An Investigation of the Role of Vibrio Vulnificus rbdA and Vibrio Parahaemolyticus sypA in Biofilm Formation

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LOYOLA UNIVERSITY CHICAGO

AN INVESTIGATION OF THE ROLE OF VIBRIO VULNIFICUS RBDA AND VIBRIO PARAHAEOMOLYTICUS SYPA IN BIOFILM FORMATION

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN INFECTIOUS DISEASE AND IMMUNOLOGY

BY

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ABSTRACT

*Vibrio vulnificus* and *Vibrio parahaemolyticus* are both bacteria that cause human infection. *V. vulnificus* has a polysaccharide locus, *rbd*, responsible for bacterial aggregation, a form of biofilm. This locus is conserved in *V. parahaemolyticus* and in the non-pathogen *V. fischeri*.

In *V. fischeri*, the polysaccharide locus, *syp*, has been extensively characterized and shown to be important for biofilm formation. In *V. fischeri*, the first gene, *sypA*, is critical for biofilm formation. *V. fischeri* biofilm-proficient strains form wrinkled colonies. In contrast, *sypA* mutants form smooth colonies, indicating a lack of biofilm formation.

To understand the function of RbdA and SypA<sub>VP</sub>, proteins encoded by the first genes in the respective polysaccharide loci, in biofilm formation, we hypothesized we could use the well-characterized *V. fischeri* model of biofilm formation. I found that *rbdA* and *sypA<sub>VP</sub>* are able to promote biofilm formation in *V. fischeri*. Additionally, RbdA and SypA<sub>VP</sub> function appear to be controlled by SypE, the negative regulator of SypA in *V. fischeri*. 
CHAPTER ONE

LITERATURE REVIEW

I. Introduction

*Vibrio vulnificus* is a halophilic bacterium naturally occurring in marine and estuarine environments. *Vibrio parahaemolyticus* is also a halophilic bacterium. It is found naturally in warm, brackish saltwater in coastal waters around the United States and Canada. Both bacteria are opportunistic human pathogens that can cause gastrointestinal and severe wound infections [1]. Many factors allow these bacteria to cause disease in humans. One likely factor is the ability to form a biofilm. A biofilm may form in the environment, for example, on an oyster. Biofilms that are not removed from oysters that are harvested for human consumption may increase the transmission of bacteria to the human host [2]. A biofilm may also form in the human host, causing persistent infection [3]. Both bacteria encode genes for the production and transport of a polysaccharide that may contribute to the formation of the biofilm, but the individual genes of these loci have not been characterized [4].

*Vibrio fischeri* is a related, non-pathogenic *Vibrio* species that is a symbiont of the Hawaiian bobtail squid, *Euprymna scolopes*. To efficiently colonize the squid, *Vibrio* form a biofilm. Biofilm formation is dependent on an 18-gene locus called *syp* [5, 6].
Genes involved in forming the biofilm and associated behaviors have been extensively characterized. The first gene in the syp locus encodes a STAS domain protein, which is a regulator of biofilm formation. The first genes in the *V. vulnificus* and *V. parahaemolyticus* loci also encode STAS domain proteins. Therefore, *V. fischeri* biofilm formation serves as a model for the study of biofilm formation in other related *Vibrio* species. Biofilms are of clinical significance and how a model organism forms a biofilm could provide insight into the pathogenesis of bacteria that form biofilms.

**II. *Vibrio vulnificus***

**Clinical significance**

*Disease associated*

*V. vulnificus* is an opportunistic pathogen that can cause disease in healthy individuals in two ways: via ingestion of contaminated food such as raw seafood, particularly oysters, or via exposure of an open wound to infected seawater. In healthy individuals, ingestion of *V. vulnificus* can cause infection with symptoms ranging from vomiting to diarrhea to abdominal pain. The initial gastrointestinal infection can become systemic, causing a serious disease with symptoms including fever, chills, decreased blood pressure, and culminating in septic shock. Infection can also cause a blistering dermatitis. Systemic infection is more likely to occur in immunocompromised individuals than healthy patients. *V. vulnificus* can also cause infection when bacteria living in seawater come into contact with an open wound. In the wound route of infection, disease can lead to skin breakdown and ulceration. Systemic infection stemming from a wound
infection can result in complications with symptoms similar to the systemic gastrointestinal infection [1].

Immunocompromised individuals are susceptible to a variety of infections that normally pose little risk to healthy individuals. Certain medical conditions, such as liver dysfunction and other syndromes that lead to increased iron deposition, including as chronic cirrhosis, hepatitis, thalassemia major, hemochromatosis, and heavy alcohol consumption, predispose individuals to infection by *V. vulnificus* through either route of infection, ingestion or contact [7]. In immunocompromised individuals with *V. vulnificus*-infected wounds, there is a higher risk for bloodstream invasion, and individuals can suffer from serious complications, including potentially death. Infections that become systemic can have a mortality rate as high as 50%. [1].

**Incidence**

*V. vulnificus* is an underreported cause of disease, in part because reporting is only required in a subset of states in the U.S.. Most cases occur in the Gulf Coast. The Center for Disease Control reports there were more than 900 cases between 1998 and 2006 in the Gulf Coast [1]. The number of infections in this time period increased 78%. There are an average of 50 culture-confirmed cases per year. On average, there are 45 hospitalizations and 16 deaths reported per year just from the Gulf Coast, which includes the states of Alabama, Florida, Louisiana, Mississippi, and Texas. For the nation, there are approximately 95 cases reported per year; however, only half of those cases are culture-confirmed. Nationwide, there are 85 hospitalizations and 35 deaths [1].
**V. vulnificus** infections and outbreaks occur worldwide. In Japan, there is an estimated occurrence of 425 cases of *V. vulnificus* per year, although the infection is not reportable in that country [8]. Japan’s warm coastal waters make it an ideal environment for the growth of *V. vulnificus*. Japan also has a higher rate of raw seafood consumption increasing the likelihood of potential infection [9].

**Pathogenesis**

Those with pre-existing medical conditions, including those with chronic liver disease, are 80 times more likely to develop bloodstream infections [1]. Septicemic infections occur upon bacterial invasion of the intestinal mucosa. From the intestinal mucosa, bacteria can enter the bloodstream [7]. There are two hallmarks of *V. vulnificus* disease: extreme destruction of host tissues and rapid proliferation of the bacteria. While virulence factors associated with the bacteria have been identified, little is known about what directly contributes to the hallmarks of disease [10].

**Virulence factors**

The most important virulence factor for *V. vulnificus* is the production of capsular polysaccharide (CPS), which protects the bacteria from host responses such as complement and phagocytosis by host immune cells [10]. *V. vulnificus* strains that do not form a capsule are attenuated in mouse models. These strains are also susceptible to human serum that contains bactericidal activity. Unfortunately, the antigenicity of the capsule can vary among different strains, making the capsule a poor vaccine target [9].

The ability to acquire iron from the environment is considered another important virulence factor of *V. vulnificus*. There is increased infection susceptibility and disease
severity in hosts with increased iron levels. To cause severe infection, the bacterium relies on iron imbalance or on factors than cause an iron imbalance [11]. Work in the role of iron in *V. vulnificus* is ongoing, but evidence suggests that iron manipulation by the bacteria can have two effects. One effect is that high iron levels can inhibit host responses, such as inhibiting neutrophil function. High iron can also significantly increase bacterial replication in skin tissue [12]. How either of these mechanisms occurs has yet to be elucidated.

*V. vulnificus* uses a flagellum for motility. Mutants defective flagellar components have been assessed for virulence and a range of defects have been observed. One such mutation in the flagellar gene *flgC* resulted in a strain that was attenuated when orally inoculated in mice. Attenuation could be a result of decreased motility or a result of decreased adherence. *flgC* mutant strains also have decreased adherence to cells [13]. Another flagellar mutant, *fliP*, was also non-motile and attenuated in mice. This mutant was able to cause severe skin infection but not systemic infection [9]. The difference between the two mutants and their ability to cause infection suggests that there may be other factors that contribute to the pathogenicity of the organism.

Other virulence factors of *V. vulnificus* include hemolysins, cytolysins, and metalloproteases [9]. When these secreted proteins are purified and injected into animals, some of the pathology of infection can be replicated. Injection of purified hemolysin causes skin damage by forming pores in host cell membranes. The formation of pores can lead to vasodilation and edema in blood vessels, symptoms that resembles those that occur during infection. When a purified metalloprotease was injected into mice, it caused
dermal necrosis, increased vascular permeability, and edema. However, when the gene for either the metalloprotease or a hemolysin or cytolysin was mutated in *V. vulnificus*, the mutant showed little attenuation in an animal model [9].

Other putative virulence factors have been identified, including the RtxA toxin and pili. The RtxA toxin causes depolymerization of actin in HeLa cells in a contact-mediated manner. The toxin causes pore formation in red blood cells and necrosis in hepatic cells. In a mouse model, however, an *rtxA* mutant was not attenuated. The presence of pili is associated with epithelial cell adherence. Pili mutants no longer produce pili or, surprisingly, hemolysin or cytolysin. Chitinase secretion is also inhibited in pili mutants. This mutant was attenuated in mice but the reason for attenuation may be due to the lack of hemolysin or cytolysin or chitinase secretion [9].

