Investigating the Role of Cysteine in Biofilm Formation by Vibrio fischeri

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LOYOLA UNIVERSITY CHICAGO

INVESTIGATING THE ROLE OF CYSTEINE IN BIOFILM FORMATION BY

VIBRIO FISCHERI

A THESIS SUBMITTED TO

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ABSTRACT

Biofilms are structured multicellular communities of microorganisms attached to a surface and embedded in a matrix generally comprised of polysaccharides, proteins and DNA. Biofilms are an important area of medical research because bacterial biofilms can form on implants such as catheters and are highly resistant to antibiotics and other antimicrobials. One good model to study biofilm formation is the mutualistic symbiosis between *Vibrio fischeri* and its host, the Hawaiian bobtail squid *Euprymna scolopes*, because genes known to be important for biofilm formation are also critical for colonization. Specifically, *V. fischeri* has an 18 gene polysaccharide locus, *syp*, that is required for biofilm formation and colonization.

A recent mutant study suggested the possibility that cysteine biosynthetic genes were involved in biofilm formation by *V. fischeri*. In this study, several mutants were obtained that had transposon insertions in cysteine metabolic genes, including *cysH*, *cysJ*, *cysK*, and *cysN*. To investigate the possible requirement for *cys* genes in biofilm formation, we generated in-frame deletion mutants of *cysK* and *cysH* and assessed biofilm formation by evaluating the development of wrinkled colonies over time. My results supported a role for *cysK* and, to a lesser extent, *cysH* in biofilm formation. The addition of extra cysteine to the medium restored wrinkled colony formation to the *cysK*
mutant strain. These data confirmed the requirement for cysteine in wrinkled colony formation.

My subsequent characterization of the cysK and cysH mutants revealed that these mutants exhibit a minor growth defect in a complex medium and a severe growth defect in minimal medium; both defects were complemented by the addition of 1 mM exogenous cysteine. It is formally possible that this growth defect accounts for the biofilm defect. However, our current evidence indicates that, while all the cys mutants exhibit similar growth defects, only the cysK mutant has a severe defect in wrinkled colony formation. I thus conclude that cysK is more important than any other cys genes for biofilm formation by V. fischeri.

I have also observed that cysK mutant forms colonies that are self-adherent; a phenotype that we predict is due to the production of the syp polysaccharide. Thus, the cysK mutant appears to grow well enough for syp production. Thus, these observations indicate that the growth defect due to the lack of cysteine does not fully account for the defect in wrinkled colony formation by V. fischeri but, rather, these observations indicate that cysteine plays some important role in biofilm formation by V. fischeri.

Finally, I have data that suggest a role for cysK in early symbiotic colonization by V. fischeri. When squids were inoculated with a V. fischeri cysK mutant for a short incubation time, little to no colonization occurred. In contrast, when the squid were inoculated with the cysK mutant for an extended period, they became readily colonized. These data suggest a requirement for cysteine in the early stages of colonization by V. fischeri.
Thus, my work has identified non-syp genes that play an important role in biofilm formation. This work thus provides insight into additional factors required for biofilm formation, and provides an explanation for an old observation that a cysteine auxotroph of *V. fischeri* was defective for squid colonization, by demonstrating that cysteine promotes biofilm formation, which is required for colonization.
CHAPTER ONE

LITRATURE REVIEW

I.A. Introduction

Bacteria are found within a wide variety of environments. Some bacteria cause infections, but most of them are harmless. Many of these harmless bacteria live in harmony with a host, and provide beneficial functions e.g., bacteria in the gut of the human body help in the digestion of food and help to maintain the immune system (O’Hara et al., 2006). Biofilm formation is one of the mechanisms by which bacteria interact with a host. Biofilms are multicellular microbial communities surrounded by an extracellular matrix. Bacteria use biofilms to protect themselves from environmental insults such as antibiotic treatments (Absalon et al., 2011) and to escape the immune system of the host (Costerton 1999). Many pathogens and symbionts use biofilms to colonize, survive, and persist in their host. Therefore, to understand the interaction between host and bacteria, it is important to understand how biofilms are formed and regulated by bacteria. One good model system to study biofilm formation and its regulation is the symbiosis between the marine bacterium Vibrio fischeri and its host, the Hawaiian bobtail squid Euprymna scolopes. To efficiently colonize the squid, V. fischeri must be able to form a biofilm.
In the introduction of this thesis, I will first describe the process of biofilm formation in general. Then, I will discuss biofilm formation and the process of colonization by *V. fischeri*. Lastly, because my work focused to confirm and probe for the requirement for cysteine biosynthetic genes in biofilm formation, I will describe the cysteine biosynthetic pathway.

### I.B. Biofilms

A biofilm is a group of microorganisms in which cells stick to each other and adhere to a surface. Bacteria within biofilms have an increased ability to survive and persist in the environment or on/in their host. Bacterial biofilms can form on biotic surfaces such as teeth, or on abiotic surfaces such as indwelling catheters. It is estimated that 80% of bacterial infections involve biofilms (Fux *et al.*, 2005; Davies, 2003; Hancock *et al.*, 2010). Biofilms can also form inside water pipes, and clog them. Bacteria within biofilms are difficult to eradicate, because they can easily evade the immune system and have high resistance to antibiotics (Costerton 1999) Due to high medical and industrial relevance, it is important to understand the process of biofilm formation, so that we can have better methods to treat biofilms.

### I.C. Process of biofilm formation

Biofilm formation is an organized, stepwise, and tightly regulated process. This process initiates when bacteria sense some signal(s) from the environment, and involves 3 main stages (Hall-Stoodley & Stoodley 2002; Hall-Stoodley *et al.*, 2004) Attachment to
a surface b) Growth and maturation c) Dispersal (Fig. 1.). The specific signals that initiate the transitions between different stages are largely unknown; however, it has been shown that chemical, mechanical, nutritional, quorum-sensing, and host-derived signals can help the biofilm to transition into different stages.

(a). Attachment

The first step in biofilm formation is attachment. The process of bacterial attachment to living and nonliving surfaces is different, and is accomplished by different bonds. The attachment between bacteria and a non-living surface is accomplished by nonspecific interactions, for example, hydrophobic interactions. In contrast, the attachment between bacteria and living surfaces is mediated by strong molecular bonds that involve multiple cellular molecules (Dunne, 2002; Kline et al., 2009; Koczan et al., 2011).

Conditioning of nonliving surfaces is an important phenomenon by which bacteria form a biofilm on nonliving surfaces, e.g., Teflon and stainless steel (Carpentier and Cerf 1993; O’Toole et al., 2000; Costerton et al., 1995). The conditioned layer has many organic and inorganic particles (Garrett et al., 2008). Conditioning of the surface helps bacteria to overcome the repulsive forces of the surface (Donlan, 2002). Bacteria are capable of sensing these surfaces, and once bacteria sense the surface, they can attach to it (O’Toole et al., 2000). The initial attachment to the surface is weak and is strengthened by flagellar interactions with the surface. Examples of organisms for which initial attachment has been studied include Listeria monocytogenes (Lemon et al., 2007) and Pseudomonas aeruginosa (O’Toole et al., 2000). Later in this process, the bacteria uses
other appendages like pili, and fimbriae to help remain attached to the surface. Even at this stage with all these attachments where pili, flagella and other appendages are involved to provide tight interaction with surface, the bacterial cell retains its ability to leave the surface and re-enter the environment as a free-living microbe. For a more strong, robust and irreversible attachment, bacteria produce exopolysaccharides (EPS) and other adhesion molecules (Zottola, 1991). The next process in the biofilm formation is growth and maturation of biofilm (Fig. 1.).

(b). Growth and Maturation

Once the bacteria irreversibly attach to the surface, they continue to multiply and produce more extracellular biofilm matrix, leading to the expansion of micro-colonies. The biofilm matrix is mainly composed of exo-polysaccharides, proteins, dead cells, lipids and sometimes nucleic acids (Valle et al., 2012). The matrix eventually extends throughout the whole colony and permits the colony to develop into a multi-layer 3D structure. These structures often look umbrella shaped or mushroom shaped, and are recognized as the form of the mature biofilm. Bacteria within the biofilm can be connected to each other via cell to cell contact. The matrix plays a protective role: it protects bacteria from environmental stress, host immune cells, antibiotics and antimicrobial therapies (Flemming and Wingender, 2010). The matrix also helps bacteria to interact with host cells (Valle et al., 2012). After growth and maturation, dispersal signals are initiated and bacterial cells can now leave the biofilm (Fig.1.).
(c). Dispersal

Dispersal is the stage of biofilm development when cells leave the biofilm structure and go back into the environment to live as free floating cells or attach to a new surface and start the biofilm process all over again. Dispersal of bacteria from the biofilm can be divided into two broad groups 1) Active and 2) Passive dispersal (Kaplan, 2010). The bacteria themselves trigger the mechanism of active dispersal, typically in response to an environmental condition. A change in pH is one of the factors that triggers self-dispersal from a biofilm structure (Schofield et al., 2007) In contrast, the passive dispersal is due to external forces, such as mechanical, chemical and environmental (Wang et al., 2014; Choi & Morgenroth 2003; Ymele-Leki &. Ross, 2007). Dispersal is a complex stage of this very complex process of biofilm formation. There is still a lot left to be learned about this process (Fig. 1.).

