA	3.53 ± 0.49	2.07 ± 0.16*	3.24 ± 0.45
Heat Shocked Animals TCBS	0.74 ± 1.04	3.66 ± 0.31	3.88 ± 0.01
Not Heat Shocked Animals TCBS	0.74 ± 1.04	0.74 ± 1.04	2.79 ± 0.66

July

	Starting Amount	Post Heat Shock	12hrs	36hrs	48hrs
Heat Shocked Animals TSA	2.73 ± 0.49	3.59 ± 0.16*	3.70 ± 0.01*	3.70 ± 0.01	3.39 ± 0.44
Not Heat Shocked Animals TSA	2.73 ± 0.49	2.84 ± 0.12*	3.04 ± 0.06*	2.87 ± 0.60	2.99 ± 0.12
Heat Shocked Animals TCBS	1.75 ± 0.64	1.94 ± 0.90	3.17 ± 0.75*	3.70 ± 0.01	2.83 ± 0.23*
Not Heat Shocked Animals TCBS	1.75 ± 0.64	1.69 ± 0.55	0*	1.92 ± 0.88	0*

Asterisk indicates significant differences between CFU/ml (*P* value ≤ 0.05)

Analyzing the bacterial populations in July, again there was high average of total bacteria present in the tissues of the oysters, although slightly less than what was observed in June (Table A.1). Following the heat treatment and after 12hrs of depuration, animals that received the heat shock had significantly more bacteria present than those that did not receive the heat shock (*P* value = 0.03) and that trend continued throughout depuration. By the end of depuration, the heat treatment group had a considerably higher average total bacterial population than at the start of the experiment while only a slight difference between the starting and ending total populations in the non-heat shocked group. Both June and July exhibited a similar trend with an increase in overall average bacteria present in the tissues of the heat treatment group over the non-heat treated group (Table A.1). On the TCBS plates, the average amount of bacteria present at the start of the experiment was higher than the average isolated in June. The trend for TCBS isolates in July was similar to what was observed in June with an increase in the average amount recovered with time but at 12hrs and 48hrs there were significantly more TCBS isolates present in the heat shocked animals than the non-heat shocked (Table A.1).

When comparing the total bacterial population with the subpopulation isolated on the TCBS plates, some striking differences emerge. In both June and July, the average amount of the total population was relatively high (~2.7-3.5 log CFU/g) and average amount of bacteria recovered on the TCBS plates was considerably less (0.74-1.75 log CFU/ml). In June, only approximately 21% of the total bacterial population was a TCBS isolate at the start of the experiment. In July, the proportion

of the total population at the start of the experiment recoverable on TCBS did increase to approximately 64%. However, at the end of the summer experiments, over 80% of the total bacterial population isolated was also found on TCBS. This was evident for both June and July, although the non-heat treated group did not have any isolates on TCBS at the end of the experiment in July (Table A.1). These results suggest that there was change in the composition of the bacterial population, for at least the heat shocked animals, over the duration of this experiment, with regards to the increase in average TCBS isolates without a tremendous increase in the average overall bacterial population.

Hemolymph is the "blood" of an oyster and contains antimicrobial factors, including hemocytes (reviewed in (Canesi, Gallo et al. 2002)). Stress is known to reduce immune functions and an oyster with a compromised immune system may have a large number of bacteria present in their hemolymph. To determine if oysters naturally have bacteria in their hemolymph and if a heat shock treatment has any effect on bacterial presence in the circulatory system, hemolymph was drawn from each treatment group and examined for total bacterial populations (non-selective media TSA) as well as the subpopulation of TCBS isolates (Table A.2). High numbers of the average total bacteria were isolated on TSA from the hemolymph at the start of the experiment. Following the heat shock, there was an increase in the total amount of bacteria in the hemolymph of heat shock animals while the number of bacteria present in the non-heat shocked animals decreased. After 12hrs of depuration, there was a decrease in the total bacterial population in the heat shocked

animals and by 36hrs, there were significantly more bacterial present in the hemolymph of heat shocked animals than non-heat shocked (*P* value = 0.02). After 48hrs of depuration, there were more bacteria, on average, in the hemolymph of the heat shocked animals (Table A.2). Interestingly, no TCBS isolates were isolated from the hemolymph of the animals at the start of the experiment. There was a slight increase in the amount of TCBS isolates following the treatment, although no difference was observed between the hemolymph of heat shocked and non-heat shocked animals. No TCBS isolates were recovered from the oyster hemolymph at 12hrs of depuration. After 36hrs, there was an increase in the number of TCBS isolates found in the heat shocked animals, markedly more than in the non-heat shocked animals, and by 48hrs there were more TCBS isolates present in the heat shocked compared to the non-heat shocked animals (Table A.2).

Table A.2. Natural Flora (Log CFU/g of Tissue) of Whole Oyster Homogenates of Heat Shocked (37°C) and Not Heat Shocked Animals (18°C), Summer

	Starting Amount	Post Heat Shock	12hrs	36hrs	48hrs
Heat Shocked Animals TSA	1.78 ± 0.77	2.06 ± 0.32	1.45 ± 2.05	2.71 ± 0.57*	1.25 ± 0.63
Not Heat Shocked Animals TSA	1.78 ± 0.77	0.83 ± 0.25	0.80 ± 1.13	0*	0.77 ± 1.09
Heat Shocked Animals TCBS	0	0.25 ± 0.35	0	1.07 ± 1.52	1.25 ± 0.63
Not Heat Shocked Animals TCBS	0	0.33 ± 0.46	0	0	0.73 ± 1.03

Asterisk indicates significant differences between CFU/ml (*P* value ≤ 0.05)

Comparing the selective (TCBS) and non-selective (TSA) isolates from the hemolymph, the bacteria naturally occurring in the hemolymph of these animals the start of the experiment were non-vibrios due to the lack of TCBS isolates from this time point. Following the heat shock, TCBS isolates began to appear and the population transitioned to predominately TCBS isolates after 48hrs of depuration, regardless of the heat treatment or not. These results are congruent with the findings for the whole animal for both June and July. However, almost 100% of the isolates

from the hemolymph were isolated on TCBS and TSA, suggesting that vibrios were the dominant circulating bacteria isolated from the hemolymph of both heat-treated and non-heat treated animals.

Overall, during the summer, the heat shock resulted in a higher average total bacterial population as well as TCBS subpopulation in the heat treated group than animals that did not receive the heat shock. In addition, during the course of the experiment, the bacterial population changed from a low percentage of TCBS isolates to a considerably high percentage of TCBS isolates at the end, constituting a shift in the bacterial population in the oyster tissue and hemolymph to predominately *Vibrio* sp.

Effects of heat shock on artificially infected V. parahaemolyticus in the Pacific oyster during the summer

There is an increased incidence of *V. parahaemolyticus* infections associated with shellfish during the summer, particularly oysters (www.cdc.gov). *V. parahaemolyticus* is more prevalent in the marine environment during the warmer, summer months (Kaneko and Colwell 1973; DePaola, Nordstrom et al. 2003; Johnson, Bowers et al. 2012) and are naturally found associated with oysters (Blackstone, Nordstrom et al. 2003; DePaola, Nordstrom et al. 2003; DePaola, Ulaszek et al. 2003; Nordstrom and DePaola 2003). In addition, during the summer, oysters may be exposed to a sudden increase in temperatures, particularly in intertidal zones, subsequently creating a heat shock. Moreover, heat shock as been shown to alter immune functions of shellfish contaminated with *V. parahaemolyticus* (Cheng,

Hsiao et al. 2004). Therefore, we examined the effects of a heat shock on oysters artificially contaminated with *V. parahaemolyticus* during the summer. Hemolymph and whole animal homogenates were sampled and Fig. A.1 shows the results for A) whole oyster homogenates and B) hemolymph extractions. After the 18hr bacterial exposure, *V. parahaemolyticus* concentrations in the animals were approximately 4 log CFU/g of tissue, comparable to levels obtained in previous studies (Su, Yang et al. 2010; Phuvasate, Chen et al. 2012). After the 2hr heat shock, heat-treated animals had significantly more *V. parahaemolyticus* (*P* value = 0.004) present than non-heat treated animals (Fig. A.1A). In addition, the group that did not receive the heat shock significantly decreased the amount of *V. parahaemolyticus* (*P* value 0.03), although the heat-treated group did not change (*P* value = 0.12). During depuration, the amount of *V. parahaemolyticus* in the tissues of the heat shocked animals remained significantly higher than in the animals that did not receive the heat shock (*P* values = 0.003, 0.007, 0. 0004, 0.008 for 12, 24, 36, and 48hrs, respectively). A similar trend was also found for the hemolymph (Fig. A.1B). Approximately 1.75 log CFU/ml of *V. parahaemolyticus* was present in the hemolymph directly after the 18hr exposure and the amount of *V. parahaemolyticus* present in the hemolymph of the heat shocked animals remained around that level throughout the experiment, while the animals that were not heat shocked had significantly less bacteria in their hemolymph at the time corresponding to the treatment (post heat shock) and after 36hrs of depuration (*P* values = 0.002 and 0.05 respectively). Overall, there appeared to be a steady reduction in the amount of *V. parahaemolyticus* in the hemolymph of non-heat

shocked animals while the heat shocked animals maintained a relatively similar amount of *V. parahaemolyticus* in the hemolymph over the 48hrs sampled.

