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Investigating the Regulatory Mechanisms by Which Arabinose and Sypa Control Biofilm Formation in *Vibrio fischeri*

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LOYOLA UNIVERSITY CHICAGO

INVESTIGATING THE REGULATORY MECHANISMS
BY WHICH ARABINOSE AND SYPA CONTROL BIOFILM
FORMATION IN *VIBRIO FISCHERI*.

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

PROGRAM IN
MICROBIOLOGY AND IMMUNOLOGY

BY
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CHICAGO, IL

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ABSTRACT

Biofilms are surface-associated microbial communities surrounded by an extracellular matrix. Through biofilm formation, many pathogens and symbionts are able to colonize, survive, and persist in their host. A model system used to study biofilm formation is the symbiotic bacterium *Vibrio fischeri*, which colonizes its host, the squid *Euprymna scolopes*. Complex signaling between the squid and the bacteria is essential for the proper regulation of biofilm formation as well as for persistent colonization.

The signal(s) that promote host-relevant biofilm formation are as-yet unknown, but recently it was discovered that the sugar, L-arabinose, serves as a unique signal to promote biofilm formation in *V. fischeri*. Wild type *V. fischeri* cells grown in the presence of L-arabinose form a pellicle at the air-liquid interface of static cultures that does not occur in the absence of this sugar. However, although arabinose can induce biofilm formation *in vitro*, it inhibited host colonization *in vivo*. For my thesis, I sought to identify genes required for the cellular response to arabinose. I performed a random transposon mutagenesis, screened for mutants that were unable to form the biofilm in response to arabinose, and identified the transposon location within the genome. Through this approach, I was able to identify various genes that are involved in the production of the arabinose-induced biofilm.

In addition to the arabinose-induced biofilm, *V. fischeri* produces a second distinct biofilm that is critical for host colonization. The formation of this biofilm

requires the 18-gene symbiosis polysaccharide (*syp*) locus. Previous results have indicated that one of the regulatory proteins, SypA, is critical for biofilm formation. Deletion of *sypA* prevents biofilm formation *in vitro* and colonization *in vivo*. Current evidence suggests that SypA controls biofilm formation at some unknown level downstream of *syp* transcription. However, the mechanism by which SypA contributes to biofilm formation and host colonization remains unknown. For my thesis, I investigated mechanisms by which SypA may be contributing to the *syp*-dependent biofilm formation. I attempted to identify downstream protein targets of SypA that are involved in the *syp*-dependent biofilm formation through a random transposon mutagenesis screen. In a second approach, I used sigma factor over-expression assays to determine if SypA acted as an anti-sigma factor antagonist as predicted by its STAS (sulfate transporter and anti-sigma factor antagonist) domain. To determine whether SypA interacts with membrane bound Syp Proteins, I attempted to establish an inner membrane isolation protocol that could be used for co-Immunoprecipitation assays. Finally, I identified critical amino acid residues in SypA that are required for *syp*-dependent biofilm formation. These various approaches led us to develop various tools that will be used for further analysis of SypA's role in *syp*-dependent biofilm formation.

CHAPTER 1

INTRODUCTION

Overview

Biofilms are surface-associated aggregates of microbial communities surrounded by an extracellular matrix. A model system used to study biofilm formation is the symbiotic bacterium *Vibrio fischeri*, which colonizes its host, the squid *Euprymna scolopes*. I investigated mechanisms by which arabinose and SypA may be contributing to the formation of two distinct biofilms in *V. fischeri*. L-arabinose serves as a unique signal to promote the formation of a brittle biofilm located at the air-liquid interface of liquid *V. fischeri* cultures and SypA is a small protein essential for *syp*-dependent biofilm formation. In this chapter, I will give a brief overview of biofilms, the *Vibrio*-squid symbiosis, and the two biofilms formed by *V. fischeri*.

Biofilms, a Background

Bacteria are a diverse set of microorganisms found in almost every environment on earth. They range in function from essential decomposers and recyclers of nutrients, to harmful pathogens and even beneficial symbionts living peacefully within a host organism. Bacteria are found in various states such as free-living organisms or within a bacterial community. Complex bacterial communities known as biofilms are surface-associated aggregates that allow bacteria to colonize almost every abiotic and biotic

surface, including air-liquid interfaces (Branda *et al.*, 2005). More than 90% of bacteria are found within biofilms, protected by their extracellular matrix composed primarily of polysaccharides, nucleic acids, and proteins (Branda *et al.*, 2005; Flemming & Wingender, 2010; Petrova & Sauer, 2012).

The production of the extracellular matrix provides many advantages to the bacterial community residing within the biofilm. This important matrix, also known as EPS (Extracellular Polymeric Substances), provides adhesive properties allowing the biofilm to attach to a surface and therefore colonize the particular environment and allows the cells within the biofilm to directly interact and communicate (Flemming & Wingender, 2010). The EPS contributes to the mechanical stability of the biofilm and provides a unique 3D structure, which allows for the formation of molecular gradients throughout the biofilm and contributes to the development of micro-niches. The cells within the micro-niches are exposed to different environmental conditions such as oxygen and nutrient availability as well as signaling molecules and environmental stresses, all of which contribute to variation in gene expression and protein production within the biofilm (Vlamakis *et al.*, 2008; Flemming & Wingender, 2010; Vlamakis, 2011).

The formation of biofilms provides many advantages to the cells within the biofilm. Since the bacteria are located in such close proximity, bacteria within biofilms have a greater rate of gene transfer, especially from the DNA released from dying cells within the biofilm (Fux *et al.*, 2005). This close proximity of bacteria within biofilms also increases the rate of bacterial communication and cooperation and thus, the bacteria

can more readily respond to changing environmental conditions (Li & Tian, 2012). Biofilm formation may also help bacterial communities compete for environmental space and resources. Biofilms aid in the protection of the bacterial communities and allow the bacteria to persist within the environment despite external stresses such as desiccation, oxidation, host defense mechanisms, and even the presence of antimicrobials or toxins (Flemming & Wingender, 2010; Vlamakis, 2011; Petrova & Sauer, 2012). This persistence is due to the heterogeneity within biofilm communities. Many cells within biofilms are metabolically inert and therefore unaffected by various environmental stresses (Rani *et al.*, 2007; Vlamakis, 2011). Bacterial aggregation and production of the matrix thus provide a dynamic and heterogeneous population of cells able to adapt to changing environmental conditions, evade their host's immune system, and thus persist despite the presence of these various environmental stresses (Hall-Stoodley & Stoodley, 2009; Joo & Otto, 2012).

The strength and integrity of biofilms is a great advantage to symbiotic bacteria that have a mutualistic relationship with their host, but can be very detrimental to the host organism if the bacteria are pathogenic. Due to their ability to persist, biofilms are associated with many chronic bacterial infections such as chronic otitis media, native valve endocarditis, gastrointestinal ulcers, urinary tract infections, and chronic lung infections (Costerton *et al.*, 1999; Hall-Stoodley & Stoodley, 2009; Joo & Otto, 2012; Petrova & Sauer, 2012). Bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and enteropathogenic *Escherichia coli* cause chronic infections by forming biofilms that allow them to persist in their hosts and acquire antimicrobial resistance

(Costerton *et al.*, 1999; Hall-Stoodley & Stoodley, 2009). These pathogens are a major problem in clinical settings since biofilms can form on catheters and indwelling devices and cause persistent infections within a hospital setting or operation (Costerton *et al.*, 1999; Joo & Otto, 2012). Biofilms are also a major problem in industrial settings where these persistent communities clog equipment and cause biofouling (Vlamakis *et al.*, 2013).

Bacteria form biofilms in response to environmental signals and these signals can be products of the bacteria, the host, or extracellular molecules (Vlamakis, 2011). The biofilm inducing signals are usually species specific and depend on the environment in which the bacteria colonize. Some of the signals include environmental stressors, quorum-sensing, two component signaling mechanisms, or complex host-microbe interactions (Karatan & Watnick, 2009; Li & Tian, 2012). These signaling molecules induce the expression of matrix genes (Vlamakis *et al.*, 2013). Some of the signaling molecules that induce biofilm formation in specific bacteria have been identified. For example, the presence of two stimuli, monosaccharides and indole from the host gastrointestinal tract, induce the expression of *Vibrio* polysaccharide (*vps*) genes, which are required for biofilm formation in *Vibrio cholerae* (Kierek & Watnick, 2003; Mueller *et al.*, 2007). The monosaccharide glucose has various effects on the biofilm formation of different bacterial species. In *S. aureus*, increasing glucose levels promotes biofilm formation (Lim *et al.*, 2004; Sutrina *et al.*, 2007), but in *B. subtilis*, the opposite is true: biofilm formation is increased under low glucose concentrations. Glucose has even been

shown to inhibit biofilm formation in *E. coli* (Jackson *et al.*, 2002; Karatan & Watnick, 2009; Stanley *et al.*, 2003).

There is a need for a greater understanding of biofilm formation in bacteria and insights into ways to treat biofilm-forming pathogens. Teasing out the mechanisms behind biofilm formation may provide better ways to prevent or disrupt persistent biofilms. Therefore, it is important to study biofilms, to know the signals necessary to induce biofilm formation, the signaling pathways required to establish biofilms, as well as the cellular responses responsible for biofilm persistence.

***V. fischeri*, model for studying biofilms**

For my thesis, I investigated the formation of two distinct biofilms found in the marine bacterium *V. fischeri*, the arabinose-induced biofilm and the Syp biofilm. *V. fischeri* is a great model to study biofilm formation due to the requirement for biofilm formation during development of the symbiotic relationship between this marine bacterium and its host the Hawaiian Bob-tailed squid *E. scolopes* (McFall-Ngai, 2014; Stabb & Visick, 2013). Since bacterial behavior varies based on environmental conditions, it is best to study bacteria in their natural surroundings. Simulated lab conditions are often artificial and may fail to mimic the natural environmental factors that contribute to the growth and development of a particular bacterial strain. The *Vibrio*-squid symbiosis is unique in that it allows us to study the bacterium as well as its biofilm formation within its natural host without leaving the laboratory (Stabb & Visick, 2013).

This symbiosis makes a good model system because both *V. fischeri* and its host can be maintained independently of one another without detrimental effects to either

partner (Stabb, 2006). Juvenile *E. scolopes* hatch aposymbiotic, or free from their bacterial symbiont. Since *E. scolopes* acquires its symbiont from the surrounding seawater through horizontal transfer (Boettcher & Ruby, 1990; Wei & Young, 1989) it is possible to control when, where, amount and type of *V. fischeri* that are introduced to the juvenile *E. scolopes*.

V. fischeri is not required for the survival of the host, but it is essential for light organ development and light production (McFall-Ngai & Ruby 1991). These two features are analyzed as a read out for proper host colonization. One can genetically manipulate the bacteria, inoculate the host, and observe the effects of the mutant strain on the host either by monitoring luminescence, calculating symbiont colony forming units (CFU), visualizing bacterial colonization through fluorescence microscopy, or analyzing the light organ for developmental markers (Stabb, 2006). The manipulated genes will then give insight into the bacterial molecules required for host interaction, colonization, and persistence.

Another reason this model is useful in studying biofilm formation is because the symbiotic *Vibrio*-Squid relationship is monospecific (Wei & Young, 1989; McFall-Ngai & Ruby, 1991). *V. fischeri* are the only bacteria to form a biofilm on and colonize the deep crypts of the squid's light organ. It is easier to understand the exact molecular interactions between just two organisms as opposed to a relationship among multiple organisms or interpreting the effects of various organisms competing for colonization.

Finally, this unique relationship between the squid host and bacterial symbiont is also a great model due to the ease with which *V. fischeri* can be genetically manipulated

(Stabb & Visick, 2013). The ease of genetic manipulation is enhanced by the availability of the *V. fischeri* genome sequence (Ruby *et al.*, 2005). In this work, I took advantage of the many tools available to generate *V. fischeri* mutants. Some of these techniques include conjugation, transposon mutagenesis, in-frame gene deletions, over expression plasmids, and even the transformation of DNA directly into *V. fischeri* cells. These tools as well as the sequenced genome allow us to identify the genes within *V. fischeri* that are critical for biofilm formation.

The *Vibrio*-Squid symbiosis

In this relationship, the squid host provides a niche and nutrients to its symbiont and the bioluminescent, Gram-negative *V. fischeri* in turn provides light for its nocturnal host. Once *V. fischeri* colonizes the light organ, *E. scolopes* uses the light produced by its bioluminescent symbiont for nocturnal camouflage in a mechanism known as counter illumination: that is, they are able to use the light produced by *V. fischeri* to mimic the moonlight and emit the light on their ventral surface, thus eliminating their shadow and evading detection by both predators and prey (McFall-Ngai & Montgomery, 1990; Jones & Nishiguchi, 2004).

How are *V. fischeri* able to colonize the light organ of the squid, which is located deep within the mantle body? Colonization of the light organ occurs solely in the deep crypts of the light organ that are only accessible through six small pores, three on either side of the bi-lobed light organ. These six pores each open into ciliated ducts followed by toxic antechambers that eventually lead to the epithelium-lined deep crypts of the light organ (McFall-Ngai & Montgomery, 1990; Montgomery & McFall-Ngai, 1993; Sycuro

et al., 2006). Each morning, the squid vents (expels) out 90-95% of its symbiont, which helps renew the population of *V. fischeri* in the seawater as well as inoculate other juvenile squid (Boettcher *et al.*, 1996; Nyholm & McFall-Ngai, 1998). During the day, the remaining *V. fischeri* cells continue to grow and reproduce. By dusk, they have reached a high cell density and the bacteria are able to produce enough autoinducer, or signaling molecule, to stimulate the production of light for the squid (Boettcher *et al.*, 1996; Miyashiro & Ruby, 2012).

Once *E. scolopes* ventilates seawater containing various kinds of marine bacteria, including its symbiont *V. fischeri*, these bacteria are able to enter the mantle cavity and travel towards the light organ due to the beating motion of the ciliated fields located just outside the light organ. These structures create a current drawing the seawater towards the pores (Nyholm *et al.*, 2000). The presence of peptidoglycan and LPS on the Gram-negative bacteria triggers the production of mucus shed by the epithelial cells lining the ciliated field and pores of the light organ (Nyholm & McFall-Ngai, 2004; Nyholm *et al.*, 2000). *V. fischeri* is able to aggregate and attach to the cilia and mucus just outside the light organ pores and form a biofilm-like aggregate (Altura *et al.*, 2013). This bacterial aggregate forms with astonishing speed, between 2-4 hours after exposure to *V. fischeri* (Nyholm & McFall-Ngai, 2004). The formation of this symbiont-specific biofilm indicates that the host must have an early means of detection, communication, and selection for its symbiont. After the aggregate is formed, individual cells disperse from the biofilm and enter the pores of the light organ. They must actively swim against the current of the mucus- and cilia-lined ducts, enter and survive the toxic environment of the

antechamber, slip through the narrow bottleneck, and finally colonize, proliferate, and persist in the epithelium-lined deep crypts of the light organ (Sycuro *et al.*, 2006). *V. fischeri* use flagellar motility and chemotaxis to leave the biofilm and travel throughout the light organ to finally reside in the deep crypt spaces (Graf *et al.*, 1994; Mandel *et al.*, 2012). Although this seems like a tremendous feat for *V. fischeri* to accomplish, colonization usually occurs as quickly as 12 hours after hatching (Nyholm & McFall-Ngai, 2004).

V. fischeri is able to overcome many challenges in order to colonize and persist in the light organ in *E. scolopes*. One such factor is competition with other bacteria for exclusive colonization of the light organ. Gram-negative bacteria, in general, are able to aggregate and attach to the cilia and mucus just outside the light organ pores (Altura *et al.*, 2013), but *V. fischeri* are able to outcompete the other Gram-negative bacteria for exclusive colonization. Even though less than 0.1% of the bacteria in the squid environment are *Vibrio fischeri*, they soon dominate and become the sole species within the bacterial aggregate (Nyholm *et al.*, 2000; Nyholm & McFall-Ngai, 2003). A second challenge for *V. fischeri* is motility. These bacteria must be able to change their flagellation state from free-living motile organisms, to non-motile bacteria within a biofilm community, followed by dispersed, free-living bacteria and finally to non-flagellated, permanent residents within the deep crypts of the light organ (Graf *et al.*, 1994; Ruby & Asato, 1993). Another challenge is that *V. fischeri* must be able to evade the host immune system, including phagocytosis from macrophage-like hemocytes (Nyholm *et al.*, 2009) and exposure to oxidative stress from molecules that induce

bactericidal effects such as reactive nitrogen species like nitric oxide (NO) and halide peroxidases that contribute to the production of hypohalous acid (Davidson *et al.*, 2004; Rader & Nyholm, 2012; Wang *et al.*, 2010). A final challenge in regulating and sustaining this symbiosis is communication. Individual *V. fischeri* cells must also be able to communicate with each other through quorum sensing, or density-dependent signal transduction that coordinates genes expression, to induce light production for their host (Stabb *et al.*, 2008). *E. scolopes*, in turn, must be able to control *V. fischeri* concentration through daily venting of bacteria. *E. scolopes* also controls light production by regulating the availability of oxygen for the luminescence reaction, and regulating the intensity of light emission using lenses, specialized reflective tissue, and the ink sac (Boettcher *et al.*, 1996; Jones & Nishiguchi, 2004; McFall-Ngai & Montgomery, 1990). These various regulatory steps must require an intricate signaling network between the host and the symbiont (Rader & Nyholm, 2012).