I.D. Biofilm formation by V. fischeri

Biofilm formation is one of the most common strategies used by bacteria to colonize, survive, persist and cause infection. It has been estimated that 65-80% of human infections involves biofilm formation (Coneye and Nelis, 2010). Due to its clinical relevance, it is very important to have a better understanding of this complex process. Numerous models have been developed to study this complex process. One of these models is the symbiosis between the marine bacterium V. fischeri and its host, the squid E. scolopes. This is a very good model to study biofilm because the genes involved in biofilm formation are also involved in host colonization (Yip et al., 2006). To
efficiently colonize its host, *V. fischeri* forms a biofilm-like aggregate. Then, the bacteria within these aggregates disperse and enter into the light organ to colonize the squid (Nyholm et al., 2000). The genes known to be required for biofilm formation are also required to form these aggregates (Yip et al., 2006). Strains that fail to form biofilms under laboratory conditions also exhibited defects in the host colonization (Yip et al., 2005; Morris et al., 2011).

Under normal growth conditions in lab, *V. fischeri* forms negligible biofilms: it can adhere to glass or plastic surfaces, but only poorly relative to conditions in which transcription of *syp* locus is induced by overexpression of RscS or SypG (Yip et al., 2005; Hussa et al., 2008). It may be possible that *V. fischeri* receives/recognizes some host-specific signal or condition to form a biofilm. At this time, nothing is known about host specific signals that promote biofilm formation by *V. fischeri*. However, in a laboratory setting, biofilms can be induced by overexpression of the sensor kinase (SK) RscS or the response regulator (RR) SypG. These are the 2 key two-component regulators involved in the transduction of a putative environmental signal to promote biofilm formation. In this part, I will briefly explain two-component signal transduction, then I will discuss some important regulators of biofilm formation by *V. fischeri*. 
Figure 1. Model of Biofilm Development. There are three main stages of biofilm development. (A). Attachment: Bacterial cells attach to the surface. The initial attachment is reversible and later cells attach irreversibly. (B). Growth and Maturation: At this stage, cells start to multiply and to release components of the matrix, results in the production of the 3D architecture of the biofilm. (C). Dispersal: Single motile cells disperse from large colonies and start biofilm formation at a new surface. This figure is modeled after that found in Hall-Stoodley et al., 2002.
I.E. Biofilm regulation by *V. fischeri*

The symbiosis between *V. fischeri* and *E. scolopes* has been studied for more than 25 years. *V. fischeri* first forms a biofilm-like aggregate outside the symbiotic organ of the squid (called the light organ) and then disperses from this aggregate to enter inside the light organ of the squid. Once *V. fischeri* enters the light organ, it starts to multiply and colonize the squid. During the process of biofilm formation and colonization, *V. fischeri* out-competes other bacteria and becomes the only colonizer of the light organ. This process is beneficial to both organisms: *V. fischeri* gets nutrients and a protected shelter to live. In return, *V. fischeri* produces light called bioluminescence and helps the squid: it is predicted that squid uses the luminescence produced by the bacteria as a camouflage, in a phenomenon called counter-illumination, to save itself from its predators at night (McFall Ngai and Ruby, 1991; Jones and Nishiguchi, 2004). The first step, in which *V. fischeri* forms a biofilm-like aggregate outside the light organ, is regulated at multiple levels and requires many proteins. The ability of *V. fischeri* to form a biofilm-like aggregate depends upon production of EPS. The production of this EPS requires the *symbiosis polysaccharide* (*syp*) locus. The *syp* locus is an 18-gene locus, and the products of these 18 genes contribute to biofilm formation (Yip *et al.*, 2005; Shibata *et al.*, 2012) (Fig. 3.).
Two Component Signal Transduction

Bacteria use two-component signal transduction to adapt to a wide variety of environments, stressors and growth conditions (Laub and Goulian, 2007). The simplest two-component signal transduction systems are composed of a histidine sensor kinase (SK), which has a conserved histidine kinase core, and a response regulator (RR), which has a conserved regulatory domain (West and Stock, 2001). The SK senses the environmental stimulus, autophosphorylates on a conserved histidine kinase residue, then donates its phosphoryl group to the RR (West and Stock, 2001). Specifically, the RR transfers this phosphoryl group to an aspartate residue present in the receiver domain of the RR (Bourret et al., 1990, Bourret, 2010). The transfer of phosphoryl group to the RR activates its downstream effector, which elicits a specific response. There are other, more complex, types of two-component signal transduction systems that promote the transfer of the phosphoryl group multiple times; these more complex pathways are called a phosphorelay. One of the most common phosphorelay systems involves a hybrid sensor kinase, which contains multiple sites of phosphorylation. In *V. fischeri*, RscS is a hybrid sensor kinase and contains 2 conserved histidine residues, as well as an aspartate residue, predicted to be sequentially phosphorylated (Visick and Skoufos, 2001).

For the purpose of this thesis, I will discuss 3 main regulatory proteins, the hybrid sensor kinase, RscS, and the response regulators, SypG and SypE.
(a) RscS

RscS is a hybrid sensor kinase. It contains two conserved histidine residues and a conserved aspartic acid residue predicted to be involved in a phosphorelay. The \textit{rscS} gene was first identified as a gene required for colonization of \textit{E. scolopes} (Visick and Skoufos, 2001): a \textit{V. fischeri} mutant defective for \textit{rscS} exhibited a host colonization defect. It was subsequently determined that the colonization defect was due to a failure of the \textit{rscS} mutant to aggregate (Yip \textit{et al}., 2006). This was the first bacterial determinant identified as necessary for aggregation of \textit{V. fischeri} on the light organ. Thus, this work demonstrated that this process is critical for colonization. Furthermore, the introduction of the \textit{rscS} gene into a strain of \textit{V. fischeri} isolated from fish was sufficient to promote colonization by this otherwise colonization-defective strain (Mandel \textit{et al}., 2009). An \textit{rscS} overexpression allele, \textit{rscS1}, increases transcription of \textit{syp} about 100-fold higher than does the vector control (Yip \textit{et al}., 2006). The same overexpression strain displayed biofilm phenotypes, including wrinkled colony formation on solid agar media. Finally, cells overexpress \textit{rscS1} exhibited increased aggregation on the surface of the light organ (Yip \textit{et al}., 2006).

(b) SypF

Another putative hybrid sensor kinase is SypF. SypF functions between the SK RscS and the RR, SypG, to control polysaccharide production (Norsworthy and Visick, in press). SypF regulates transcription of the \textit{syp} locus, apparently by donating a phosphoryl group to SypG (Norsworthy and Visick, in press). A \textit{sypF} mutant fails to colonize the squid, a result that demonstrates its critical role in \textit{syp} regulation.
(c). SypG

SypG is an RR encoded in the *syp* locus. Like RscS, SypG is critical for biofilm formation. SypG is a member of the NtrC family of RRs and is predicted to contain 3 domains, the N-terminal REC domain, the central $\sigma^{54}$-interaction domain, and the C-terminal DNA binding domain (Yip *et al*., 2005). It has been hypothesized that RscS, upon sensing unknown signal(s), auto-phosphorylates and then transfers this phosphoryl group to the REC domain of SypG (Hussa *et al*., 2008). Phosphorylation of SypG is predicted to activate it such that it binds to sequences upstream of *syp* promoter sites to activate transcription of the *syp* locus, thus inducing production of the polysaccharide necessary for biofilm formation (Shibata *et al*., 2012, Yip *et al*., 2005, Ray *et al*., 2013)

(d). SypE

Overexpression of either *sypG* or *rscS* in otherwise wild-type cells induces *syp* transcription to significant levels (Hussa *et al*., 2008). However, the biofilm phenotype exhibited by these two strains (expressing either *rscS* or *sypG*) is totally different: RscS induces wrinkled colony formation and pellicle formation, whereas SypG fails to induce wrinkled colony formation (smooth colonies formed) and promotes only weak pellicle formation. Subsequently, it was found that overexpression of *sypG* in cells that lack *sypE* can promote wrinkled colony formation and strong pellicle formation, like overexpression of *rscS* in wild-type cells (Hussa *et al*., 2008). This is how the regulatory role of SypE was uncovered.

SypE is novel: it consists of centrally-located REC domain flanked by a serine kinase domain in the N-terminus and a serine phosphatase domain in the C-terminus
(Morris et al., 2011; Morris and Visick, 2013 a & b) It has been documented that SypE regulates biofilm formation by controlling the function of SypA, but it is still unknown how SypA functions to regulate biofilm.
**Figure 2. Regulation of biofilm formation by *V. fischeri*.** Two component regulators control transcription of the *syp* locus in biofilm formation by *V. fischeri*. The sensor kinase RscS senses some yet unknown environmental signals and serves as phosphodonor to the downstream response regulators SypG and SypE. Phosphorylation activates SypG and starts the transcription of *syp* locus. My work indicates that cysteine functions somewhere below the level of *syp* transcription. This image is inspired by Visick, 2009.
I.F. Host colonization by *V. fischeri*

The mutualistic symbiosis between *V. fischeri* and squid is a very good model to study biofilm, because both the animal host and its bacterial partner can be studied separately. Furthermore, *V. fischeri* is the sole colonizer of squid and can be genetically modified. In addition, genes required for biofilm formation are also required for host colonization and the formation of biofilms in a lab setting can be directly correlated to host colonization. (Stabb, 2006; Ruby, 1996). Together these features make this model one that can be easily studied in the lab and in natural settings.

*V. fischeri* represents a very small proportion of the bacterial population present in seawater (less than 0.1%), between 200 and 1500 bacteria/ml of seawater (Ruby and Lee 1998; Lee and Ruby, 1994). In the laboratory, 200-250 colony forming units (CFU)/ml is sufficient to colonize 50% of juvenile squid and when the number of colonies is increased to 1000 CFU/ml, 100% of the juveniles get colonized (McCann *et al.*, 2003). In spite of being present in such a low concentration in seawater, *V. fischeri* outcompetes other bacteria and colonizes the light organ of the squid (Nyholm and McFall Ngai, 2003). This information indicates that there is a very specific selection process to promote colonization by *V. fischeri*.