**Economic and environmental burden**

One of the primary vectors of infection with *V. vulnificus* is the oyster. Oysters can only be legally harvested from waters free from fecal contamination. However, *V. vulnificus* can be present in non-fecal contaminated waters as it is a natural inhabitant of marine environments. The presence of the bacteria does not alter the appearance, taste, or odor of the oysters. Infections are reported to the Food and Drug Administration, which identifies the source of infection, preventing further infection in humans. These efforts can help predict environmental factors that increase the chance that oysters carry pathogens [1]. The increase in reported infections could be due to climate change resulting in increased marine temperatures. While there have been more cases of infection, the rate of infection has not increased [14].
Studies determining differences between clinical and environmental isolated strains are ongoing. Studies have found that oyster isolates have the ability to cause disease in mice, although clinically isolated strains had a higher prevalence of causing systemic infection in mice. In the clinic however, wound infections do not always progress to systemic disease. These results suggest that there is a difference between strains that cause systemic infection and those that cause wound and gastrointestinal infections only. Thus, there appear to be different populations of *V. vulnificus*, a population that can cause destruction and localized infection and a population that causes systemic infection. The factors that distinguish the two bacterial populations have yet to be identified [9]. The differences in host populations that become infected with either wound infections or gastrointestinal infections have yet to be identified as well.

III. *Vibrio parahaemolyticus*

**Clinical significance**

**Disease associated**

*V. parahaemolyticus* can cause disease in humans who ingest seafood, including raw or undercooked shellfish particularly oysters, contaminated with the bacteria. Symptoms of the illness caused by *V. parahaemolyticus* include abdominal cramping, nausea, vomiting, fever, and chills. Symptoms usually occur 24 hours after ingestion of contaminated food. Infection is self-limiting lasting on average three days. Immunocompromised individuals and those with weakened immune systems can have more severe and prolonged illnesses. Medical conditions including alcoholism and liver
disease may increase the risk of severe and prolonged disease. Wound infections can also occur when an open wound is exposed to infected warm seawater; however, this route of infection is less common. Infection rarely spreads to the bloodstream [15].

Incidence

*V. parahaemolyticus* was first discovered after a food poisoning outbreak in Japan in 1950. The outbreak affected 272 individuals making *V. parahaemolyticus* one of the leading causes of foodborne illnesses [16]. In Asian countries such as Taiwan and Japan, consumption of raw seafood is not uncommon leading to higher rates of infection [2].

In 1971, the United States had its first *V. parahaemolyticus* outbreak in Maryland after individuals consumed infected crabmeat [17]. The CDC estimates that there are 4500 cases of *V. parahaemolyticus* infection each year. The number of cases is underreported as the disease is self-limiting, preventing many people from seeking medical attention. Additionally, laboratories rarely use the selective media required to identify *V. parahaemolyticus*. There are approximately 215 culture-confirmed cases, 30 hospitalizations, and 1-2 deaths reported each year to the CDC [15].

Pathogenesis

*V. parahaemolyticus* causes infection by invading the intestinal epithelia of humans after ingestion of contaminated seafood such as shellfish or oysters [15]. Bacteria adhere to the intestinal epithelia [18]. The bacteria then inject toxins into the host cells via a type III secretion system; these toxins can cause a change in the ion flux in the cell, ultimately leading to cell death. Cell death and alterations in the ion flux of the cell cause the symptoms that manifest in the clinic [2].
Virulence factors

The thermostable direct hemolysin (TDH) is an important virulence factor of *V. parahaemolyticus*. This hemolysin is responsible for the beta-hemolysis seen when bacteria are grown on blood agar plates [19]. TDH is a pore-forming toxin that alters the ion flux in intestinal cells. This alteration causes diarrhea. Other TDH-related hemolysins have been identified. These toxin-like proteinaceous substances can cause death in mice at high concentrations and diarrhea at low concentrations. Both toxins have been isolated in clinical strains of *V. parahaemolyticus* that cause gastroenteritis; however, the genes for these toxins are missing from most environmental isolates [2].

The ability to hydrolyze urea is another important virulence factor of *V. parahaemolyticus*. Although the role of urease activity in pathogenicity is unclear, urease activity has been correlated with the presence of TDH [2]. Like *V. vulnificus*, an important virulence factor for *V. parahaemolyticus* is the ability to acquire iron. Vibrioferin is a novel siderophore that may provide bacteria with a competitive advantage for surviving in an iron-limited environment [20]. It has been previously shown that strains grown in iron-limiting conditions exhibited greater adherence, increased hemolysis, and higher rates of proliferation [21, 22].

Investigators hypothesize that bacteria first adhere to host cells and then produce toxins, thus causing disease. *V. parahaemolyticus* produces heat-stable somatic O antigens (lipopolysaccharide), heat-labile capsular K antigens (capsular polysaccharide), and H antigens (flagellar antigens) [18]. Recent studies have shown that strains grown in the presence of bile or deoxycholate had increased capsule size and exhibited increased
adherence to epithelial cells [18]. The in vitro concentrations of bile and deoxycholate used in this study are within the physiologic range found in humans, suggesting that the presence of bile and deoxycholate could have effects on *V. parahaemolyticus* capsular size, and therefore adherence, during human infection. In other enteric bacteria, it has been previously shown that increased capsular size correlates to increased virulence. In *V. parahaemolyticus*, the increased capsule and increased adherence could allow the bacteria to aggregate and adhere to intestinal cells, thus promoting disease [18].

Emerging data suggest that the presence of a type III secretion system within a pathogenicity island plays a role in *V. parahaemolyticus* virulence. Type III secretion systems have been shown in other bacteria to function in the export of bacterial virulence factors into host cells. *Vibrio cholerae* is a related pathogenic bacterium that, like *V. parahaemolyticus*, causes diarrheal diseases. In *V. parahaemolyticus* infection, diarrhea is considered an inflammatory response [2]. In *V. cholerae*, the response is non-inflammatory [23], suggesting there could be two different mechanisms of action causing diarrhea by *Vibrio* species. This difference could in part be explained by the presence of a type III secretion system. *V. cholerae* does not encode a type III secretion system. It is hypothesized that the presence of the type III secretion system is important in disease that results in a non-inflammatory response [2].

**Environmental and economic burden**

*V. parahaemolyticus* infections occur seasonally with 70% of infections occurring between the months of May and October due to warmer water temperatures, which are optimal for bacterial growth. Large outbreaks have been linked to the consumption of
oysters. Environmental factors, such as warm water and moderate salinity, caused an increase in the number of *V. parahaemolyticus* organisms in shellfish and thus played a role in the outbreaks in 1997, 1998, and 2006 [15].

Several techniques are employed to ensure the removal of potentially harmful bacteria from shellfish populations. Relaying, a technique used to purify oysters, involves transferring contaminated shellfish from restricted areas to approved natural biological purification areas. Depuration, another oyster purifying technique, involves the same process except contaminated shellfish are transferred to a controlled aquatic environment instead of a natural environment. Several techniques have been employed to decrease bacterial load post-harvest as the bacteria can rapidly replicate while shellfish are held at room temperature. Mild heat treatment, low temperature pasteurization, high-pressure processing, and low dose irradiation can reduce the number of bacteria associated with oysters. Strains of *V. parahaemolyticus* have different sensitivities to treatments. Finding a treatment or combination of treatments that eliminates all strains of *V. parahaemolyticus* is ongoing in the oyster industry [2].

IV. *Vibrio fischeri*

**Symbiotic relationship**

*Vibrio fischeri* is a non-pathogenic *Vibrio* species. The bacterium is a symbiont of the Hawaiian bobtail squid, *Euprymna scolopes*. The bacterium colonizes the light organ of the juvenile squid. The colonization is highly specific as *V. fischeri* is the only colonizer of the squid organ despite the number of the closely related bacteria present in
the seawater [24]. One step in early colonization involves the formation of a biofilm on the surface of the squid’s symbiotic organ, the light organ [6, 25, 26]. Biofilm formation is required for *V. fischeri* to efficiently colonize its symbiotic host, and depends on a cluster of genes called the *syp* locus, for *symbiosis polysaccharide locus* [5, 6].

V. Bacterial Biofilm Formation

Stages of biofilm formation

A biofilm is a complex community of microorganisms that adhere to each other and, typically, to a surface. Bacteria within the biofilm are embedded in a matrix consisting of polysaccharides and other molecules such as proteins and DNA. The matrix can protect commensal and pathogenic bacteria from environmental stresses, such as antibiotics, as well as from host responses [27, 28]. Other polysaccharides, such as capsular polysaccharides, produced by bacteria can protect the bacteria from host responses and allow them to stick to host cells [14].

Biofilms form when free-living bacterial cells respond to environmental cues that trigger changes in gene expression that allow bacteria to attach to surfaces. The initial interaction between the bacterial cells themselves and between the cells and a surface occurs when any of a variety of cell structures ranging from pili to polysaccharides promote attachment. After attachment, the biofilm matures. This stage is marked by two properties: increased exopolysaccharide production and increased antibiotic resistance. During biofilm maturation, bacteria may develop additional properties such as increased resistance to UV light, increased rates of genetic exchange, increased secondary
metabolite production, and altered biodegradative capabilities. The last stage of biofilm formation involves detachment from the biofilm. In a patient, this detachment can allow bacteria to disseminate to another site, form a biofilm, and cause persistent disease. The molecular mechanisms underlying these changes are an active area of research [29].

**Clinical relevance of biofilm formation**

Biofilms allow bacteria not only to colonize human hosts growing on prosthetic devices and catheters, causing persistent infection, but also promote their survival. Bacteria within biofilms pose a threat in the clinical setting because bacteria exhibit a decreased susceptibility to antimicrobial agents. This could be an intrinsic property of the bacteria or it could be an acquired property. Transfer of antibiotic resistance genes between different populations of bacteria within the biofilm could give the bacteria a survival advantage when faced with antimicrobial agents. Bacteria within a biofilm are often more resistant to antibiotics than planktonic cells. The differences in antibiotic susceptibilities between cell types can be problematic in the laboratory setting as antimicrobial susceptibility testing is done on planktonic cells. The microdilution testing that is used in the clinical laboratory does not accurately determine the susceptibility of bacteria within the biofilm [3].