Another unique feature of the *Vibrio*-squid relationship is the ability of *V. fischeri* to signal developmental changes in the squid's light organ. The presence of *V. fischeri* within the light organ is necessary to coordinate these developmental changes (Stabb & Visick, 2013). *V. fischeri* use MAMPs or microbe associated molecular patterns, to signal morphological changes in its host. For example, various components of peptidoglycan and lipopolysaccharide are signals that induce hemocyte trafficking, apoptosis and regression of the ciliated epithelial fields, a decrease in NO production, and the eventual loss of mucus production (Altura *et al.*, 2011; Rader & Nyholm, 2012). Once *E. scolopes* is able to recognize these MAMPs and the presence of *V. fischeri*

within its light organ, then it is able to make specific developmental changes to the light organ that, in turn, help prevent the unnecessary colonization of other bacterial species (McFall-Ngai *et al.*, 2010).

***Vibrio fischeri* Biofilms**

The Arabinose-Induced Biofilm

In the laboratory, *Vibrio fischeri* forms two distinct types of biofilms. One of the biofilms is an arabinose-induced biofilm (Visick *et al.*, 2013), while the second is the *syp*-dependent biofilm (Visick, 2009). These two biofilms are distinct in their *in vitro* phenotype as well as their involvement in host colonization. *V. fischeri* is able to form a pellicle at the air-liquid interface of static liquid cultures when grown in LBS supplemented with 0.2% L-arabinose. The presence of the pellicle is an indication of biofilm formation *in vitro*; however, L-arabinose is not sufficient to induce the formation of wrinkled colonies, another indication of *in vitro* biofilm formation (Visick *et al.*, 2013). The arabinose-induced pellicle has a brittle phenotype and is easily disrupted by a pipette tip (Figure 1). The biofilm phenotype induced by arabinose is very distinct from those controlled by the *syp* (symbiosis polysaccharide) locus, which result in the production of a pellicle that is thick and sticky and the formation of a wrinkled colony on solid plates (Visick, 2009) (Figure 2).

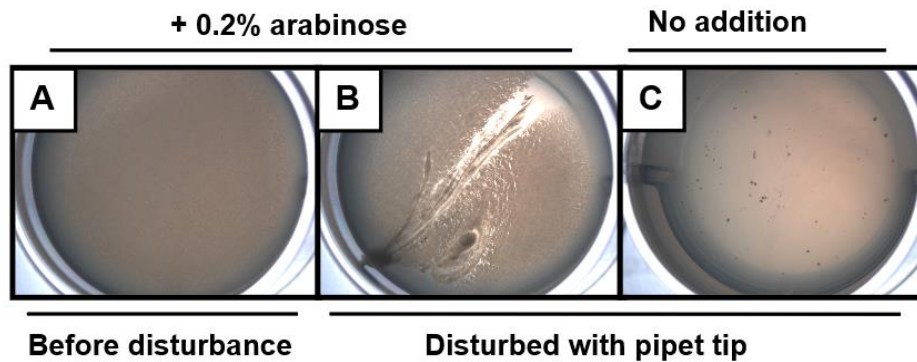


Figure 1: *V. fischeri* pellicle production in the presence of 0.2% arabinose. This figure is from Visick *et al.* (2013) and shows wild type *V. fischeri* grown in the presence (A and B) or absence (C) of arabinose. The pellicle has a brittle phenotype, which is best seen when disrupted with a pipet tip (B).

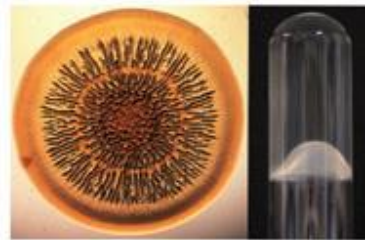


Figure 2: *V. fischeri* *in vitro* *syp*-dependent biofilm formation. This figure was modified from Visick (2009) and shows a rugose or wrinkled colony (A) and pellicle (B) formed by *V. fischeri* upon the overexpression of the sensor kinase, *rscS*.

Vibrio fischeri's response to arabinose is unique to this sugar as even structurally similar sugars failed to induce it. This response is also surprising since *V. fischeri* does not use arabinose as a sole carbon source. However, based on experiments using the acid/base indicator phenol red, *V. fischeri* is able to respond to and metabolize arabinose (Visick *et al.*, 2013): *V. fischeri*, when grown in media containing phenol red and of 0.2%

arabinose, caused a slight, but reproducible color change from red to orange. Therefore, arabinose is inducing a change in the metabolic activity of *V. fischeri*. This interesting relationship between arabinose and *V. fischeri* requires further study. For my thesis I attempted to identify the genes involved in the formation of this arabinose-induced biofilm.

The syp-Dependent Biofilm

In contrast to the arabinose-induced biofilm, which does not promote host colonization, the biofilm required for host colonization is the *syp*-dependent biofilm (Nyholm *et al.*, 2000; Shibata *et al.*, 2012; Yip *et al.*, 2005). Studies have shown that this biofilm is dependent on the symbiosis polysaccharide (*syp*) gene locus, which is regulated by the sensor kinase RscS, or the regulator of symbiotic colonization sensor (Figure 3) (Visick & Skoufos, 2001). The *syp*-locus is an 18-gene locus that encodes both regulatory proteins and structural proteins involved in synthesis and transport of the Syp polysaccharide (Yip *et al.*, 2005).

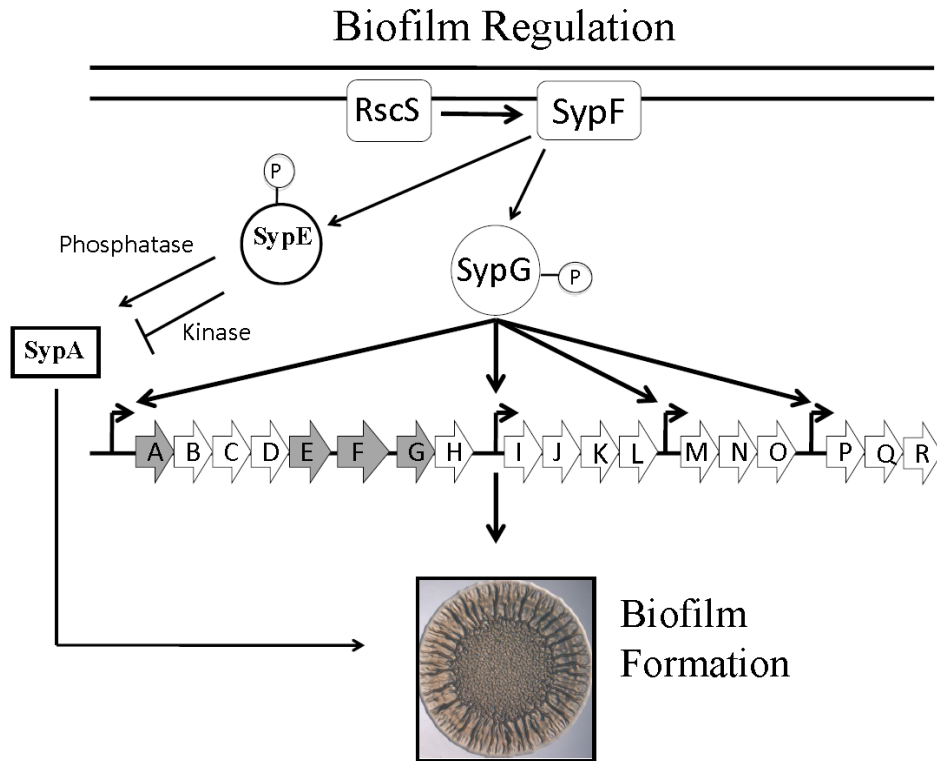


Figure 3: A proposed and simplified model for *syp*-dependent biofilm regulation in *V. fischeri*. This figure was modified from Visick (2009). RscS acts as a Sensor Kinase, initiating a phosphorelay, transferring the phosphate group to a second SK, SypF, which then phosphorylates the response regulators SypG and SypE. Phosphorylated SypG promotes *syp* transcription while SypE acts at a level downstream of *syp* transcription by interacting with and regulating the phosphorylation of SypA. SypA is critical for *syp*-dependent biofilm formation in *V. fischeri*. Phosphorylated SypA fails to promote biofilm formation, while unphosphorylated SypA promotes biofilm formation. The genes for the 4 known regulators of Syp biosynthesis are colored in grey while the genes associated with Syp polysaccharide production and transport are pictured in white.

In vivo, the *syp*-dependent biofilm forms at the aggregation stage of symbiosis, just outside of the light organ (Yip *et al.*, 2006). This biofilm forms during the earliest stage of host-symbiont recognition and the subsequent stages of colonization are dependent on this biofilm formation. *In vitro*, the *syp*-dependent biofilm can result in the formation of strong, thick pellicles at the air-liquid interface of liquid culture or wrinkled

colonies on solid media (Yip *et al.*, 2006). The strong, sticky *in vitro* phenotype (Figure 2) as well as the *in vivo* biofilm-like aggregate is dependent on the *syp* locus that encodes genes (*sypA-sypR*) responsible for the regulation, production, and transport of the Syp polysaccharide (Yip *et al.*, 2006). These genes encode proteins that include four known regulators (SypA, SypE, SypG, and SypF), six glycosyltransferases (SypH-J, SypN, and SypP-Q), two putative export proteins (SypC and SypK), and six proteins thought to be involved in polysaccharide modification (Shibata *et al.*, 2012).

The *syp* locus is regulated by means of a two-component signal transduction pathway. Two-component signal transduction pathways generally contain a sensor kinase that detects an environmental signal, autophosphorylates, and then initiates a phosphorelay, which eventually phosphorylates a downstream response regulator. The phosphorylated response regulator is then able to elicit a cellular response, such as regulating gene transcription, all as a result of the initial environmental stimuli (Wuichet *et al.*, 2010). The two known sensor kinases responsible for the transcription of the *syp* locus and production of the Syp polysaccharide are RscS and SypF (Hussa *et al.*, 2008; Norsworthy & Visick, 2015; Yip *et al.*, 2006). SypK, detects an as-yet unknown environmental signal and initiate a phosphorelay to phosphorylate the second sensor kinase, SypF. SypF then phosphorylates the response regulators SypG and SypE (Norsworthy & Visick, 2015). These response regulators then aid in the production of the Syp-polysaccharide either at the level of transcription (in the case of SypG) or downstream of *syp* transcription (for SypE). Phosphorylated SypG acts as a σ^{54} -dependent transcription factor and promotes the transcription of the *syp*-locus (Ray *et al.*,

2013). The response regulator SypE plays a dual role in biofilm formation, both promoting and inhibiting *syp*-dependent biofilms depending on its phosphorylation state. Phosphorylated SypE acts as a phosphatase to remove the phosphoryl group and thus activate the SypA protein, which promotes biofilm formation. Unphosphorylated SypE acts as a kinase to add a phosphoryl group to SypA, which inhibits biofilm formation (Morris & Visick 2013a; Morris & Visick 2013b). Unphosphorylated SypA is critical for biofilm formation, but the direct role it plays in formation of the *syp*-biofilm is as-yet unknown (Figure 3).

Transcription of this locus is also dependent on the alternate sigma factor, σ^{54} , as well as the response regulator and the σ^{54} -dependent activator, SypG (Ray *et al.*, 2013; Yip *et al.*, 2005). Although the signaling molecule needed to induce *syp*-dependent biofilm formation is unknown, overexpression of regulators such as RscS, SypG, and SypF* (a mutated, active form of SypF) induces *syp* transcription and subsequent biofilm formation.

SypA: a STAS domain protein

Another Syp regulatory protein that is important for *syp*-dependent biofilm formation is SypA. Deletion of *sypA* prevents biofilm formation *in vitro* and colonization *in vivo*. Under biofilm-inducing conditions, RscS and SypF promote phosphorylation of the response regulator SypE, which acts as a phosphatase to dephosphorylate SypA. Phosphorylation of serine 56 controls the activity of SypA. Unphosphorylated SypA promotes biofilm formation, but phosphorylated SypA fails to do so (Morris & Visick, 2013a; Morris & Visick, 2013b). Evidence indicates that SypA activity is working at a

level downstream of *syp* transcription and may be working by promoting Syp polysaccharide production (Figure 3) (Morris & Visick, 2013b). However, the mechanism by which SypA contributes to biofilm formation and host colonization remains unknown.

SypA is a small, 105 amino acid protein that contains a single STAS (sulphate transporter and anti-sigma factor) domain, which is commonly found in regulatory proteins that function as anti-sigma factor antagonists and anion transporters (Aravind & Koonin, 2000; Sharma *et al.*, 2011). The STAS domain usually contains four beta-strands and five alpha-helices. Proteins that contain a STAS domain can either be single STAS domain proteins or multi-domain proteins. Single STAS domain proteins are highly conserved among bacteria and many are anti-sigma factor antagonists (Mittenhuber, 2002). The multi-domain STAS proteins are conserved across multiple domains and are found in bacteria, plants and even humans (Alper & Sharma, 2013). Many of these multi-domain STAS proteins are found in the family of SulP/SLC26 anion transporters, but others (such as the LOV-STAS protein, YtvA, of *B. subtilis*) are involved in phototransduction (Jurk *et al.*, 2011). These multi-domain STAS proteins, such as YchM from *E. coli*, contain a transmembrane N-terminal domain with a cytoplasmic C-terminal STAS domain (Babu *et al.*, 2010).

Two of the best-studied single STAS domain proteins in bacteria are SpoIIAA (Seavers *et al.*, 2001) and RsbV (Igoshin *et al.*, 2007), which are both found in the Gram-positive bacterium, *Bacillus subtilis*. These proteins act as anti-sigma factor antagonists to regulate sporulation and the cellular stress response to harsh environmental stimuli.

SypA shows sequence similarity to RsbV and SpoIIAA in *B. subtilis* as well as to PA3347 in *P. aeruginosa*, which is also proposed to be an anti-anti-sigma factor that plays a role in virulence by regulating bacterial swarming and flagella synthesis (Bhuwan *et al.*, 2012). This sequence similarity raises the question as to whether SypA is also acting as an anti-sigma factor antagonist.

Many anti-sigma factor antagonists are involved in a signaling cascade that controls the activity of alternate sigma factors such as ECF (extracytoplasmic factors) sigmas, which generally respond to environmental stimuli or stress (Helmann, 2011). ECF sigma factor activities are primarily controlled by anti-sigma factors and these anti-sigma factors in turn can be regulated by anti-anti-sigma factors whose genes are often located in or near the anti-sigma factor operon (Ho & Ellermeier, 2012). *V. fischeri* contains eleven sigma factors. Some of these sigma factors have known cellular functions that do not seem to be affected by SypA activity. For example, RpoD (sigma 70) is the essential housekeeping sigma factor and RpoH (sigma 32) is the heat shock sigma factor. Since $\Delta sypA$ only seems to affect biofilm formation and does not appear to be detrimental to any other cellular functions, it is unlikely that SypA acts as an anti-anti-sigma factor for either of these two sigmas. RpoN (sigma 54) controls *syp* transcription and is not a likely target of SypA since mutations in *sypA* do not affect *syp* transcription (Morris & Visick, 2013a; Morris & Visick, 2013b). FliA (sigma 28) controls motility and is not a likely candidate since *sypA* mutants are motile (Morris and Visick, unpublished data). RpoS (sigma S) controls gene transcription for cellular stress response. This may appear to be the most likely candidate, since biofilms formation may

result due to environmental stresses, but Jakob Ondrey in the Visick lab has shown that overexpressing RpoS actually leads to a diminished biofilm (Ondrey and Visick unpublished data).

There are, however, six sigma factor candidates in *V. fischeri* that could be part of a SypA-anti-sigma factor cascade. *V. fischeri* contains five ECF sigma factors, two of which are associated with known anti-sigma factors. *V. fischeri* also contains one sigma factor encoded close to the *syp* locus, RpoQ (VF_A1015). Therefore, it is reasonable to hypothesize that one of these six sigma factors may be indirectly regulated by SypA.

Although many single STAS domain proteins control sigma factor activity, not all single STAS domain proteins act as anti-sigma factor antagonists (Sharma *et al.*, 2011). BtrV is an example of a single STAS domain protein found in the Gram-negative bacterium *Bordetella bronchiseptica*, where it regulates type III secretion (Kozak *et al.*, 2005). Other bacterial species such as *Chlamydia trachomatis* (Hua *et al.*, 2006) contain STAS-domain proteins whose functions are unknown, but that may play roles in host interactions or virulence. SypA, in turn, may not be acting as an anti-sigma factor antagonist to promote biofilm formation. An alternative hypothesis is that SypA may interact with other regulatory proteins or structural proteins within *V. fischeri* to promote biofilm formation. SypA may even interact with inner membrane proteins to regulate transport of molecules across the membrane similar to the multi-domain STAS proteins.

Determining the mechanism by which SypA promotes biofilm formation in *V. fischeri* may contribute to a greater understanding of biofilm formation in pathogenic bacteria and may shed light on the biofilm formation of closely related biofilm-

dependent, pathogenic bacteria. The goal of my thesis was to investigate mechanisms by which arabinose and SypA contribute to the formation of two distinct biofilms in *V. fischeri*.