Newly hatched juvenile squid are aposymbiotic (Ruby; 1996; McFall-Ngai and Ruby, 1991). Within hours of hatching, juvenile squid get colonized by *V. fischeri* present in the surrounding seawater (Boettcher *et al.*, 1996; Nyholm and McFall-Ngai, 1998). *Vibrio fischeri* colonizes a specific organ of the squid called the light organ. The
light organ is located in the center of the mantle cavity. The squid’s light organ has a bi-
lobed structure (McFall-Ngai and Ruby, 1991) and each lobe has 3 pores and a pair of
appendages, covered with cilia (McFall-Ngai and Ruby, 1991) (Fig. 2). It has been
proposed that these cilia help the bacteria to colonize the light organ by directing their
way into the pores (McFall-Ngai and Ruby, 1991; Montgomery and McFall-Ngai, 1993.
However, the ability of the bacteria to form a biofilm on the surface of the light organ is
also critical, as biofilm-defective strains colonize poorly (Yip et al., 2006)

This squid-Vibrio model has provided a lot of information about biofilm
formation, the process of colonization by bacteria, and different environmental conditions
experienced by bacteria all along the process of colonization (Ruby, 1996; Visick 2009;
Nyholm et al., 2000; Yip et al., 2006). The process of colonization has been divided into
Ruby and McFall-Ngai, 1999).

(a). Initiation

The light organs of newly hatched juvenile squid are uncolonized initially and
become colonized by V. fischeri symbionts as soon as 3 hours following exposure to V.
fischeri in a lab setting (Altura et al., 2013) and generally within 12 hours in natural
seawater. In the absence of V. fischeri, the light organ remains un-colonized by any other
bacteria in the seawater (McFall-Ngai and Ruby, 1991). The initiation stage of
colonization appears to be a three step process (Nyholm et al., 2000; Yip et al., 2006;
Altura et al., 2013): first, bacteria attach to the host squid in the ciliated area of the light
organ; d second, bacteria form a biofilm-like aggregate near the pore; and third, they disperse from the aggregate into the crypt spaces.

**Attachment:** Bacteria come in the contact with host and attach to the host. The attachment of bacteria on the cilia of the light organ is not specific to *V. fischeri*, as other marine bacteria, for example *V. parahaemolyticus*, are also able to attach (Altura *et al.*, 2013). This initial attachment is independent of the *syp* locus (Altura *et al.*, 2013).

**Biofilm:** In contrast, *syp* is required for the subsequent aggregation, which appears to be a type of biofilm. Those *V. fischeri* cells that are unable to form a biofilm in culture also fail to aggregate and fail to efficiently colonize the host (Yip *et al.*, 2005; Yip *et al.*, 2006; Morris *et al.*, 2011; Shibata *et al.*, 2012; Morris and Visick, 2013a, 2013b). This aggregation step appears to be promoted by mucus secretion by the squid, an event that is triggered by contact with either Gram-positive or Gram-negative bacteria (Nyholm *et al.*, 2002). Additional experiments showed that peptidoglycan, a very common component of the bacterial cell envelope, is sufficient to induce mucus secretion by squid (Nyholm *et al.*, 2002).

**Dispersal:** It has been observed that *V. fischeri* cells migrate towards the pores of the light organ of the squid in a controlled fashion. The migration towards the crypts spaces is facilitated by chemotaxis and motility genes present in *V. fischeri* (Lupp and Ruby, 2005; Brennan *et al.*, 2013). It is believed that more than one of these chemotaxis proteins may be involved in the migration of the bacteria towards the pores.
(b). Accommodation

Once *V. fischeri* cells have dispersed from the aggregate, the next step in host colonization is accommodation. This stage encompasses the processes (following dispersal) that result in the ability of *V. fischeri* to reach the crypt spaces and colonize to a high cell density. First, *V. fischeri* cells travel through ducts and antechambers before colonizing the crypt spaces. The ability to enter into and colonize the crypt spaces is highly specific to *V. fischeri*: other bacteria fail to do so. Within the crypt spaces, the bacteria multiply to a high cell density (Montgomery and McFall-Ngai, 1993; Graf and Ruby, 1998; Ruby and Asato, 1993).

When *V. fischeri* cells travel through the ducts and antechambers towards the crypt spaces, they are exposed to a variety of host defenses, including antimicrobial compounds and nitric oxide. It is believed that *V. fischeri* has developed ways to evade these host defenses and, thus, can easily migrate through these regions of toxicity and can reach and colonize the crypt spaces (Davidson et al., 2004; Wang et al., 2010). The light organ of the squid has macrophage-like immune cells called hemocytes. These immune cells do not recognize *V. fischeri* cells as foreign and let them travel through the crypt spaces safely. There are studies that suggest that these hemocytes develop tolerance for *V. fischeri* cells (Nyholm et al., 2009; McFall-Ngai, et al., 2010).

Once *V. fischeri* successfully reaches the crypt spaces, colonization takes place. The squid provides nutrients to *V. fischeri* and thus facilitates its colonization. Studies have shown that several amino acid auxotrophs of *V. fischeri* colonize the crypt spaces of squid, a result that provides evidence that the squid provides nutrients to the bacteria
(Graf and Ruby, 1998). Mutants that don’t colonize to a high cell density have been identified, and are considered accommodation mutants e.g., pgm (DeLoney et al., 2002).

**c. Persistence**

After successful colonization of light organ, *V. fischeri* induces morphological changes to the light organ. These changes are thought to reduce the likelihood that other bacteria will colonize the light organ. As they grow to high cell density, the bacteria produce light, which is used by the squid for protection from predators. In this way, these organisms mutually help each other, as the squid provides food and shelter to *V. fischeri* and, in return, *V. fischeri* helps the squid to escape from predators. Every morning, the squid expel approximately 95% of their bacterial symbionts into the seawater (Boettcher et al., 1996; Ruby, 1996) The remainder of the bacteria repopulate the light organ. This dynamic partnership is maintained for the rest of the life of the squid. Regulation of bioluminescence is dependent upon the cell density in a process called quorum sensing. Quorum sensing is the mechanism by which bacterial cells sense chemical signals called auto inducers (Miller and Bassler, 2001). Bioluminescence is important for host colonization. *V. fischeri* cells that are defective for light production fail to persist in the crypt spaces and appear to be more readily expelled out of the squid (Visick et al., 2000, Bose et al., 2008).

**Conclusions**

Biofilm formation by *V. fischeri* is a complex, highly controlled process. With the availability of a good animal model and with the availability of tools to genetically modify these bacteria, it is possible to learn a lot about the complex process of biofilm
formation by *V. fischeri*. Here, in this brief overview, we learned that there are several regulators of biofilm and they work at different levels of this process.

Furthermore, we have also learned that mutants that fail to form a biofilm in the lab setting also fail to colonize the squid in the natural environment. The colonization process of the squid is a complex process in which *V. fischeri* cells out-compete other bacteria and evade host defenses for the exclusive colonization in the crypts spaces of the light organ. However, more remains to be understood about biofilm formation, its regulation and its role in host colonization using this useful Vibrio-squid model.
Figure 3. Host colonization by *V. fischeri*. (A). Juvenile squid, depicting bilobed light organ. (B). Enlarged view of the squid’s light organ colonized by *V. fischeri*. a. Bacteria attach and form an aggregate near the pore in the light organ, and then travel through ciliated duct in the deep crypt spaces. b. Colonization and persistence of *V. fischeri* in crypt spaces; here, they grow in high cell density and produce light.
I.G. Other genes, besides *syp*, are required for biofilm formation by *V. fischeri*

In addition to the *syp* genes, biofilm formation depends upon a number of other genes. In particular, we recently discovered the involvement in biofilm formation of a set of genes required for cysteine biosynthesis. A transposon (Tn) mutant screen for biofilm-defective strains identified mutants with insertions in the genes depicted in red (Fig. 4.). Cysteine biosynthetic genes are associated with biofilm formation in other bacteria as well. For example, in *Staphylococcus aureus*, a mutation in the *cysM* gene caused a defect in biofilm formation (Soutourina *et al*., 2009). In both *Escherichia coli* and *Providencia stuartii*, a mutation in *cysE* enhanced biofilm formation (Sturgill *et al*. 2004). This information suggests that cysteine can be a positive regulator of biofilm in some bacteria and a negative regulator in others. In *V. fischeri*, it seems that cysteine is a positive regulator of biofilm formation.

Because my project investigated the involvement of cysteine in biofilm formation by *V. fischeri*, I will first discuss the literature about cysteine in general and then I will briefly describe the pathway of cysteine biosynthesis as determined in other bacteria.
I.H. Introduction

Cysteine plays an important role in all life (Plants, animals and bacteria). Cysteine is non-essential amino acid because it is synthesized in the body of an organism and is not needed as a supplement. Cysteine is required for various functions in the body, e.g., metal binding, enzyme catalysis, structure, and proper growth. Cysteine residues serve as a source of sulfur for many cellular metabolites such as coenzyme A (CoA), thiamin, biotin, S-adenosyl methionione, molybdopterin and lipoic acid (Seiflein and Lawrence, 2006; Kolko et al., 2001) and importantly glutathione (without glutathione the cell cycle gets arrested). Cysteine is also an important component of antibiotics and siderophores. If hydrogen sulfide (H\textsubscript{2}S) is present in the environment, then synthesis of cysteine is just a two-step process. In the first step, L-serine is converted into O-acetyl serine (OAS) by the action of serine transacetylase. In the second step, OAS and H\textsubscript{2}S together enzymatically synthesize L-cysteine. However, a non-enzymatic reaction favors the formation of N-acetyl serine (NAS) (Fig. 4.).