Most bloodstream infections are caused by the release of bacteria from the biofilm on an indwelling catheters, prosthetics, or other indwelling medical devices. Bloodstream infections originating from an infection of an indwelling catheter are most often caused by a biofilm forming on the indwelling medical device. Greater understanding of how biofilms form on indwelling devices can provide insight on how to treat infections [3].
Biofilm formation and aggregation in V. vulnificus

Biofilm formation by bacteria in a human host can have serious consequences in the clinical setting. The production of polysaccharides plays a large role in biofilm formation, and phenotypes of biofilm formation can be measured in the lab by the production of rugose colonies, pellicles, or aggregate formation [4]. V. vulnificus has two polysaccharide loci that have been studied. One polysaccharide locus, designated rbd for regulation of biofilm development, is responsible for bacterial aggregation [4]. Wild-type strains of V. vulnificus are able to form biofilms on glass slides and on the surface of a crab shell. However, when the rbd locus was disrupted, V. vulnificus strains formed a less extensive biofilm on either surface suggesting the rbd polysaccharide locus is important for the formation of the biofilm. In contrast, disruption of the rbd locus did not impact rugose phenotype, suggesting that the rbd locus is not important for this phenotype. Indeed, disruption of a second polysaccharide locus, brp (for biofilm and rugose polysaccharide), which was previously shown to be involved in biofilm formation, abolished the rugose phenotype [4].

Further studies determined the rbd locus was, instead, important for aggregation. To evaluate aggregation, researchers grew wild-type stains and rbd-overexpressing strains overnight, and then allowed the cultures to sit for 5 seconds or 5 minutes. Wild-type stains of V. vulnificus formed little to no ring on the sides of the culture tube, while the rbd-overexpressing strains produced a biofilm ring on the sides of the culture tube. Strains overexpressing rbd also formed a visible cell aggregate. The same experiments tested the ability of brp-overexpressing strains to form aggregates. In combination with
the rugose phenotype experiments, it was concluded that \textit{rbd} has a more dramatic effect on aggregation, while \textit{brp} has greater impact on rugosity [4]. These studies suggest that \textit{V. vulnificus} can form different biofilms using different polysaccharides.

In other studies, investigators sought to determine the contribution of the \textit{rbd} polysaccharide locus to disease in a mouse model. It was found that when mice were infected with a \textit{V. vulnificus} strain in which the \textit{rbd} locus was disrupted, there was no effect in attenuation in mouse lethality. Although work to date has not revealed a role for \textit{rbd} in mouse colonization, it remains possible that this locus may be one of the factors that contribute to persistence in the oyster population [30]. \textit{rbd} may be triggered to enhance colonization of the oyster, or other shellfish, by enabling the dissemination of aggregates of bacteria that can be easily retained by the filter-feeding host or captured from the water environment [4].

\textbf{Conservation and role of polysaccharides in biofilm formation}

To produce a biofilm, bacteria secrete an extracellular matrix that holds the bacterial community together. One component of the extracellular matrix is polysaccharide [31]. The \textit{rbd} locus, which encodes genes for the production and transport of a polysaccharide, in \textit{V. vulnificus} has been shown to be involved in aggregation. This polysaccharide locus is conserved in \textit{V. parahaemolyticus} as well as in the non-pathogenic \textit{V. fischeri}. When induced to form a biofilm, \textit{V. fischeri} produces the Syp polysaccharide, and this biofilm formation in \textit{V. fischeri} depends on Syp polysaccharide production [6]. When the \textit{syp} locus is expressed, \textit{V. fischeri} produces wrinkled colonies, pellicles, and extracellular matrix. When any of the \textit{syp} genes are lost, the wild-type
phenotype is restored as no wrinkled colonies are produced, and no extracellular matrix appears to be present [25].

Additionally, disruption of the *syp* locus abolishes colonization [6, 25, 26]. The *syp* cluster can be over-expressed *in vitro* to permit the study of biofilm formation [26]. When a regulator of the *syp* locus is over-expressed, strains of *V. fischeri* form wrinkled colonies when spotted on to agar plates and form pellicles in static liquid culture (Figure 1).
Figure 1. Transcriptional regulation of the syp locus. The histidine kinase RscS acts upstream of the syp-encoded response regulator SypG to control syp transcription. RscS is activated and autophosphorylates upon sensing an unidentified signal and serves as a phosphodonor for SypG. Phosphorylated SypG activates transcription of the individual operons. Transcription of the locus leads to polysaccharide production, which in turn leads to the formation of wrinkled colonies when cells are grown on agar plates.
The first gene in the polysaccharide locus in *V. fischeri* is *sypA*, which encodes the protein SypA, a regulator of biofilm formation. SypA has a sulfate transporter and anti-sigma factor antagonist (STAS) domain. Although the specific function of SypA is unknown, this protein is required for biofilm formation by *V. fischeri* [32, 33]. The activity of SypA is regulated by another protein encoded by the polysaccharide locus, SypE. SypE phosphorylates a conserved serine residue, serine 56, within a consensus sequence of SypA [32].

### VI. STAS Domain Proteins

The first gene encoded in the *syp* locus is *sypA*. Interestingly, a STAS domain is encoded in the first gene of the *rbd* locus, *rbdA*, in *V. vulnificus* and in the first gene of the *V. parahaemolyticus* polysaccharide locus, *sypAvP*. While the individual genes of the *V. vulnificus* locus, including *rbdA*, have not been well characterized [4], there is much known about the *V. fischeri* STAS domain protein, SypA. In *V. fischeri*, the STAS domain protein, SypA, is a regulator of biofilm formation and is required for polysaccharide production and biofilm formation [32, 33]. Although its function is unknown, evidence suggests it functions differently from the best studied STAS domain proteins.

**Characteristics of STAS domain proteins**

Sulfate transporter and anti-sigma factor antagonist (STAS) domain proteins that have been well characterized are typically involved in stress responses in the cell, and the
function of STAS domain proteins has been studied in many different bacterial models [34]. The first studied STAS domain proteins were single domain anti-sigma factor antagonists. This type of STAS domain protein indirectly activates transcription by inactivating an inhibitor of transcription such as an anti-sigma factor kinase. The inactivation of the anti-sigma factor kinase allows sigma factors to activate transcription [35].

STAS domain proteins have been shown to have a variety of other functions. STAS domain proteins can also bind nucleotides. Some interact within a multi-domain protein to sense light, oxygen, or other proteins. STAS domain proteins exhibit a conserved fold of 4 β sheets interspersed among 5 α helices [35].

**Role of STAS domain proteins in non-*Vibrio* species**

i. **SpoIIAA**

Bacteria encounter a wide variety of stresses. Some stresses are extreme, and in the presence of extreme stress can cause the bacteria to sporulate, as is the case with *Bacillus subtilis*. When *B. subtilis* is faced with stress, such as starvation, cells begin to sporulate. *B. subtilis* sporulation, the small STAS domain protein, SpoIIAA, interacts with the anti-sigma factor SpoIIAB to promote activation of transcription of σ^F_ genes that lead to sporulation. In this pathway, the anti-sigma factor SpoIIAB binds to σ^F_, preventing it from binding polymerase. SpoIIAA binds SpoIIAB. This binding between SpoIIAA and SpoIIAB promote a steric and electrostatic clash that leads to the dissociation of SpoIIAB from σ^F_, thus permitting activation of sporulation gene transcription. SpoIIAA is controlled via phosphorylation. When SpoIIAA is
phosphorylated, it dissociates from SpoIIAB. When SpoIIAA is unphosphorylated, the protein is able to form a tight complex such that SpoIIAB can no longer bind to $\sigma^F$ [35].

It is known that when cells are induced to sporulate, the concentrations of GTP and GDP significantly decrease. SpoIIAA acts not only as an anti-anti-sigma factor but also as a GTP-binding protein. It was also found that SpoIIAA has GTPase and ATPase activity. When the site of phosphorylation is mutated in SpoIIAA, the GTPase activity was diminished [36].

ii. RsbV

Bacteria also encounter less extreme stresses more frequently. *B. subtilis* can also respond to frequent, moderate stresses it encounters by controlling gene expression through use of an anti-sigma factor, RsbW, and an anti-sigma factor antagonist, RsbV. When *B. subtilis* is not stressed, the sigma factor $\sigma^B$ is held inactivated in a complex with the anti-sigma factor kinase RsbW. When the cell encounters stress in the form of temperature, pH, osmolarity, ethanol, blue light changes or cell wall stress, the anti-anti sigma factor RsbV can inhibit RsbW [37, 38]. RsbV itself is controlled via phosphorylation by RsbW, releasing $\sigma^B$ to induce stress response genes. This inhibits the anti-anti-sigma activity of RsbV. It can be further dephosphorylated by RsbU which restores the anti-anti-sigma activity of RsbV (Figure 2) [35].

In addition to the RsbV STAS domain protein, there are other STAS domain proteins in this *B. subtilis* stress response pathway, including RsbR and RsbS. RsbU, which is one of the regulators of RsbV activity, is controlled by a complex of proteins that integrates several stress signals to effect a single outcome called the stressosome
(Figure 2) [35]. The signal transduction cascade increases the expression of genes that respond to stress [34]. The stressosome consists of 40 copies of the multidomain STAS protein RsbR, 20 copies of the simple STAS domain protein RsbS, and 20 inactive, sequestered copies of the switch kinase RsbT [37]. RsbR has a C-terminal STAS domain and an N-terminal globulin fold domain that has an as of yet unknown function [35].