CHAPTER 2

MATERIALS AND METHODS

Strains and Media

The *V. fischeri* strains used in this study are listed in Table 1. *V. fischeri* strain ES114, an isolate from *E. scolopes*, was used as the parental strain throughout this study (Boettcher & Ruby, 1990). The *V. fischeri* derivatives were constructed through conjugation (Stabb & Ruby, 2002) or transformation (Pollack-Berti *et al.*, 2010). *E. coli* strains Tam1 λ pir (Active Motif, Carlsbad, CA), DH5 α (Invitrogen, Carlsbad, CA), and GT115 (Invivogen, San Diego, CA) were used for cloning and conjugative purposes. *V. fischeri* strains were usually grown in the complex medium Luria-Bertani salt (LBS) (Graf *et al.*, 1994). In some experiments, *V. fischeri* cells were grown in complex Sea Water Tryptone (SWT) medium (Yip *et al.*, 2005). For motility assays, TB-SW was used (DeLoney-Marino *et al.*, 2003). *E. coli* strains were cultured in Luria Bertani medium (LB) (Davis *et al.*, 1980). For solid media, agar was added to a final concentration of 1.5%. When necessary, the following antibiotics were added to LBS medium at the indicated concentrations: chloramphenicol (Cm) 2.5 μ g ml⁻¹, erythromycin (Erm) at 5 μ g ml⁻¹, and tetracycline (Tc) at 5 μ g ml⁻¹. When necessary, the following antibiotics were added to LB, media at the indicated concentrations: ampicillin (Ap) at 100 μ g ml⁻¹, Cm at 25 μ g ml⁻¹, Erm, 150 μ g ml⁻¹, kanamycin (Kan) at 50 μ g ml⁻¹, or Tc at 15 μ g ml⁻¹.

Transposon Mutagenesis

To perform transposon (Tn) mutagenesis, we used a triparental conjugation technique (Stabb & Ruby, 2002) using an *E. coli* strain containing the mini-Tn5 transposon on plasmid pEVS170 (Lyell *et al.*, 2008) an *E. coli* strain (KV5066) containing the helper plasmid pEVS104 (Stabb & Ruby, 2002) and a recipient *V. fischeri* strain. The pEVS170 plasmid contains multiple elements that facilitate plasmid DNA transfer, maintenance, and Tn delivery, including *oriT*, an origin of transfer to allow the plasmid to enter *V. fischeri*; R6K, an origin of replication that allows for replication within *E. coli* and not in *V. fischeri*; an erythromycin resistant cassette (*erm*^R) located within the transposon; and a kanamycin resistant cassette (*kan*^R) located outside of the transposon. We grew overnight cultures of the desired strains, sub-cultured the strains, concentrated the cells through centrifugation, spotted them on an LBS plate, and allowed them to incubate for 3 hours at 28°C. The cells were then re-suspended, plated onto LBS containing Erm, and incubated overnight at 28°C. Colonies that arose were isolated and their phenotypes analyzed either through pellicle or wrinkled colony assays. To identify the location of the transposon, chromosomal DNA was isolated and digested with a restriction enzyme, and then the resulting DNA fragments were self-ligated and transformed into *E. coli* cells (Lyell *et al.*, 2008). The location of the Tn was determined by sequencing the DNA flanking the Tn insertion with primer 908.

Arabinose-induced Pellicle Assay

To observe arabinose-induced pellicle formation by the transposon mutants, we first selected for mutant colonies and inoculated them into wells of a 96-well plate

containing LBS and 0.2% arabinose. The cultures were incubated at room temperature (about 23 or 24°C), and observed after 24 and 48 h. To determine if a pellicle had formed, we disrupted the air-liquid interface of each well with a pipette tip and imaged the well using a Zeiss Stemi-C dissecting microscope with an attached camera as previously described (Visick *et al.*, 2013). To confirm the pellicle phenotype, we grew *V. fischeri* cells in LBS medium with shaking overnight at 28°C and then diluted the cells 1:200 in 2 ml of fresh LBS medium containing 0.2% arabinose. We placed the diluted cultures into 24-well plates (Greiner Bio-One), incubated them at room temperature, and observed them after 24 and 48h.

Motility Assay

Because one of the arabinose-induced pellicle mutants we obtained contained an insertion in a gene required for motility, we subsequently tested other pellicle mutants for defects in motility. We grew overnight cultures in SWT with shaking, and then we inoculated TB-SW soft agar plates, containing 0.25 – 0.30% agar, with 10 µl aliquots of the culture. We monitored the migration of cells every hour through the soft agar. Cells that failed to migrate beyond the point of inoculation within 6 to 8 h were scored as non-motile.

β-galactosidase Assay

Plasmid pKV143, which contains the *lacZ* gene under the control of the arabinose-inducible pBAD promoter, was introduced into *V. fischeri* ES114 and specific mutant strains. Triplicate cultures of cells were grown with shaking in LBS containing Cm overnight at 28°C and diluted 1:200 in 2 ml LBS containing Cm and 0.2% arabinose.

The cultures were grown statically for 24 h at room temperature, and then 1 ml aliquots were concentrated by centrifugation, re-suspended in Z buffer and lysed with chloroform. β -Galactosidase activity was measured using *o*-nitrophenyl- β -D-galactopyranoside (ONPG), and reactions were stopped using sodium carbonate as described previously (Miller, 1972). Lowry assays were performed to standardize the β -galactosidase activity to protein concentration (Lowry *et al.*, 1951).

Plasmids and Molecular Biology Techniques

Plasmids and primers used in this study are listed in Tables 2 and 3. We used the method of Le Roux *et al.* (Le Roux *et al.*, 2007) to generate unmarked, in-frame deletions to remove VF_1812. We generated DNA fragments, between 500 to 600 bp in length, upstream and downstream of the gene of interest through PCR by using KOD HiFi DNA polymerase (Novagen), template chromosomal DNA from *V. fischeri* ES114, and the appropriate primers indicated in Table 3. The resulting upstream and downstream fragments were fused in overlap extension PCRs (Horton *et al.*, 1989). Each resulting PCR product was then cloned into pJET1.2/blunt (Fermentas) and sub-cloned into the suicide plasmid pKV363 (Shibata & Visick, 2012). The deletion constructs were then introduced into *V. fischeri* strain ES114, and the resulting recombinants were confirmed using PCRs with *Taq* polymerase (Promega) and primers outside each deletion region.

DNA was introduced into *V. fischeri* using triparental conjugations (DeLoney *et al.*, 2002; Stabb & Ruby, 2002) or transformation using the pLostfoX plasmid (protocol from Ned Ruby's Lab). The presence of the gene *tfoX* expressed on this multicopy plasmid allows *V. fischeri* to take up exogenous DNA (Pollack-Berti *et al.*, 2010). To

back-cross the *yhdP*::Tn5 mutation from KV6941 into the parent *V. fischeri* background (Δ *sypA* Δ *sypE*; KV4716), I used the method of Brooks et al, 2014 (Brooks *et al.*, 2014). Through this method, I conjugated the pLostfoX plasmid (from strain KV6834) into KV4716, generating strain KV6944. I then isolated the genomic DNA of KV6941, made the cells of strain KV6944 competent and mixed 500 μ l of the competent culture with 2.4 μ g of genomic DNA from KV6941 and incubated the mixture at room temperature for 30 min. I added 1 ml LBS to the transformation mixture, incubated the transformation culture at 25°C with shaking for 1 hr., then plated 50 μ l of the transformation culture onto LBS-Erm plates and incubated the plates overnight at 28°C. To ensure the isolation of pure transformants, I performed two rounds of picking single colonies and re-streaking them onto the selective media. To ensure that the strains had lost the pLostfoX plasmid and retained the transposon, I picked single colonies and patched them on to LBS-Cm plates, LBS-Erm plates, and LBS-Erm liquid culture and grew the strains overnight. I saved strains that were Erm resistant and Cm sensitive and verified that the Tn was inserted into *yhdP* gene using PCR and *Taq* polymerase (Promega).

The *sigma* factor alleles used in this study were generated by PCR using primers listed in Table 3, KOD HiFi DNA polymerase (Novagen), and ES114 template DNA. The PCR products were cloned into pJET1.2/blunt (Fermentas) and then sub-cloned into pVSV105. The sigma factor sequences were confirmed through sequence analysis by ACGT, Inc (Wheeling, IL). The multicopy plasmids were introduced into *V. fischeri* through triparental conjugations.

To generate site-directed mutations in *sypA-HA*, I used the Gibson Assembly Kit (New England Biolabs) along with mutagenic primers (Table 3) and produced the mutant alleles through PCR. Generation of the desired mutations were confirmed by sequence analysis using ACGT, Inc (Wheeling, IL). PCR products were cloned into the plasmid pARM47, and inserted into the Tn7 site using tetraparental conjugations (McCann *et al.*, 2003).

Wrinkled Colony Assay

To observe wrinkled colony formation, I streaked the indicated *V. fischeri* strains onto LBS agar plates containing the necessary antibiotics. I picked single colonies and cultured them with shaking in LBS broth containing antibiotics overnight at 28°C. The following day, I sub-cultured the strains in 5 ml of fresh medium. Following growth to early log phase, the cultures were back-diluted in LBS to an OD₆₀₀ of 0.2, spotted (10 µl) onto LBS agar plates containing the necessary antibiotics, and grown at 28°C. Images of the spotted cultures were acquired at the indicated time points using a Zeiss stemi 2000-C dissecting microscope.

Western Blot Analysis of *V. fischeri* Cell Lysates and Coomassie Staining

The indicated *V. fischeri* strains were cultured in LBS containing the appropriate antibiotics overnight at 28°C. Typically, 1 ml of cell cultures was spun down and the cells were lysed in 500 µl of 2X sample buffer (4% SDS, 40 mM Tris pH 6.3, 10% glycerol). The strains expressing point mutant versions of SypA were sub-cultured in fresh media and grown to an OD₆₀₀ of 0.2 prior to lysis in 2X sample buffer. Prior to loading the samples on the SDS-PAGE gel, I boiled each sample for 5 min. Samples

were resolved on 10-12% 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. The indicated Syp proteins were detected by Western blot analysis using either a rabbit anti-FLAG antibody or 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).

Inner Membrane Isolation

V. fischeri cultures were grown overnight in LBS at 28°C with shaking. We used 2 mL of the overnight culture and through centrifugation concentrated the cells, saved the pellet, and discard the supernatant. The pellet was resuspended in 1 mL lysis buffer (Aeckersberg *et al.*, 2001; McCarter & Silverman, 1987), which contains 50 mM Tris pH 8, 50 mM EDTA, 15% sucrose, 1:200 dilution of 0.1 M phenylmethylsulphonyl fluoride (PMSF), 0.5 mg/mL lysozyme, 10 µL of 5 mg/mL DNase, and water to volume. The solution was incubated for 5 min at room temperature with gentle rotation. The sample underwent a series of 3 freeze-thaw cycles where the solution was frozen for 20 min at -80° C and then thawed at room temperature with gentle rotation. The sample was spun-down in a centrifuge at 14,000 rpm for 5 min at 4° C. The soluble cell fraction, located in the supernatant, was decanted and saved. The remaining pellet was washed two times with 1 mL of phosphate-buffered saline (PBS) and centrifuge at 14,000 rpm for 5 min at 4°C. I resuspended the pellet in 500 µL of Cytoplasmic Membrane Solubilization Buffer (Arnold & Linke, 2008), which contains 1% sarkosyl, 100mM NaCl, 50mM Tris·Cl pH

8.0, and 1:200 dilution of 0.1M PMSF. The solution was incubated for 30 min at room temperature with gentle rotation. The sample was centrifuged at 14,000 rpm for 30 min at 4° C. I decanted and saved the supernatant, which contained the inner membrane cell fraction. The soluble and inner membrane cell fractions were resolved using SDS-PAGE. Samples were then transferred to PVDF membrane and proteins were detected using rabbit anti-FLAG and anti-HA antibodies followed by an HRP-conjugated secondary antibody.

Co-Immunoprecipitation

Rabbit anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO) was coupled to magnetic Dynabeads (5 mg, Invitrogen) according the manufacturer's protocol from the Dynabeads Co-immunoprecipitation Kit (Invitrogen, Oslo, Norway). Antibody-coupled beads were incubated with the inner membrane cell fractions at 4°C with rocking for 1 h following the Co-Immunoprecipitation for Western Blotting protocol (Invitrogen, Oslo, Norway). Eluted samples were diluted with 2X sample buffer and resolved using SDS-PAGE. Samples were then transferred to a PVDF membrane and proteins were detected using rabbit anti-FLAG and anti-HA antibodies followed by an HRP-conjugated secondary antibody.

Bioinformatics

Amino acid sequences of the SypA homologs from the various *Vibrio* species as well as RsbV and SpoIIAA from *B. subtilis*, PA3347 from *P. aeruginosa*, BtrV from *B. bronchiseptica*, RsbV_1 and RsbV_2 from *C. trachomatis* were obtained from the National Center for Biotechnology Information (NCBI) database. Amino acid sequence

alignments were generated using the Clustal Omega multiple-sequence alignment program from EMBL-EBI (<http://ebi.ac.uk/Tools/msa/clustalo>)(Sievers *et al.*, 2011). The SpoIIAA tertiary structure 1AUZ_A (Kovacs *et al.*, 1998) was downloaded from the Molecular Modeling Database (MMDB) (Madej, 2012) from the NCBI website and visualized using Cn3D (Wang *et al.*, 2000).

Table 1: *V. fischeri* Strains used in this study

Strains	Genotype	Reference or Source
ES114	Wild-type isolate from <i>Euprymna scolopes</i>	(Boettcher & Ruby, 1990)
KV4238	pVSV105, pCLD56/ Δ sypE (KV3299)	Visick lab, unpublished
KV4453	pARM9/ Δ sypE (KV3299)	Visick lab, unpublished
KV4716	Δ sypA Δ sypE	(Morris & Visick, 2013b)
KV4724	pARM9/ Δ sypA Δ sypE (KV4716)	Visick lab, unpublished
KV5452	pVAR45/ Δ sypE (KV3299)	Visick lab, unpublished
KV5804	VFA0685::Tn5	(Visick <i>et al.</i> , 2013)
KV5805	<i>ptsI</i> ::Tn5 VF1896	(Visick <i>et al.</i> , 2013)
KV5807	VF1812::Tn5*	(Visick <i>et al.</i> , 2013)
KV5942	VF1812::Tn5*	(Visick <i>et al.</i> , 2013)
KV6591	pCLD56/ Δ sypA Δ sypE attTn7::sypA-HA (KV6580)	Visick lab, unpublished
KV6834	endA1 hsdR17 (rK- mK+) supE44 thi-1 recA1 relA Δ (lacIZYA-argF)U169 phoA [ϕ 80dlac Δ (lacZ)M15]	(Pollack-Berti <i>et al.</i> , 2010)
KV6941	Δ sypA Δ sypE VF0377::Tn5 pt mutant, pARM9	This Study
KV6942	Δ sypA Δ sypE VF0377::Tn5 pt mutant	This Study
KV6943	Δ sypA Δ sypE VF0377::Tn5 pt mutant, pARM9	This Study
KV6944	Δ sypA Δ sypE, pLosTfoX	This Study
KV6945	Δ sypA Δ sypE VF0377::Tn5	This Study
KV6946	Δ sypA Δ sypE VF0377::Tn5, pARM9	This Study
KV6994	Δ sypA Δ sypE attTn7::sypA-HA K67A, pCLD56	This Study
KV6999	Δ sypA Δ sypE attTn7::sypA-HA Q84A, pCLD56	This Study
KV7004	Δ sypA Δ sypE attTn7::sypA-HA R68A, pCLD56	This Study
KV7009	Δ sypA Δ sypE attTn7::sypA-HA R93A	This Study
KV7018	Δ sypA attTn7::sypA-HA R93A, pCLD56	This Study
KV7034	Δ sypA Δ sypE, pSMM4, pCLD56	This Study
KV7037	Δ sypA Δ sypE, pSMM6, pCLD56	This Study
KV7040	Δ sypA Δ sypE, pSMM8, pCLD56	This Study
KV7042	Δ sypA Δ sypE attTn7::sypA-HA (erm), pCLD56, pKV476	This Study
KV7043	Δ sypA Δ sypE, pCLD56, pARM9	This Study
KV7044	Δ sypA Δ sypE, pVSV105, pCLD56	This Study

*These strains contain two independent insertions.