I.I. Cysteine Biosynthesis

Cysteine can be synthesized by three different pathways, namely 1) Transulfuration, 2) Sulfuration and 3) Cystathionine. The transulfuration pathway synthesizes cysteine from inorganic sulfur, which is converted into methionine through cystathionine as an intermediate (Rabeh and Cook, 2004). Very few bacteria and fungi use the transulfuration pathway to synthesize cysteine e.g., Corynebacterium glutamicum
(Hwang et al., 2002). Fungi, *Saccharomyces cerevisiae*, also use this pathway to synthesize cysteine (Cherest et al., 1993; Ono et al., 1999).

Most bacteria and fungi use either the sulfuration (also called sulfhydrylation) (direct use of sulfur) or cystathionine pathway to synthesize cysteine. The sulfuration pathway does not need cystathionine as an intermediate and uses sulfur directly to synthesize cysteine. For example, *Mycobacterium tuberculosis* uses this pathway (Hatzios et al., 2011; Rivera-Marrero et al., 2002). The cystathionine pathway requires a high amount of sulfur for cysteine synthesis. *Klebsiella pneumoniae* uses the cystathionine pathway and utilizes methionine to synthesize cysteine (Seiflein and Lawrence, 2006).

As cysteine is the most predominant form of sulfur, the requirement for cysteine is different in different bacteria. Some bacteria require less cysteine for growth and some need more cysteine for growth. Thus, they synthesize different amounts of cysteine. For example, *Salmonella* and *E. coli* need only small amounts of cysteine and hence they only reduce only small amounts of sulfur into cysteine (Peck, 1961). Some anaerobic bacteria respire sulfur instead of oxygen and they utilize more sulfur, thus more sulfur is synthesized into cysteine.

In the above description of the cysteine pathway, the conversion of serine to cysteine is a two-step process, but the conversion of sulfur to cysteine is a much longer pathway. First, sulfate and thiosulfate are taken up by bacteria through a single periplasmic transport system (Sirko et al., 1990; Pardee et al., 1966) by the action of sulfate-thiosulfate permease. Sulfate-thiosulfate permease is encoded by *cysP, cysT,*
cysW, cysA, cysM and sbp (Sirko et al., 1990; Hellinga and Evans, 1985; Mansilla and de Mendoza, 2000).

The first step of sulfur reduction after its uptake is the conversion of sulfur into adenosine 5-phosphosulfate by the action of ATP sulfurylase. This process is called adenylation, and adenosine 5-phosphosulfate is the activated form of sulfate. The genes cysN and cysD encode the enzyme ATP sulfurylase. The reaction consumes a lot of energy. Since so much energy is consumed, this reaction is highly unfavorable. Therefore, to maintain the equilibrium, hydrolysis of the $\beta$-$\gamma$ bond of GTP takes place. Hydrolysis of $\beta$-$\gamma$ bonds and shifts the equilibrium towards sulfate assimilation (Wie et al., 2002). Activation of sulfate gives adenosine 5-phosphosulfate (APS) and PPi. (Satishchandran and Markham, 1989; Robbins and Lipmann, 1958). Further, in bacteria, yeast, and fungi, APS is reduced into 3-phosphoadenosine5-phosphosulfate (PAPS) by the action of APS kinase. In this step, 1 molecule on ATP is utilized and one molecule of ADP is released (Conrad and Woo, 1980; Robbins and Lipmann, 1956). The enzyme APS-kinase is encoded by the cysC gene. PAPS further gets reduced to sulfite.

In the conversion of PAPS to sulfite, the enzyme PAPS-sulfo-transferase transfers a sulfo group from PAPS to a sulfo group accepter (Negishi et al., 2001), in this case thio-redoxin. The enzyme PAPS-sulfotransferase is encoded by cysH. Sulfite is further reduced to sulfide by the action of an enzyme called NADPH-sulfite reductase, encoded by cysI, cysJ and cysG. The sulfide is reacts with hydrogen to get reduced to hydrogen sulfide (H$_2$S).
In the biosynthesis of cysteine, L-serine reacts with Acetyl-CoA to form OAS, by the action of enzyme called serine trans-acetylase, encoded by cysE. Once both H$_2$S and OAS is present, cysteine formation is just one-step ahead. The enzyme O-acetyl serine A or B, encoded by cysK and cysM respectively, converts H$_2$S and OAS into L-cysteine and makes it available for further cellular functions.

Cysteine is an important amino acid required for many functions in many living organism. The defect in genes required for cysteine biosynthesis could lead to poor growth and incorrect folding of proteins. Recently, we have found that the disruption in cysteine biosynthetic genes in V. fischeri leads to a defect in biofilm formation. In Staphylococcus aureus, a defect in the cysM gene decreases the ability of this organism to survive in various environments. On the contrary, in E. coli, a defect in the cysE gene increases its ability to form biofilm.
**Figure 4. Cysteine biosynthesis.** The proteins, CysK, CysN, CysH, CysJ, shown in red here, appear to be important in biofilm formation, as mutations in their respective genes appeared to disrupt biofilm formation by *V. fischeri*. This model shows that various Cys proteins functions at different steps of the cysteine metabolism pathway. The CysK protein is at the bottom of the pathway and functions to convert O-Acetyl L-serine and H$_2$S into cysteine.
I.J. Objective

Biofilm formation is a complex process and is highly regulated in bacteria. Because of the medical and industrial relevance of biofilms, we need to develop a better understanding of biofilm formation so that we can develop better measures to treat problems caused by biofilm formation. The objective of my research was to investigate the role played by genes, other than syp, in biofilm formation by V. fischeri. Our collaborators found that mutations in genes involved in cysteine biosynthesis caused a defect in biofilm formation by V. fischeri.

The first goal of my thesis was to find the relative importance of various cys genes in biofilm formation by V. fischeri. Then, I determined if there were any growth defects of these mutants, because cysteine is also important for growth of the cells. Next, I wanted to understand that if cysteine has any negative impact of syp transcription, because this can uncover if cysteine functions at or above the level of syp transcription. Then, I determined if a mutation in a cysteine biosynthesis gene caused a defect in host colonization by V. fischeri.

During the course of this study I found that E.coli could induce wrinkled colony formation by a V. fischeri cysK mutant strain. This observation led us to determine which E. coli Keio collection mutant failed to suppress the biofilm defect of the V. fischeri cysK mutant.
CHAPTER TWO

MATERIAL AND METHODS

II.A. Bacterial strains and media

All strains used in this study are listed in Table 1. *V. fischeri* strains utilized in this research are derived from strain ES114, a bacterial isolate from *Euprymna scolopes* (Boettcher and Ruby, 1990) and MJM1198, a derivative of ES114 that overexpresses rscS due to the insertion of a transposon upstream of the *rscS* gene. *V. fischeri* strains were grown in LBS (Luria Bertani-Salt) (Stabb *et al*., 2001) and TMM (Tris minimal media). All the derivatives of *V. fischeri* were generated via natural transformation (Pollack-Berti *et al*., 2010) or conjugation (DeLoney *et al*., 2002). To generate Tn7 insertions in *V. fischeri*, tetraparental mating were carried out as previously described (McCann *et al*., 2003). For the overexpression of *sypG*, plasmid pEAH73 was used. *E. coli* strains π3813 (Le Roux *et al*., 2007) and CC118 (Herrero *et al*., 1990) were used for cloning. All *E. coli* strains were grown in LB medium (Davis *et al*., 1980). All solid media were made using agar to a final concentration of 1.5%.

The following antibiotics were added to liquid and solid media where required for growth of *V. fischeri*: tetracycline (Tet) 5 μg ml⁻¹, chloramphenicol (Cm), 5 μg ml⁻¹, kanamycin (Kan) 100 μg ml⁻¹, and erythromycin (Erm) at 5 μg ml⁻¹. Cysteine was added
to *V. fischeri* cells to a final concentration of 1 mM. As needed, antibiotics were added to *E. coli* cell culture media and agar plates as follows: Tet at 15 μg ml⁻¹, Cm at 25 μg ml⁻¹, and Kan at 5 μg ml⁻¹. Thymidine was added to a final concentration of 0.3 mM for *E. coli* strain π3813.

**II.B. Wrinkled colony formation**

To evaluate wrinkled colony formation, LBS medium containing appropriate antibiotics was inoculated with a single colony of the appropriate *V. fischeri* strain. Cultures were grown overnight (14-16 h) at 28°C with shaking, then diluted 1:100 into fresh LBS and grown under same conditions for 2-3 h the next day. The sub-cultures were then standardized to an optical density of 600 nm (OD₆₀₀) of 0.2 and 10 μl aliquots were spotted onto LBS Tet agar plates, or LBS Tet agar plates containing 1 mM cysteine. Then, strains containing pRscS plasmid pKG11 were incubated at 23°C and strains containing pSypG plasmid pEAH73 were incubated at 28°C. Spots were monitored over time for wrinkled colony development. Images of wrinkled colony development were acquired at the indicated time points using a Zeiss Stemi 2000-C dissecting microscope at a fixed setting to avoid any discrepancies. Each experiment was repeated at least 3-4 times. For each experiment, all strains were spotted on same plate to avoid discrepancies.
II.C. Evaluation of *V. fischeri* growth

Growth of wild-type and mutant *V. fischeri* was assayed in LBS or TMM, lacking or containing 1 mM cysteine. For both conditions, a single colony of the appropriate *V. fischeri* strain was used to inoculate LBS medium and cultures were grown overnight at 28°C with shaking. Then, the cultures were diluted to an OD$_{600}$ of 0.005 (LBS cultures) or 0.02 (TMM cultures) in 20 ml of medium and incubated at 28°C with shaking. After every 60 min, the OD$_{600}$ was measured using a spectrophotometer until the growth of the culture reached a plateau. The OD$_{600}$ values were plotted over time. These experiments were repeated at least 3 times for each set of strains. The same batch of culture medium was used for each set of experiments to avoid any discrepancies.