In response to stress, RsbT phosphorylates RsbR and RsbS, promoting the release of RsbT from the stressosome. Free RsbT activates RsbU phosphatase. Active RsbU is then able to remove the inhibitory phosphoryl group from RsbV, permitting RsbV to bind to RsbW. RsbW releases σB, allowing it to activate RNA polymerase and transcribe the σB regulon. The stressosome is inactivated by the dephosphorylation of the stressosome proteins RsbS and RsbR by the phosphatase RsbX. This activation of stress response genes is highly sensitive with high cooperativity [37, 39].
Figure 2. The Rsb Stressosome. The stressosome, made up of STAS proteins RbsR and RbsS, sequesters RsbT. Under stress, RsbT phosphorylates RsbR and RsbS. RsbT is then released and free to activate RsbU. The RsbT/RsbU complex dephosphorylates RsbV which is then able to bind the anti-sigma factor RsbW. RsbV binding to RsbW liberates $\sigma^B$ from the inactivating complex with RsbW. Free $\sigma^B$ can then bind RNA polymerase to promote gene expression [35].
iii. Rv1739c

STAS domain proteins also play a role in anion transport. *Mycobacterium tuberculosis* has a sulfate or anion transporter that is comprised of a SulP-type transmembrane domain and a C-terminal cytoplasmic STAS domain, Rv1793c. When purified, Rv1793c was found to bind guanine nucleotides. It was also found that the protein had modest GTPase activity. Interestingly, this protein does not serve as a phosphorylation target [35]. It has been shown that the Rv1793c undergoes conformational changes when bound to either GTP or GDP. The differences in the conformation of Rv1793c when bound to either GTP or GDP give more insight into the interaction between nucleotides and STAS domain proteins [36].
CHAPTER TWO
MATERIALS AND METHODS

Strains and Media. Bacterial strains used in this study are listed in Table 1. *V. fischeri* strains were derived by conjugation. *Escherichia coli* GT115 (Invivogen, San Diego, CA) was used for cloning and conjugation experiments [40, 41]. *V. fischeri* strains were cultured in Luria-Bertani salt (LBS) [42]. The following antibiotics were added to LBS medium at the indicated concentrations: chloramphenicol (Cm) 2.5 µg ml⁻¹, erythromycin at 5 µg ml⁻¹, and tetracycline (Tc) at 5 µg ml⁻¹ in LBS. *E. coli* strains were cultured in Luria Bertani medium (LB) [43]. The following antibiotics were added to LB medium at the indicated concentrations: Cm at 25 µg/ml⁻¹, kanamycin (Kan) at 50 µg ml⁻¹, Tc at 15 µg/ml⁻¹, or ampicillin (Ap) at 100 µg ml⁻¹. For solid media, agar was added to a final concentration of 1.5%.

Bioinformatics. Amino acid sequences for *V. vulnificus* RbdA, *V. parahaemolyticus* SypA V P, and *V. fischeri* SypA were obtained from the National Center for Biotechnology Information (NCBI) database. Alignments of RbdA, SypA V P, and SypA were generated using the Clustal Omega multiple-sequence alignment program from EMBL-EBI (http://ebi.ac.uk/Tools/msa/clustalo) [44].

Molecular and genetic techniques. The *rbdA* and *sypA* V P alleles used in this study were generated by Polymerase Chain Reaction (PCR) using primers listed in Table 2. PCR
products were cloned into the plasmid pARM47, a vector for chromosomal insertion at the Tn7 site containing the lac promoter [45], that was digested to remove the sypE gene. For dual promoter constructs, the homologous sypA genes were cloned downstream of the V. fischeri sypA promoter. The Gibson Assembly kit (New England Biolabs) was used to generate plasmid constructs for the homologous sypA genes. Alleles were inserted into the chromosomal Tn7 site of V. fischeri strains using tetraparental conjugation. To generate site-directed mutations in sypA, mutated alleles of sypA were generated by PCR using mutagenic primers (Table 3) and the Gibson Assembly kit. The mutations were confirmed by sequence analysis using ACGT, inc (Wheeling, IL).

**Wrinkled colony formation assay.** To observe wrinkled colony formation, the indicated V. fischeri strains were streaked onto LBS agar plates containing the necessary antibiotics. Single colonies were then cultured with shaking in LBS broth containing antibiotics overnight at either 24°C (for rscS overexpressing strains) or 28°C (for sypG overexpressing strains). The strains were then sub-cultured the following day in 5 ml of fresh medium. Following growth to early log phase, the cultures were back-diluted in LBS to an OD$_{600}$ of 0.2. 10 µl of diluted cultures were spotted onto LBS agar plates, containing necessary antibiotics, and grown at either ~ 24°C (for rscS overexpressing strains) or 28°C (for sypG overexpressing strains). Images of the spotted cultures were acquired over the course of the developmental process of biofilm formation at the indicated time points using a Zeiss stemi 2000-C dissecting microscope.

**Western blot analysis of V. fischeri lysates.** V. fischeri strains were cultured in LBS containing the appropriate antibiotics overnight at 24 °C. Cultures were standardized to
the same amounts using OD_{600} measurements, concentrated by centrifugation, and lysed
in 500 µL 2X sample (4% SDS, 40 mM Tris pH 6.3, 10% glycerol) buffer. Samples were
resolved on either 10 or 15% SDS-PAGE gels (10% 29:1 acrylamide: N, N’-methylene-
bis-acrylamide, 375 mM Tris pH 8.6, 0.1% SDS), and transferred to PVDF membranes.
SypA and SypA-like proteins were detected by western blot analysis using rabbit anti-HA
antibody (Sigma-Aldrich, St. Louis, MO) followed by a secondary, donkey anti-rabbit
IgG antibody (Sigma-Aldrich, St. Louis, MO) conjugated to horseradish peroxidase
(HRP), and visualized using SuperSignal West Pico Chemiluminescent Substrate
(Thermo Fischer Scientific, Rockford, IL).

**Assessment of SypA phosphorylation in vivo.** Two plasmids were introduced into *E.
coli*, one that expressed either *rbdA* or *sypA_{VP}* and one that expressed *V. fischeri sypE* and
the phosphorylation state of either the RbdA protein or the SypA_{VP} protein was evaluated
using the Phos-tag™ reagent (Wako Chemicals USA, Inc., Richmond, VA). The Phos-
tag™ reagent retards the migration of phosphorylated proteins. The reagent preferentially
binds to phosphorylated proteins, thus separating phosphorylated proteins from
unphosphorylated proteins and, resulting in a band shift observable following gel
electrophoresis and western blotting. *E. coli* strains were cultured overnight with shaking
in LB containing the appropriate antibiotics at 37°C. Cells were sub-cultured the
following day in 5 ml of fresh medium and grown for 8 hours. Aliquots of cells were
standardized to the same amounts using OD600 measurements, then concentrated by
centrifugation. Samples were lysed in 2X SDS sample buffer and resolved on SDS-
PAGE gels containing 25-30 µM Phos-tag™ acrylamide (WAKO Chemicals USA, Inc.,
Richmond, VA) and 50-60 µM MnCl₂. Gels were fixed for 15 min in standard transfer buffer containing 1 mM EDTA, and then incubated an additional 20 min in transfer buffer without EDTA. Proteins were transferred to a PVDF membrane and the proteins were detected by western blot analysis using either an anti-HA or anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO).
Table 1: Strains used in this study

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<th>Reference or source</th>
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\(^1\)All derivatives of pARM47 contain the indicated gene and lack the sypE gene; except where indicated as “lac promoter only”, all pARM47 derivatives carry both the lac promoter and the sypA promoter.
Table 3: Oligonucleotides used in this study

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CHAPTER THREE
EXPERIMENTAL RESULTS

Ability of the sypA-like genes from Vibrio pathogens to promote biofilm formation by V. fischeri

Introduction

V. vulnificus and V. parahaemolyticus are two human pathogens that can cause serious infections in humans and can have particularly high mortality rates in the case of V. vulnificus. Several factors have been hypothesized to contribute to pathogenicity in both organisms, although the cause for the high mortality rates remains unclear. One hypothesis is that both bacteria can form a biofilm that in the human host can lead to persistent infection.

The V. vulnificus genome includes a polysaccharide locus, rbd, that has been previously shown to be involved in aggregation, although individual genes of the locus have not been well characterized [4]. The rbd locus is conserved in the pathogen V. parahaemolyticus and in the non-pathogen V. fischeri, where it has been extensively characterized. In V. fischeri, this polysaccharide locus, called syp, is important in biofilm formation. The deletion of genes within the syp locus results in strains that no longer form a biofilm. Specifically, these strains do not produce wrinkled colonies, one readout of biofilm formation.
One of the regulators of biofilm formation in *V. fischeri* is SypA, a homolog of both RbdA, encoded by the first gene in the *V. vulnificus rbd* locus, and SypAvP, encoded by the first gene in the *V. parahaemolyticus* locus. The *V. vulnificus* RbdA protein has 55% identity and 73% similarity to *V. fischeri* SypA (E value=4e-39) [48], which is required for *syp*-dependent biofilm formation in *V. fischeri*. The *V. parahaemolyticus* SypA-like protein has 58% identity and 73% similarity to *V. fischeri* SypA (E value=e-43) [48] (Figure 3). When *sypA* is deleted in *V. fischeri*, biofilms do not form. The activity of SypA is negatively regulated via phosphorylation by SypE: when *V. fischeri* SypA is phosphorylated, no biofilm forms.