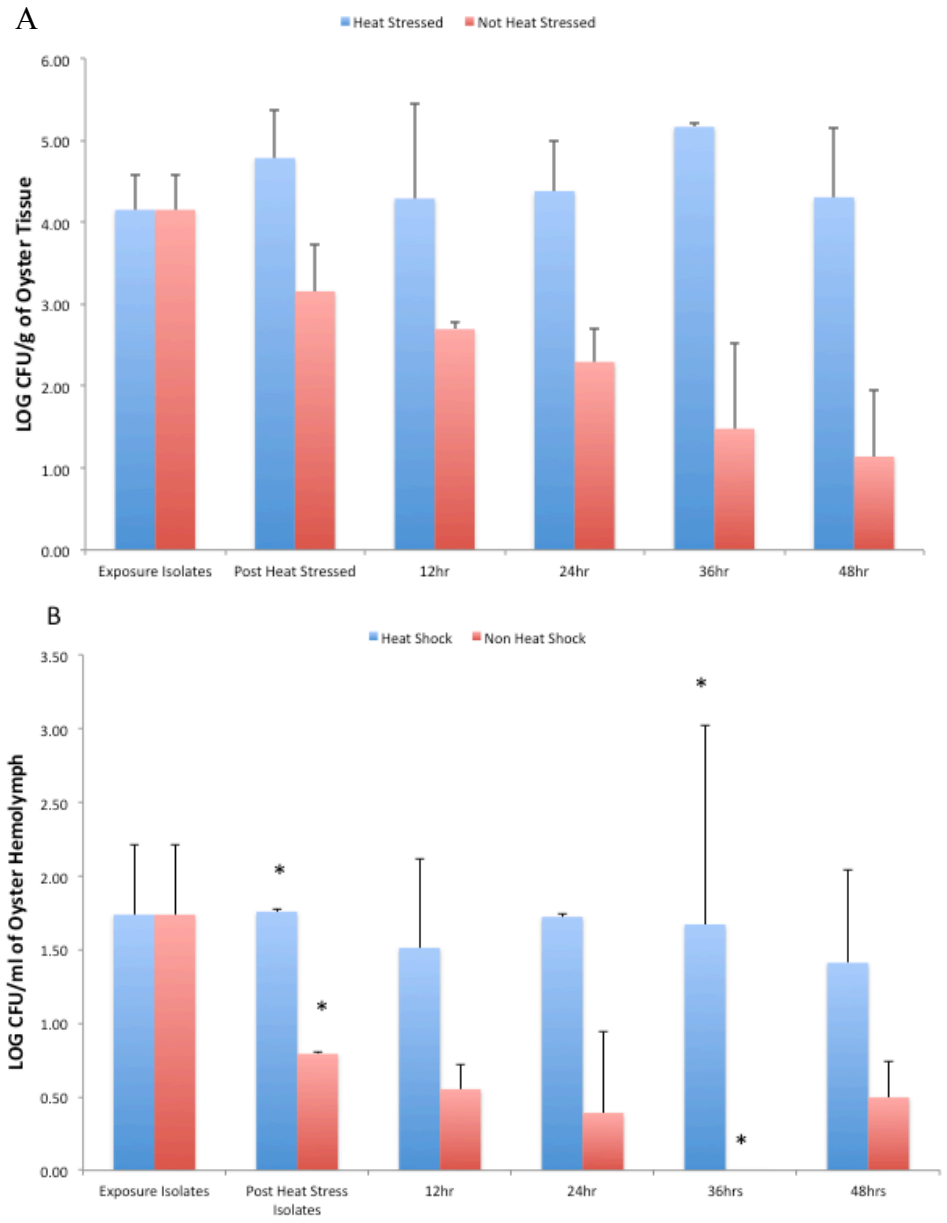


Figure A.1. Response of Pacific oysters exposed to *V. parahaemolyticus* in the summer. A) Whole oyster homogenates or B) hemolymph from oysters exposed to *V. parahaemolyticus* and either heat shocked (37°C) or not heat shocked (18°C) during the summer (June and July). Asterisk indicates significant difference between the heat shocked and not heat shocked animals (P value ≤ 0.05).

Effects of heat shock on naturally occurring bacteria in the Pacific oyster during the fall.

Seasonal fluctuations in the abundance of bacteria in the marine environment have been observed (Kaneko and Colwell 1973; DePaola, Nordstrom et al. 2003; Johnson, Bowers et al. 2012). Therefore, we repeated the study in the fall and examined oysters for the effects of heat shock on their natural microflora (Table A.3). At the start of the experiment performed in October, the average amount of total bacteria recovered on non-selective media (TSA) was considerably high (~3.6 log CFU/g). In general, the average total bacterial population remained relatively high with slight fluctuations at various time points. The heat treated animals had significantly more total bacteria than non-heat treated after 16 and 28hrs of depuration (*P* value = 0.005 and 0.02, respectively). Overall, the trend observed was that heat shocked animals generally had a higher average total bacterial population recovered than the non-heat shocked (Table A.3). When examining the bacteria recovered on the selective medium, TCBS, the average amount of isolates at the start of the experiment was relatively high (~1.95 log CFU/g). The animals that received the heat shocked had an increase in the average TCBS isolates while the non-heat shocked had a decrease. During depuration, there were significantly more TCBS isolates in the heat shocked animals at 28hrs of depuration (*P* value = 0.02) than the non-heat shocked and the general trend during the entire depuration was that heat shocked animals maintained higher average TCBS isolates that non-heat shocked.

Table A.3. Natural Flora (Log CFU/g of Tissue) of Whole Oyster Homogenates of Heat Shocked (37°C) and Not Heat Shocked Animals (18°C), Summer

October

	Starting Amount	Post Heat Shock	6hrs	16hrs	28hrs	40hrs
Heat Shocked Animals TSA	3.60 ± 0.55	3.67 ± 0.58	3.26 ± 0.31	3.86 ± 0.01*	3.87 ± 0.01*	3.98 ± 1.27
Not Heat Shocked Animals TSA	3.60 ± 0.55	4.23 ± 0.03	1.63 ± 2.30	3.02 ± 0.09*	2.71 ± 0.28*	2.84 ± 0.16
Heat Shocked Animals TCBS	1.95 ± 0.48	2.95 ± 0.61	3.03 ± 0.29	3.56 ± 0.44	3.72 ± 0.21*	4.03 ± 1.19
Not Heat Shocked Animals TCBS	1.95 ± 0.48	1.48 ±0.43	3.06 ± 0.43	2.46 ± 0.19	2.62 ± 0.09*	2.62 ± 0.09

November

	Starting Amount	Post Heat Shock	6hrs	16hrs	28hrs	40hrs
Heat Shocked Animals TSA	4.00 ± 0.01	4.69 ± 0.44	4.65 ± 0.16*	3.83 ± 0.07	2.79 ± 0.08	3.88 ± 0.01
Not Heat Shocked Animals TSA	4.00 ± 0.01	3.63 ± 0.34	3.73 ± 0.20*	3.68 ± 0.28	2.18 ± 1.00	3.41 ± 0.30
Heat Shocked Animals TCBS	3.18 ± 0.45	3.26 ± 1.36	0*	3.54 ± 0.22	2.35 ± 0.04	3.68 ± 0.27*
Not Heat Shocked Animals TCBS	3.18 ± 0.45	2.69 ± 0.62	2.74 ± 0.12*	3.28 ± 0.13	2.35 ± 0.24	2.33 ± 0.21*

December

	Starting Amount	Post Heat Shock	6hrs	16hrs	28hrs	40hrs
Heat Shocked Animals TSA	4.00 ± 0.01	3.36 ± 0.91	2.45 ± 0.96	3.52 ± 0.49	3.88 ±0.01	3.88 ±0.01
Not Heat Shocked Animals TSA	4.00 ± 0.01	3.88 ± 0.01	3.21 ± 0.07	2.65 ± 0.46	3.88 ±0.01	3.49 ± 0.45
Heat Shocked Animals TCBS	0.40 ± 0.92	0	0	0	2.17 ± 0.12*	0.98 ± 1.38
Not Heat Shocked Animals TCBS	0.40 ± 0.92	0.74 ± 1.04	0	0	0*	0

Asterisk indicates significant differences between CFU/ml (*P* value ≤ 0.05)

Comparing the total population of bacteria recovered with those isolated on TCBS, there were significantly more total bacteria isolated on TSA than TCBS (*P* value 0.009), although the TCBS population contributed to close to half of the overall bacteria loads (Table A.3). In addition, while the total bacterial population fluctuated slightly during the experiment, there was a noticeable increase in the number of TCBS isolates with time for both the heat shocked and non-heat shocked groups, contributing to more than 90% of the bacteria recovered.

When this experiment was repeated in November, the average amount of total bacteria recovered was considerably high at the start of the experiment (~4.0 log CFU/g). After the heat treatment, there was a higher average of total bacteria in the heat shocked group than the non-heat shocked after 6hrs of depuration (*P* value = 0.04), and although the average concentrations fluctuated during depuration, this trend remained consistent throughout the 40hrs of depuration (Table A.3). As for the TCBS isolate populations, the average amount at the beginning of the experiment was considerably high (~3.18 log CFU/g). Following the heat shock, there were more

TCBS isolates, on average, in the heat treated group than those that remained at 18°C except for the 6hrs of depuration, where no bacteria was isolated on TCBS for the heat treated group while isolates were obtained from the non-heat treated group (*P* value = 0.009). However, after 40hrs of depuration, there were significantly more TCBS isolates from the heat shocked animals than the non-heat shocked.