II.D. Squid Colonization assay

To perform squid colonization assays, juvenile *E. scolopes* were incubated with 1000-1500 *V. fischeri* cells per ml of artificial sea water (ASW) (Instant Ocean; Aquarium systems, Mentor, OH) for 3-4 hours. After incubation, juvenile squid were washed in freshly made artificial seawater and placed in *V. fischeri*-free artificial seawater for another 12-15 h. The next day, juvenile squid were put in small glass vials containing 5 ml of artificial seawater and the bioluminescence of each animal was measured using a scintillation counter or luminometer. Then, the squid were homogenized to release the contents of the light organs. The homogenates were diluted using artificial seawater and plated onto SWT plates to determine the colony forming units (CFU) per squid. The graphs are plotted as CFU/squid.
**II.E. Pellicle Assay**

Strains were grown (in triplicate) with shaking in LBS-Tet containing or lacking 1 mM cysteine at 28°C overnight and then diluted to an OD$_{600}$ of 0.2 in 2 ml of fresh medium in 24 well microtiter dishes. Cultures were then incubated statically at 28°C for 48 h for strains containing pSypG plasmid pEAH73 were incubated at 28°C and strains containing pRscS plasmid pKG11 were incubated at 23°C. The strength of each pellicle was evaluated by disrupting the air/liquid interface with a sterile toothpick after 48 h of incubation. Images of pellicles were acquired at the indicated time point using a Zeiss Stemi 2000C dissecting microscope. Medium from one batch was used for each set of experiments to avoid any discrepancies. The experiment was repeated at least 3 times.

**II.F. Stickiness assay.**

*V. fischeri* cells were cultured as indicated above for the wrinkled colony assay. Then, strains were standardized to an OD$_{600}$ of 0.2 and several 10 μl spots were placed on LBS tet plates. Then plates were incubated statically at 28°C for strains containing pSypG plasmid pEAH73 and strains containing pRscS plasmid pKG11 were incubated at 23°C. At the indicated times, the spots were disrupted using a sterile toothpick. A spot was considered sticky if it exhibited biofilm-like properties (either self-adherent or adherent to the plate). Images of the spots were acquired at the indicated time point using a Zeiss Stemi 2000-C dissecting microscope. Each experiment was repeated at least 3 times.
II.G. E. coli - V. fischeri mixing assay.

*V. fischeri* and *E. coli* cells were grown at 28°C and 37°C, respectively, overnight. The next day, 50 μl of the *E. coli* culture was mixed with 50 μl of the *V. fischeri* culture. For the Keio collection experiment, mixing was carried out in 96 well plates and these mixes were spotted onto LBS plates using a 48 well replicator. For other experiments, 10 μl volumes were inoculated onto plates. Finally, the plates were incubated at 28°C and wrinkling was observed over time.

II.H. β-galactosidase assay

Cultures of reporter strains carrying the *sypG* overexpression plasmid pEAH73 were grown in triplicate with vigorous shaking in LBS medium containing Tet at 28°C overnight, then sub-cultured into TMM medium containing tet for 24 h. After 24 h, the OD₆₀₀ of each culture was determined, then 1 ml aliquots of cells were concentrated by centrifugation, and the pellets were re-suspended in 1 ml Z-buffer. The reactions were started by adding 0.2 ml substrate, O-nitrophenyl-β-D-galactosidase (ONPG; 4 mg/mL), and the reaction start time was noted. Reactions were stopped by the addition of sodium carbonate after sufficient yellow color appeared and the reaction stop time was noted. Then, the OD₄₂₀ and OD₅₅₀ were determined. As a measure of *syp* transcription, β-galactosidase activity was calculated as previously described (Miller, 1972).
II.I. Transposon mutagenesis and identification of wrinkling mutants.

Transposon mutants were generated as previously described (Lyell et al., 2008). The plasmid containing the mini Tn5 transposon was introduced into *V. fischeri* strain KV6509 and KV6991, via tri parental conjugation. The spot was re-suspended in 1 ml LBS, which was plated on LBS erm–tet plates that were then incubated at room temperature. The ex-conjugates were screened for their ability to form wrinkled colonies.
Table 2. Strains used in this study

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<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
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<td>CC118λpir</td>
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<td><strong>V. fischeri</strong> (KV #)</td>
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### TABLE 3. Oligonucleotides used in this study

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CHAPTER THREE
EXPERIMENTAL RESULTS

Introduction

The symbiotic relationship between Hawaiian bobtail squid *Euprymna scolopes* and the marine bacterium *Vibrio fischeri* is a well-established model to study biofilms. During the colonization process, *V. fischeri* cells first form a biofilm-like aggregate on the surface of the symbiotic organ, then disperse from it and enter the organ where they grow to high cell density (Nyholm, *et al.*, 2000; Nyholm and McFall-Ngai, 2004). This biofilm formation by *V. fischeri* is tightly regulated by a set of 18 genes, called the *syp* locus, that is predicted to be involved in the regulation, production, and transport of the polysaccharide component of the biofilm (Fig. 1) (Yip *et al.*, 2004; Yip *et al.*, 2005). In addition to the *syp* genes, biofilm formation depends upon a number of other genes. Recently, a transposon (Tn) mutant screen for biofilm-defective strains performed by Mark Mandel’s lab identified mutants with insertions in the cysteine biosynthetic genes that encode the enzymes depicted in red in Fig. 4. Disruption of any of those genes resulted in a delayed or defective wrinkled colony phenotype. Cysteine biosynthetic genes are associated with biofilm formation in other bacteria as well. For example, in *Staphylococcus aureus*, a defect in the *cysM* gene results in a defect in biofilm formation (Soutourina *et al.*, 2009). In *E. coli* and *Providencia stuartii*, a mutation in *cysE* enhances
biofilm formation (Sturgill, et al. 2004). This information suggests that cysteine can be a positive regulator of biofilm in some bacteria and a negative regulator in others. In *V. fischeri*, it seems that cysteine may be a positive regulator of biofilm formation.

The goal of my project was to determine the mechanism of action of the cysteine biosynthetic genes in promoting biofilm formation by *V. fischeri*. Understanding the requirement for cysteine will give us insight into the complex control over biofilm formation.
**TABLE 1. The cys::Tn mutants of biofilm-forming *V. fischeri*.**

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Relative importance of specific cys genes in biofilm formation

by V. fischeri.

To determine the relative defects in biofilm formation of the cys::Tn mutants, I evaluated biofilm formation using a sensitive time course assay of wrinkled colony formation (Ray et al., 2012). I examined all of the cys::Tn mutants available in the lab, namely cysH, cysJ, cysK, and cysN. These strains overexpress RscS due to a Tn insertion positioned upstream of rscS in the chromosome; RscS induces the syp locus and thus biofilm formation. For my controls, I used the cys+ parent strain MJM1198, which wrinkles within 24 hours, and wild type strain ES114, which never wrinkles.

All the cys::Tn mutants did indeed show a delay in biofilm formation by V. fischeri (Fig. 5). From these data, it appeared that the cysN::Tn mutant was the most defective, followed by cysK::Tn and cysH::Tn. However, for reasons described below, I reconstructed the cysN::Tn mutant via natural transformation (NT) (backcross) into the parent stain and found that the new strain had little to no defect (Fig. 5).

From these data, it appeared that the cysK::Tn mutant was the most defective in biofilm formation by V. fischeri. I thus hypothesized that cysK was the most critical cys biosynthetic gene because CysK functions at the bottom of the biosynthetic pathway to convert OAS and H2S into cysteine (Fig. 4).
Figure 5. Wrinkled colony formation by cys transposon mutants. A time course assay of wrinkled colony formation was performed using strains that overexpress rscS from the chromosome. 10 μL of freshly growing cultures were spotted on LBS plates, which were incubated at room temperature. Wrinkled colony formation was observed over time. (A) cys+ parent strain (MJM1198), (B) WT (ES114), (C) cysH::Tn (MJM1964), (D) cysJ::Tn (MJM1965), (E) cysK::Tn (MJM1966), (F) cysN::Tn (MJM1967), (G) cysN::Tn [NT] (KV7409) These data are representative of at least three independent experiments.
Addition of exogenous cysteine suppresses the biofilm defect.

I hypothesized that, if the biofilm defects of the \textit{cys} mutants are due to lack of cysteine, then addition of exogenous cysteine should suppress the defects. To answer this question, I spotted the \textit{cys::Tn} mutants onto LBS plates contains exogenous cysteine and evaluated wrinkled colony formation over time. My results indicated that exogenous addition of cysteine suppresses the biofilm defect (Fig. 6). One exception to this was for the \textit{cysN::Tn} mutant; biofilm formation by this mutant was not restored by addition of exogenous cysteine. These data thus indicated that original \textit{cysN::Tn} mutant may have some secondary mutation. Indeed, the \textit{cysN::Tn} mutant generated by backcross into its parent strain, MJM1198 (\textit{cysN::Tn [NT]}) exhibited only a small delay in wrinkled colony formation that was suppressed by the addition of cysteine (Fig. 6). Finally, during these experiments, I was also able to determine that 1 mM is the minimum concentration of cysteine required to suppress the biofilm defect. Together, the results of these experiments indicated that \textit{cys::Tn} mutants have a delay and defect in biofilm formation.
Figure 6. Complementation of wrinkled colony formation by exogenous addition of cysteine. The ability of exogenous cysteine to complement the wrinkled colony formation defect of cys::Tn mutants was assayed by adding 1 mM cysteine to the agar plate. (A) cys + parent strain (MJM1198) (B) cysH::Tn (MJM1964), (C) cysJ::Tn (MJM 1965), (D) cysK::Tn (MJM1966), (E) cysN::Tn (MJM1967), (F) cysN::Tn [NT](KV7409). These data are representative of at least three independent experiments.
Pellicle formation by \textit{cys::Tn} mutants

It has been observed in the Visick lab that mutants that fail to form biofilms on a solid agar surface also fail to form pellicles at the air/liquid interphase of static cultures. Keeping this observation in mind, I performed pellicle assays and hypothesized that, in this absence of cysteine, the \textit{cysK::Tn} mutant would not form a pellicle and the addition of cysteine would suppress the pellicle defect.