The experimental goal is to elucidate the function of *V. vulnificus* RbdA and *V. parahaemolyticus* SypAvP. Because of the high degree of conservation between RbdA, SypAvP, and SypA and the tractability of the *V. fischeri* genome, this model of biofilm formation can be used to further our understanding of the function of *rbd*-encoded proteins and those encoded by *V. parahaemolyticus*. It is my hypothesis that because of the substantial sequence similarity, these proteins, RbdA and SypAvP, and SypA are orthologs. If the ability to promote biofilm formation is conserved in *rbdA* from *V. vulnificus*, and *sypAvP* from *V. parahaemolyticus*, then *V. fischeri* biofilm-deficient *sypA* mutant strains will form biofilms (wrinkled colonies) when the non-native genes are expressed. These experiments will give insight into the function of the SypA-like proteins in their own species and the role of STAS domain proteins in biofilm formation.
Figure 3. Alignment of SypA-like proteins. To determine the similarity between RbdA, SypA<sub>v</sub>, and SypA, I aligned the three proteins [44]. The amino acid sequence is highly conserved around serine 56 which in <i>V. fischeri</i> SypA is phosphorylated by SypE, another protein encoded in the <i>syp</i> locus.
Ability of *V. vulnificus* *rbdA* and *V. parahaemolyticus* *sypA*VP to promote biofilm formation

To being to determine the function of *rbdA* and *sypA*VP in biofilm formation in their own species, I assessed the ability of the two genes to promote biofilm formation by *V. fischeri*. I separately introduced a copy of the *rbdA* gene and the *sypA*VP gene at the Tn7 site in the chromosome of a *V. fischeri* strain deleted for *sypA*. I then induced strains to form a biofilm and assessed wrinkled colony formation. In these experiments, expression of *rbdA* and *sypA*VP was driven from the *lac* promoter alone. These constructs did not induce production of wrinkled colonies (Figure 4). I hypothesized that the lack of complementation may be due to insufficient levels of either RbdA or SypAVP.

In biofilm formation assays, a Δ*sypA* *V. fischeri* strain that was complemented with *sypA* at the Tn7 site of the chromosome was used as a positive control for complementation. The *sypA* complemented strain produced wrinkled colonies; however, the *sypA* gene was expressed from two promoters, the *lac* promoter and the native *sypA* promoter. I therefore hypothesized that the *lac* promoter alone was insufficient to induce levels of SypA necessary for complementation. In support of this idea, when I expressed the native *V. fischeri* *sypA* from the *lac* promoter alone, I similarly did not observe wrinkled colony formation (Figure 5).

Therefore, I engineered constructs in which the *rbdA* gene and the *sypA*VP gene were driven from the *lac* and *sypA* promoters, and I found that the complemented strains were able to form wrinkled colonies (Figure 6). The wrinkling appeared similar to a control strain that expressed the native *V. fischeri* *sypA* from the Tn7 site. However,
strains expressing \textit{sypA}_{VP} exhibited a slight delay in wrinkling. The SypA_{VP} protein may function less efficiently due to changes in amino acids that in the native SypA are important for function.

The data from the biofilm assay suggested that a threshold of SypA is required for biofilm formation. Western blotting was performed to compare SypA_{VP} protein levels and native \textit{V. fischeri} SypA levels (Figure 7). \textit{V. fischeri} SypA appears to be produced more abundantly than SypA_{VP}. Three microliters of extract containing SypA was loaded on the gel while six microliters of extract containing SypA_{VP} was loaded on the gel. Interestingly, there was a difference in the migration of the \textit{V. fischeri} and \textit{V. parahaemolyticus} proteins, a finding that will be discussed in the Discussion section.

Additionally, SypA protein was not visualized by Western blotting when expression of the \textit{sypA} gene was driven by the \textit{lac} promoter alone, a result that likely accounts for its inability to complement.

In the laboratory, biofilm induction is usually induced in two ways: over-expression of the response regulator, \textit{sypG} or over-expression of the sensor kinase, \textit{rscS}. In the above biofilm assays, I induced strains to form a biofilm by overexpressing \textit{sypG}. In these strains, SypE, the negative regulator of SypA is absent. I also induced biofilm formation by overexpressing the sensor kinase \textit{rscS}. In these strains, SypE is absent, and the \textit{sypA} genes were expressed from the \textit{lac} and \textit{sypA} promoters. These strains, when induced to form a biofilm, did not form the robust wrinkled colonies that I previously observed using overexpression of \textit{sypG}. The strains produced smooth colonies (Figure 8). Strains expressing \textit{sypA}_{VP} began to wrinkle, but even after prolonged incubation, these
strains did not appear similar to a positive control strain expressing *V. fischeri sypA*. These results suggest that over-expression of the sensor kinase *rscS* might not lead to sufficient production of SypA to produce wrinkled colonies.

Although the exact function of SypA is unknown, it is likely that SypA interacts with other proteins to promote biofilm formation, potentially forming a complex with itself and/or other proteins. In strains that express RbdA or SypA<sub>VP</sub>, this hypothetical protein complex may not form correctly and/or may not work as efficiently as SypA-expressing cells to promote biofilm formation. To test the hypothesis that the presence of RbdA or SypA<sub>VP</sub> may disrupt the interaction between SypA and itself or between SypA and other proteins in promoting biofilm formation, I moved either *rbdA* or *sypA<sub>VP</sub>* into a strain containing the native *V. fischeri sypA*. In these experiments, strains not expressing *rbdA* or *sypA<sub>VP</sub>* wrinkle due to the presence of *V. fischeri sypA*. I expected that a strain that expressed *sypA* and *rbdA* or *sypA* and *sypA<sub>VP</sub>* would produce smooth colonies if the homologous proteins impaired the function of SypA. However, I found that *rbdA* and *sypA* expressing strains produced wrinkled colonies (Figure 9). Wrinkling appeared similar to strains expressing two alleles of *V. fischeri sypA*. Additionally, there was no difference in the timing of wrinkling. These data suggest that if SypA is interacting with itself or other proteins to promote biofilm formation, RbdA and SypA<sub>VP</sub> do not disrupt this interaction. This level of regulation could help to ensure biofilm formation occurs at the proper place and time in the environment.
Figure 4. Neither *rbdA* nor *sypA* <sub>VP</sub>, when expressed from a single promoter, promotes biofilm formation. A wrinkled colony assay was performed with strains deficient for *sypA* and *sypE* and expressing *rbdA* and *sypA* <sub>VP</sub> from the chromosome. Gene expression was driven by the *lac* promoter. Biofilm induction was achieved by over-expressing the gene for the response regulator, *sypG*. Pictures were taken at the indicated time points.
Figure 5. Biofilm production depends on sypA expression from two promoters. A wrinkled colony assay was performed with strains deficient for sypA and sypE expressing sypA from either the lac promoter alone or the lac and sypA promoters. Biofilm induction was achieved by over-expressing the gene for the response regulator, sypG. Pictures were taken at the indicated time points.
Figure 6. *rbdA* and *sypA*<sub>VP</sub> expressed from two promoters promote biofilm formation. A wrinkled colony assay was performed with strains deficient for *sypA* and *sypE* and expressing either *rbdA* or *sypA*<sub>VP</sub> from the chromosome. Gene expression was driven by the *lac* and *sypA* promoters. Biofilm induction was achieved by over-expressing the gene for the response regulator, *sypG*. Pictures were taken at the indicated time points.
Figure 7. SypA migrates to a higher molecular weight than SypA<sub>vp</sub>. Whole cell lysates were electrophoresed on an SDS-PAGE gel. Proteins were visualized using rabbit anti-HA primary antibody and a donkey anti-rabbit IgG HRP-conjugated secondary antibody. The first lane contains an extract from V. fischeri ΔsypA expressing SypA-HA (3 µl loaded). The second lane contains an extract from V. fischeri ΔsypA expressing untagged SypA (3 µl loaded). The third lane contains an extract from V. fischeri ΔsypA expressing V. parahaemolyticus SypA<sub>vp</sub>-HA (6 µl loaded).
Figure 8. Biofilm induction by RscS does not promote biofilm formation of strains expressing \textit{rbdA} or \textit{sypA}_{VP}. A wrinkled colony assay was performed with strains deficient for \textit{sypA} and \textit{sypE} and expressing \textit{rbdA} or \textit{sypA}_{VP} from the chromosome. Gene expression was driven by the \textit{lac} and \textit{sypA} promoters. Biofilm induction was achieved by over-expressing the gene for the sensor kinase, \textit{rscS}. Pictures were taken at 41, 51, and 66 hours.
Figure 9. Co-expression of *rbdA* or *sypA*VP with *sypA* does not impact biofilm formation. A wrinkled colony assay was performed with strains deficient for *sypE* and expressing *rbdA* and *sypA* or *sypA*VP and *sypA*. Gene expression of *rbdA* and *sypA*VP was driven by the *lac* and *sypA* promoters. Biofilm induction was achieved by over-expressing the gene for the response regulator, *sypG*. Pictures were taken at the indicated time points.
Summary

The goal of this study was to elucidate the function of *V. vulnificus* RbdA and *V. parahaemolyticus* SypA<sub>VP</sub>. I hypothesized that because of the substantial sequence similarity, these proteins, RbdA and SypA<sub>VP</sub>, and SypA are orthologs. If the ability to promote biofilm formation is conserved in *rbdA* from *V. vulnificus*, and *sypA<sub>VP</sub>* from *V. parahaemolyticus*, then *V. fischeri* biofilm-deficient *sypA* mutant strains will form biofilms (wrinkled colonies) when the non-native genes are expressed. I determined that *rbdA* and *sypA<sub>VP</sub>* have the ability to promote biofilm formation in *V. fischeri* as both genes were able to functionally complement a *sypA*-deficient *V. fischeri* strain for biofilm formation. These results suggest that RbdA and SypA<sub>VP</sub> may play a similar role in promoting biofilm formation in their own species. Complementation appears to depend on the presence of a minimal level of protein as expression of genes driven from the lac promoter alone was not enough to produce wrinkled colonies. Strains only produced wrinkled colonies when gene expression was driven from the lac and *syp* promoters. Wrinkled colonies were only observed when strains were induced to form a biofilm by overexpression of *sypG*. Cells overexpressing *rscS* did not produce the same wrinkled colonies. These experiments indicate there may be differences between SypA proteins, and provide insights into SypA function. Overall, these results validate this approach as one that can provide insights into the function of homologous genes from related species.
Ability of the SypA-like proteins from *Vibrio* pathogens to serve as a substrate for phosphorylation by *V. fischeri* SypE