Comparing the starting bacterial amounts for both selective and non-selective media, a large percentage (~80%) of the total population isolated from oysters at the beginning of the experiment was most likely from the *Vibrio* genus. The number of TCBS isolates remained high throughout the experiment constituting upwards of 70% or more of the total bacteria isolated. Overall, there was no dramatic difference between the amount of total starting bacteria in the heat shocked animals and the amount recovered at the end while the non-heat shocked appeared to have a slight decrease in total bacteria and the majority of the bacteria recovered were vibrios.

The experiment was repeated in December and the average amount of total bacteria present at the start of the experiment was quite high (~4.0 log CFU/g). Although this high amount of total bacteria remained throughout the experiment, some fluctuations did occur during depuration and the heat shock did not appear to dramatically impact the total bacterial populations between heat shocked and non-heat shocked animals (Table A.3). As for the TCBS isolates, the amount of vibrios recovered was relatively low in the beginning. Following the heat shock, very low levels of vibrios were recovered but heat shocked animals had significantly more TCBS isolates after 28hrs of depuration (*P* value = 0.001) and no TCBS isolates were

found from non-heat shocked animals throughout the 40hrs of depuration. Comparing the total population with the TCBS isolates, a very small percentage (~10-20%) of the total bacteria isolated were vibrios at any time during the experiment and the population composition did not appear to drastically change except for after 28hrs of depuration where TCBS isolates constituted ~55% of the total population.

When comparing the total bacterial population as well as the TCBS isolate subpopulation for the fall, marked differences were observed between early fall (October), mid fall (November), and late fall (December). Overall, the average amount of total bacteria isolated from animals at the beginning of the experiments was fairly consistent amongst October, November, and December. As for the heat shock, animals in October and November responded similarly to the heat shock with the trend of higher averages of total bacteria found compared to the non-heat shocked animals. In contrast, animals in December did not exhibit the consistent trend of heat shocked animals maintaining higher averages of bacteria and more fluctuations in averages between the groups did occur. With the TCBS isolates, each month exhibited a different amount of starting bacteria. November had the highest starting averages, followed by October, and December had the lowest starting amounts overall, significantly less than either October (*P* value = 0.03) or November (*P* value = 0.0009) (Table A.3). In addition, both October and November shared a similar trend where heat shocked animals had more TCBS isolates than non-heat shocked. Strikingly, the average amount of TCBS isolates increased dramatically by the end of the experiment in October, with an average increase of over 2 log CFU/g, and more

than 90% of the final population was most likely vibrios. This trend was also similar for November, albeit less dramatic, with only an average 0.5 log CFU/g increase and the percentage of vibrios in the total population increased from 80% at the start to 90% at the end. December had the most dramatic differences in the TCBS isolates of all, with relatively low numbers of TCBS isolates over the entire experiment and a very small portion of only ~25% of the total population of bacteria were vibrios. Taken together, these data suggest that animals can have a different response to a heat shock, dependent on the time of the year and that depuration studies can select for vibrios in a mixed population.

In addition to whole oyster homogenates, hemolymph was also examined during the fall experiments to determine the amount of TCBS isolates and overall total bacteria present and the effects of heat shock on the number of bacteria in the hemolymph at this time of the year (Table A.4). At the start of the experiments, the average amount of total bacteria in the hemolymph was considerably high (~2.7 log CFU/ml). Following the heat shock, more total aerobic bacteria were recovered in the hemolymph of heat shocked animals than the non-heat shocked group (*P* value = 0.04) as well as after 40hrs of depuration (*P* value = 0.05). As for the TCBS isolates, the starting average was relatively low (~1.0 log CFU/ml) in the hemolymph. The heat shocked group did not have increases in the TCBS isolate averages until 16hrs of depuration. From there, the average TCBS isolates were higher for the heat shocked animal than the non-heat shocked. In addition, the average amount of TCBS isolates in the hemolymph was considerably less at the start of the experiment than the overall

total bacterial population, only about 37%. At the end of the experiment, a large percentage of the total bacteria recovered in the hemolymph were vibrios, upwards of 70%. With time, the number of vibrios in the hemolymph increased in the heat shocked animals but was relatively similar for the non-heat shocked animals. Overall, the hemolymph data reflects the overall trend observed in the whole animal results for October and November, where heat shocked animals maintained higher averages of total bacteria and vibrios than non-heat shocked. In addition, the population in the hemolymph changed with time with an increase in the *Vibrio* population with no dramatic overall increase in total bacterial populations.

Table A.4. Natural Flora (Log CFU/ml) of Oyster Lymph of Heat Shocked (37°C) and Not Heat Shocked Animals (18°C), Fall

	Starting Amount	Post Heat Shock	6hrs	16hrs	28hrs	40hrs
Heat Shocked Animals TSA	2.69 ± 1.00	2.44 ± 0.35*	1.98 ± 0.06	1.74 ± 1.26	1.15 ±1.63	3.01 ± 0.16*
Not Heat Shocked Animals TSA	2.69 ± 1.00	1.14 ± 0.8*	2.30 ± 0.30	1.94 ± 0.52	0.62 ± 0.17	1.14 ± 0.57*
Heat Shocked Animals TCBS	1.00 ± 0.54	0.25 ± 0.35	0.69 ± 0.06	1.25 ± 1.78	1.15 ± 1.62	2.17 ± 0.67
Not Heat Shocked Animals TCBS	1.00 ± 0.54	0.48 ± 0.67	0.94 ± 1.33	0.89 ± 1.26	0.25 ± 0.35	0.42 ± 0.60

Asterisk indicates significant differences between CFU/ml (*P* value ≤ 0.05)

Effects of heat shock on artificially infected V. parahaemolyticus in the Pacific oyster during the fall

The presence of *V. parahaemolyticus* in the marine environment declines after the summer when the temperatures decrease (Kaneko and Colwell 1973; DePaola, Nordstrom et al. 2003) and oysters move out of the reproductive phase of gametogenesis and move into the resorption phase (Kennedy and Krantz 1982). To examine the effects of resorption, we again tested the effects of heat shock on oysters artificially contaminated with *V. parahaemolyticus* during the fall when animals are in this different physiological phase of the year. Figure A.2 shows the amount of *V. parahaemolyticus* in A) whole oyster homogenates and B) hemolymph from heat

shocked and non-heat shocked animals. After the 18hr exposure to *V. parahaemolyticus*, there was approximately 4.83±0.85 log CFU/g of tissue present in the tissue of the oyster. Following a 2hr heat shock, there was a slight but significant (*P* value 0.02) increase in the amount of *V. parahaemolyticus* in the heat shocked animals, significantly more than the non-heat shocked animals (*P* value 0.04), while the non-heat shocked animals maintained *V. parahaemolyticus* levels (4.76±0.08 log CFU/g of tissue) similar to the starting amount (Fig. A.2A). After 6hrs of depuration, there was a significant decrease in the amount of *V. parahaemolyticus* present in the tissues of both heat shocked (*P* value 0.009) and non-heat shocked animals (*P* value 0.003) but there was significantly more *V. parahaemolyticus* present in the tissues of heat shocked animals (*P* value 0.04). Over time, there was no dramatic decline in the amount of *V. parahaemolyticus* in either heat shocked or non-heat shocked animals but there were significantly more *V. parahaemolyticus* present in the heat shocked animals after 40hrs of depuration than the non-heat shocked animals (*P* value 0.05).