To test this hypothesis, the parent and \textit{cys::Tn} mutants were grown in LBS statically in the absence or presence of cysteine. Pellicle formation was observed after 45 hours. All of the \textit{cys::Tn} mutants except the \textit{cysK} mutant exhibited pellicle formation in the absence and presence of cysteine (Fig. 7). However, pellicle formation by all the \textit{cys} mutants except \textit{cysK::Tn} was ~ 3 hours faster in the presence of cysteine than in the absence of cysteine. Thus, cysteine is involved in pellicle formation by all the other \textit{cys::Tn} mutants but not by the \textit{cysK::Tn} mutant. The positive control (parent) had no difference in pellicle formation in either case. In contrast, the \textit{cysK::Tn} mutant grew very poorly in static culture regardless of the presence or absence of cysteine and did not form pellicles (Fig. 7). These data suggest that the pellicle defect of the \textit{cysK} mutant is not due to the lack of cysteine. Instead, it may be caused by the accumulation of products like OAS and sulfide. This hypothesis remains to be tested.
Figure 7. Pellicle formation by cys::Tn mutants. Pellicle formation by the cys::Tn mutants was observed in the absence or presence of cysteine. The top panel shows pellicles formed without added cysteine and the bottom panel shows pellicles formed in the presence of 1 mM cysteine. (A) cys<sup>+</sup> parent (MJM1198) (B) cysH::Tn (MJM1964), (C) cysJ::Tn (MJM1965), (D) cysN::Tn (MJM1967), (E) cysK::Tn (MJM1966) (F) cysN::Tn [NT](KV 7409). These data are representative of at least three independent experiments.
The *cys::Tn* mutants have a growth defect.

Cysteine is required for biofilm formation. Cysteine is also an important amino acid required for growth. Therefore, it is possible that the biofilm defect of the *cys* mutants is caused by a growth defect. Here, I asked whether the *cys* mutants have a growth defect.

To determine if the *cys::Tn* mutants had a growth defect due to their inability to synthesize cysteine, I first performed a growth curve experiment in LBS liquid medium in the presence or absence of cysteine. I monitored the growth of the *cys::Tn* mutants by measuring the optical density (Fig. 8 A and B). In this complex medium, the *cys::Tn* mutants exhibited relatively mild growth defects, a result that indicates that LBS supplies cysteine in amounts that are nearly sufficient for growth under these conditions. However, there is an apparent growth defect on LBS agar plates, which matches the lack of growth in liquid medium.

To gain a better understanding of the growth of the *cys::Tn* mutants, I evaluated their growth in Tris minimal medium (TMM) supplemented with 0.03% casamino acids. All the *cys::Tn* mutants grew very poorly in this medium (Fig. 9A) but grew fairly well when 1 mM cysteine was added (Fig. 9B). These data indicated that all the *cys* mutants has almost same growth defect in the absence of cysteine.

However, they displayed different wrinkled colony patterns during the wrinkled colony time course. Because the different *cys* mutants exhibited similar growth defects,
yet quite different wrinkled colony defects, I conclude that the wrinkled colony phenotypes are not due solely to the growth defects of these strains.
Figure 8. Growth of the cys transposon mutants in LBS medium containing or lacking added cysteine. Cells were grown in LBS medium lacking or containing added 1 mM cysteine. The strains assayed are as follows: Parent strain MJM1198 (light blue), cysH::Tn, MJM1964 (Orange), cysJ::Tn, MJM1965 (Gray) cysN::Tn [NT] (KV7409)(Yellow) and cysK::Tn MJM1966 (dark blue). These data are representative of at least three independent experiments.
Figure 9. Growth of the *cys* transposon mutants in Tris minimal medium lacking or containing added cysteine. Cells were grown in LBS medium and then sub-cultured into minimal medium (A) lacking or (B) containing added 1 mM cysteine. The strains assayed are as follows: Parent strain MJM1198 (light blue), *cysH*::Tn (MJM1964) (orange), *cysJ*::Tn (MJM1965)(gray), *cysN*::Tn (MJM1967) (yellow), *cysK*::Tn (MJM1966)(dark blue) and *cysN*::Tn [NT] (KV7409)(green). These data are representative of at least three independent experiments.
Wrinkled colony assay with unmarked deletion mutants.

It was somewhat surprising that the cys::Tn mutants exhibited similar growth defects yet very different wrinkled colony phenotypes, especially the cysK::Tn mutant. It seemed possible that the cysK::Tn mutant harbored a secondary mutation that caused the biofilm defect, or else that the Tn was polar on genes required for wrinkled colony development. To assess these possibilities, we generated in-frame deletions of the cysH and cysK genes in a ΔsypE background, then induced syp transcription by overexpression of sypG from a plasmid. These strains were spotted onto LBS-tet plates, and wrinkled colony development was observed over time (Fig. 10).

These data indicated that the wrinkled colony phenotype of the ΔcysH mutant was similar to that of the cysH::Tn mutant strain (Fig. 10). However, relative to the cysK::Tn mutant, the ΔcysK mutant exhibited an even more dramatic defect in wrinkled colony formation, displaying a biofilm defect even after prolonged incubation (Fig. 10). This severe defect could be suppressed by addition of 1 mM exogenous cysteine (data not shown).
Figure 10. Wrinkled colony assay with unmarked deletion mutants. Wrinkled colony assays were performed using cysH and cysK deletion mutants. Transcription of the syp locus was induced by overexpression of sypG from a plasmid. [A] Positive control (KV3937). [B] Overexpression of sypG in the ΔsypE ΔcysH background (KV6309). [C] Overexpression of sypG in the ΔsypE ΔcysK background (KV6509).
The *cysK* mutation might be polar on downstream genes.

The drastic difference in the wrinkled colony phenotypes of the *cysK::Tn* and Δ*cysK* mutants suggested that the Tn insertion might be polar on genes downstream of *cysK*, that is *crr* and *ptsI*. The *crr* and *ptsI* genes are known to play a role in the phosphotransferase system in *V. fischeri* (Kotrbá *et al.*, 2001; Visick *et al.*, 2007) So the transposon might be polar on or both of these genes in the *cysK::Tn* mutants, and thus may interrupt the function of these genes; this may be a reason why the *cysK::Tn* mutants form a defective wrinkled colony after a delay. However, in the unmarked mutation, these genes are not interrupted by any Tn and fully functional to inhibit wrinkled colony formation in the absence of the *cysK* gene. Therefore, I hypothesized that one or both genes function to inhibit wrinkled colony formation. To investigate this possibility, I generated double mutants, Δ*cysK ptsI* and Δ*cysK crr*, and induced *syp* transcription by overexpressing *sypG* from a plasmid. I found that the wrinkled colony development of the double mutants was no different from the Δ*cysK* single mutant. These data indicate that the phenotypic difference between the Δ*cysK* and *cysK::Tn* mutants is not due to polarity of the Tn on *ptsI* or *crr*. It remains unclear why the Δ*cysK* and *cysK::Tn* mutant strains exhibit different wrinkled colony phenotype from one another.
Complementation with the cysK gene can restore wrinkled colony and pellicle formation in the absence of cysteine.

*V. fischeri* deleted for the cysK gene failed to form wrinkled colonies and also failed to form pellicles. As described above, the defect in wrinkled colony formation could be suppressed by the addition of 1 mM cysteine, which indicates that the role of CysK in generating cysteine is critical for biofilm formation. However, cysteine addition failed to suppress the defect in pellicle formation. Thus, it is possible either that a secondary mutation exists in this strain or else that some other function of cysK is required for pellicle formation. To verify the importance of the cysK gene in pellicle formation, it was important to complement the cysK mutant by introducing the cysK gene back into the chromosome.

To do this, I cloned the cysK gene and then moved it into a benign site, the Tn7 site, of the ΔsypE ΔcysK mutant, then induced syp transcription by overexpressing sypG from a plasmid. Then, I evaluated wrinkled colony formation and pellicle formation (Fig. 11 A and B).

The data from these experiments revealed that complementation with the cysK gene restored both wrinkled colony and pellicle formation (Fig. 11 A and B). From these results, we conclude that the smooth phenotype of the cysK deletion strain and its inability to form a pellicle is indeed due to the lack of cysK gene. They also suggest that active CysK function, and not cysteine *per se*, is required for pellicle formation.
Figure 11. Complementation of the cysK mutant with cysK. Complementation with the cysK gene restores (A) Wrinkled colony formation and (B) Pellicle formation. [A] (a) Positive control, psypG/ΔsypE (KV3937); (b) psypG/ΔcysK ΔsypE (KV6509); (c) psypG/ΔcysK ΔsypE cysK^+ (KV7523). [B] (a) Positive control, psypG/ΔsypE (KV3937); (b) psypG/ΔcysK ΔsypE (KV6509); (c) psypG/ΔcysK ΔsypE cysK^+ (KV7523).
Cysteine mutation does not affect syp transcription.