**Introduction**

It has previously been shown that SypA is regulated by phosphorylation at a specific serine residue by another protein encoded within the *syp* locus, SypE [32]. When active, SypE phosphorylates SypA, inactivating it and inhibiting biofilm formation. When SypE is inactive, SypA is unphosphorylated and can promote biofilm formation. Like SypA, RbdA and SypA<sub>VP</sub> each contain a serine residue, predicted to be the site of phosphorylation, that is highly conserved in STAS domain proteins. Not only is the serine conserved, but many surrounding amino acid residues are conserved between the three proteins. Thus, it is possible that RbdA and SypA<sub>VP</sub> are also phosphorylated. Furthermore, it is plausible that SypE can recognize and phosphorylate RbdA and SypA<sub>VP</sub> due to the high conservation not only of the serine but also the surrounding amino acids. However, neither *V. vulnificus* nor *V. parahaemolyticus* have a SypE homolog, suggesting that the ability to be phosphorylated may not be conserved. Alternatively, it is possible that another serine kinase, distinct from SypE, could phosphorylate these proteins. Given the serine conservation as well as the sequence conservation between SypA, RbdA, and SypA<sub>VP</sub>, I hypothesize that the SypA-like proteins can be phosphorylated by SypE; if so, they may also be regulated by phosphorylation in their native organism. The studies in this section are designed to test this hypothesis.
**Ability of *V. vulnificus* RbdA and *V. parahaemolyticus* SypA<sub>VP</sub> to be controlled by SypE**

My previous biofilm studies made use of strains in which the biofilm inhibitor SypE, which phosphorylates and thus inactivates SypA, was missing or inactivated. Here, I assessed the ability of SypE to control the activity of RbdA and SypA<sub>VP</sub> using a genetic approach. I assessed biofilm formation in a *sypA*-deficient strain expressing not only *rbdA* or *sypA<sub>VP</sub>* but also *sypE*. If SypE can recognize and phosphorylate these SypA homologs, smooth colonies will be produced. Alternatively, if the SypA homologs cannot be controlled by SypE, wrinkled colonies will be produced even in the presence of the biofilm inhibitor.

As previously observed, strains expressing *V. fischeri* *sypA* and its negative regulator *sypE* produce smooth colonies when induced to form a biofilm (Figure 10). In contrast, a control strain expressing *sypE* and a mutant *V. fischeri* *sypA* allele that cannot be phosphorylated produced wrinkled colonies when induced to form a biofilm. When either *rbdA* or *sypA<sub>VP</sub>* was co-expressed with *sypE* in a strain deficient for *sypA*, smooth colonies were produced (Figure 10). At later time points, strains co-expressing *rbdA* and *sypE* exhibited a partial wrinkling phenotype. These data suggest RbdA is inactivated by SypE but inactivation may be inefficient or overcome with time.

Data suggesting that RbdA is controlled by SypE led to the hypothesis that RbdA could serve as the preferred target for SypE’s inhibitory activity, thus sequestering SypE and freeing SypA to promote biofilm formation. To test if RbdA or SypA<sub>VP</sub> could be preferentially inhibited by SypE, allowing SypA to promote biofilm formation, I
expressed *sypA*, and *sypE*, along with either *rbdA* or *sypA*<sub>VP</sub>, in *V. fischeri* and induced biofilm formation using SypG (Figure 11). Strains expressing *rbdA* or *sypA*<sub>VP</sub> with *sypA* and *sypE* did not wrinkle, suggesting that the non-native proteins do not interact more strongly with SypE than SypA and that both proteins are likely inactivated by phosphorylation.
**Figure 10. Function of RbdA and SypAVP is controlled by SypE.** A wrinkled colony assay was performed with strains deficient for sypA and expressing sypE and rbdA or sypAVP. Gene expression of rbdA and sypAVP was driven by the lac and sypA promoters. Biofilm induction was achieved by over-expressing the gene for the response regulator, sypG. Pictures were taken at the indicated time points.
Figure 11. Co-expression of \textit{rbdA} or \textit{sypA}_{VP} with \textit{sypA} in the presence of \textit{sypE} does not promote biofilm formation. A wrinkled colony assay was performed with strains expressing \textit{sypE} and \textit{rbdA} and \textit{sypA} or \textit{sypA}_{VP} and \textit{sypA}. Gene expression of \textit{rbdA} and \textit{sypA}_{VP} was driven by the \textit{lac} and \textit{sypA} promoters. Biofilm induction was achieved by over-expressing the gene for the response regulator, \textit{sypG}. Pictures were taken at 25, 50, and 67 hours.
Genetic studies suggested that SypE controls RbdA. To test if this control is due to phosphorylation of RbdA, as is the case with *V. fischeri* SypA, I tried to visualize the RbdA by phos-tag gels and Western blotting. The phos-tag reagent retards the migration of phosphorylated proteins by preferentially binding to phosphorylated proteins, thus separating phosphorylated proteins from unphosphorylated proteins, resulting in a band shift observable following gel electrophoresis and western blotting. However, I have been unable to visualize bands correlating to RbdA and SypA in the presence of a vector or SypE in *E. coli* when visualizing protein by phos-tag. Furthermore, when I have observed the proteins, no shift was evident, even with my native SypA-HA control. SypA has previously been shown to be phosphorylated via phos-tag when SypA was GST-tagged [32]. If a shift can be visualized between the GST-tagged SypA in the presence of vector vs. SypE but not with untagged SypA, then it is likely that SypA, and therefore SypA<sub>VP</sub> and RbdA, will need to be GST-tagged to observe the phosphorylated state. If I obtain this result, future directions beyond the scope of my thesis would be to generate larger fusion protein with RbdA and SypA<sub>VP</sub> and then assess their phosphorylation state.

Given the sequence similarities—*V. parahaemolyticus* SypA<sub>VP</sub> also has a conserved serine that could be phosphorylated—I hypothesized that this protein may also be recognized and phosphorylated by SypE. Either phosphorylation or physical interaction between SypE and SypA<sub>VP</sub> could account for the ability of SypE to inhibit SypA<sub>VP</sub>-induced biofilm formation. To test if SypA<sub>VP</sub> is inhibited by phosphorylation by SypE, I visualized SypA<sub>VP</sub> by phos-tag. Preliminary data has shown the SypA<sub>VP</sub> may be
phosphorylated. It is difficult to tell, however, since the positive control for band shift, phosphorylated SypA, was not be visualized by Western blot.

**Summary**

Given their sequence similarity and their ability to complement a sypA mutant, I hypothesized that RbdA and SypAvp are true orthologs of SypA. If so, then they should be susceptible to control by SypE. I hypothesized that when sypE is expressed in strains complemented with either rbdA or sypAvp, the strains will no longer produce wrinkled colonies. I found this to be true; strains expressing either rbdA or sypAvp failed to form a biofilm in the presence of SypE. This result is the same as when sypE is expressed with the native sypA, because SypA becomes phosphorylated and thus inactivated. These results suggest that SypE is able to control the functions of RbdA or SypAvp potentially by phosphorylation. It is interesting that *V. vulnificus* and *V. parahaemolyticus* do not have SypE homologs, but are still sensitive to SypE. It is formally possible that SypE does not phosphorylate RbdA or SypAvp, but rather binds to these proteins, physically preventing them from interacting with other proteins to promote biofilm formation. Future work is necessary to distinguish amongst these possibilities.
Identify specific residues important for biofilm formation and for recognition and phosphorylation by SypE

Introduction

Both *rbdA* and *sypA* were able to promote biofilm formation in a strain in which the native *sypA* was absent, suggesting that amino acids important for interaction with SypA’s partner in promoting biofilm formation are conserved in all three species. In addition, RbdA and SypAVP also appeared to be susceptible to regulatory control by SypE. Thus, amino acids important for (1) biofilm formation and (2) interaction with SypE must be conserved in all three species. Mutating amino acids in SypA important for interaction with a putative downstream partner would disrupt biofilm formation, while mutations that impact its interaction with SypE could result in a protein that is blind to SypE’s inhibitory effects. Mutagenesis of SypA could result in null mutants. These mutants could provide insight into amino acids that are required for the stability of the protein.

Identification and mutation of conserved amino acids

To determine the similarity between the proteins, I first compared the amino acid sequence of *V. vulnificus* RbdA, *V. parahaemolyticus* SypA VP, and *V. fischeri* SypA and identified amino acids conserved in all three proteins (Figure 12) [44]. I then compared the conserved amino acids in the SypA-like proteins to other STAS domain proteins and chose to mutate amino acids that were not conserved in other STAS domain proteins (Data not shown). The amino acids that are conserved in *Vibrio* species, but not in other unrelated STAS domain proteins, could be critical for RbdA’s function in promoting
biofilm formation and/or in interacting with SypE. I generated point mutations in codons for conserved amino acids that I hypothesized to be important for biofilm formation in an allele of sypA and introduced copies of the genes into the Tn7 site in the V. fischeri chromosome to assess biofilm formation and interaction with SypE.
Figure 12. SypA point mutations. In red are mutations that I have made. In blue are mutations previously made in the lab. Above the amino acid sequence is the secondary structure of the SypA protein as predicted by threading the protein onto another known STAS domain protein [44].
Investigation into the roles of conserved amino acids in promoting biofilm formation

I generated eight *sypA* alleles with a single point mutation. I chose to mutate residues to alanine as alanine is non-bulky and least affects protein secondary structure. The mutations were confirmed by sequence analysis using ACGT, inc (Wheeling, IL). I then performed a sensitive time course assay and found six amino acids that are important for promoting biofilm formation. Interestingly, some amino acids appeared to be more important than others as there were a range of defects (Figure 13). Some mutants were delayed for biofilm formation while others failed to form a biofilm altogether. Strains that expressed SypA with either of two amino acid mutations, E2A and Y64A, were delayed and defective for biofilm formation. Even after days of growth, these mutants never formed wrinkled colonies similar to the positive control. Yet another strain, expressing SypA-D34A, was completely defective for biofilm formation, never producing wrinkled colonies. Strains expressing SypA-R74A or SypA-G83A, had short delays in biofilm formation, but produced normal wrinkled colonies at later time points. The SypA-E71A-expressing strain produced wrinkled colonies with a normal timing and pattern, but the colonies attached poorly to the plate, indicating some defect in biofilm development. Finally, strains expressing either SypA-G25A or SypA-P99A did not exhibit any defect, suggesting that those amino acids are not required for biofilm formation under these conditions.