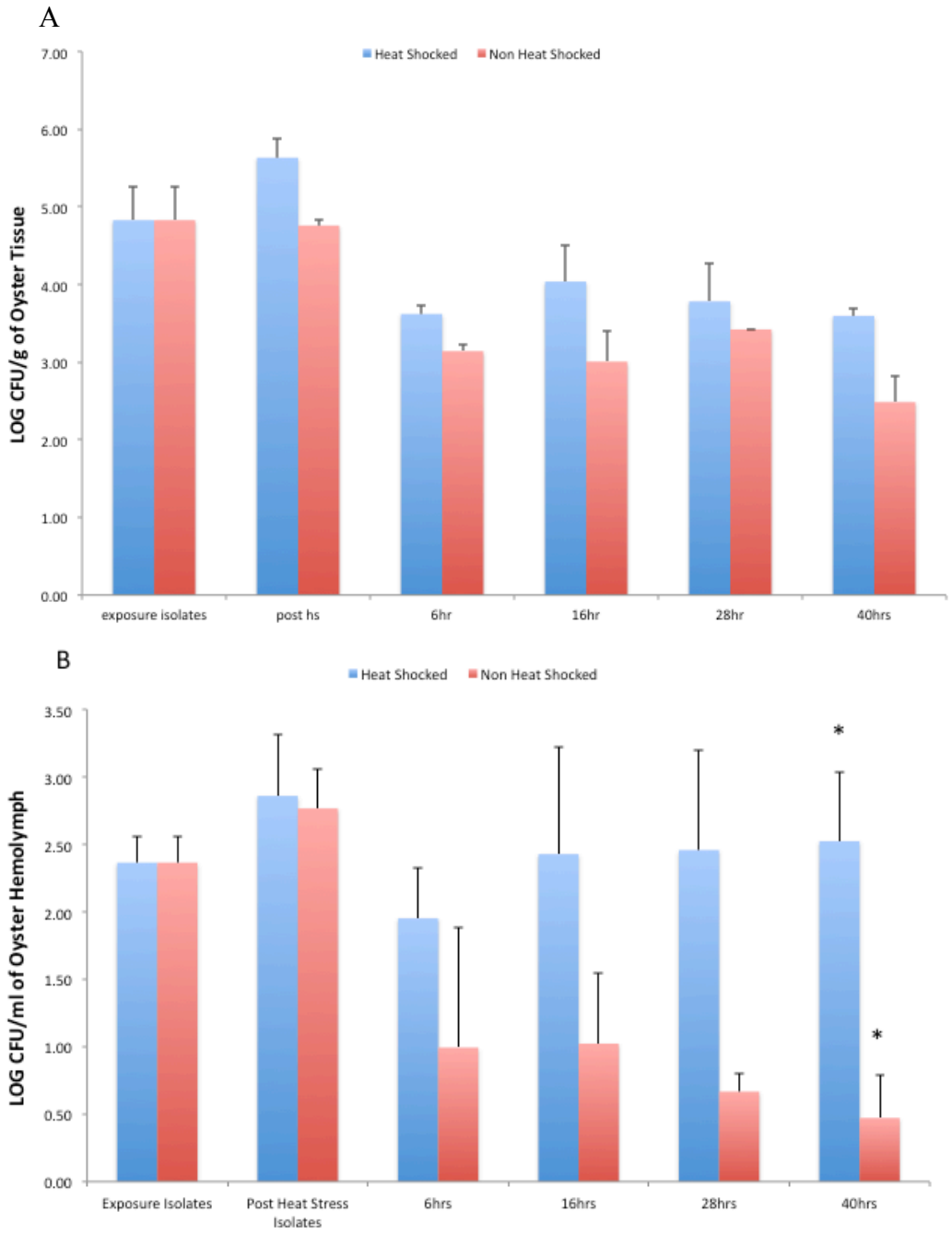


Figure A.2. Response of Pacific oysters exposed to *V. parahaemolyticus* in the fall. A) Whole oyster homogenates or B) hemolymph from oysters exposed to *V. parahaemolyticus* and either heat shocked (37°C) or not heat shocked (18°C) during the fall (October and November). Asterisk indicates significant difference between the heat shocked and not heat shocked animals (P value ≤ 0.05).

Similar results were found for the hemolymph of heat shocked and non-heat shocked animals (Fig. A.2B). Approximately 2.4±0.19 log CFU/ml of *V. parahaemolyticus* was present in the hemolymph of the oysters after an 18hr exposure. Following the treatment, there was a slight increase in the number of *V. parahaemolyticus* in the hemolymph of both heat shocked and non-heat shocked animals, although the data was not statistically significant. During depuration, there was a strong reduction in the amount of *V. parahaemolyticus* in the non-heat shocked animals while the amount of *V. parahaemolyticus* in the heat shocked animals remained similar to the initial starting amounts. By 40hrs of depuration, there were significantly more *V. parahaemolyticus* in the hemolymph of heat shocked animals compared to non-heat shocked (*P* value = 0.04) (Fig. A.2B), consistent with what was observed for the whole animal homogenates. Overall, whole animal homogenates and hemolymph isolates have congruent findings where a heat shocked group maintained higher amounts of *V. parahaemolyticus* than a non-heat shocked group.

Effects of heat shock on natural oyster flora, summer versus fall

To identify seasonal differences in natural oyster flora and the effects of heat shock on these bacterial populations, we compared the experiments conducted in the summer with those completed in the fall and some striking differences emerged. When examining the amount of vibrios recovered in the oyster tissues at the start of the experiment, June (Table A.1) and December (Table A.3) had similarly low concentrations while July (Table A.1) and October (Table A.3) were comparable with

somewhat higher concentrations. November (Table A.3) had the highest recovery of vibrios of all months examined. After the heat shock, June (Table A.1) had the most dramatic difference in vibrios recovered from heat shocked versus non-heat shocked animals and the most dramatic increase of starting amounts. The other months did not exhibit such dramatic differences between vibrios recovered from heat shocked and non-heat shocked nor the increase in overall concentrations of vibrios after the heat shock. Throughout depuration, the number of vibrios recovered for summer and fall fluctuated with time but by the end of depuration, all months showed increased amounts of vibrios present than at the end of the experiments and heat shocked animals had higher averages of vibrios recovered overall than the non-heat shocked animals.

Interestingly, the amount of total bacteria recovered at the start of the experiments was fairly consistent, although July (Table A.1) showed lower averages of bacterial isolates than the other months. June, July, October, and November had similar trends regarding the differences between bacteria present in heat shocked and non-heat shocked animals. Overall, there appeared to be more bacteria present in the heat shocked animals as opposed to the non-heat shocked animals. December had some variations in this trend but in the end, heat shocked animals also had higher averages of bacteria present. When comparing the percentage of total bacterial populations recovered in the oysters over the study, at the beginning of the experiments, vibrios accounted for less than half of the total bacteria recovered in June (Table A.1) and December (Table A.3), approximately half of the bacterial

population in July (Table A.1) and October (Table A.3), while they were a large majority of the total bacteria population in November (Table A.3). By the end of depuration, the bacterial population appeared to always switch where the majority of the bacteria present in all months examined were vibrios, except for December, which had a very small percentage. This held true for heat shocked and non-heat shocked animals.

When comparing the amount of vibrios isolated from the hemolymph during the summer with the fall, vibrios were only isolated at the start of experiment from animals during the fall. Heat shock did not affect the average amount of vibrios isolated from the hemolymph during the summer (Table A.2) or fall (Table A.4). However, there were more vibrios in the hemolymph of heat shocked animals by the end of the experiment than at the start of the experiments. Total bacteria numbers in the hemolymph of animals in the fall (Table A.4) were higher than in those examined in the summer (Table A.2). In contrast to the vibrios, heat shock significantly increased the amount of bacteria isolated from animals in the fall and a similar trend was observed in the summer. Over the course of depuration, this trend was fairly consistent where heat shocked animals retained higher amounts of bacteria than non-heat shocked animals with significantly more at the end of the experiments in the fall and a similar tendency existed for the summer. Similar to what was observed in whole animal homogenates, vibrios were a small percentage of the total bacteria recovered from the hemolymph at the start of the experiments for both summer (Table A.2) and fall (Table A.4) while at the end, the population switched to

primarily vibrios, which was more evident in the summer (Table A.2) than the fall (Table A.4).

Effects of heat shock on V. parahaemolyticus infected oysters, summer versus fall

When comparing artificial *V. parahaemolyticus* infections of oysters in the summer and in the fall, some dramatic differences did occur. While each set of experiments had similar starting amounts of *V. parahaemolyticus* in the whole oyster homogenates (Fig. A.1A and A.2A), after a heat shock there were more *V. parahaemolyticus* present in the tissues of heat shocked animals than non-heat shocked. The most dramatic difference between the summer and fall experiments was the reduction of bacterial concentrations in the non-heat shocked animals. In the summer, there were more than a 3 log CFU/g difference in *V. parahaemolyticus* recovered between heat shocked and non-heat shocked animals, while there was only a little over a 1 log CFU/g difference in the fall. The summer heat shocked animals maintained approximately the same amount of *V. parahaemolyticus* by 48hrs of depuration compared with the starting amount. In contrast, the summer non-heat shocked animals had ~3 log CFU/g reduction between starting amounts and 48hrs of depuration, similar to previous reports (Su, Yang et al. 2010; Phuvasate, Chen et al. 2012). In the fall, the heat shocked animals only decreased by about 1.2 log CFU/g of *V. parahaemolyticus* over the 40hrs of depuration while the non-heat shocked animals had a more robust (~2.3 log CFU/g of oyster tissue) reduction in *V. parahaemolyticus* (Fig. A.2A). Thus, it appears that during the summer, heat-

shocked animals' retained higher amounts of *V. parahaemolyticus* than non-heat shocked animals. In addition, non-heat shocked animals were more efficient at clearing the *V. parahaemolyticus* from their tissues in the summer compared to the fall.

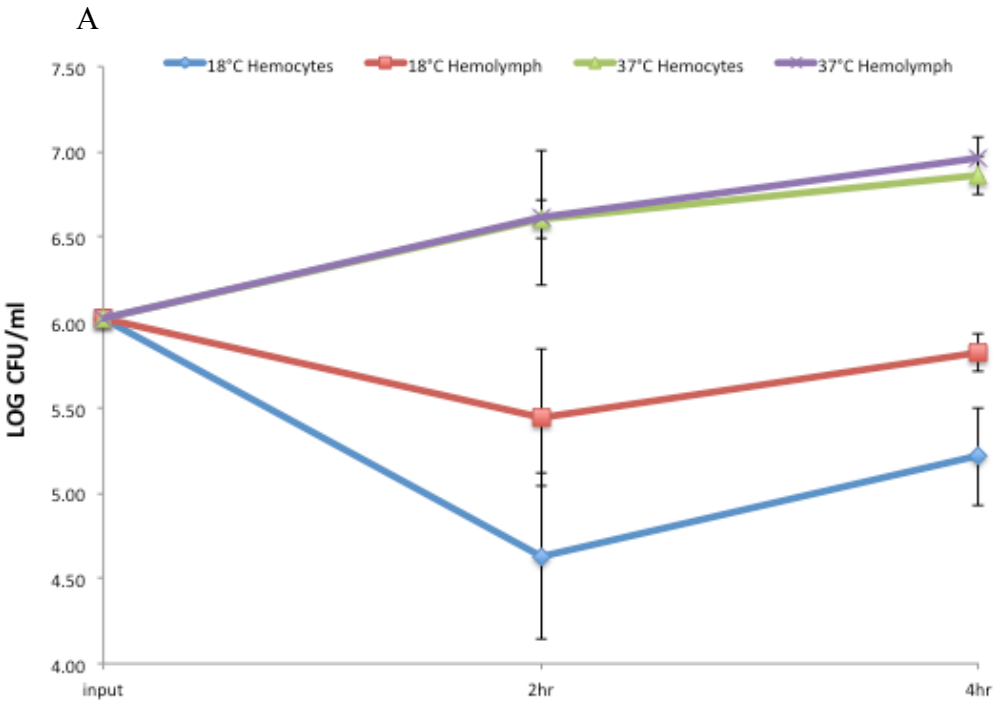
As for the hemolymph, there appeared to be more *V. parahaemolyticus* in the hemolymph of animals in the fall compared to the summer (Figs. A.1B and A.2B). For both times of the year, the heat shocked animals retained a fairly consistent amount of *V. parahaemolyticus* in the hemolymph over the time course of the experiment, while non-heat shocked reduced their bacterial counts considerable throughout the experiment. Moreover, during the fall, more *V. parahaemolyticus* were found in the hemolymph after the 18hr exposure than during the summer. Thus, seasonality appears to affect *V. parahaemolyticus* colonization of the Pacific oyster overall as well as their ability to contain bacteria in their hemolymph.