Cysteine plays an important role in biofilm formation by *V. fischeri*, but the mechanism involved in this process is unknown. I hypothesized that cysteine could be necessary for the expression or function of factors known to be required for biofilm formation. In particular, I hypothesized that cysteine could impact transcription of the *syp* genes, which play an important role in biofilm formation by *V. fischeri* (Yip *et al.*, 2005; Yip *et al.*, 2006; Hussa *et al.*, 2008). If cysteine impacts *syp* transcription, then the deletion of the *cysK* gene should negatively impact *syp* transcription.

To address this question, I introduced PsypA-lacZ (the *sypA* promoter fused to the lacZ reporter gene) at the Tn7 site of the ΔsypE ΔcysK strain. I then induced *syp* transcription by overexpressing *sypG* from a plasmid. For my positive controls, I used a pSypG/ΔsypE strain that contained PsypA-lacZ. I also used a pSypG/ΔsypE ΔcysH strain that contained PsypA-lacZ; this strain had been previously tested in the lab. As my negative controls, I used the respective empty vector-containing strains. I then assayed β-galactosidase activity (Fig. 12).

These data from the experiments revealed that the *cysH* and *cysK* mutants exhibited β-galactosidase activity similar to the positive control, suggesting that the *cys* mutation does not impact *syp* transcription (Fig. 12). These results are similar to an observation previously made by Dr. Ray for the *cysH* mutant.

From these data, I conclude that the defect in cysteine biosynthesis does not exert any effect on *syp* transcription and, thus, cysteine contributes to biofilm formation at a
level distinct from *syp* transcription, perhaps somewhere below the level of *syp* transcription.

**Figure 12. Measurement of *syp* transcription.** The first 2 bars are empty vector (KV7517) control and positive control (KV5514), respectively. The middle 2 bars are empty vector (KV7518) for ΔcysH and ΔcysH carrying PsypA-lacZ (KV6311) and last 2 bars are empty vector for ΔcysK (KV7515) and ΔcysK carrying PsypA-lacZ (KV7514).
Search for mutant(s) that can suppress the wrinkled colony defect of the unmarked *cysK* strain using transposon mutagenesis.

Cysteine plays an important role in biofilm formation by *V. fischeri*, but the mechanism involved in this process is yet unknown. One readily testable hypothesis for the role of cysteine in biofilm formation is that cysteine inhibits a biofilm inhibitor. If so, then it should be possible to restore biofilm formation to the *cysK* mutant through disruption of the downstream inhibitor. To address this hypothesis, I performed transposon mutagenesis in a biofilm-induced *cysK* mutant background (psypG/ΔsypE ΔcysK), then screened for mutants that can form wrinkled colonies. To search for mutants that regained the ability to form wrinkled colonies, I screened almost 80,000 mutants. I did not find any mutants that suppressed the wrinkled colony defect. This indicates that this is the wrong hypothesis.

From these observations I concluded that cysteine does not act as an inhibitor, and may be act through some other as-yet unknown mechanism.
Stickiness property of cys mutants

Recently, it has been observed by the Visick lab that there is a correlation between Syp polysaccharide (Syp-PS) production and the stickiness of smooth-appearing colonies: smooth but sticky colonies retain their ability to produce Syp-PS. Therefore, I wanted to determine if the cysK mutants are sticky, as this may suggest if Syp-PS production is defective or not. If cysK mutants are sticky, then we can speculate that cysK mutants can produce Syp-PS.

To evaluate colony stickiness, I spotted the sypG-overexpressing ΔsypE ΔcysK mutant on an LBS tet plate and evaluated the stickiness over time by disrupting the spots with a flat toothpick. During the course of these experiments, I observed an unusual pattern of stickiness. I clearly saw a 3-step development of biofilm formation. In the first step, between 10-12 hours, neither the WT nor the cysK mutant was sticky (Fig. 13). In the second step, between 12-24 hours, both WT and cysK mutants adhered to the surface of the agar plate (Fig. 13). Surprisingly, the cysK mutant adhered to the surface more than the WT. Finally, in the third step, between 24-44 hours, the WT cells no longer attached to the surface but instead, appeared to stick to other cells such that the cells and surrounding material could be lifted away from the plate largely intact. In contrast, the cysK mutant both adhered to the surface and to other cells (Fig. 13).

From these data, we determined that cysK mutant is sticky and thus concluded it is likely producing Syp-PS. These data can be further verified by examining Syp-PS production using a Western blot approach with anti-Syp-PS antibodies (Shibata and
Visick, 2012; Shibata et al., 2012). Furthermore, these data also revealed that there is a stage in biofilm formation that was previously unknown, adherence to the agar surface. This stage is very brief, in the case of the WT strain, but prolonged in the case of the cysK mutant. Therefore, investigation of this phenotype further may provide insights into the process of biofilm formation in *V. fischeri*.

**Figure 13. Stickiness phenotype of the cysK mutant.** (A) Undisturbed spots, psvpGΔsypE (KV3937) (B) spots disrupted at indicated time point (KV3937). (C) Undisturbed spots of the cysK mutant, psvpGΔcysKΔsypE (KV6509) (D) ΔcysK mutant spots disrupted at indicated time (KV6509). All the strains were cultured at 28°C for 14 hours, then sub-cultured for 3 hours. Then standardized to an OD$_{600}$ of 0.2 and then spotted 10 μL on LBS tet plate. The spots were disrupted with a toothpick at the indicated time points.
Escherichia coli provides something to the cysK mutant

Cysteine plays an important role in biofilm formation by *V. fischeri*, but the mechanism involved in this process is yet unknown. As described above, one readily testable hypothesis for the role of cysteine in biofilm formation is that cysteine inhibits a biofilm inhibitor. If so, then it should be possible to restore biofilm formation to the cysK mutant through disruption of the downstream inhibitor. To address this hypothesis, I performed transposon mutagenesis in a biofilm-induced cysK mutant background (pSypG/ΔsypE ΔcysK). Then, I screened for mutants that could form wrinkled colonies.

To introduce transposon into the cysK mutant, I performed conjugation. To perform conjugation, I mixed *V. fischeri* with donor and helper *E. coli*, and then screened for wrinkled colonies. I never found wrinkled colonies despite screening over 80K mutants. However, I found a surprising result in the course of these experiments. When these strains were spotted alone, they formed smooth colonies, while the conjugation mixture resulted in wrinkled spot. I predicted that either the donor or helper *E. coli*, or both, provides something to the *V. fischeri* cysK mutant to suppress the wrinkled colony defect. To test this possibility, I mixed the helper and donor *E. coli* strains individually with the psypG/ΔsypE ΔcysK strain and spotted the mixture on an LBS agar plate. Both spots promoted wrinkled colony formation, suggesting that *E. coli* provides something to *V. fischeri*, perhaps cysteine as *E. coli* is known to secrete cysteine.

To determine the mechanism by which *E. coli* promotes *V. fischeri* biofilm formation, we tested the ability of mutants in the *E. coli* Keio collection (obtained from
Dr. Alan Wolfe’s lab) to promote biofilm formation by \( \text{psypG/ΔsypE ΔcysK} \). Of the approximately 5000 \( E. \text{coli} \) Keio mutants screened, a single \( E. \text{coli} \) mutant, cysE, failed to induce wrinkled colony formation of the \( V. \text{fischeri} \) cysK mutant (psypG/ΔsypE ΔcysK).

Screening the 5K library with the mixing spot approach was very labor intensive and thus was performed only once. To verify this result, I collected all of the \( E. \text{coli} \) cys mutants in the Keio collection and assayed their ability to promote wrinkled colony formation of \( V. \text{fischeri} \). I found that, again, only the cysE mutant failed to promote wrinkling (Fig. 14).
Figure 14. Mixing assay with *E. coli* Keio collection cys mutants. The *V. fischeri* cysK (KV6509) mutant was mixed with approx. 5000 individual *E. coli* mutants, and the resulting wrinkled colony formation was evaluated. This figure shows only the results for mixing with *E. coli* cys mutants.
The ΔcysK mutant exhibits a defect in colonizing squid.

A biofilm-induced strain fails to form a biofilm (either wrinkled colonies or pellicles) in the absence of the cysK gene. It has been observed in the lab that mutants that fail to form wrinkled colonies and pellicles also fail to colonize the squid host. Thus, I hypothesized that the cysK mutant would exhibit a colonization defect.

To test this hypothesis, I performed a colonization assay. I inoculated newly hatched squid with either the cysK or WT strain for 3 h or for overnight. Regardless of the amount of inoculation time, I incubated all animals for approximately 24 hours, then I homogenized the squid, plated them on agar plates, and counted the resulted V. fischeri colonies (Fig. 15 A and B).

Data from the 3 h inoculation experiments indicated that the cysK mutant is defective for colonization relative to the WT control. However, when the inoculation took place overnight, the cysK mutant was able to colonize the squid similar to the control. From these data, I was able to conclude that the cysK mutant has an early colonization defect and it is able to overcome this defect when incubated for a longer period of time.
Figure 15. Squid colonization by the cysK mutant. To determine if cysK is required for colonization, squid were inoculated with the ΔcysK mutant, its parent (ΔsypE), or the wild-type strain for 3 hours or 16 hours. Every symbol represent one animal. The blue diamonds represent apo symbiotic animals, the orange squares represent WT-inoculated animals, yellow circles represent the ΔsypE parent, and the gray and green triangles represent ΔsypE ΔcysK.
CHAPTER FOUR

DISCUSSION

The goal of my project was to determine if cysteine plays a role in biofilm formation by *V. fischeri*, and if so, describe that role and begin to probe the requirement for cysteine. In *V. fischeri*, it is known that the *syp* locus and its regulators play very important roles in biofilm formation and they are very well characterized (Yip *et al.*., 2005; Hussa *et al.*, 2008; Shibata *et al.*, 2012). To identify genes other than *syp* that are important for biofilm formation, our collaborators performed a transposon mutagenesis and screened for mutants with defects in wrinkled colony formation. Subsequently, sequence analysis identified several independent insertions in genes involved in cysteine biosynthesis, including *cysH, cysJ, cysN,* and *cysK*.