Table 2: Plasmids used in this study

Plasmids	Description	Reference or Source
pARM9	pVSV105 containing 1.7 kb <i>sypG</i>	(Morris & Visick, 2013b)
pARM40	<i>VF_A1015 (rpoQ)</i> in pVSV105	Visick lab, unpublished
pARM47	Derivative of Tn7 delivery plasmid pEVS107 that contains <i>sypE</i> , KanR, ErmR	(Morris <i>et al.</i> , 2011)
pCLD56	pKV282 containing 1.7 kb <i>sypG</i>	(Morris & Visick, 2013b)
pEVS104	Conjugal helper plasmid (<i>tra trb</i>), kanR	(Stabb & Ruby, 2002)
pEVS107	Mini--Tn7 delivery plasmid; <i>oriR6K</i> , <i>mob</i> ; KanR, EmR	(McCann <i>et al.</i> , 2003)
pKV143	pEVS78 containing arabinose-inducible <i>lacZ</i> derived from pBAD/His/LacZ	(Visick, <i>et al.</i> , 2013)
pKV363	Suicide plasmid (<i>ori-R6K</i>); Cm ^R	(Shibata & Visick, 2012)
pKV476	<i>sypK</i> -FLAG in pVSV105	Visick lab, unpublished
pLosTfoX	995 bp of <i>V. fischeri</i> ES114 DNA containing the <i>tfoX^{VF}</i> ORF cloned into pEVS79	(Pollack-Berti <i>et al.</i> , 2010)
pSMM2	<i>VF_1812</i> in pVSV105	(Visick, <i>et al.</i> , 2013)
pSMM4	<i>VF_2498</i> in pVSV105	This Study
pSMM6	<i>VF_A0820</i> in pVSV105	This Study
pSMM8	<i>VF_0972</i> in pVSV105	This Study
pSMM10	<i>sypA</i> K67A point mutant in pARM47(Δ <i>sypE</i>) (#4) amplified from pARM163	This Study
pSMM11	<i>sypA</i> Q84A point mutant in pARM47(Δ <i>sypE</i>) (#1) pEVS107 amplified from pARM163	This Study
pSMM12	<i>sypA</i> R68A point mutant in pARM47(Δ <i>sypE</i>) (#6) pEVS107 amplified from pARM163	This Study
pSMM13	<i>sypA</i> R93A point mutant in pARM47(Δ <i>sypE</i>) (#2) pEVS107 amplified from pARM163	This Study
pVAR45	<i>sypG</i> -FLAG in pVSV105	(Ray <i>et al.</i> , 2013)
pVSV105	Mobilizable vector, R6Kori(<i>pES213</i>) RP4 <i>oriT</i> Cm ^R	(Dunn <i>et al.</i> , 2006)

Table 3: Oligonucleotides used in this study

Gene/Promoter	Sequence (5'to 3')	Primer
VF1812-F	GGT CGG ATT TGT CGT CTA TG	1478
VF1812-R	CCT AGT CCC TTG AAG CGA AG	1479
VF0377- F	GAGTGAAGCTGAGATCTCAC	1610
VF0377-SOE-R	TAGGCGGCCGCACTTAGTATGTAAAAATACCAGCAGCA ATAATCC	1611
VF0377-SOE-F	CATACTAAGTGC GGCCGCTAG AATATCAATTACCTGAA AAAACG	1612
VF0377- R	GTCTGAGACAGTTCCTGCTG	1613
VFA0766 - F	GATTTTGTGATCATTCAACTATGC	1614
VFA0766 - R	CACCGCTAATGATTCGGGTA	1615
VFA0820-F	GCTGTTCAACGGATTCAAGC	1616
VFA0820-R	AGCCGACTATTAATGTCCGGC	1617
VF2540-F	TCCGCCTTTCACCTGCAGTC	1618
VF2540-R	GAACTCTTTC AAGTTCAGCC	1619
VF2498-F	GACAAACACTTCAACGCCAG	1620
VF2498-R	ACGAAATAGCAGAGCGCAAC	1621
VF0377-F	CCAAGAGCAGTTTGACGTCG	1623
VF0377-R	CCCCGAAGTCATCTGAACAA	1624
VF2093-F	GAATAACATGGGAGTACCCG	1625
VF2093-R	TCTTACCGTTTGACCGTTG	1626
VFA0820-R	CAAGAAGCAAGCGTTCTCGT	1627
VF2540 + SalI F	GTCGACTCCGCCTTTCACCTGCAGTC	1651
VF2540 + SpeI R	ACTAGTGA ACTCTTTC AAGTTCAGCC	1652
VFA0766 + SalI F	GTCGACGATTTTGTGATCATTCAACTATGC	1653
VFA0766 + XbaI R	TCTAGACACCGCTAATGATTCGGGTA	1654
VFA0820 + SalI F	GTCGACGCTGTTCAACGGATTCAAGC	1655
VFA0820 + XbaI R	TCTAGACAAGAAGCAAGCGTTCTCGT	1656
VF2093 + SalI F	GTCGACGAATAACATGGGAGTACCCG	1657
VF2093 + XbaI R	TCTAGATCTTACCGTTTGACCGTTG	1658
VF2093 + SalI	GTCGACAGTTAATCGTGGTATAGCTGG	1674
<i>sypA</i> K67A F	TTATCTATATGCACGACTTATAGAGAAAGATCGTA	1729
<i>sypA</i> K67A R	CTATAAGTCGTGCATATAGATAAACAATAGCGCAA	1730
<i>sypA</i> Q84A F	TGCACATGGCGCGCCACTAGAGTTACTAAA ACTTC	1731
<i>sypA</i> Q84A R	ACTCTAGTGGCGCGCCATGTGCATTTTAACTCTGC	1732
<i>sypA</i> R68A F	TCTATATAAAGCACTTATAGAGAAAGATCGTACTAT	1733
<i>sypA</i> R68A R	TCTCTATAAGTGCTTTATATAGATAAACAATAGCGCC	1734

<i>sypA</i> R93A F	AAAACCTTCTAGCCATTGAAAACGCAATTCCTGTAA	1735
<i>sypA</i> R93A R	CGTTTTCAATGGCTAGAAGTTTTAGTAACTCTAGTG	1736
GA A0766 F	GCCTGCAGGTCGACTCTAGAGTGATCATTCAACTATGCT CAA	1741
GA A0766 R	TATAGGGCGAATTCGAGCTCTCTTTGGGATGGTAAGTGA TC	1742
GA 2093 F	GCCTGCAGGTCGACTCTAGAGAATAACATGGGAGTACC CG	1743
GA 2093 R	TATAGGGCGAATTCGAGCTCCTTACCCGTTTGACCGTTG G	1744

CHAPTER 3

RESULTS

Genetic Characterization of the Arabinose-induced biofilm

Introduction

The presence of L-arabinose, but not other similar sugars, in LBS induces *V. fischeri* to form a pellicle at the air-liquid interface of static cultures (Visick *et al.*, 2013). This arabinose-induced biofilm appears to be independent of the Syp biofilm since *syp* mutants retain the ability to form a pellicle in static cultures containing arabinose. Arabinose is also inhibitory to host colonization. Although *V. fischeri* is unable to use arabinose as a carbon source, somehow this small molecule can act as a signal to induce this unique biofilm phenotype. The presence of this biofilm is clear, but the mechanism behind the formation of this novel biofilm as well as the genes that control the formation of this biofilm are unknown. To identify the genes involved in arabinose-induced biofilm formation, we performed a random transposon mutagenesis and screened for mutants that fail to form biofilms in response to arabinose. I subsequently verified the involvement of one of these genes in the phenotype using a complementation analysis. Finally, I assessed one possible mechanism of the role of the identified genes, namely, arabinose uptake.

Identifying genes involved in the formation of the arabinose-induced biofilm

To identify the genes involved in the arabinose-induced biofilm formation, members of the Visick lab performed random transposon mutagenesis on wild type *V. fischeri* cells and screened for colonies that were unable to form a biofilm in the presence of arabinose. We reasoned that if the transposon interrupts a gene essential for arabinose-induced biofilm formation, then the cells would be unable to produce the biofilm in the presence of arabinose. The transposon contains an Erythromycin (erm) marker and thus, we selected for colonies that contained the transposon by initially growing the cells on LBS media containing erm. Following our initial selection, we screened for mutants that failed to form a pellicle in static culture in the presence of arabinose by inoculating 96 well plates with the selected colonies. We monitored the pellicle formation after 24 and 48 h post inoculation. To check for pellicle formation, we disturbed the air-liquid interface of the static culture using a pipet tip. We disregarded the strains that were still able to form a biofilm and further analyzed the strains that were unable to form a biofilm.

We further characterized mutants that failed to form a pellicle in the presence of arabinose by their motility phenotype. Kevin Quirke previously showed that motility was required for arabinose-induced biofilm formation. Thus, to identify new unknown genes involved in arabinose-induced biofilm formation, we performed soft-agar motility assays to distinguish the motile from the non-motile mutants. From the set of mutants I evaluated, only one exhibited a defect in motility and was eliminated from further consideration.

After eliminating motility mutants, we took the remaining mutants and identified the location of the transposon to determine the genes or operon disrupted. To identify the exact location of the transposon within the mutants, we cloned the transposon with flanking DNA from the chromosome; the transposon contains both an origin of replication that can permit the replication of the Tn-containing DNA and an erm-resistance cassette that we used to select for erm-resistant colonies that contain the transposon. We obtained clones and sequenced from the ends of the transposon. Sequence analysis of the transposon-containing plasmids revealed the DNA sequence within which the transposons inserted.

Through this screen of 8,000 mutants from 6 independent trials, 50 mutants were reproducibly defective in producing an arabinose biofilm. Of those 50 mutants, 21 had normal motility. The locations of the Tn insertions in the remaining genes were identified (Table 4). The wide variety of genes apparently involved indicates that the cellular response to arabinose is quite complex and a more in-depth analysis is necessary for a complete understanding of the mechanism behind the arabinose-induced biofilm formation. Although we found multiple mutations in a few genes, we did not reach saturation with our transposon mutagenesis screen and therefore this may not be a comprehensive list of the genes involved in the arabinose induced biofilm phenotype. This work was completed by Karen Visick, Kevin Quirke, Shikhar Tomur and myself.

Table 4: Locations of Transposon Insertions in mutant strains unable to form a pellicle in the presence of Arabinose.

Strain	Location of Transposon Insertion	Predicted Gene Function
KV5944	<i>VF_0311 (cysI)</i>	Sulfite reductase subunit beta
KV6001	<i>VF_0360 (mshM)</i>	MSHA biogenesis protein MshM
KV6000	<i>VF_0361 (mshN)</i>	MSHA biogenesis protein MshN
KV5948	IG (<i>VF_0365-0366 (mshB-mshA)</i>)	Between MSHA pilin protein MshB and and MshA
KV5999	<i>VF_0435 (gshB)</i>	Glutathione synthetase
KV5629	<i>VF_0696 (acfD)</i>	Accessory colonization factor AcfD-like protein
KV5635	<i>VF_0804 (asnB)</i>	Asparagine synthetase B
KV5943	<i>VF_0819 (sdhC)</i>	Succinate dehydrogenase cytochrome b556 large membrane subunit
KV6002	<i>VF_1037 (ainS)</i>	C8-HSL autoinducer synthesis protein AinS
KV5998	IG (<i>VF_1631-1632 (hns-mipA)</i>)	Between global DNA-binding transcriptional dual regulator H-Ns and scaffolding protein for murein synthesizing machinery
KV5807 KV5942	<i>VF_1812</i>	Long-chain fatty acid transport protein
KV5805 KV5633	<i>VF_1896 (ptsI)</i>	phosphoenolpyruvate-protein phosphotransferase
KV5634	<i>VF_2291 (aroB)</i>	3-dehydroquinate synthase
KV5945	<i>VF_A0351 (yidK)</i>	Transporter
KV5804	<i>VF_A0685 (talB)</i>	Transaldolase B
KV5632	<i>VF_A0703 (gevP)</i>	Glycine dehydrogenase
KV5630	<i>VF_A0859</i>	Methyl-accepting chemotaxis protein
KV6028	<i>VF_A0860</i>	Hypothetical protein
KV5631	<i>VF_A1015 (rpoQ)</i>	Sigma Factor

I personally screened about 600 mutants and identified three previously uncharacterized mutants with independent transposon insertions that were unable to form a biofilm in the presence of arabinose but maintained their motility. In one mutant, the transposon interrupted the gene *VF_A0685*, which encodes a transaldolase. In the second mutant, the transposon disrupted the gene *VF_1896*, which encodes a phosphoenolpyruvate-protein phosphotransferase. The third gene disrupted by the transposon was *VF_1812*, which codes for a putative long-chain fatty acid transport protein precursor.

Throughout the entire mutagenesis assay, the gene *VF_A0685* was disrupted only once but *VF_1812* and *VF_1896* were each disrupted in independent mutagenesis screens performed by Kevin Quirke. The two transposons inserted at two different sites within the *VF_1812* gene and the *VF_1896* gene, but caused the same loss of biofilm phenotype. These two independent insertions gave us confidence that disruption of these genes could be the cause of the loss of the arabinose-induced biofilm and may be essential for arabinose-induced biofilm formation. However, we did not perform complementation experiments on the mutant strains that disrupted genes *VF_A0685* or *VF_1896* and therefore, we cannot be sure whether these disrupted genes actually are responsible for the mutant phenotype or if there is a secondary mutation within the genome.

To confirm that the disruption in *VF_1812* was responsible for the mutant phenotype and that the loss of biofilm formation was not caused by a secondary mutation within the genome, we performed complementation assays and reintroduced the *VF_1812* gene into the mutant strains. The *VF_1812* gene introduced on plasmid pSMM2 was able

to restore the wild type phenotype, as the complemented mutants again formed a pellicle in the presence of arabinose (Figure 4).

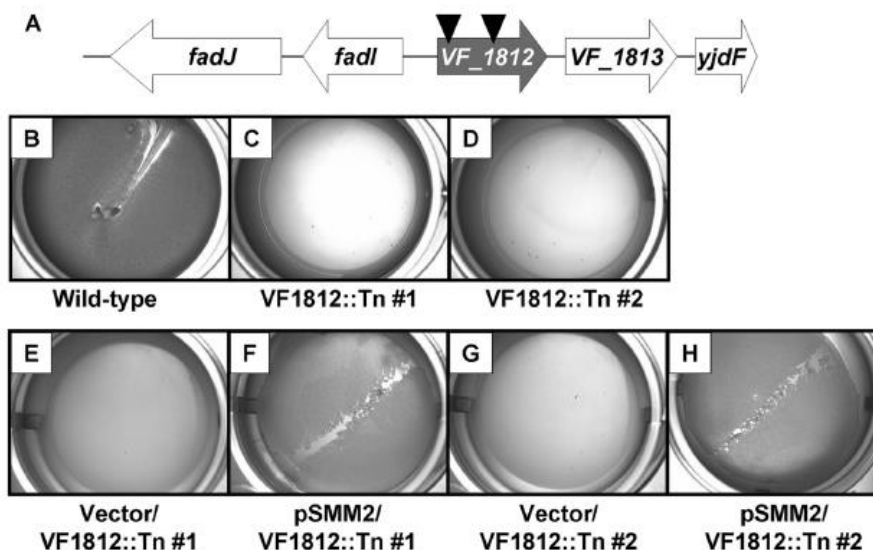


Figure 4: Reintroducing the *VF_1812* gene on pSMM2 resulted in the restoration of the WT pellicle phenotype. (A) The two mutants KV5807 and KV5942 had independent transposon insertions in the *VF_1812* gene that resulted in the loss of the biofilm phenotype. The black triangles depict the approximate locations of the transposon insertions. (B-H) shows the pellicle formation of various *V. fischeri* strains grown statically in LBS and 0.2% arabinose. The air-liquid interface of each well was disrupted with a pipette tip to show the presence or absence of a pellicle. This figure is from Visick *et al.*, 2013.

Identifying the Arabinose Transporter

Although we predict that these disrupted genes are involved in the arabinose induced biofilm phenotype, we do not know how these genes are required for biofilm formation. One hypothesis is that these proteins could affect arabinose uptake into the cell. To determine whether the disrupted genes encode a transporter of arabinose, we introduced an arabinose-inducible *lacZ* reporter construct into our three mutant strains

and performed β -galactosidase assays to indirectly measure whether arabinose was present in the cells. We hypothesized that mutants defective in arabinose uptake would have reduced β -galactosidase activity compared to wild type *V. fischeri* cells grown in the presence of arabinose. We measured β -galactosidase activity from aliquots of cells grown statically in the presence of arabinose and performed Lowry assays to standardize the β -galactosidase activity to the total protein concentration. All three newly identified mutants had similar β -galactosidase activities to that of wild type cells. Thus, arabinose was entering the cells and we concluded that these three mutants are not defective in arabinose uptake (Quirke unpublished data).

Summary

Wild type *V. fischeri* forms a pellicle at the air-liquid interface of static cultures that contain 0.2% arabinose. By performing a random transposon mutagenesis assay, we were able to identify various genes that contributed to the arabinose-induced biofilm phenotype. Three of the genes required for pellicle formation in the presence of arabinose were *VF_1812*, which encodes a putative long-chain fatty acid transport protein, *VF_A0685*, which encodes a transaldolase, and *VF_1896*, which encodes a phosphoenolpyruvate-protein phosphotransferase. Both *VF_1896* and *VF_1812* were identified in multiple independent trials leading us to believe that these genes are essential for arabinose induced biofilm formation. We were able to introduce an intact copy of *VF_1812* into the mutant strains and restore biofilm formation indicating that *VF_1812* is in fact necessary for this biofilm phenotype. We have not yet complemented the other two mutant strains and therefore have not confirmed that genes *VF_1896* and

VF_A0685 are necessary for the biofilm phenotype. Finally, the three mutants I investigated did not exhibit a defect in arabinose uptake into the cells, and thus some other gene(s) must be responsible for arabinose uptake.

Identifying downstream targets of SypA in *syp*-dependent biofilm formation.

Introduction

It is well-known that the Syp polysaccharide and Syp proteins are important for biofilm formation and squid colonization, and that the *syp* locus is controlled at the level of *syp* transcription (Norsworthy & Visick, 2015; Ray *et al.*, 2013; Yip *et al.*, 2006). However, *syp*-dependent biofilm formation is also controlled at an important but unknown level below *syp* transcription. This second control mechanism depends upon the SypA protein, but the exact function of the SypA protein in biofilm formation remains unknown. Currently, the only known regulator of SypA is the response regulator, SypE. Phosphorylated SypE acts as a phosphatase to remove the phosphate group from residue S56 of SypA. Unphosphorylated SypE acts as a kinase to introduce a phosphate group on S56 of SypA. Phosphorylated SypA is unable to promote biofilm formation, while unphosphorylated SypA is essential for Syp dependent biofilm formation (Morris *et al.*, 2011; Morris & Visick, 2013a; Morris & Visick, 2013b).

SypA, a small 105 amino acid protein, contains a single STAS (sulphate transporter and anti-sigma factor antagonist) domain commonly found in regulatory proteins that function as anti-sigma factor antagonists. Some preliminary data exist that indicate that SypA may not act in the same manner, but whether it does or does not has not been conclusively demonstrated. However, due to its small size and the known role of

these types of proteins in other systems, we hypothesize that SypA exerts its effect on biofilm formation through an interaction with another protein. Since SypA positively affects biofilm formation, we hypothesize that SypA is contributing to biofilm formation either by indirectly inhibiting an inhibitor, like an anti-sigma factor or another regulator, or by directly interacting with a Syp structural protein to control biofilm formation. Here, to begin to understand the function of SypA, I used three different approaches, transposon mutagenesis, sigma factor over expression assays, and co-immunoprecipitation, to test specific hypotheses as described below. Although my results did not reveal an interacting partner, they nevertheless provide insights into the possible function of SypA.