Hemocyte interactions under heat shock (37°C) and non-heat shock (18°C) conditions

Since heat shocked animals had more bacteria present in their tissues and hemolymph than non-heat shocked animals, we examined the effects of heat shock on oyster hemocyte interactions with *V. parahaemolyticus*. Hemocytes are macrophage-like phagocytic innate immune cells capable of eliminating bacteria from the oyster (reviewed in (Canesi, Gallo et al. 2002)). Differences in temperature have been shown to affect hemocyte killing abilities (Harris-Young, Tamplin et al. 1993; Cheng

and Chen 2000) and this could account for the differences found for heat shocked and non-heat shocked animals. Figure A.3 shows the results of *C. gigas* hemocyte interactions with *V. parahaemolyticus* following exposure to a heat shock or no heat shock. Hemocytes that were exposed to a 2hr heat shock (37°C) and then placed at 18°C for 2hrs were unable to reduced the amount *V. parahaemolyticus*, while the non-heat shocked hemocytes were capable of decreasing *V. parahaemolyticus* numbers by approximately 1.5 log CFU/ml (Fig. 3A). *V. parahaemolyticus* were also exposed to cell-free hemolymph under both conditions. While there was no difference between *V. parahaemolyticus* exposed to hemocytes or hemolymph with a 37°C heat shock, there was a significant difference between *V, parahaemolyticus* survival in non-heat shocked hemocytes and hemolymph after 4hrs at 18°C (*P* value = 0.02). This data showed that a heat shock affected the ability of hemocytes to reduce the number of *V. parahaemolyticus*, possibly explaining the differences observed in the whole animal and hemolymph (Figs. A.1 and A.2).

Fig. A.3. Hemocyte interactions with *V. parahaemolyticus.* Oyster hemocytes were exposed to *V. parahaemolyticus* and subjected to a heat shock (37°C) or no heat shock (18°C) during the A) spring (April), B) summer (July), and C) fall (September). Asterisk indicates significant difference between lymph from heat shocked and not heat shocked animals (*P* value ≤ 0.05).



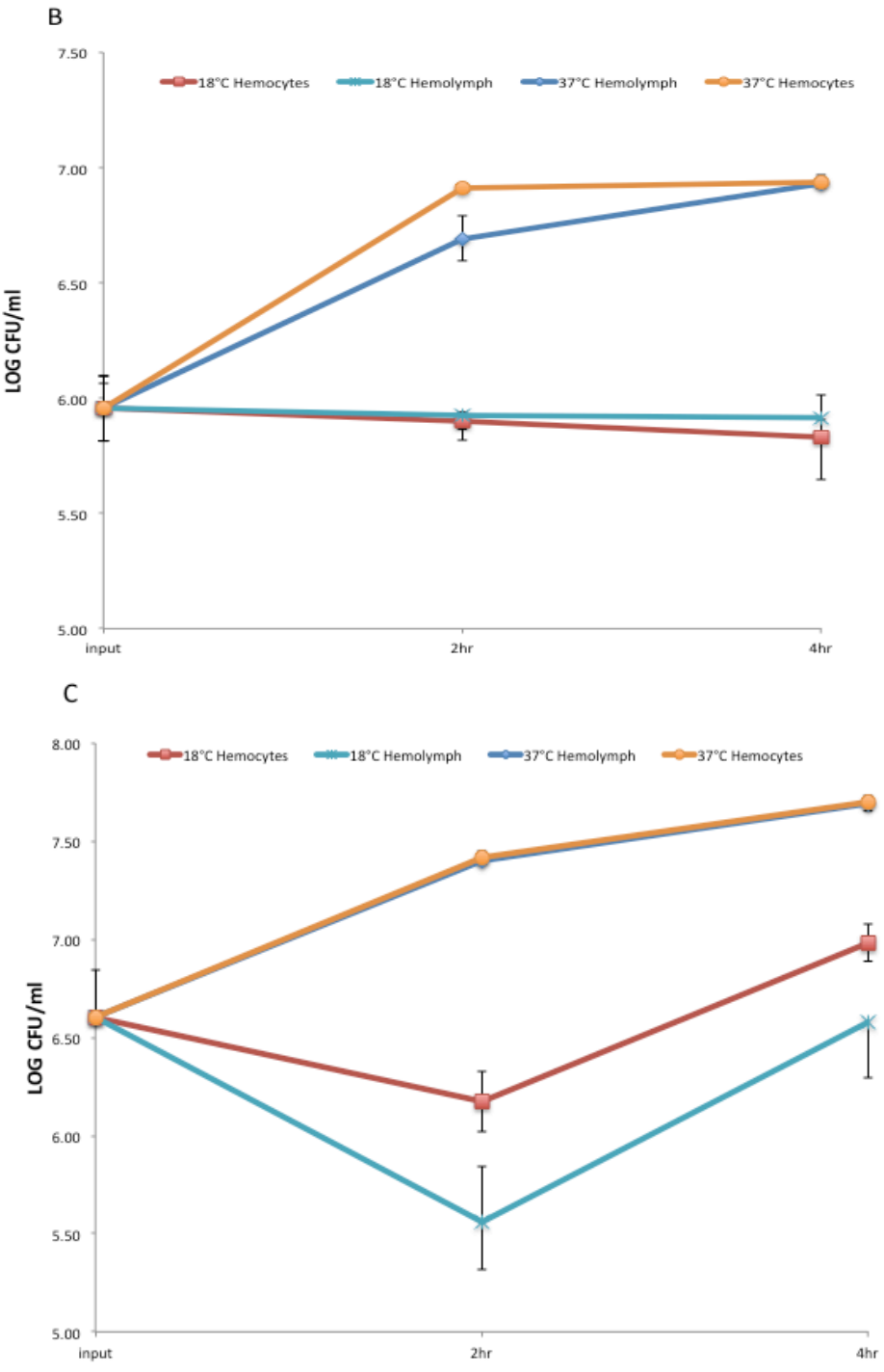


Fig. A.3. Hemocyte interactions with *V. parahaemolyticus.*

This hemocyte data was collected during the spring (April) and since we did observe a seasonal difference in bacterial contaminates in animals either exposed or not exposed to a heat shock, the hemocyte assay was repeated in the summer (July) and fall (September) to determine if hemocyte interactions with *V. parahaemolyticus* also had a seasonal aspect. During the summer, oysters are typically in a mature, spawning physiological state (Kennedy and Krantz 1982; Barber 1996; Huvet, Normand et al. 2010), which is most likely a stress to the animal, including its immune system. In Fig.A.3B, the *V. parahaemolyticus* interactions with hemocytes exposed to a heat shock of 37°C responded similarly in July to what was observed in the spring (Fig. A.3A). In contrast, the *V. parahaemolyticus* interactions with hemocytes that were maintained at 18°C for the entire experiment did not exhibit any difference in bacterial log CFU/ml at either 2hrs or 4hrs. This is significantly different than what was observed in the spring where non-heat shocked hemocytes were successful in reducing *V. parahaemolyticus* by ~1.5 log CFU/ml. The only difference between the spring and summer assays were the time of the year and considering that the animals were in spawning conditions during the summer, but not spring, our data suggests that there may be some impairment of hemocyte-*Vibrio* interactions during the summer even without an additional temperature stress.

Based on the spring and summer hemocyte findings, we examined the hemocyte interactions with *V. parahaemolyticus* during early fall (September) to determine if the late summer/early fall hemocyte killing was most similar to spring or summer. Fig. A.3C shows that while the non-heat shocked hemocytes appeared to

produce a slight decrease in *V. parahaemolyticus* numbers, it did not reflect the robust reduction in *V. parahaemolyticus* observed in the spring and there appeared to be an increased in *V. parahaemolyticus* concentrations by 4hrs, suggesting bacterial growth (Fig. A.3A). Moreover, the cell-free hemolymph actually had a dramatic reduction in the amount of *V. parahaemolyticus* (~1 log CFU/ml) (*P* value = 0.0008) at 2hrs, only to recover by 4hrs (Fig. A.3C). Overall, the hemocytes exposed to *V. parahaemolyticus* and the heat shock exhibited the same trend as the spring (Fig. A.3A) and summer (Fig. A.3B) hemocyte assays. Acidic vacuole formation and reactive oxygen species production were measured and there was an observable difference in these immune parameters for heat shocked and non-heat shocked hemocytes (data not shown), similar to previous observations (Cheng, Hsiao et al. 2004; Cho and Jeong 2005). Taken together our data suggests that a heat shock promotes higher amounts of bacteria in the oyster, possibly due to immune system impairment. In addition, seasonality appeared to impact the hemocyte interactions of non-heat shocked hemocytes, possibly as a result of immune system impairment due to gametogenesis. Total hemocyte counts were higher from animals examined in the summer and fall compared to the spring (data not shown), further suggesting that the depuration and hemocyte results observed for the summer and fall may be the result of seasonal physiological changes in the animals.