In addition to the eight mutations I made, four other mutations have been made in SypA by a previous member of the lab. Three of the four mutations resulted in strains that no longer produced wrinkled colonies. When assessed in conjunction with the
mutants I have generated, we can determine what part(s) of the protein is important for function in forming the biofilm. Using Cn3D [49], an application used to view the 3-dimensional structures of proteins, I visualized the location of the mutations in SypA that resulted in a biofilm-defective phenotype. Many of these mutations are located on the same face of two alpha helices. This face of the protein may interact with another protein to promote biofilm formation. I hypothesize that, when these amino acids are changed, SypA can no longer interact with its putative partner.
Figure 13. SypA point mutants have a range of effects on biofilm formation. A wrinkled colony assay was performed with strains deficient for sypA and sypE and expressing the mutant sypA. Biofilm induction was achieved by over-expressing the gene for the response regulator, sypG. Pictures were taken at the indicated time.
Investigation into the roles of conserved amino acids in recognition and phosphorylation by SypE

The activity of SypA is negatively controlled via phosphorylation by SypE. To date, Serine 56 is the only residue known to be critical for the ability of SypE to recognize and control SypA’s activity. I predicted that other residues are also important for the interaction between SypA and SypE. I anticipated that mutations that change amino acids in SypA important for recognition by SypE will result in a SypA protein no longer recognized and/or phosphorylated by SypE. When introduced into a strain that expresses SypE, a mutant SypA that fails to interact with SypE will overcome its inhibition activity, permitting biofilm formation under conditions in which it typically does not occur.

I expect SypA mutants with changes in amino acids that are important for recognition and phosphorylation by SypE will promote the formation of wrinkled colonies even when the genetic background results in the production of inhibitory SypE. However, thus far strains expressing SypA with any of the eight mutations I have generated have produced smooth colonies in the presence of SypE, suggesting these mutant SypA proteins are still susceptible to control by SypE (Figure 14).
Figure 14. SypA point mutations are susceptible to control by SypE. A wrinkled colony assay was performed with strains deficient for sypA and expressing the mutant sypA as well as sypE. Biofilm induction was achieved by over-expressing the gene for the response regulator, sypG. Pictures were taken at 24 and 48 hours.
Summary

I hypothesized that amino acids important for biofilm formation must be conserved in the three homologous STAS domain proteins, RbdA, SypAvP, and SypA as all three proteins are able to promote biofilm formation. I identified conserved residues and generated mutations in which these residues were changed to alanine. When assessed for biofilm formation in a *V. fischeri ΔsypA* strain, some mutations result in delayed and defective wrinkled colonies. Other mutations only resulted in delayed wrinkled colony formation. Changes in SypA residues important for interaction with other proteins may prevent interactions from occurring thereby by inhibiting biofilm formation. The amino acids mutations I have made thus far do not have any effects on the ability of SypE to recognize and phosphorylate SypA. These mutants can be used in further studies to elucidate the function of SypA and identify other proteins SypA may interact with to promote biofilm formation.
CHAPTER FOUR

DISCUSSION

In this thesis, I investigated the function of the SypA-like proteins encoded by the pathogenic Vibrio species V. vulnificus and V. parahaemolyticus using the non-pathogenic relative, V. fischeri. The V. fischeri model of biofilm formation utilizes a readily visualized phenotype, wrinkled colonies, to determine the contribution of specific genes to biofilm formation. When genes important for biofilm formation such as sypA are deleted, strains produce smooth colonies. Genes, such as rbdA or sypAVP, can easily be moved into the chromosome to assess their ability to promote biofilm formation. This work has revealed that RbdA and SypAVP function similarly to V. fischeri SypA in promoting biofilm formation, producing wrinkled colonies. The ability to promote biofilm formation and produce wrinkled colonies must be conserved in the proteins of all three organisms. In the course of this work, I also obtained unexpected findings that have permitted a greater understanding of the role of the STAS domain protein, SypA, in biofilm formation.

**Single promoter vs. dual promoter expression of SypA**

My work revealed that expression of V. fischeri sypA (and, similarly, of rbdA or sypAVP) from the lac promoter alone was insufficient to promote biofilm formation. This was somewhat surprising, as the lac promoter (alone) has been sufficient to promote gene
expression of other genes in previous complementation studies, resulting in biofilm formation [50]. In contrast, when sypA was expressed from the lac promoter as well as the sypA promoter, strains produced wrinkled colonies; whether both promoters are required for optimal expression of the non-native syp genes, or merely the native V. fischeri sypA promoter is yet unknown.

There are several possibilities that may account for the results I observed. sypA expression from the lac promoter alone may not complement a V. fischeri sypA deletion strain because there may not be sufficient levels of sypA transcript present needed to produce high levels of protein. In support of this possibility, I was unable to detect SypA protein from V. fischeri expressing native sypA from the lac promoter alone via Western blot. However, these data don’t exclude alternative possibilities. For example, the lac promoter may produce sufficient levels of sypA messenger RNA, but the sypA messenger RNA transcripts may be unstable and degraded before the transcript can be translated into SypA protein. Such instability could be due to the presence, in the lac-driven sypA transcript, of a sequence that signals degradation of the transcript or that forms a secondary structure, such as a stem loop, that prevents translation. Another possibility is that there’s temporal control over the lac promoter: it is presumed to be constitutively active, producing sypA transcripts and thus SypA protein during log and lag phase, but perhaps it doesn’t function well during stationary phase, the phase during which biofilm formation occurs. It remains to be determined which of these various possibilities accounts for the failure of the lac promoter-driven rbdA and sypAVP to complement the sypA mutant.
Aberrant migration of *V. fischeri* SypA

I found that the migration of the SypA-HA in a SDS gel differed from that of SypA\textsubscript{VP}-HA, with SypA\textsubscript{VP} migrating to the position expected for both proteins. For SDS-PAGE and Western blot preparation, SypA samples are lysed in 2X sample buffer containing sodium dodecyl sulfate (SDS) and β-mercaptoethanol and boiled, after which samples are run on SDS gels. SDS denatures proteins by disrupting non-covalent bonds causing proteins to lose their native conformation. β-mercaptoethanol is a reducing agent that has the ability to disrupt the structure of proteins by breaking disulfide bonds between proteins. Thus, the altered migration of SypA may not be due to a non-covalent interaction, or else not due to a protein-protein interaction.

Based upon the observation that SypA protein migrates to a higher molecular weight than predicted, I speculate that SypA may be forming a covalently bonded complex with itself, or potentially be modified by another molecule such as a lipid or polysaccharide. Various treatments can be applied to the cells, prior to evaluating the migration of SypA, to determine the nature/stability of a putative interaction or modification. This could provide insight into what SypA is interacting with and how it is functioning to promote biofilm formation. This interaction may not be important for biofilm formation, however, as SypA\textsubscript{VP} migrates as predicted for its molecular weight yet still promotes biofilm formation. Thus, this analysis could identify a previously unknown function of SypA.

**SypA interaction with other molecules**
Previous studies have determined that SypA is unlikely to act as an anti-sigma factor antagonist (Sheila McEwen and Karen Visick, personal communication), and thus how SypA functions remains unclear. Because there appears to be a minimum amount of SypA required to promote biofilm formation, I speculate that SypA is a structural protein, rather than a regulator. Potentially, SypA could interact with other proteins within a stressosome complex, like RsbR and RsbS [35]. Identifying the other protein(s) with which SypA interacts is an important step in understanding SypA function. I anticipate that, if SypA functions within a stressosome, or as part of another complex, the same will be true for RbdA and SypaVP since these non-native proteins can promote biofilm formation in *V. fischeri*.

One method that can be used to elucidate SypA’s binding partner is a Far-Western [51]. In this method, SypA would be used as a non-antibody probe to identify protein-protein interactions in a cell lysate. *V. fischeri* cell lysate is electrophoresed on a native gel. Purified, epitope-labelled SypA that can be detected using an antibody is used to probe and detect a target on the membrane. The resulting band on the gel observed after probing corresponds to the protein interacting with SypA. Alternatively, specific candidate partners can be tested more directly using co-immunoprecipitation. When *sypA* is not active, there is no polysaccharide production (Visick, personal communication), suggesting SypA interacts with other proteins within the Syp pathway. Thus, co-immunoprecipitation assays with epitope-tagged Syp proteins can be performed to determine direct interactions between SypA and other Syp proteins.
In addition to interacting with a partner to facilitate biofilm formation, it is possible that SypA may also bind GTP and/or may have GTPase activity. STAS domain proteins from other bacterial species have been shown to bind and hydrolyze GTP in addition to performing other functions. For example, the STAS domain protein Rv1739c from *M. tuberculosis* has GTP binding and hydrolyzing capabilities [36]. The STAS domain protein SpoIIAA of *B. subtilis* also has reported GTP binding and hydrolyzing activity, although the consequence of this activity has yet to be determined [52]. Comparisons can be made between SypA and STAS domain proteins known to bind GTP to determine if amino acids known to be shifted upon GTP binding are conserved in SypA. If the ability to bind GTP is a common function of STAS proteins, then SypA might behave similarly. If SypA binds GTP, then one of the mutants that I generated may be defective for this activity. Future work can determine if SypA binds GTP and, if so, whether that activity is important for biofilm formation and/or interactions with SypE.