Perform transposon mutagenesis as a random screen to identify genes involved in SypA-dependent biofilm formation that act as downstream targets of SypA.

We first hypothesized that SypA may act as a regulator by inhibiting an inhibitor of biofilm formation. One way to identify downstream inhibitory genes is to perform transposon mutagenesis and screen for biofilm restoration in a *syp*-induced $\Delta sypA$ strain. This strain on its own is unable to produce a biofilm, but through this by-pass suppressor screen, we could identify specific genes that when disrupted by a transposon restored biofilm formation. We could then determine whether the predicted proteins encoded by those genes interact with SypA.

To search for an inhibitory downstream target of SypA, I performed a random transposon mutagenesis to disrupt genes and screened for the restoration of biofilms in a *sypA* mutant background ($\Delta sypA, \Delta sypE, psypG$). I screened more than 30,000 mutants

and identified one transposon mutant that displayed a wrinkled phenotype (KV6941). This mutant exhibited a wrinkled phenotype that was greater than the negative control but both delayed and diminished compared to the wrinkled phenotype of a biofilm promoting strain containing wild type SypA ($\Delta sypE$, $psypG$) (Figure 5). This wrinkled phenotype was dependent on the *syp* locus since curing the mutant of $psypG$ resulted in a complete loss of wrinkled colony formation (Figure 6). To identify the exact location of the transposon within the mutants, we sequenced the chromosomal DNA flanking the transposon. Sequence analysis of the transposon-containing plasmid revealed that the transposon inserted in the gene *VF_0377* or *yhdP*, a large 4 kb gene that encodes a putative membrane associated transport protein.

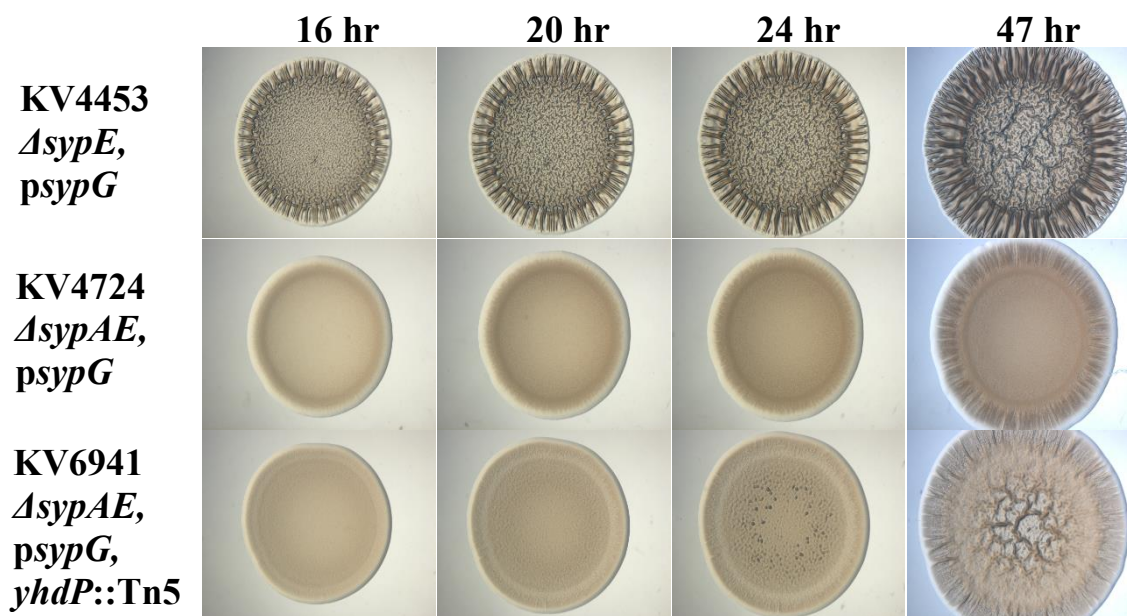


Figure 5: Wrinkling time course of *yhdP::Tn5* mutant at 16, 20, 24, and 47 hours. The transposon mutant strain, KV6941, shows a wrinkled phenotype compared to the negative control, KV4724

To determine if the wrinkled phenotype was due to the disruption of *VF_0377* and not a secondary mutation, I performed a backcross to move the transposon disrupted *VF_0377* gene into the parent background ($\Delta sypA \Delta sypE$). To accomplish this, I used a transformation technique that is relatively new for *V. fischeri*. Transformation by *V. fischeri* is facilitated by over expression of the *tfoX* gene on the plasmid plostfox (Pollack-Berti *et al.*, 2010). I used this new technique to transform my parental strain ($\Delta sypA, \Delta sypE$) with chromosomal DNA from my *VF_0377* mutant. I selected for colonies that were erythromycin resistant indicating that the cells had taken up the transposon and that recombination of the transposon and flanking DNA had occurred. I used PCR to confirm that the transposon inserted in the proper location within the *yhdP* gene. I then reintroduced *psypG* into my strain to induce *syp* transcription and assessed the wrinkled colony phenotype. If the wrinkled phenotype was due to the transposon insertion in *VF_0377*, then the transformed strain now containing the transposon in the same *VF_0377* site should also display the wrinkled colony phenotype. This however was not the case (Figure 6), indicating that a secondary mutation may have been responsible for this wrinkled phenotype. In order to identify the secondary mutation and locate the mutated gene responsible for this phenotype, full genome sequencing would have to be performed.

Through these mutagenesis experiments, I was unable to identify a downstream target of SypA. Although it is possible that the screen was simply not saturating, another possibility is that SypA is acting as a regulator by positively controlling another positive regulator of biofilm formation. SypA may also be playing a structural role interacting

with other proteins to promote biofilm formation. A Tn mutagenesis screen would not be helpful in determining if these two possibilities are correct.

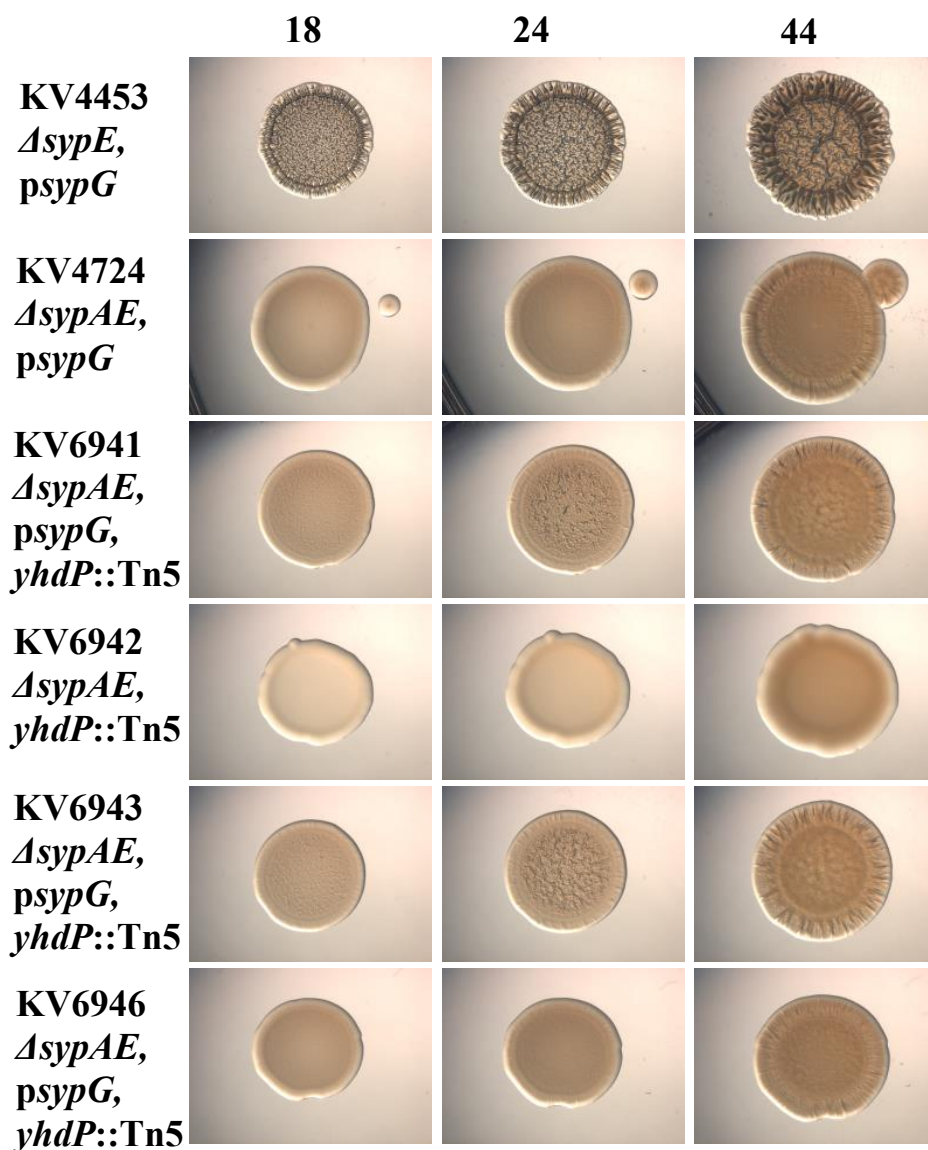


Figure 6: Wrinkling time course of various *yhdP::Tn5* strains at 18, 24, and 44 h. KV4453 is the positive control while KV4724 is the negative control strain. KV6941 is the original transposon mutant that displays a delayed wrinkled phenotype. KV6942 contains the original transposon mutant, but is cured of the SypG plasmid. KV6943 contains the original transposon mutation derived from KV6942 but contains the restored SypG plasmid. KV6946 contains the transposon transformed into a fresh background.

Determine if SypA acts as an anti-anti sigma factor for V. fischeri ECF sigma factors

I next tested the hypothesis that SypA is acting like an anti-sigma factor antagonist similar to other single STAS domain proteins such as SpoIIAA (Sharma, *et al.*, 2011). SypA may function as an anti-anti-sigma factor by inhibiting the activity of an anti-sigma factor that in turn inhibits the ability of a sigma factor essential for *syp*-dependent biofilm formation. If SypA functions as an anti-sigma factor antagonist, then deleting *sypA* would release the inhibition of the anti-sigma factor, which in turn would inhibit the activity of a particular sigma factor that helps regulate biofilm formation. If this is the case, then overexpressing the particular sigma factor could overcome the inhibitory effect of the anti-sigma factor in a $\Delta sypA$ strain.

To determine if SypA is acting as an anti-anti sigma factor, I cloned various sigma factors onto plasmids containing a constitutively active promoter and introduced these plasmids into a biofilm-inhibited strain ($\Delta sypA$, $\Delta sypE$, *psypG*). The sigma factors I chose to overexpress were the five ECF sigma factors as well as RpoQ (encoded by *VF_AI015*), which is located near the *syp* locus (Table 5) and was identified in the arabinose biofilm screen (Table 4). If SypA is acting as an anti-sigma factor antagonist for one of these six sigma factors, then overexpressing the downstream sigma factor should overcome the biofilm defect of a *sypA* mutant and we should see restoration of the wrinkled colony phenotype.

I was able to clone and overexpress 4 out of the 6 chosen sigma factors into a biofilm-inhibited strain ($\Delta sypA$, $\Delta sypE$, *psypG*), but I did not observe a restoration of the wrinkled phenotype in any of the overexpression strains (Figure 7). I was unable to clone

VF_2093 or VF_A0766, the two ECF sigma factors that are known to be controlled by specific anti-sigma factors. Expression of these two sigma factors seemed to be detrimental to *E. coli* and thus difficult to clone without transformation directly into *V. fischeri*.

Table 5: Sigma factors found in *V. fischeri*¹

	Gene	Sigma factor	Description	Known sigma factor antagonist	Over Expression Assay
1	VF_0387	σ^{54} (RpoN)	Controls motility, <i>syp</i> transcription, and bioluminescence		Unattempted
2	VF_0972	σ^E (RpoE2)	ECF sigma factor		Complete (KV7040)
3	VF_1834	σ^{28} (FliA)	Flagellar sigma factor that controls motility		Unattempted
4	VF_2067	σ^S (RpoS)	Controls cellular stress response and catalase activity RpoS over expression diminishes biofilms		Previously attempted
5	VF_2093	σ^E (RpoE)	ECF sigma factor	VF_2092 (RscA)	Incomplete
6	VF_2254	σ^{70} (RpoD)	Housekeeping sigma factor and essential gene		Unattempted
7	VF_2450	σ^{32} (RpoH)	Putative heat shock sigma factor		Unattempted
8	VF_2498	σ^E (RpoE5)	ECF sigma factor		Complete (KV7034)
9	VF_A0766	σ^E (RpoE4)	ECF sigma factor	VF_A0765 (ChrR)	Incomplete
10	VF_A0820	σ^E (RpoE3)	ECF sigma factor		Complete (KV7037)
11	VF_A1015	σ^Q (RpoQ)	Controls luminescence, chitinase activity, and motility Located close to the <i>syp</i> locus		Complete (KV7043)

¹Bold lettering was used to highlight the sigma factor genes that I attempted to overexpress

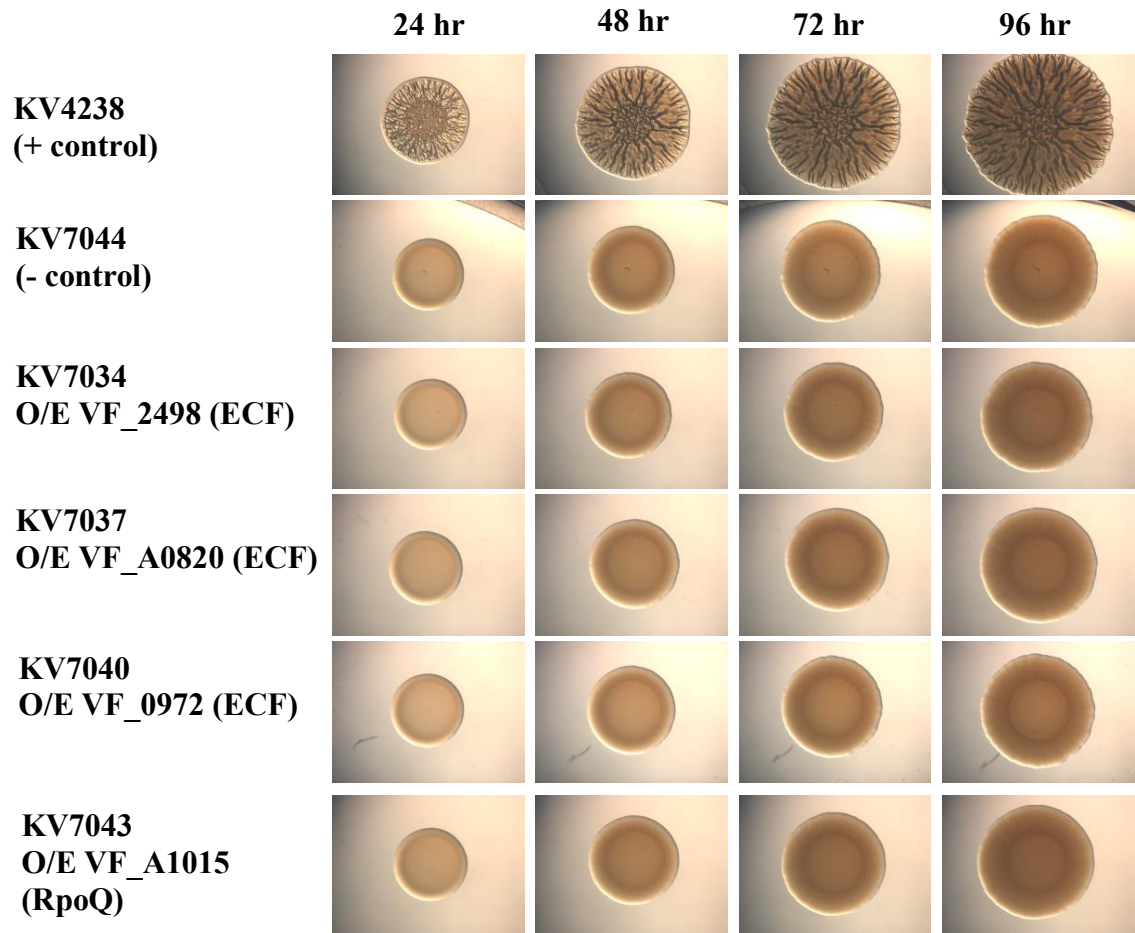


Figure 7: Wrinkling time course of the sigma factor over expression (O/E) assays. Strains KV7034, KV7037, KV7040, and KV7043 each contain the non-biofilm promoting background, $\Delta sypA$, $\Delta sypE$, $psypG$, as well as an overexpression plasmid containing a sigma factor.

Establish inner membrane isolation and co-immunoprecipitation protocols to determine if Syp proteins directly interact with SypA

Finally, I tested the hypothesis that SypA interacts with one or more Syp proteins to promote biofilm formation. To date, SypA is known only to interact with SypE, which controls its activity (Morris & Visick, 2013a; Morris & Visick, 2013b). SypA is predicted to act within the Syp pathway but downstream of *syp* transcription (Morris &

Visick, 2013a; Morris & Visick, 2013b). Syp polysaccharide production is essential for *syp*-dependent biofilm formation; *syp* mutants defective in producing the Syp polysaccharide have a non-sticky colony phenotype (Ray *et al.*, 2015). $\Delta sypA$ mutants also exhibit this non-sticky phenotype and therefore we predict that SypA may regulate the production of the Syp polysaccharide by interacting with another Syp protein(s). If this were the case, then SypA should physically interact with a downstream protein target, and we can identify such a protein. SypA is predicted to interact with other proteins to promote biofilm formation, but these downstream targets of SypA are unknown. Since SypA is predicted to act downstream of *syp* transcription and is essential for biofilm formation, it is not unreasonable to predict that SypA may interact with a Syp protein that is also required for biofilm formation and that these two proteins, or complex of proteins, may interact to promote biofilm formation.