Discussion

The marine environment can experience fluctuations in the composition of bacterial populations present at different locations in the water column during different times of the year (Kaneko and Colwell 1973; Wright, Hill et al. 1996; DePaola, Nordstrom et al. 2003; Johnson, Bowers et al. 2012). Numbers of *Vibrio* species have been shown to fluctuate in coastal waters with a seasonal aspect typically related to sea surface temperatures (Kaneko and Colwell 1973; Olafsen, Mikkelsen et al. 1993; Wright, Hill et al. 1996; Johnson, Bowers et al. 2012). During the winter months, vibrios tend to move from the water column to the sediments. In contrast, warmer summer months tend to have relatively high numbers of *Vibrio* sp. present in the water, attached to plankton and associated with oysters (Kaneko and Colwell 1973; Wright, Hill et al. 1996), presenting a potential health threat if the oyster associated vibrios are human pathogens, such as *V. vulnificus* or *V. parahaemolyticus*. Here we show that the natural oyster microflora found during the summer and winter months were a mixture of vibrios and other heterotrophic bacteria, similar to other reports (Garnier, Labreuche et al. 2007; Srivastava, Tucker et al. 2009). During the course of our depuration study, the percentage of vibrios found in the animals changed dramatically and by the end of the experiments, the bacterial populations were predominately vibrios, and this was consistent for animals sampled in the summer and fall. The only exception was in December where vibrios were only a small percentage of the total bacterial population recovered from the oyster tissue and these levels remained low even after depuration. This lower occurrence of vibrios is edible

bivalves in colder months has been suggested to correspond with the natural tendency of *Vibrio* sp. to move to the sediments with the decrease in water temperatures (Kaneko and Colwell 1973; Wright, Hill et al. 1996; DePaola, Nordstrom et al. 2003; Johnson, Bowers et al. 2012). Upon examining the hemolymph of these animals, the bacterial populations in the lymph were similar to the tissues. The amount of naturally occurring vibrios in the hemolymph was consistently less than the total bacteria recovered but by the end of depuration, there was consistently more vibrios in the hemolymph than in the beginning and the population of bacteria within the lymph was shifted towards predominately *Vibrio* sp.

A heat shock has been shown to affect shellfish interactions with bacteria (Cheng, Hsiao et al. 2004; Gagnaire, Frouin et al. 2006). Therefore, we examined effects of a heat shock on naturally occurring bacterial populations in the oyster. When comparing results for the heat treatment and non-heat treatment, a similar trend of changing bacterial population in favor of vibrios was observed for all months sampled. Animals that had the heat shock treatment appeared to consistently have higher amounts of vibrios and total bacteria recovered from their tissues compared to those that were not heat treated. When examining the hemolymph of these animals, it appeared to reflect what was observed in the whole animals. Thus, our basic depuration experiments enriched for naturally occurring vibrios, regardless of the addition of a heat stress, although the addition of a heat stress did correlate with higher total numbers of bacteria. These data also support the previous observations that vibrios tend to be not recovered in oysters during the winter months. December

was the only month sampled that did not exhibit relatively high amounts of vibrios in the tissue and no change in the bacterial population composition over the course of the experiment were observed. Taken together these observations have important implications for future post-harvest treatment schemes, as depuration is less likely to reduce *Vibrio* numbers to safe levels during certain periods of the year.

In addition to the higher incidence of vibrios, pathogenic and non-pathogenic, during the warmer months, oysters are exposed to increased water temperatures and gametogenesis, which has been linked to the phenomenon known as summer mortality (Berthelin, Kellner et al. 2000; Cheney, MacDonald et al. 2000; Huvet, Normand et al. 2010). Researchers have shown that factors that can induce summer mortality events, such as reproduction and increased water temperatures, can also leave oysters more susceptible to bacterial inoculation (Cho and Jeong 2005; Lambert, Soudant et al. 2007; Travers, Le Goic et al. 2008). Although *V. parahaemolyticus* has not be attributed to summer mortality, it is interesting that *V. parahaemolyticus* associations with oysters occur during warmer months when oysters are exposed to conditions shown to induce mass mortality events and affect the oyster immune system, (Lambert, Soudant et al. 2007; Samain, Degremont et al. 2007), possibly providing a link with the increased incidence of *V. parahaemolyticus* infections during the summer resulting from the consumption of oysters. Therefore, we subjected summer oysters artificially contaminated with *V. parahaemolyticus* to a heat shock (37°C), known to significantly affect oysters (Li, Qin et al. 2007; Samain, Degremont et al. 2007), and measured the effects of the heat stress on *V.*

parahaemolyticus populations in the tissue and hemolymph compared with animals that were maintained at 18°C. Similar to what was observed for the natural oyster microflora, animals that received the heat stress had significantly higher amounts of *V. parahaemolyticus* in their tissues and hemolymph than those that were held at ambient temperature. This suggests that a short heat shock is sufficient to impair the oyster's abilities to clear the contaminating *V. parahaemolyticus* from its hemolymph and tissues. Thus, animals that are exposed to such conditions could maintain high levels of this human pathogenic bacterium, posing significant health risks if oysters are harvested from intertidal areas during the summer where dramatic increases in temperature due to sun exposure can occur. Such a natural heat shock can then be expected to lead to high levels of *V. parahaemolyticus* in market oysters even following certain post-harvest treatments, such as depuration. Our findings may lead to important considerations for oyster producers that maintain animals in intertidal regions with regards to contamination by this human pathogen, *V. parahaemolyticus* and the timing of harvesting oysters should take potential heat stresses to the animals into consideration.

Since oysters are also under reproductive stress during the summer months and naturally exposed to warmer water temperatures, we repeated our experiments during the fall to determine how oysters that are in cooler environmental temperatures and are no longer in spawning conditions will respond to artificial contamination with *V. parahaemolyticus.* It has been shown that pre- and post-spawning animals respond differently to a heat shock (Li, Qin et al. 2007), suggesting that animals in the fall my

respond differently than animals in the summer. Consistent with the summer experiments, the heat shock resulted in significantly more *V. parahaemolyticus* in the tissue and hemolymph of the oysters at the end of the experiment, although some important differences were observed. The fold reduction between *V. parahaemolyticus* recovered from the tissues of heat shocked and non-heat shocked was lower in the fall compared to the summer. Animals that were heat shocked in the summer maintained a similar amount of *V. parahaemolyticus* in their tissues over time, while similarly treated animals in the fall did have a reduction during depuration. Despite these differences, the overall trend was consistent, suggesting a heat shock of 37°C for 2hrs was sufficient to alter the oyster's ability to clear *V. parahaemolyticus* from its tissues and hemolymph. In addition, our data suggests that seasonal changes in bacterial clearing abilities in the oysters were affected, possibly a result of gametogenesis. Animals in the fall, which are no longer in gametogenesis, responded similarly to the heat shock as the animals in the summer with mature gonads, albeit not as robust of a response, but maintained higher overall levels of *V. parahaemolyticus* compared with non-heat shocked animals. It is tempting to speculate that the differences observed between the summer and fall could be a result of the physiological state of the oyster, where heat shocked animals in the summer may take longer to clear the *V. parahaemolyticus* compared to the fall animals. Future studies are required to fully address this and determine if, with more depuration time, heat shocked animals are capable of reducing the concentration of *V. parahaemolyticus* to levels observed for the non-heat shocked animals and/or if there

are differences in extended depuration rates of heat shocked animals between the summer and the fall.

Unlike vertebrates, oysters only possess an innate immune systems consisting of humoral and cellular defenses (reviewed in (Canesi, Gallo et al. 2002)). Hemocytes, found in the hemolymph, or blood, of the oyster, are the primary phagocytic cells in bivalves, mainly responsible for the recognition, binding and internalization of invading bacteria. Hemocytes from bivalves have been shown to produce toxic metabolites and enzymes responsible for the degradation and killing of microbes. Stress is a known immunosuppressant and temperature has been shown to affect hemocyte function (Cheng and Chen 2000). Therefore, we infected hemocyte monolayers with *V. parahaemolyticus* and subjected them to a heat shock, similar to the animal experiments, to determine if the differences observed between the heat shocked and non-heat shocked animals were a result of altered hemocyte-*Vibrio* interactions. While hemocytes held at 18°C reduced the amount of *V. parahaemolyticus* in the monolayer, those held at 37°C for 2hrs, were unable to affect the amount of *V. parahaemolyticus* and the bacteria actually grew. Interestingly, there was no difference between bacteria incubated with hemocytes at 37°C and bacteria incubated 37°C in hemolymph alone, further suggesting that hemocytes exposed to a heat shock were impaired in their ability to kill *V. parahaemolyticus*. In contrast, there was no difference the amount of *V. parahaemolyticus* in cell-free hemolymph incubated at 18°C over the 4hrs tested.