Because of the conserved ability of RbdA and SypA<sub>VP</sub> to promote biofilm formation, it is plausible that any additional function of *V. fischeri* SypA is also conserved. If *V. fischeri* has GTP binding or GTPase activity, then it is possible that these functions are conserved in the proteins of other species and that RbdA and SypA<sub>VP</sub> could be have GTP binding or GTPase activity. Further studies could determine the impact(s) of these additional functions have on biofilm formation and the pathogenesis of either *V. vulnificus* and *V. parahaemolyticus*.

**SypA point mutants**
I have identified conserved amino acids that are important for the ability of SypA to promote biofilm formation. I generated point mutations in codons for conserved amino acids that I hypothesized to be important for biofilm formation, and found that a subset of these mutations did, in fact, disrupt biofilm formation in complementation experiments.

In complementation experiments, SypA point mutations had a range of effects on biofilm formation. While some mutations resulted in proteins with diminished function, others could no longer promote biofilm formation. These loss of function alleles may not promote biofilm formation for several reasons. One reason for the lack of biofilm formation may be that the mutant SypA protein may not be produced. The SypA mutants I generated are epitope tagged, and thus Western blotting can be performed to determine if the protein is made.

One approach that may provide some insight into whether SypA is folded is evaluating the ability of SypE to phosphorylate SypA; if the mutant SypA is able to be phosphorylated by SypE, it would suggest the protein is adequately folded to permit SypE to recognize and phosphorylate SypA. If the mutant SypA cannot be phosphorylated, it may be due to improper SypA folding.

The positions of amino acids that are required for biofilm formation can be analyzed using Cn3D [49], an application used to view the 3-dimensional structures of proteins. When I visualized the location of the mutations in SypA that resulted in a biofilm-defective phenotype, I found that three mutations, tyrosine 64, lysine 67, and arginine 68, are located on the same face of one alpha helix. The amino acids on this face of the protein may interact with another protein to promote biofilm formation. I
hypothesize that, when these amino acids are changed, SypA can no longer interact with its putative partner. Four additional amino acids are located on the same face of the alpha helix as these required amino acids including glycine 60, leucine 65, leucine 69, and isoleucine 70. These residues may provide points of contact between SypA and another protein, and this interaction may promote biofilm formation. Substitution of these residues with alanine may prevent contact between SypA and its partner, this inhibiting biofilm formation.

In addition to loss of function mutants, some mutations may result in proteins with enhanced function. Strains expressing alleles that have enhanced function might form biofilms at earlier time points than wild-type strains. Alternatively, point mutant strains may form an enhanced biofilm due to increased adherence of the cells. This phenotype has been observed previously in the lab (Ondrey and Visick, personal communication): cells of control strain adhere to each other during biofilm formation, but cells of strains that form enhanced biofilms adhere not only to each other but to the agar plate as well. I observed that some of my strains that expressed a mutant allele of sypA adhered to the plate, suggesting that changes in some amino acids may result in a SypA protein with an enhanced ability to promote biofilm formation. However, whether this altered adherence actually represents an enhanced or a defective biofilm has yet to be determined.

Some mutants were more defective for biofilm formation than others. These SypA mutants that produce smooth colonies when induced to form a biofilm can be used in further studies to find suppressor mutants. These strains can be mutagenized by UV
radiation, and mutagenized strains that are able to restore wrinkling contain either revertant or suppressor mutations. The genome of these mutant strains that are able to restore biofilm formation can be analyzed to determine the location of the mutation. If the mutant is a suppressor mutant, it could give insight into what protein(s) SypA is interacting with and how it is functioning.

**Function and control of RbdA and SypAvp**

In complementation experiments, *rbdA* and *sypAvp* were able to promote biofilm formation. The ability to produce wrinkled colonies in *V. fischeri* suggests that both RbdA and SypAvp are performing similar roles in promoting biofilm formation as *V. fischeri* SypA. Thus, the function of this STAS domain protein has been conserved among the species. RbdA and SypAvp may be functioning in a similar way to promote biofilm formation in their own species. Furthermore, the *V. fischeri* model of biofilm formation can be used to assess the function of genes from related species.

While *rbdA* and *sypAvp* were able to promote wrinkled colony formation in strains induced by *sypG*, they did not do so in strains induced by *rscS*. The difference observed under the two conditions may be due to the amount of transcript produced, as it is known that *sypG* is a stronger inducer of biofilm formation. Alternatively, the difference may be due to the position of the ribosomal binding site in the non-native *sypA* genes. The plasmid constructs containing the non-native genes contain the ribosomal binding sites for *V. vulnificus* and *V. parahaemolyticus*, which may not be optimal for *V. fischeri*. Overexpression of *sypG* might induce enough transcription to overcome the effects of the non-optimal ribosomal binding site, but overexpression of *rscS*, which does not induce
*syp* transcription to the same extent as does *sypG*, may not be able to overcome this potential decreased translation. Biofilms induced by *rscS* may not produce enough *rbdA* or *sypAVP* to meet the minimum amount of protein required to promote biofilm formation. Experiments are on-going to determine if this trivial explanation can account for the dramatic differences in complementation depending whether *sypG* or *rscS* is overexpressed. If it doesn’t, then I would conclude that the differences in complementation are due to differences in functionality of the protein. The non-native SypA produced may not function as well as the native SypA due to differences between the proteins. The abundance of the sub-optimally functioning protein, which occurs during *sypG* induction of biofilms, may be able to overcome deficits in biofilm formation.

My work also showed that the ability of RbdA and SypAVP to be controlled by SypE is conserved, although *V. vulnificus* and *V. parahaemolyticus* have lost the gene for the SypE homolog. However, my current results do not demonstrate that SypE retains the ability to phosphorylate these non-native proteins. An alternative hypothesis is that SypE retains the ability to bind and thus sequester the SypA homologs, preventing their biofilm-promoting activity. If SypE can phosphorylate these proteins, and phosphorylation is indeed key to controlling their activity (as assessed with mutants with changes at the conserved site of phosphorylation), then it is possible that, in *V. vulnificus* and *V. parahaemolyticus*, another kinase may perform the function of SypE.

**Significance**
My work demonstrates that the function of the SypA STAS domain protein is conserved among three Vibrio species, V. vulnificus, V. parahaemolyticus, and V. fischeri. While the precise function(s) of these STAS domain proteins remains unclear, they all can function in promoting biofilm formation by V. fischeri. It is important to understand the function of SypA as it plays a crucial role in biofilm formation. Because rbdA and sypA VP can complement a V. fischeri mutant, it is likely that RbdA and SypA VP are similarly required for biofilm formation in their own species, pathogenic bacteria whose ability to cause disease may depend on biofilm formation.

Unlike the best-characterized bacterial STAS domain proteins (e.g., SpoIIAA and RsbV [35]), SypA does not appear to function as an anti-sigma factor antagonist. Further work elucidating the function of SypA will contribute to our understanding of the role of STAS domain proteins in signaling and in other cell processes. My work has advanced our understanding of SypA as we now know that SypA must be present at sufficient levels to promote biofilm formation, suggesting it may have a structural rather than regulatory role. Additionally, my evidence suggests that SypA may have unusual properties and/or may be modified, as it runs aberrantly in an SDS gel.

Previous studies have shown that the rbd locus in V. vulnificus contributes to aggregation [4], however, individual genes of the locus have not been studied. This locus is conserved in V. parahaemolyticus, but no studies of its function have been reported. Determining the contribution of individual genes in either locus to biofilm formation is an important step in understanding how V. vulnificus and V. parahaemolyticus biofilms form. We can use the V. fischeri model of biofilm formation to determine the function of
genes from *V. vulnificus* and *V. parahaemolyticus* by complementing a mutant *V. fischeri* strain. My work has shown that rbdA and sypA VP are able to complement a *V. fischeri* mutant indicating a conservation in function between these proteins. It is likely that RbdA and SypA VP are required for biofilm formation in their own species as well.

As potential regulators of biofilm formation, RbdA and SypA VP and the complexes they may form could serve as a potential target for an inhibitor of biofilm formation. An inhibitor of *V. vulnificus* or *V. parahaemolyticus* biofilms could serve many purposes. While there exist many treatments options for removing bacteria from the oyster population, few treatments effectively remove all the bacteria. Treatments that are effective are costly. When treatments are not effective and bacteria persist in the oyster population, increased human transmission and infection due to bacterial contamination can occur. Outbreaks can lead to recall of oysters which can be financially devastating to the oyster industry. An inhibitor of biofilm formation could be used as a treatment to effectively remove bacteria from oyster populations harvested for human consumption. This inhibitor could target a potential regulator of biofilm formation such as RbdA in *V. vulnificus* or SypA VP in *V. parahaemolyticus*. Inhibitors of *V. vulnificus* or *V. parahaemolyticus* biofilms could also be used to treat humans infected with either organism. Drugs that prevent the formation of a *V. vulnificus* biofilm in a susceptible individual could drastically improve patient outcome.
References


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VITA

Cecilia Margaret Thompson was born in Springfield, Illinois, to Patricia Sorrill-Thompson and John Thompson. She earned Bachelor of Science degrees in Biology and in Clinical Laboratory Science from Loyola University Chicago in May 2012. In July 2012, Cecilia became certified by the American Society for Clinical Pathology as a medical laboratory scientist.

In August, 2012, Cecilia entered the Master of Science program for Infectious Disease and Immunology at Loyola University Chicago. She joined the laboratory of Dr. Karen Visick in 2012, where her research focused on investigating the role of STAS domain proteins from different Vibrio species in biofilm formation.