One technique that could determine if any Syp proteins directly interact with SypA is co-immunoprecipitation. The Visick lab has an established protocol to determine direct protein interactions between soluble proteins and Andrew Morris used this method to show the direct interaction between SypA and SypE (Morris & Visick, 2013b). SypA and SypE are soluble proteins, but many of the other Syp proteins, such as SypK and SypL, are membrane-associated proteins (Shibata *et al.*, 2012). I therefore attempted to establish a protocol to determine if these proteins are directly interacting with SypA by isolating membrane-associated Syp proteins and performing co-immunoprecipitation assays, followed by Western blot analysis.

While establishing this protocol, I chose to use FLAG-tagged SypK as a representative membrane bound protein to determine if I could properly lyse the inner membrane fraction of the cells as well as isolate SypK-FLAG through the use of anti-FLAG antibody-conjugated magnetic beads. I chose to use SypK since the banding pattern of the Syp polysaccharide in a *sypK* mutant is similar to the polysaccharide banding pattern of a *sypA* mutant (Shibata and Visick unpublished data). Thus, it is reasonable to predict that SypA and SypK mutants have similar effects on Syp polysaccharide production and may be working together to transport polysaccharides across the inner membrane. Therefore, I could use the inner membrane isolation protocol and co-immunoprecipitation protocol to determine if SypA directly interacts with SypK.

Unfortunately, while performing the cell fractionation and inner membrane isolation, I was unable to consistently detect FLAG-tagged SypK. I could visualize SypK-FLAG in the whole cell lysate, but only infrequently was I able to detect SypK after isolating the inner membrane fraction. I was also unable to detect SypK-FLAG through precipitation with the anti-FLAG conjugated beads.

When I tried to separate the inner membrane fraction from the soluble fraction of the cells, SypA appears to be present in the inner membrane fraction as well as the soluble fraction (Figure 8). We were unsure if SypA actually associated with proteins within the inner membrane or if this was due to contamination of the inner membrane fraction. To ensure that SypA was not in the inner membrane fraction by accident, we used SypG-FLAG as a negative control to ensure that we had a clean inner membrane fraction. SypG is a transcription factor and is not predicted to associate with the

membrane, but SypG also appeared in both fractions (Figure 9). Therefore, my inner membrane isolation protocol was not successful and further optimization is required before co-immunoprecipitation should be attempted.

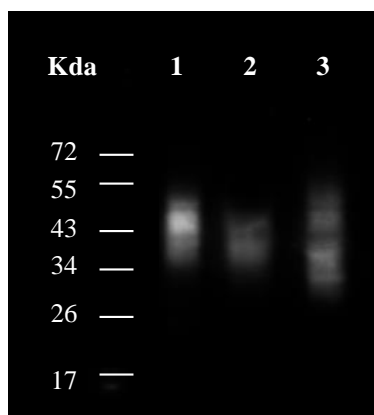


Figure 8: α HA Western blot showing the location of SypA-HA after performing the inner membrane isolation protocol. Lane 1 contains the whole cell lysate from the strain KV7042 ($\Delta sypA$, $\Delta sypE$, attTn7::*sypA*-HA, *psypG*, *psypK*-FLAG). Lane 2 contains the soluble fraction from KV7042. Lane 3 contains the Inner Membrane fraction from KV7042. 5 μ L of the WCL and 15 μ L of the soluble and the IM fractions were loaded into the wells.

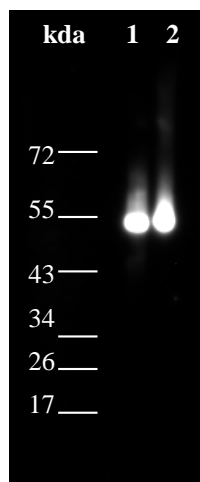


Figure 9: α FLAG Western blot showing the location of SypG-FLAG after performing the inner membrane isolation protocol. Lane 1 contains the soluble fraction of KV5452 (Δ *sypE*, *psypG*-FLAG). Lane 2 contains the Inner Membrane fraction from KV5452. 2 μ L of each sample were loaded into the wells.

Another difficulty encountered through these experiments was my Western Blot techniques. In my western blots, both the SypA-HA and SypA-FLAG tagged constructs appeared to run at a higher than predicted molecular weight. SypA is predicted to be a 17 kDa protein, which should run a little higher on a gel with an HA or FLAG tag attached. My SypA protein usually ran between 40-55 kDa, which is double the predicted size. My SypA proteins also continually ran in a large smear down the gel instead of clear and distinct bands (Figure 8).

Summary

I tried three distinct techniques to identify downstream targets of SypA with the goal of better understanding how SypA contributes to biofilm formation. I first used transposon mutagenesis to determine if SypA is acting as an inhibitor of another regulatory protein. Through the transposon mutagenesis, I was able to isolate one mutant

that had a delayed but distinct wrinkled phenotype in the absence of SypA. However, this wrinkled phenotype was not due to the transposon itself but may have been caused by a second-site mutation. Despite the large screen of transposon mutants, we cannot conclude that SypA is acting as an inhibitor.

The second tactic was to determine if SypA is acting as an anti-sigma factor antagonist for one of *V. fischeri*'s five ECF sigma factors or for RpoQ. I attempted to overexpress these various sigma factors in a *sypA* mutant strain to see if I could overcome the inhibitory effect of a putative anti-sigma factor and thus restore biofilm formation. I was successful in overexpressing three of the ECF sigma factors as well as RpoQ, but none of these overexpression strains exhibited biofilm formation. I was unable to clone and overexpress two of the ECF sigma factors (VF_2093 and VF_A0766); these two ECF sigmas are encoded adjacent to known anti-sigma factors, and it may be that the activity of these sigmas is detrimental to cell growth in the absence of their respective anti-sigma factors. It remains possible that SypA may be involved in controlling the activity of one of these two sigma factors.

The third approach to identify downstream targets of SypA and thus better understand SypA's contribution to biofilm formation was to determine if SypA directly interacts with any of the Syp structural proteins. More specifically, I asked whether SypA interacts with any of the inner membrane associated proteins. To answer this question, I attempted to establish a cell fractionation and co-immunoprecipitation protocol with SypA-HA and other FLAG-tagged Syp proteins. This approach was not successful and I was unable to properly separate the inner membrane from the soluble

fraction of the cell. Further optimization of the protocol is necessary before co-immunoprecipitation of the membrane-bound proteins can be performed.

Identify the critical amino acid residues essential for SypA activity

Introduction

The SypA primary amino acid sequence as well as the location of its gene within the *syp* locus is highly conserved among the *Vibrio* species. It was recently shown that the *sypA* homologues from *Vibrio vulnificus* and *Vibrio parahaemolyticus* are able to complement a *sypA* mutant in *V. fischeri* and restore biofilm formation (Thompson & Visick, 2015). If SypA is playing the same role within other *Vibrio* species to promote biofilm formation, then proteins with which SypA interact may also be conserved within these species. If this is true, it is reasonable to believe that the amino acid residues required for the protein interactions are also conserved. Therefore, we should be able to identify the highly conserved SypA amino acid residues among the *Vibrio* species and perform site-directed mutagenesis to confirm whether these residues are required for SypA function in promoting biofilm formation.

Identification of the amino acid residues essential for SypA activity may be used as a tool for investigating SypA's interaction with downstream proteins. To determine the amino acid residues critical for SypA activity, I performed site-directed mutagenesis on *sypA* and screened for mutants that failed to promote biofilm formation. I used site-directed mutagenesis to mutate conserved amino acid residues to discover the amino acid residues important for SypA activity.

Perform bioinformatics analysis to predict the SypA amino acid residues required for interaction with its downstream target

The STAS domain of SypA is highly conserved. To predict which amino acid residues may be important for SypA activity, I performed bioinformatics analyses to compare the amino acid sequence of SypA with SypA homologues in other *Vibrio* species and SypA homologues in other genera. We hypothesize that conserved residues in closely related *Vibrio* homologues may be more important for SypA's activity in regulating biofilm formation than the residues in non-*Vibrio* species whose STAS domain proteins do not promote biofilm formation.

I first performed a Blast search (<http://blast.ncbi.nlm.nih.gov/Blast>) to identify highly conserved SypA homologues in various *Vibrio* species (Figure 10). I then compared these conserved sequences with the amino acid sequences of more divergent STAS domain homologues of SypA in various genera whose protein function is known (Figure 11). These proteins included SpoIIAA and RsbV of *B. subtilis*, PA3347 of *P. aeruginosa*, BtrV of *B. bronchiseptica*, and RsbV of *C. trachomatis*. I aligned the sequencing using ClustalW and compared the sequences to the known SpoIIAA secondary structure (Kovacs *et al.*, 1998). I then analyzed the looped regions and alpha helices 2 and 3, which contain amino acid residues on various STAS domain homologues that are known to interact with other proteins (Babu *et al.*, 2010).

I picked four polar and charged amino acid residues that were conserved among the *Vibrio* species but not highly conserved in the other genera. These polar or charged

amino acid residues could be facing into the solvent and available to interact with other proteins. The four residues were K67, R68, Q84, and R93 (Figures 10, 11 and 12).

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Vibrio campbellii           MELRKQETAPNTLTIEFFGDLDAKGCRCQAQHQIDEVIHSDHHKEVEINLE
Vibrio harveyi ATCC BAA-1116 MELRKQETAPNTLTIEFFGDLDAKGCRCQAQHQIDEVIHSDHHKEVEINLQ
Vibrio harveyi HY01         MELRKQETAPNTLTIEFFGDLDAKGCRCQAQHQIDEVIHSDHHKEVEINLQ
Vibrio sp. HENC-02         MELRKLETPNLTIEFFGDLDAKGCRCQAQHQIDEVIHSDHHKEVEINLQ
Vibrio sp. AND4            MELRKQETANNTLTIEFFGDLDAKGCRCQAQHQIDEVIHTDHHKEVEISLQ
Vibrio alginolyticus 12G01 MELRKLEINQDKLAIIEFFGDLDAKGCRCQAQHHIDEVIHNNHKEVEINLQ
Vibrio sp. Ex25            MELRKLEINQDKLAIIEFFGDLDAKGCRCQAQHHIDEVIHNNHKEVEINLQ
Vibrio alginolyticus 40B   MELRKLEINQDKLAIIEFFGDLDAKGCRCQAQHHIDEVIHNNHKEVEINLQ
Vibrio parahaemolyticus RIMD 2210633 MELRKLEITQDKLAIIEFFGDLDAKGCRCQAQHHIDEVIHNNHKEVEINLQ
Vibrio sinoaloensis DSM 21326 MELRKIEASETILTTLVINGNLDAAGSRSAQPHIDEIADKKSEREIEIDFS
Vibrio sp. 16              MELRKIEASDTVLTLVINGNLDAAGSRSAQPHIDEIADKKSREIEIDFS
Vibrio sp. EJY3            MELRKIEINTDTVTWVINGNLDAAGSRSAQPHIDEIILAEPSTREIEIDFS
Vibrio tubiashii ATCC 19109 MELRKIEINTDTVTWVINGNLDAAGSRSAQPHIDEIILAENSREIEIDFS
Vibrio sp. N418            MELRKIEINTNELLTLVINGNLDAAGSRSAQPHIDEIISDLSHPEIEIDL
Vibrio scophthalmi LMG 19158 MELRKIEINTNELLTLVINGNLDAAGSRSAQPHIDEIISDLSHPEIEIDL
Vibrio ichtthyocenteri ATCC 700023 MEMRKIEINTNELLTLVINGNLDAAGSRSAQPHIDEIADLSHPEIEIDL
Vibrio coralliilyticus ATCC BAA-450 MELRKIEITNEILTQLIDGNDLDAAGSRSAQPHIDEIADQHG-EVEIDFS
Vibrio fischeri ES114      MELHQFESNEDIIVLSVQGDMDAIGCRDIIQPSID-SVIEQEHQVQIDLS
Allivibrio salmonicida LFI1238 MELHQFESNTNLTIVLSVQGDMDAVGCRDIQPTID-EVIEQHRQVHIDL
Vibrio nigripulchritudo ATCC 27043 MEMQRSEQSPQLLTLVLSVQGDMDAMGCKSLQPPQIDKVFEDTHHEVEIDL
**::: * .. .*: ** *.: * : * : ..*:

Vibrio campbellii           HVQFLDSSG:GAIIVVLYKRLVERERSMIIENVSQ:QPLEIMSLLRINHAIP
Vibrio harveyi ATCC BAA-1116 HVQFLDSSG:GAIIVVLYKRLVERERSMIIENVSQ:QPLEIMSLLRINHAIP
Vibrio harveyi HY01         HVQFLDSSG:GAIIVVLYKRLVERERSMSIENVSQ:QPLEIMSLLRINHAIP
Vibrio sp. HENC-02         HVQFLDSSG:GAIIVVLYKRLVERERSMIIENASG:QPLEIMSLLRINHAIP
Vibrio sp. AND4            HVQFLDSSG:GAIIVVLYKRLVERERSMSIENASG:QPLEIMSLLRINHAIP
Vibrio alginolyticus 12G01 HVQFLDSSG:GAIIVVLYKRLVERERSMSIENVSQ:QPLEIMSLLRINHAIP
Vibrio sp. Ex25            HVQFLDSSG:GAIIVVLYKRLVERERSMSIENVSQ:QPLEIMSLLRINHAIP
Vibrio alginolyticus 40B   HVQFLDSSG:GAIIVVLYKRLVERERSMSIENVSQ:QPLEIMSLLRINHAIP
Vibrio parahaemolyticus RIMD 2210633 HVQFLDSSG:GAIIVVLYKRLVERERSMSIENVSQ:QPLEIMSLLRINHAIP
Vibrio sinoaloensis DSM 21326 HVQFLDSSG:GAIIVVLYKRLIERQSRMRIENVSQ:QPLEIMNLLRIDQAIP
Vibrio sp. 16              QVQFLDSSG:GAIIVVLYKRLIERQSRMRIENVSQ:QPLEIMNLLRIDQAIP
Vibrio sp. EJY3            RVQFLDSSG:GAIIVVLYKRLIENQSRMRIENASG:QPLEIMNLLRIDQAIP
Vibrio tubiashii ATCC 19109 QVQFLDSSG:GAIIVVLYKRLVERQRNMRIENVSQ:QPLEIMNLLRIDQAIP
Vibrio sp. N418            HVGFLDSSG:GAIIVVLYKRLVERRRMRIENATG:QPLEIINLLRIDQAIP
Vibrio scophthalmi LMG 19158 RVGFLDSSG:GAIIVVLYKRLVERRRMRIENATG:QPLEIINLLRIDQAIP
Vibrio ichtthyocenteri ATCC 700023 NVGFLDSSG:GAIIVVLYKRLVERQRNMRIENATG:QPLEIINLLRIDQAIP
Vibrio coralliilyticus ATCC BAA-450 NVRFDSSG:GAIIVVLYKRLVERERQMRIENVVTG:QPLEIINLLRIDQAIP
Vibrio fischeri ES114      HVAFLDSSG:GAIIVVLYKRLIEKDRTMQIKNAHG:QPLELLKLLRIENAI
Allivibrio salmonicida LFI1238 QVAFLDSSG:GAIIVVLYKRLVEKERTMQIQNAHG:QPLELLKLLRIENAI
Vibrio nigripulchritudo ATCC 27043 QVSFLDSSG:GAIIVVLYKRLVEKKRVMRIENAHG:QPLELITLLRISNAIP
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Figure 10: SypA amino acid sequence alignment between species in the *Vibrio* genera. The wild type *V. fischeri* SypA amino acid alignment appears green and is aligned with SypA-like proteins in other *Vibrio* species. The four amino acid residues K67, R68, Q84, and R93 marked in red were chosen for site-directed mutagenesis. The purple boxed region is the highly conserved region with the S56 residue of SypA that is known to interact with and become phosphorylated/dephosphorylated by SypE.