These hemocyte experiments were conducted in the spring (April), when oysters are in the early stages of gametogenesis (Barber 1996). Considering all the animal experiments were conducted in the summer or fall when the animals are in different reproductive phages, we examined hemocyte killing during the summer (July) and fall (September) to determine if hemocyte interactions were affected by different times of the year. Surprisingly, hemocyte interactions with *V. parahaemolyticus* in the summer appeared to be completely impaired, with no change in *V. parahaemolyticus* concentrations at 18°C and there was no difference between bacteria with hemocytes or cell-free hemolymph. Not surprising was the observation that the heat shock treatment of hemocytes resulted in no observed reductions of *V. parahaemolyticus*, comparable to what was observed in the spring. In the fall, hemocyte killing started to resemble what was observed in the spring, although there was only a slight decrease in *V. parahaemolyticus* exposed to hemocytes after 2hrs at 18°C, although the bacteria appeared to recover from the initial decrease by 4hrs, and there was significantly less *V. parahaemolyticus* in cell-free hemolymph alone, contrary to what was observed in the spring. Overall, these data suggest there is seasonality associated with the differences in hemocyte interactions with *V. parahaemolyticus* under "optimal" conditions (18°C), while there did not appear to be any difference in *V. parahaemolyticus* interactions with hemocytes exposed to 37°C at any time of the year. Typically during the spring (March through May), oysters are in an early active phase of gametogenesis, where gonad production is low (Kennedy and Krantz 1982; Barber 1996). Animals generally are mature by June, where

gametes fill the gonads and animals are ready for spawning. Mid-summer (July-August), animals spawn by releasing their gametes into the water column and begin resorption of their reproductive tissue. By September and October, hemocytes usually enter the gonadal area for resorption and a majority of the animals have fully resorbed their gonads, thus entering into the inactive stage (Barber 1996). This description of typical gametogenesis in the Pacific oyster generally reflects the seasonal aspects of the hemocyte assays. In the spring, animals are in the initial stages of gametogenesis and based on the hemocyte assay, appear to have fully functioning hemocyte activity. The summer assays coincide with the height of the spawning season and it is possible that spawning impairs hemocyte activity, thus leading to reduced killing of *V. parahaemolyticus*, in which would explain the observation that hemocytes did not reduce the amount of *V. parahaemolyticus* in the summer. Finally, in the fall, animals are recovering from the spawning season and their hemocytes may begin to regain their killing abilities, similar to what was observed in our September assays. Our data does reflect what was found for the Taiwan abalone, *Haliotis diversicolor supertexta*, artificially infected with *V. parahaemolyticus* and exposed to different temperatures for 24, 72 and 120hrs (Cheng, Hsiao et al. 2004). The authors concluded that a 4°C increase in temperature was sufficient to impair the immune functions of the abalone, resulting in increased susceptibility to *V. parahaemolyticus*. Increased temperatures have shown to significantly alter lysosomal membrane integrity in hemocytes of oysters (Zhang, Li et al. 2006), and we did observe differences in acidic vacuole formation between heat

shocked and non-heat shocked hemocyte monolayers with or without *V. parahaemolyticus* added (data not shown). Compared with the abalone study, our experimental design had a much more dramatic increase in temperature for a shorter period of time, although it implies that a sudden but much less dramatic increase in temperature may also result in impaired immune function. This is an interesting observation and examining different parameters of the heat shock, temperatures and duration of exposure, may identify natural conditions in which oysters may experience in the environment as well as the recoverability of the animals during depuration to identify modifications to the post-harvest strategies currently employed to reduce bacterial contaminants in the oysters. In addition, a detailed study examining any connection between gametogenesis and hemocyte killing of *V. parahaemolyticus* could provide some insight into the ecology of this natural association between *V. parahaemolyticus* and the Pacific oyster, with some emphasis on seasonality associated with summer outbreaks of human disease and/or conditions known to induce summer mortality episodes in oysters.

In summary, our findings suggest that during the summer, oysters may experience one or multiple stresses, ranging from gametogenesis to increased temperatures and other various factors that have been shown to alter the physiological status of the animal and increase susceptibility to bacteria infection. In addition, animals that are exposed to high levels of *V. parahaemolyticus* and an increase in temperature, be it the summer or the fall, may be unable to effectively remove the contaminating bacteria to levels safe for oyster consumers. Overall, it appears that *V.*

parahaemolyticus is more of an opportunist that may be effectively "kept in check" by an immunocompetent animal but should that animal be subjected to stress, such as a heat shock, gametogenesis or combination of these, *V. parahaemolyticus* may overcome the oyster cellular defenses and monopolize the situation. Further studies defining this balance between *V. parahaemolyticus* and the Pacific oyster could provide some insight into *Vibrio*-oyster interactions as well as open up new areas of research for effective depuration strategies to ultimately reduce the contamination of this bacterium and possibly other naturally occurring *Vibrio* sp., reducing seafood-borne illness in the United States and worldwide.

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Toxicity of purified *Vibrio tubiashii* protease to *Crassostrea gigas* larvae

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Unpublished

Introduction

The shellfish production industry in the United States, particularly on the East and West Coasts, is dependent on hatchery and nursery production of large quantities of high quality larvae and juvenile bivalves mollusks (e.g. oysters, clams, mussels). In the past few years, bacterial diseases, particularly vibriosis, was a major cause of mortality in such hatcheries and nurseries resulting in major losses and great expense for shellfish growers (Tubiash et al. 1965; Takahashi et al. 2000; Estes et al. 2004). Although first identified as a causative agent of such mortalities in 1965, *Vibrio tubiashii* (Tubiash et al. 1965) has re-emerged in 2006 and 2007 and has been responsible for mass larval mortalities of eastern oysters, hard clams, and Pacific oysters, causing business failures in east coast states and resulting in a crisis in seed production in all West Coast states. Seed production of hard clams in Florida and Pacific oyster production in west coast states were severely damaged in 2006 and 2007 due to *V. tubiashii* contamination. The problem was so severe in 2007 that one Oregon hatchery was not able to produce any oyster larvae for extensive periods of the year (personal communications, Sue Cudd, Whiskey Creek Hatchery, Tillamook, OR). Growers have reported serious difficulties in obtaining sufficient seed due to these hatchery problems. Such seed shortages will have far more serious carry-over economic effects in subsequent years because oyster farmers will be unable to plant grounds with seed and meet their production goals and market demands for shellfish.

Bacterial infections of adult mollusk bivalves only rarely lead to disease while larval and juvenile bivalves are highly susceptible to *Vibrio* infections (Tubiash et al.

1965; Delston et al. 2003). Vibriosis is considered to be the most serious disease of hatchery-reared oyster larvae (Tubiash et al. 1965; Estes et al. 2004). The disease is characterized by a rapid onset of generalized reduction in larval motility, detached vela, and necrotic soft tissue, accompanied by high mortality rates, often exceeding 90% within 24 hours of infection.

In spite of the economic importance of *Vibrio* sp. in the cultivation of larval and juvenile bivalves in shellfish hatcheries, very little is known about the virulence mechanisms employed by these pathogens. This gap in our knowledge prevents the design of targeted intervention strategies to effectively eliminate this pathogen from shellfish hatcheries. Furthermore, specific tests to detect toxins and/or to distinguish pathogenic from non-pathogenic vibrios are lacking. From our research we anticipate the exciting possibility to significantly increase our understanding of the molecular mechanisms underlying the pathology of vibriosis and to provide the shellfish industry with useful information to help them manage bacterial diseases in their hatcheries.

Materials and Methods

V. tubiashii metalloprotease isolation

To isolate active *V. tubiashii* metalloprotease, we cloned the protease gene into the pBAD-TOPO expression vector, under the control of an arabinose-inducible promoter and containing a C-terminal 6xHis-tag. The protease expression vector was transformed into *Escherichia coli* TOP10 cells. In the presence of arabinose, the

protease gene is expressed on the pBAD-TOPO vector and active protease is produced by TOP10. The protease contains a 6xHis-tag on the C-terminus that allows for column purification using the QIAexpress Ni-NTA Fast Start kit, designed to purify recombinant 6xHis-tagged proteins. From here, the Bradford assay will determine the protein concentration of isolated protease. Protease previously isolated using this method does retain its activity and larval toxicity.

Toxicity bioassays

Freshly harvested eggs and sperm from multiple oysters are fertilized following the spawning protocol established by Dr. Langdon and the Molluscan Broodstock Program. Fertilized eggs are counted and 50 eggs per ml are added to 100mls of clean, filtered seawater at 25°C in a 250ml erhlenmeyer flask containing different concentrations of protease. Control flasks contain 1X phosphate buffered saline or sterile media. Flasks are incubated for 18 hours until the eggs develop into D-stage larvae. The water is changed and the larvae are rinsed to remove residual protease or control buffer. The larvae are thinned to 5 larvae per ml and transferred to a 250ml baffled flask to provide efficient aeration while on an orbital shaker. Formalin is added to the remaining D-stage larvae to determine percent survival of the egg bioassay. The larvae are fed a mixture of cultured algae at a concentration of 10,000 algal cells/ml. The flasks are set to rotate on the orbital shaker at the lowest setting, less than 1000 rpm, for two days. The larvae are fed every day and the water is changed every two days until day 7. On day 7, formalin is added to the larval culture to determine 7 day percent survival.

Enzymatic assays

We have successfully used the azocasein assay, as previously described by Halpern et al. (7), to determine *V. tubiashii* protease activity. Briefly, 100 µl of protease supernatants are incubated with 400 µl of 1% azocasein for 30 minutes at 37°C. The reaction is stopped by the addition of 600 µl of 10% trichloroacetic acid and incubated on ice for 30 minutes before being centrifuged at 13,000 rpm for 5 minutes. 800 µl of the supernatants from the centrifuged reactions are added to 200 µl of 1.8 N sodium hydroxide and the absorbance at 420 nm are measured in a Bio Rad SmartSpec Plus spectrophotometer.

Determining concentration of protease toxic to C. gigas larvae

To determine the toxicity to larvae, protease will be diluted to specific concentrations and assayed for larval toxicity. Azocasein assays will be used to measure protease activity for each predetermined concentration. Larval toxicity assays will be performed to identify the minimum concentration of protease toxic to larvae. This will allow for correlation between protease concentration, azocasein activity and larval toxicity.

Previous work

In our previous work, we examined the roles of the *V. tubiashii* extracellular hemolysin and protease in the toxicity of culture supernatants to Pacific oyster (*Crassostrea gigas*) larvae. We have concluded that the metalloprotease (VtpA), but

not the hemolysin (VthA), acts as one of the critical factors for the toxicity of *V. tubiashii* supernatants on Pacific oyster larvae based on the following evidence: (i) treatment of *V. tubiashii* culture supernatants with metalloprotease inhibitors severely diminished the toxicity to Pacific oyster larvae whereas other classes of protease inhibitors or a specific inhibitor of hemolysin did not affect lethality; (ii) strains of *V. cholerae* expressing the *vtpA* gene, but not *vthA* or the vector plasmid, caused high larval mortality (Fig. B.1); (iii) a VtpA$^-$ mutant strain of *V. tubiashii* showed a dramatic loss of toxicity to oyster larvae (Fig. B.2) (Hasegawa et al. 2008).

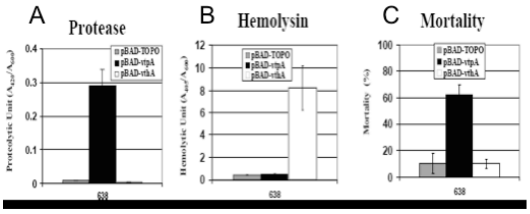


Fig. B.1. Expression of *vtpA* and *vthA* in *V. cholerae*. Protease (A) and hemolysin (B) production, as well as toxicity to oyster larvae (C) analyzed from *V. cholerae* strain 638, a protease deleted strain.

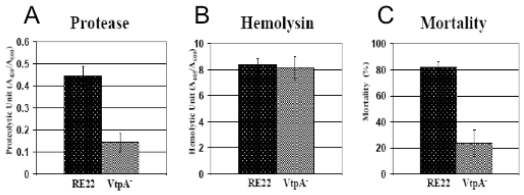


Fig. B.2. Analyses of supernatants from a VtpA$^-$ mutant of *V. tubiashii* strain RE22. Protease (A) and hemolysin (B) production, as well as toxicity to oyster larvae (C) were compared to the wild type strain.

Objective

Protease plays an important role in *V. tubiashii* pathogenicity of *C. gigas* larvae. Although our previous work showed that the *V. tubiashii* metalloprotease is toxic to *C. gigas* larvae, nothing is known about the concentration of the protease needed for toxicity. Determining the concentration required for toxicity could lead to development of a sensitive assay that would detect protease in seawater at hatcheries. Accurate detection of the toxic protease would allow the hatchery to better predict survivability of larvae. It is costly to proceed with larval culture if it is not known whether they will survive or continue to develop properly in the presence of sub-

lethal concentrations of *V. tubiashii* protease. Understanding the relationship between protease concentration and toxicity will aid in development of treatment strategies to assist struggling oyster hatcheries in overcoming *V. tubiashii* toxicity.

To purify the V. tubiashii metalloprotease and to determine the concentration required for toxicity of C. gigas larvae

The aims of this objective are to isolate and purify active *V. tubiashii* metalloprotease in order to then use these preparations to determine the concentration toxic to *C. gigas* larvae. This will provide the groundwork for developing a sensitive detection assay to measure concentrations of *V. tubiashii* protease in larval hatchery water.

Results

Once protease protein concentrations can be correlated to proteolytic activity and larval toxicity, specific assays can be developed to allow for sensitive protease detection. The lower limit of detection for protease will need to be determined, allowing for hatcheries to test their waters with a highly sensitive protease assay. Measuring the concentration of protease in the water will allow for an estimation of larval survival in response to protease exposure.

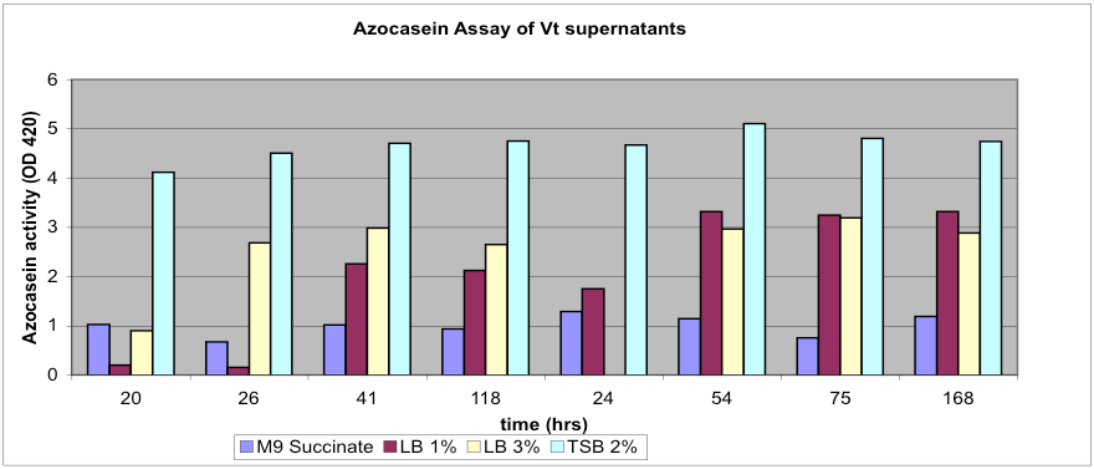
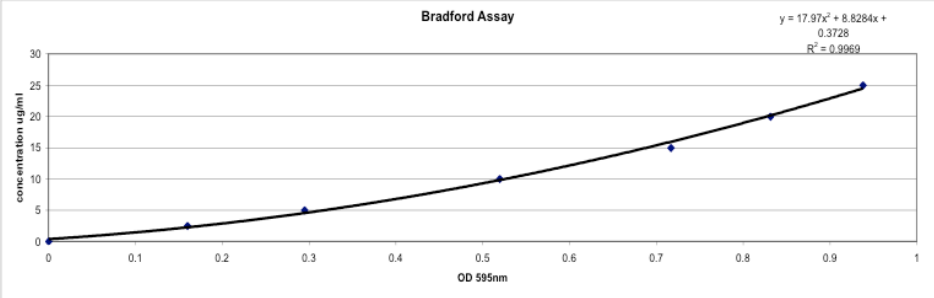


Fig. B.3 Azocasein assay on supernatants from *V. tubiashii* grown in different media



sample	OD595	Minus Blank	Azocasein OD 420	Concentration BSA ug/ml
Blank	0.319	0		0
purified protease	0.562	0.243	0.048	3.57
LB 3% Vt supernatants	1.280	0.964	0.068	25.58
LB 3% media control	1.255	0.939	0	24.51

Fig. B.4 Bradford assay on *V. tubiashii* purified protease. A) Bradford assay standard curve and B) protein concentration for purified *V. tubiashii* protease, LB 3% NaCl broth *V. tubiashii* supernatants, and LB 3% NaCl media control based on Bradford assay standard curve.

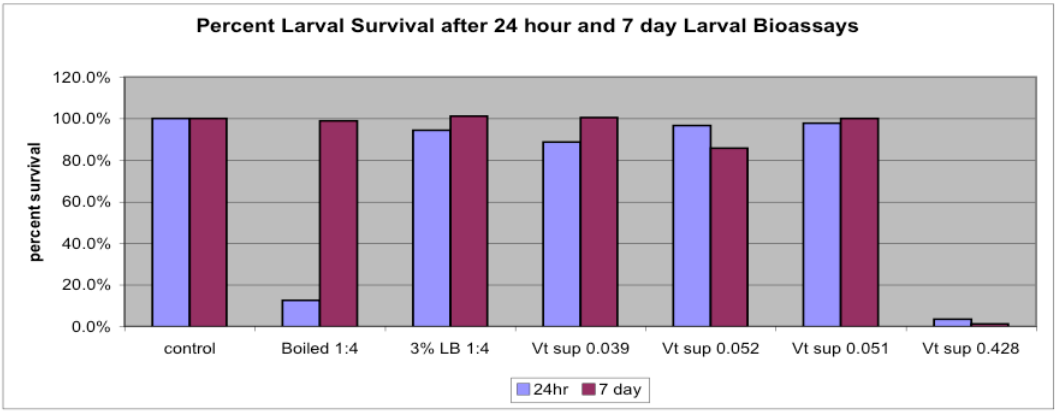


Fig. B.5 Larvae survival presented as a percentage of the control for the 24 hour and 7 day bioassay. Boiled 1:4 is *V. tubiashii* supernatants diluted 1:4 and boiled for 10 minutes to deactivate the metalloprotease. 3% LB 1:4 is a the media control diluted 1:4 to account for any proteins present in the media which may effect larval development. Vt sup 0.039, 0.052, 0.051, and 0.428 are azocasein measurements of various dilutions of supernatants from *V. tubiashii* grown in LB supplemented with 3% NaCl.

Conclusions

The 24hr egg bioassay and 7 day bioassay have been established, although the chronic assays need to be repeated with purified protease that can be quantified using the Bradford assay so a correlation can be made between azocasein activity, protein concentration, and larval toxicity. Unfortunately, water quality issues during the spawning season of 2010 prevented bioassays from being repeated with purified *V. tubiashii* protease and confirm previous findings. Future experiments are required to expand these experiments to establish accurate detection assay for use in the hatcheries.

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