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gi|16077538|RsbV|NP_388352.1|      MNINVDVKQNENDIQVNIAGEIDVYSAPVLREKLVPLAEQG--ADLRICL
gi|16079404|SpoIIAA|NP_390228.    MSLGIDMNVKESVLCIRLTGELDHHTAETLKQKVTQSLEKDDIRHIVLNL
gi|33600631|BtrV|NP_888191.1|     --MKLTMDKIDGMLIACLQGVVNSANAELAEELAAQVDKGE--RRVVLDL
gi|385244511|RsbV_1|YP_0058123    -MSNFQKEEQGQTGILHLQKLDGVSSPAVQESISESLSNG-MKNIILDC
gi|385244852|RsbV_2|YP_0058126    --MEWIAREYKNIFIVSLKGDMDAVTVPALEEFITKSIKAG-RVNVLLNM
gi|15598543|PA3347|NP_252037.1    MAITALPSADGQELTIQIQGRFDFAHQDFRDAYERVAITP--RRYVVDL
gi|59714203|SypA|YP_206978.1|     -MELHQFESNEDILVLSVQGDMDAIGCRDIQPSIDSVIEQEH-HQVQIDL
                                     : * ..      :
gi|16077538|RsbV|NP_388352.1|      KDVSYMDSTGLGVFVGTFKMVKKQGGSLKLENLSERLIRLFDITGLKDII
gi|16079404|SpoIIAA|NP_390228.    EDLSFMDSSGLGVILGRYKQIKQIGGEMVVCAISPVKRLFDMSGLFKII
gi|33600631|BtrV|NP_888191.1|     GRLDYISSAGLRVLLVAKQLRQVQGEVLVCELKPHVREVEFISGFLSIF
gi|385244511|RsbV_1|YP_0058123    GDLDYISSAGIRVLLQSYHQVGKNAGKIALTSVSKTVEQTLYVTGFLSYF
gi|385244852|RsbV_2|YP_0058126    EKVFYMSSAGLRLLSLAKLTQNHNGKLCICCLRDEVADIIRIAGLDKVI
gi|15598543|PA3347|NP_252037.1    RNATYLDSSALGMLLLLRDHAGGENAQISLANCSPEVRKILAIISNFEQLF
gi|59714203|SypA|YP_206978.1|     SHVAFLDSSGIGAIVYLYKRLIEKDRMTQIKNAHQPLELLKLLRIENAI
                                     ...*:::  : : : : :
gi|16077538|RsbV|NP_388352.1|      DISAKSEGGVQ-----
gi|16079404|SpoIIAA|NP_390228.    RFEQSEQQALLTLGVAS--
gi|33600631|BtrV|NP_888191.1|     PVANSREAAAAAFKTALPR
gi|385244511|RsbV_1|YP_0058123    KVFDVNEALQALAKENS-
gi|385244852|RsbV_2|YP_0058126    TIRKAEQESFSDF-----
gi|15598543|PA3347|NP_252037.1    KIS-----
gi|59714203|SypA|YP_206978.1|     PVNKTTH-----

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Figure 11: SypA amino acid sequence alignment with orthologs from various species in different genera. The wild type *V. fischeri* SypA amino acid alignment appears green and is aligned with SypA-like proteins in other non-*Vibrio* species. The amino acid residues marked in red (K67, R68, Q84, and R93) are not highly conserved between these strains and were chosen for site-directed mutagenesis.

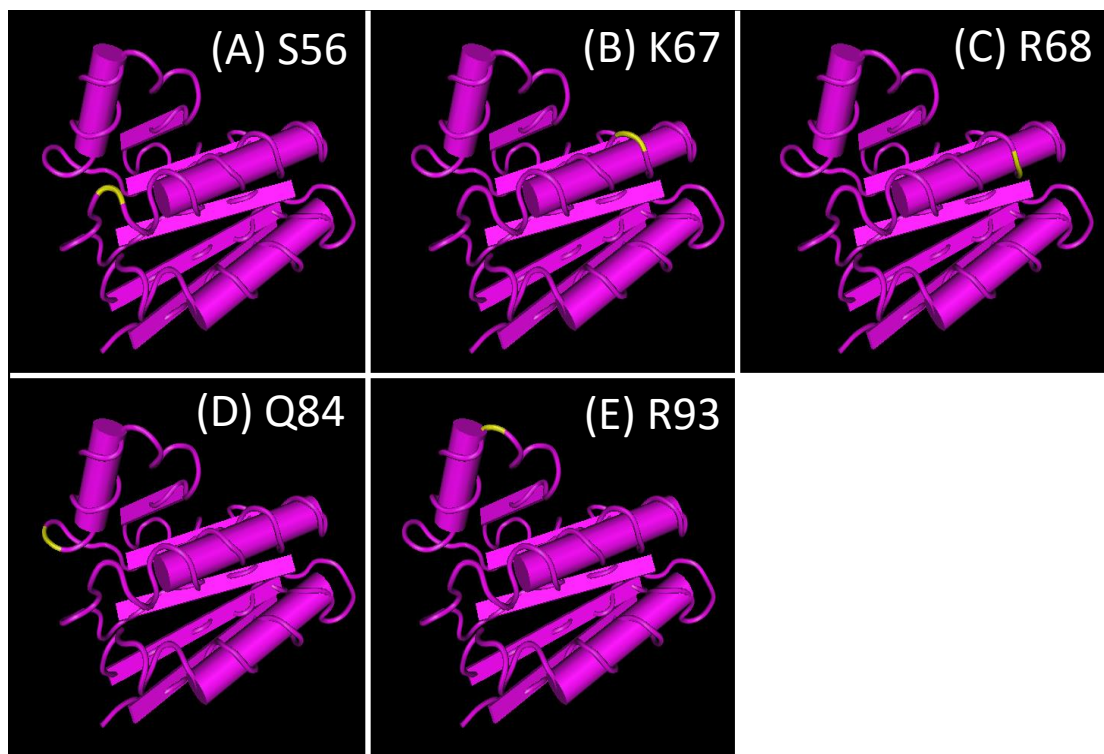


Figure 12: Predicted SpoIIAA tertiary structure. Panel (A) shows the location of the amino acid residue S56, which is highly conserved and known to interact with SypE. Panels (B-E) show the predicted location of the four amino acid residues selected for site directed mutagenesis. These polar or charged amino acid residues appear in yellow and are predicted to face the solvent and may be in an available position to interact with other proteins.

Perform site-directed mutagenesis to generate SypA proteins with altered amino acid residues

To determine if these amino acid residues are essential for SypA activity, I performed site-directed mutagenesis and converted the codons for these residues to encode alanine in the context of an HA-tagged *sypA* gene. To analyze the function of the mutant SypA proteins, I cloned the mutated *sypA* constructs onto plasmids and introduced the mutant alleles into the chromosome at the Tn7 site of a non-biofilm

producing $\Delta sypA \Delta sypE$, $psypG$ strain. If these amino acid residues are important for SypA activity, then the mutant *sypA* should not be able to complement the $\Delta sypA$ strain and the strain should not be able to form biofilms. However, non-complementation does not mean that those amino acid residues were important for SypA function. Changing specific amino acid residues may disrupt SypA protein stability. Therefore, to determine if the mutant SypA proteins are made *in vivo*, I used Western blot analysis, probing for the HA-tagged SypA protein. If I can detect HA-tagged SypA, then I can conclude that the mutant protein is produced *in vivo* and that the amino acid residues may be responsible for the mutant SypA's inability to complement a $\Delta sypA$ strain. However, further analysis will be necessary to confirm that the protein is properly folded.

One of the four mutants, *sypA*^{R93A}, was able to complement a *sypA*-deleted strain and form wrinkled colonies (Figure 13). Although this mutant had a slightly delayed wrinkled phenotype, *sypA*^{R93A}'s ability to restore wrinkled colony formation leads us to believe that this conserved arginine residue is not essential for SypA's ability to promote biofilm formation.

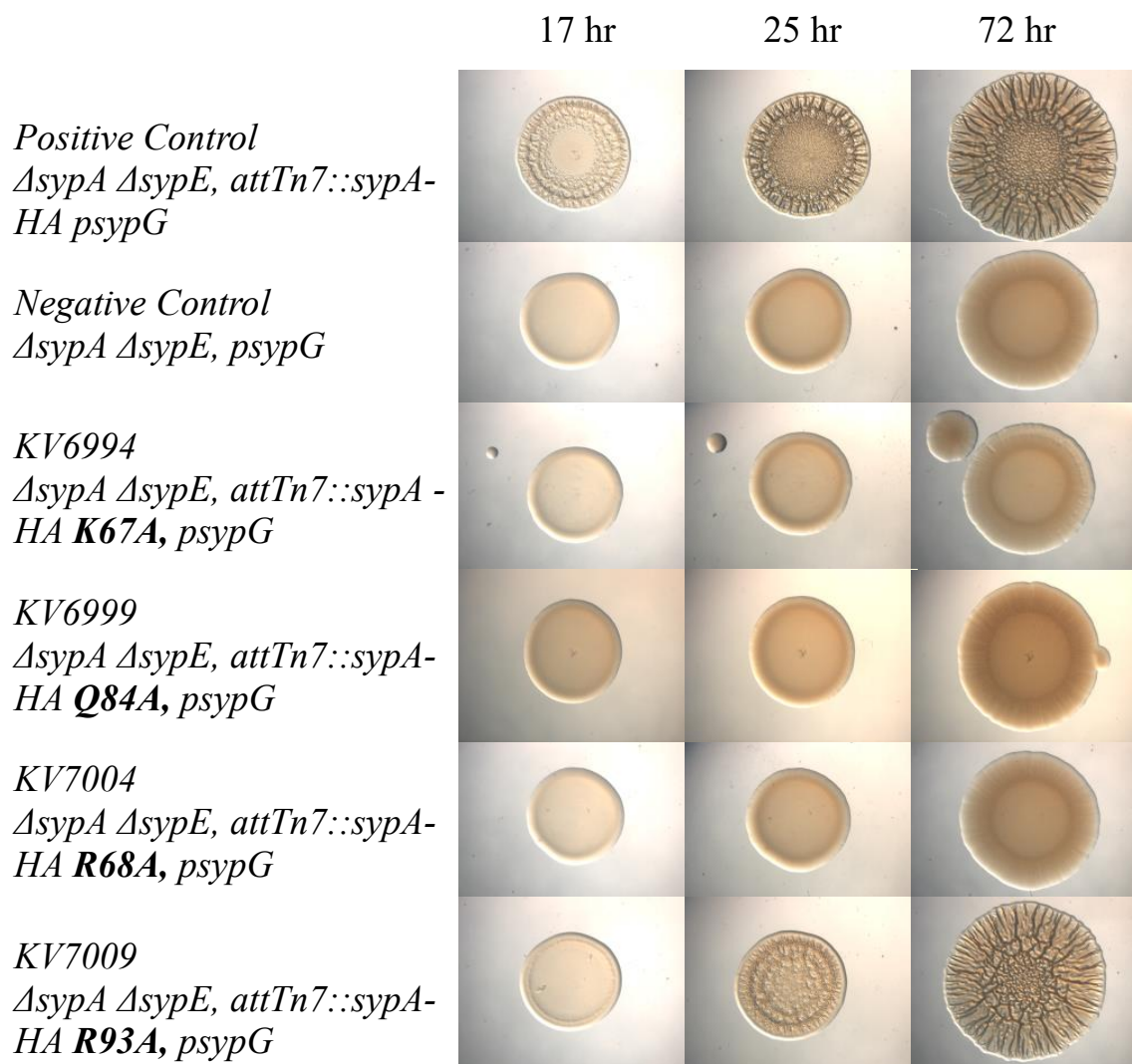


Figure 13: Wrinkling time course of SypA point-mutant complementation assays.

Two of the mutants, *sypA^{K67A}* and *sypA^{R68A}*, failed to form wrinkled colonies and were thus unable to complement a *sypA* deletion (Figure 13). I was able to detect these proteins by Western blot (Figure 14) and therefore, we can conclude that the proteins are made *in vivo*. The inability of these mutant SypA proteins to complement a *sypA* deletion

indicates that these two residues are required for SypA's ability to promote biofilm formation. These two mutants can now be used as genetic tools to identify second-site suppressor mutations in *Vibrio fischeri*.

The fourth mutant, *sypA*^{Q84A}, also failed to form wrinkled colonies (Figure 13), but when detected by Western blot, the protein appeared to be produced in a smaller quantity than the other SypA point-mutants or wild type SypA (Figure 14). Coomassie staining indicates that relatively similar amounts of protein were loaded in each lane (Figure 15).

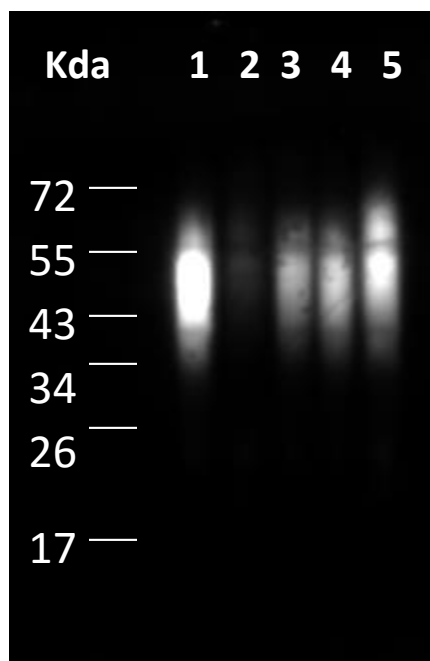


Figure 14: α HA Western Blot of SypA point mutants. The samples are standardized to an OD₆₀₀ and resolved on a 12% gel. Lane 1 contains KV6994 (Δ *sypA* Δ *sypE*, *attTn7::sypA-HA K67A*, *psypG*). Lane 2 contains KV6999 (Δ *sypA* Δ *sypE*, *attTn7::sypA-HA Q84A*, *psypG*). Lane 3 contains KV7004 (Δ *sypA* Δ *sypE*, *attTn7::sypA-HA R68A*, *psypG*). Lane 4 contains KV7009 (Δ *sypA* Δ *sypE*, *attTn7::sypA-HA R93A*, *psypG*). Lane 5 contains the positive control KV6591 (Δ *sypA* Δ *sypE*, *attTn7::sypA*⁺-*HA*, *psypG*).

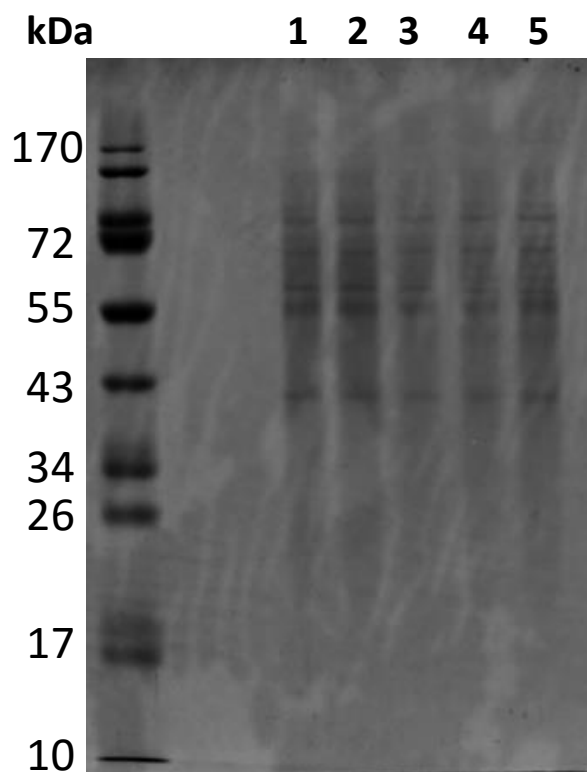


Figure 15: Coomassie-stained gel of cell extracts containing SypA point mutants. The samples are standardized to an OD_{600} and run on a 12% gel. Lane 1 contains KV6994 ($\Delta sypA \Delta sypE$, *attTn7::sypA-HA K67A*, *psypG*). Lane 2 contains KV6999 ($\Delta sypA \Delta sypE$, *attTn7::sypA-HA Q84A*, *psypG*). Lane 3 contains KV7004 ($\Delta sypA \Delta sypE$, *attTn7::sypA-HA R68A*, *psypG*). Lane 4 contains KV7009 ($\Delta sypA \Delta sypE$, *attTn7::sypA-HA R93A*, *psypG*). Lane 5 contains the positive control KV6591 ($\Delta sypA \Delta sypE$, *attTn7::sypA⁺-HA*, *psypG*).

Determine if SypE recognizes SypA R93A

Arginine 93 of SypA is not essential for promoting biofilm formation since the mutants, *sypA^{R93A}*, was able to complement a *sypA*-deleted strain and form wrinkled colonies, but it may be required for SypE recognition. SypE is required for phosphorylating SypA, which prevents SypA from promoting biofilm formation. If this R93 residue is required for SypE recognition, then mutating the arginine 93 to an alanine

should prevent SypE recognition and prevent SypA phosphorylation. Normally, a $\Delta sypA, psypG$ strain fails to form wrinkled colonies because SypE is present and able to phosphorylate SypA. We predicted that if the R93 residue is important for SypE recognition then SypE should not be able to recognize SypA^{R93A} and SypA^{R93A} should be able to promote biofilm formation regardless of the presence of SypE. To test whether R93 is required for SypE recognition, I introduced the *sypA*^{R93A} allele into the Tn7 site of a $\Delta sypA$ strain that was over expressing *sypG*. Contrary to our hypothesis, this strain was unable to form wrinkled colonies (Figure 16). We therefore believe that the R93 residue is not required for SypE's recognition of SypA.

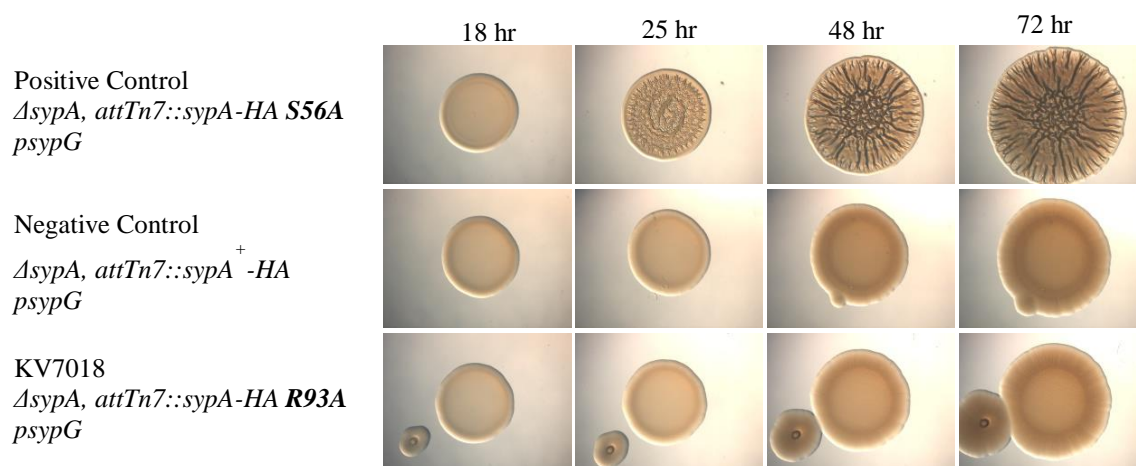


Figure 16: Wrinkling time course of the SypA R93A mutant in the presence of SypE.

Summary

Through the use of bioinformatics and site-directed mutagenesis, I was able to identify three amino acid residues on SypA that appear to be required for biofilm

formation. These residues are K67, Q84, and R68. These polar or charged amino acids may be required for SypA to interact with other proteins to promote biofilm formation in *V. fischeri*. Further analysis must be performed to determine if these SypA mutants are properly folded, but once that is determined, these mutants can be used in by-pass suppressor screens to identify downstream targets of SypA.

CHAPTER 4

DISCUSSION

Overview

I investigated mechanisms by which arabinose and SypA may be contributing to the formation of two distinct biofilms in *V. fischeri*. L-arabinose serves as a unique signal to promote the formation of a brittle biofilm located at the air-liquid interface of liquid *V. fischeri* cultures. I performed a random transposon mutagenesis to identify genes required for this cellular response to arabinose and I was able to identify various genes that are involved in the production of the arabinose-induced biofilm. SypA is a small protein essential for *syp*-dependent biofilm formation. I attempted to identify downstream protein targets of SypA that are involved in the *syp*-dependent biofilm formation through a random transposon mutagenesis screen. In a second approach, I used sigma factor over-expression assays to determine if SypA acted as an anti-sigma factor antagonist. I attempted to establish an inner membrane isolation protocol that could be used for co-Immunoprecipitation assays to determine whether SypA interacts with membrane bound Syp Proteins. Finally, I identified critical amino acid residues in SypA that are required for Syp-dependent biofilm formation.

Genetic characterization of arabinose-induced biofilms

The arabinose-induced biofilm is a newly discovered biofilm with a brittle phenotype found at the air/liquid interface of static cultures. The Visick lab attempted to characterize this biofilm and identify the genes involved in this response to arabinose. We used transposon mutagenesis and screened for mutants that failed to display this biofilm in the presence of arabinose. From a screen of over 8,000 mutants, we were able to identify 21 distinct genes that were necessary for the production of this biofilm.

Although a few genes were identified in independent screens and a few operons had multiple independent insertions, this study was not a complete characterization of the genes involved. For a saturating study, we should have seen multiple hits in each of the genes. There are close to 4,000 genes in the *V. fischeri* genome and although we found genes involved in motility, pilus production, and possibly arabinose transport, we did not find genes involved in transcription or arabinose metabolism. These missing pieces are still unknown and further analysis is required to fully characterize the genes required for the biofilm production.

While a Tn screen is a useful tool to identify necessary genes, it cannot be used to identify either redundant or essential genes. Therefore, there are many other genes that could possibly be involved in this particular phenotype, but other tools/approaches are needed to identify them. Another problem with the use of a transposon is that many of the genes identified were located within or near operons. Therefore, it is unclear whether the specific gene identified is responsible for the phenotype, or if the transposon is polar on another gene(s) within the operon.

In this study, I identified three genes that contribute to the arabinose-induced biofilm phenotype and that do not appear to be involved in arabinose uptake. I did not complement *VF_1896* or *VF_A0685*, so it is unclear if these genes are involved in the biofilm phenotype or if a secondary mutation somewhere in the chromosome is responsible for the loss of biofilm formation. We did identify *VF_1896* in two independent mutagenesis screens; thus, we are more confident that this gene encoding a phosphoenolpyruvate-protein phosphotransferase could be involved in biofilm formation. *VF_1812* was the only gene I was successfully able to complement, but we do not understand the role a long-chain fatty acid transport protein plays in biofilm formation. Also, the *VF_1812* mutants appear to have a slight growth defect in the presence of arabinose. This phenotype raises the question of whether *VF_1812* is required for the production of the biofilm, if *VF_1812* protects the cells against the harmful effects of arabinose, or if the biofilm defect is an artifact stemming from the relatively poorer growth of the mutant.

In the laboratory, arabinose is used as a signaling molecule to induce the *ara* promoter, which is often used in promoter fusions to control the expression of specific genes. If arabinose can induce other signaling cascades besides the promoter under investigation, then the presence of arabinose may alter the phenotype of the cells in question and confuse or cause misinterpretation of the data. As a result, it is important to understand the impact of arabinose on the physiology of *V. fischeri*.

Transposon mutagenesis of SypA: Bypass Suppressor screen

To further characterize the role of SypA in biofilm formation, I performed a series of Tn mutagenesis experiments to identify bypass suppressors of a *syp*-induced $\Delta sypA$ strain. In theory, this screen would work if SypA is playing a regulatory role and acting to inhibit an inhibitor of biofilm formation. This, however, does not seem to be the case. I screened more than 30,000 mutants and was able to find only one Tn insertion that restored biofilm formation in the absence of *sypA*. However, the biofilm phenotype also occurred when I moved the Tn-disrupted gene into a fresh background.. This indicated that a secondary mutation is most likely responsible for the restoration of the biofilm phenotype.

What could this second site mutation be? One possibility is that the mutation is within a protein that interacts with SypA and that somehow the mutation compensates for the lack of SypA. A second possibility is that the mutation occurs within one of the STAS domain proteins encoded by *V. fischeri* such as the NTP-binding protein *VF_0399* or the sulfate transporter *VF_AI052*. Although much less likely, it is formally possible that this mutation alters one of the homologues such that it can mimic SypA and restore biofilm formation. A full sequence analysis of the strain KV6941 should be performed to identify this mutation.

Through these mutagenesis experiments, I was unable to identify a downstream target of SypA. Consequently, SypA does not appear to inhibit an inhibitor of biofilm formation. Therefore, we are left with two other possibilities, SypA may be acting as a regulator by positively controlling another positive regulator of biofilm formation or

SypA may be playing a structural role by interacting with other proteins to promote biofilm formation. These possibilities would not be recognized in a transposon mutagenesis screen, but a full sequence analysis of the strain KV6941 should shed light on one of these two possibilities.

SypA not a likely anti-anti sigma factor

SypA contains only a single STAS domain and most of the single STAS domain proteins studied to date, such as SpoIIAA and RsbV in *B. subtilis*, act as anti-sigma factor antagonists. We hypothesized that SypA was also acting as an anti-sigma factor antagonist and tested this hypothesis by overexpressing sigma factor genes encoded by *V. fischeri*; we anticipated that high levels of the target sigma factor, achieved by overexpression of its gene, could “overwhelm” the anti-sigma factor in a $\Delta sypA$ strain to restore biofilm formation. None of the putative target sigma factor genes that I overexpressed, however, was able to restore biofilm formation to the *sypA* mutant.

Specifically, I attempted to overexpress six sigma factor genes, including those that encode the five ECF sigmas as well as RpoQ. I successfully cloned and overexpressed the genes for RpoE2, RpoE3, RpoE5, and RpoQ, but the presence of excess sigma factor was unable to restore biofilm formation in these strains. I was unable to successfully clone the genes for the two remaining ECF sigmas, RpoE and RpoE4. These two troublesome sigmas are the only sigma factors in *V. fischeri* associated with known anti-sigma factors. Thus, one possible explanation for the inability to clone these sigma factor genes is that, without their respective anti-sigma factors present, the sigma factors were harmful to the *E. coli* strains. In support of this possibility, the only colonies

able to survive the transformation encoded a mutated version of the *V. fischeri* ECF sigma. In order to overcome the problem of cloning in *E. coli*, we could consider separately cloning the anti-sigma factor gene on a compatible plasmid.

Although there is a slight possibility that SypA acts as an anti-sigma factor antagonist for either RpoE or RpoE4, it is unlikely due to the Tn screen mentioned above. If SypA was an anti-sigma factor antagonist, then its associated anti-sigma factor should have appeared in our bypass suppressor screen. These data further support our view that SypA is not acting as an inhibitor of an inhibitor of biofilm formation.

If SypA is not an anti-sigma factor antagonist as its single STAS domain predicts, what role could it play in promoting biofilm formation? Not all proteins that contain STAS domains are anti-anti-sigmas. RsbR and RsbS are single STAS domain proteins that help form the stressosome in *B. subtilis* (Sharma *et al.*, 2011). One single STAS domain protein, YP_749275.1 from *Shewanella frigidimarina*, appears to be associated with a lipid bilayer, although its function is unclear (Kumar *et al.*, 2010). Other STAS domains are found within multi-domain proteins and act as sensors and anion transporters across the membrane such as the SulP/Slc26 family of proteins (Aravind & Koonin, 2000).

Removing SypA from the category of anti-anti-sigma factor opens up a broad range of functional possibilities. One possibility is that SypA acts as a signaling molecule and activates one of the other Syp proteins. Alternatively, SypA could bind to a protein within the inner membrane and, together with that target, operate in a similar

fashion to a Slc26/SulP transporter. If this is the case, then phosphorylation of the S56 residue of SypA may interfere with these interactions and prevent biofilm formation.

The *syp* locus contains many membrane-associated and transmembrane proteins such as glycosyltransferases and the flippase SypK. Perhaps SypA associates with one of these Syp proteins to promote biofilm formation by regulating the production and/or export of the Syp polysaccharide. The presence of Syp polysaccharide is essential for *syp*-dependent biofilm formation. SypA may be involved in Syp polysaccharide production or export since *sypA* mutants display a non-sticky colony phenotype, indicating an absence of Syp polysaccharide.

Attempted inner membrane isolation protocol

To determine if SypA interacts with any of the Syp proteins associated with the membrane, I attempted to establish an inner membrane isolation protocol for the lab that could be used for future co-immunoprecipitation assays. My attempt at cell fractionation was not successful. I used FLAG-tagged SypK as my target protein of choice, but was never able to properly separate the inner membrane fraction from the whole cell lysate since SypG-FLAG appeared in both fractions. Why was this attempt unsuccessful? Perhaps I was unable to properly lyse the cells and intact cells remained in the pellet fraction, thus contaminating the inner membrane fraction with soluble proteins. Another possibility is that I did not thoroughly wash the pellet fraction and some soluble protein remained in the inner membrane fraction.

Another difficulty encountered through these experiments was my Western blot techniques. My SypA proteins consistently appeared as a large smear on the Western blot

instead of clear and distinct bands. In my Western blots, both the SypA-HA and SypA-FLAG tagged constructs appeared to run at a higher than predicted molecular weight. SypA is predicted to be a 17 kDa protein, which should run a little higher on a gel with an HA or FLAG tag attached. My SypA protein usually ran between 40-55 kDa, which is double the predicted size. I am unsure as to why my SypA proteins runs in such a strange fashion, but Cecilia Thompson in the Visick lab has shown that the type of gel affects the way SypA runs on a Western Blot. When Cecilia uses a pre-made commercial gel, her SypA protein runs close to the predicted size and runs/appears as a tight band instead of my smears (Thompson & Visick, 2015).

Identify the critical amino acid residues essential for SypA activity

To determine the amino acid residues critical for SypA activity, I compared the amino acid sequence of SypA to homologues found in other similar *Vibrio* species as well as those found in more divergent genera. I picked four polar and charged amino acid residues, K67, R68, Q84, and R93, which were conserved among the *Vibrio* species but were not highly conserved in the other genera. These four residues were located on the looped regions and alpha helices 2 and 3, which contain amino acid residues that in some STAS domain homologues are known to interact with other proteins (Babu *et al.*, 2010). I performed site-directed mutagenesis on these residues in SypA, replacing them with alanine and screened for mutants that failed to promote biofilm formation.

In my analysis, one mutant, SypA^{R93A}, did not appear to affect either biofilm formation or SypE inhibition. We can conclude that this residue is not essential for promoting biofilm formation. I was, however, able to identify three amino acid residues,

K67, Q84, and R68, which appear to be required for biofilm formation since the mutations disrupted the ability of *V. fischeri* to form a biofilm. These polar or charged amino acids may be required for SypA to bind to or interact with other proteins and their absence may be interfering with these necessary interactions. These mutants can be used in future bypass suppressor screens to identify downstream targets of SypA.

Through Western blot analysis, I was able to show that these Syp mutants were present in the cells, but I am unable to determine, with this approach, if these proteins are properly folded. Therefore, these mutants might prevent biofilm formation by preventing the proper folding of SypA. One future approach to determine if these proteins are properly folded is to perform a Phos-tag experiment as described in Morris & Visick, 2013. If the mutant Syp proteins are able to be phosphorylated, then we know that they are folded enough to be recognized by SypE.

The mutated protein SypA^{Q84A} appeared to be present in a lower quantity than the other Syp mutants. Therefore, it is unclear if the mutation itself is interfering with biofilm formation or if SypA is not abundant enough to perform its required role in biofilm formation. Perhaps this mutation may impact the stability of SypA.

SypA questions regarding GTP binding and hydrolysis

Many STAS domain proteins such as SpoIIAA are able to bind to and hydrolyze GTP (Mahmoud *et al.*, 1996; Sharma *et al.*, 2011). Since SypA contains a STAS domain, there is a possibility that it also binds to and hydrolyzes GTP. Although we have not yet looked into this possibility, the idea that SypA interacts with GTP raises many interesting questions.

First, could GTP/GDP binding cause conformational changes to SypA and whatever other protein it binds to? Some proteins that contain a STAS domain, such as the SulP/Slc26 transport protein Rv1739c from *M. tuberculosis* (Sharma *et al.*, 2011; Sharma *et al.*, 2012; Sharma, Ye, *et al.*, 2011), change conformation due to GTP binding. These SulP/Slc26 transport proteins contain an N-terminus trans-membrane domain and a C-terminus cytoplasmic STAS domain and are involved in anion transport across the membrane. It has been shown that the conformational changes in the STAS domain of SulP/Slc26 transport protein YeSlc26A2 from *Yersinia enterocolitica* are required for anion transport (Compton *et al.*, 2011). Could SypA act in a similar way? If SypA does bind to GTP causing conformational changes to the SypA-protein complex, could these changes in SypA aid in either the production or transport of Syp-polysaccharide? One hypothetical possibility is if SypA binds to an inner membrane protein (like SypK) creating a complex or a faux/hybrid SulP/Slc26 transport protein, then GTP hydrolysis from SypA might help power/control enzymatic activity (*e.g.*, activate the SypK flippase).

My second main question is, when SypA is phosphorylated, could this interfere with the ability of GTP to bind? If GTP binding or hydrolysis is necessary for Syp-polysaccharide production or transport and phosphorylated SypA is unable to promote biofilm formation, then the phosphate may interfere with GTP binding and inhibit Syp-polysaccharide production. The GTP binding site of SpoIIAA is located near the conserved serine residue (Kovacs *et al.*, 1998; Mahmoud *et al.*, 1996). It is not unreasonable to suggest that the phosphate on S56 of SypA may inhibit GTP binding.

Third, do any of the point mutations interfere with GTP binding or hydrolysis?

Assuming our point mutants are folded correctly, and if these mutations are found to inhibit GTP binding, then we can hypothesize that GTP binding to SypA and or hydrolysis is required for biofilm formation.

Although these are intriguing questions, we would first have to verify whether GTP does bind to SypA *in vivo*.

Significance

For my thesis, I studied *V. fischeri* and its ability to form two distinct biofilms, the arabinose-induced biofilm and the *syp*-dependent biofilm. Biofilm formation is an important stage in the life cycle of many bacteria. It is required for them to survive and persist in harsh environments as well as to colonize particular habitats. Studying the arabinose-induced biofilm allowed us to identify various genes that are required to respond to the arabinose signal and form a biofilm. This mutagenesis and gene screening approach led us to more questions than answers, but it allowed us to appreciate the complex interaction between environmental stimuli and the bacterial response. Does *V. fischeri* form this biofilm in the wild? Is the formation of this biofilm a consequence of how *V. fischeri* evolved to protect itself against the toxic effects of arabinose (or a similar, more environmentally-relevant molecule)? Or is the biofilm a remnant of an evolutionary pathway that once allowed a distant ancestor of *V. fischeri* to colonize sources that contain arabinose in order to metabolize arabinose and use it as a carbon source? These and many more questions have yet to be answered, but this study helped

open the doors to these new ideas about the relationship between arabinose and *V.*

fischeri.

The study of SypA and its role in the *syp*-dependent biofilm has led us down new pathways for the possible function of this STAS protein. As a result of these experiments, we have de-emphasized the hypothesis that SypA is acting as an anti-sigma factor antagonist, or any inhibitor of an inhibitor of biofilm formation. Instead, we hypothesize that SypA is playing a positive role, perhaps by directly interacting with a Syp protein to aid in the production of the Syp polysaccharide. I was able to find, although not identify, a bypass suppressor of SypA and identify three amino acid residues within SypA that appear to be required for biofilm formation. These mutant strains can be used as tools to further analyze SypA's ability to promote biofilm formation in *V.*

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VITA

Sheila McEwen is originally from Ingleside, IL. She attended The Willows Academy for high school graduated as the Salutatorian of the class of 2004. She later attended The University of Dallas where she graduated *Cum Laude* in 2008 with a major in Biochemistry and a French concentration. After college, she taught biology and chemistry for three years at Oackrest School in McLean, VA.

Sheila began her graduate studies at Loyola University of Chicago in the fall of 2011. She joined the lab of Dr. Karen Visick in the spring of 2012 and focused her research on the role of arabinose and SypA in *V. fischeri* biofilm formation.

She moved to Miami, FL in the summer of 2013 where she taught high school biology at Our Lady of Lourdes Academy. She currently lives in Texas with her husband and daughter. In the future, Sheila hopes to continue to raise her family and to once again teach high school biology.