Cysteine is an important amino acid for all living organisms. Cysteine residues are required for various functions in the body of any living organism, including metal binding, proper growth, and building and proper folding of proteins. As a result, it is not surprising that I found that the *V. fischeri cys* mutants fail to grow in minimal medium that contains limiting amounts of cysteine. However, the *cys* mutants only exhibited a minor defect in growth on LBS, the complex medium used to assay wrinkled colony formation. This result indicates that cysteine is supplied in amounts that are largely sufficient for growth. This minor defect in growth could account for the delayed biofilm phenotype of most of the *cys* mutants, except *cysK*. This latter mutant exhibited a much
greater defect in wrinkled colony formation than the other mutants, but only a slightly greater defect in growth. Thus, I conclude that the growth defect does not account for the defect in wrinkled colony formation by *V. fischeri*.

The wrinkled colony defect that *cys* mutants exhibit could be suppressed by the addition of 1 mM cysteine to the solid agar plate. However, it could not be suppressed by the addition of cysteine at any of a variety of concentrations when it was added directly to the cell culture just prior to spotting (data not shown). It remains unclear why this form of cysteine addition failed to suppress the biofilm defect. Furthermore, the addition of cysteine suppressed the slight delay in pellicle formation by all the *cys* mutants except the *cysK::Tn* mutant. The *cysK::Tn* mutant exhibited a severe defect in pellicle formation that could not be suppressed by the addition of cysteine, regardless of whether this amino acid was added during the prior shaking growth of the cultures and/or during the static growth conditions used to grow pellicles. In contrast, the defect in pellicle formation could be complemented with a wild-type copy of *cysK* inserted at a benign site in the chromosome.

These results suggest two alternative possibilities. First, pellicle formation may be inhibited by the build-up of substrate. The *cysK* gene functions at the bottom of cysteine pathway and converts OAS and H₂S into cysteine. I speculate that, in the absence of *cysK*, there is a lot of OAS and H₂S built up. In addition, OAS can be non-enzymatically converted into N-acetyl serine (Kredich and Tomkins, 1966). One or more of these substrates could inhibit pellicle formation. Indeed, there is evidence in the literature that
addition of OAS suppresses biofilm formation by *E. coli*, presumably by activating inhibitory genes (Sturgill *et al.*, 2004).

Second, it may be the CysK protein itself, rather than cysteine (or the build-up of substrates), that is required for pellicle formation. The CysK is known to physically interact with at least two proteins, CysE and CdiA. The interaction between CysK and CysE (serine acetyltransferase) results in the formation of the cysteine synthase complex that produces cysteine (Wirtz *et al.*, 2010, Zhao *et al.*, 2006). CysK binding to CdiA-cytotoxin (CdiA-CT) is required for contact dependent growth inhibition in *E. coli* (Diner *et al.*, 2012, Ruhe *et al.*, 2013). *V. fischeri* does not encode the components required for contact-dependent growth inhibition. However, the fact that CysK can form complexes with other proteins makes it possible to speculate that CysK may perform other functions, potentially interacting with a protein that is required to promote pellicle formation, or with one that inhibits it.

Once my data verified the involvement of cysteine (and *cysK*) in biofilm formation, I began to investigate its specific role. Because the *syp* locus is important for biofilm formation by *V. fischeri*, it seemed logical to determine if cysteine was required for *syp* transcription and/or Syp polysaccharide (Syp PS) production. My subsequent experiments provided no evidence that *cys* mutations had any impact on transcription of *syp* locus. Furthermore, during the course of this project, I found that the *cysK* mutant attaches to the agar surface very tightly. Other lab members had also observed that some mutants form colonies that are very sticky, either self-adherent or strongly attached to the agar surface. Moreover, there is evidence that the Syp PS is required for both attachment
phenotypes. I thus predicted that the ability of the \textit{cysK} mutant colonies to be self-adherent or attached to the agar surface could be due to the production of Syp PS. Although this possibility still needs to be tested for the \textit{cysK} mutant, these data largely eliminate the possibility of cysteine functions at the level of \textit{syp} transcription and Syp PS production.

I speculate that the three stages of wrinkled colony formation in the laboratory can be correlated to biofilm formation in the natural environment and/or in medically relevant settings such as biofilm formation on/in indwelling catheters. The first stage, in which the bacteria are not attached to anything and the spot can be easily disrupted, can be correlated to the initial attachment that occurs when bacteria are reversibly attached to the indwelling catheter and can easily move back into environment as free living bacteria. The second stage, in which bacteria attach to the agar surface and start to attach to each other and are resistant to perturbation, can be correlated with the growth and maturation stage of biofilm development, in which the cells make a complex 3D structure. The last stage, when wrinkled colonies can be pulled apart from the surface, due to loosening of attachment to the agar surface, could be correlated to dispersal of bacteria from the catheter to a new surface, due to induction of dispersal signals from environment and/or from bacteria itself. The \textit{\textDelta cysK} mutant exhibits the first stage biofilm development and spot does not stick to anything. In the second stage, the spot sticks to the agar surface tightly and it is difficult to disrupt these spots. Here, it is difficult to interpret if the cells within the spots stick to each other or not. However, it is very clear that \textit{\textDelta cysK} mutant lacks the third stage of biofilm development. From these observations, I speculate that the
ΔcysK mutant does not form wrinkled colony because it fails to reach the 3rd stage of biofilm development.

It should be noted that these stages of biofilm development were observed using a sypG overexpressing ΔsypE strain of V. fischeri grown at 28°C. Despite an exhaustive time course, I did not observe attachment to the plate by an rscS overexpressing strain (grown at room temperature). I speculate that this phenotype is specific to sypG-dependent transcription of the syp locus.

Next, I proposed that cysteine might be an inhibitor of a biofilm inhibitor, and performed transposon mutagenesis to screen for mutant colonies with a disruption in a putative inhibitor gene that regained the ability to form a wrinkled colony. I screened almost 80,000 colonies, but I did not find any mutant with restored biofilm formation. One possibility for the absence of wrinkled colony mutants could be that the target gene is very small, and thus rarely disrupted. An alternative possibility is that cysteine activates a positive biofilm regulator; such a positive regulator would not have been identified by my screen. Identifying a putative positive regulator will require a different approach, such as ultraviolet light mutagenesis.

To perform the transposon mutagenesis experiment, I introduced the transposon via conjugation, a method in which helper and donor E. coli are mixed with V. fischeri cells. To my surprise, the mixed spot became wrinkled while the spots of the individual strains remained smooth. I hypothesized that E. coli might provide cysteine to V. fischeri, as E. coli is known to secrete cysteine (Franke et al., 2003). Alternatively, E. coli might provide a factor, missing in V. fischeri cysK mutants, that promotes biofilm formation. To
obtain insight into the interaction between these organisms, I performed mixing experiments of *V. fischeri* cys*K* with the ~5000 strains of the *E. coli* Keio mutant collection to identify which mutant(s) fail to induce wrinkled colony formation by *V. fischeri*. Through this experiment, I identified a single *E. coli* mutant, cys*E*, that failed to induce wrinkling of *V. fischeri* cys*K* mutant. All of the other cys mutants in the Keio collection retained the ability to promote wrinkling. Thus, the next interesting question is, why don’t all of the *E. coli* cys mutants fail to induce wrinkled colony formation of the *V. fischeri* cys*K* mutant? One speculation could that maybe other cysteine mutants can overcome the mutation with some yet unknown pathways. Alternatively, in the absence of cys*E*, but not other cys mutants, the substrate L-serine is built-up; perhaps L-serine can inhibit wrinkled colony formation.

In both *E. coli* and *Providencia stuartii*, cysteine negatively regulates biofilm formation. In these bacteria, mutations in cys*E* result in robust biofilm formation and higher biomass production (Sturgill *et al*., 2004). Furthermore, the enhanced biofilms of the *E. coli* cys*E* mutant were reduced by the exogenous addition of 10 mM of OAS, the product of Cys*E* (Sturgill *et al*, 2004). Thus, it is hypothesized that OAS and other cysteine metabolites may play a physiological role, presumably by activating genes whose expression inhibit biofilm (Sturgill *et al*, 2004).

Given the importance of cys*E* in the ability of *E. coli* to promote biofilm formation by *V. fischeri* and its known role in biofilm formation in *E. coli* and *P. stuartii* (Sturgill *et al*., 2004), I wanted to investigate the role of the cys*E* gene in *V. fischeri* biofilm formation. To this end, I generated a cys*E* deletion construct and tried to obtain
an unmarked deletion in *V. fischeri*, but I was unsuccessful. Furthermore, our collaborator also failed to find any cysE::Tn mutant in screens in his lab (Brooks *et al.*, 2014). These observations lead me to speculate that maybe CysE is essential for *V. fischeri* survival.

Next, I proposed that the *V. fischeri* cysK mutant would exhibit a colonization defect. Mutants that fail to form wrinkled colonies typically also exhibit a colonization defect. To test this hypothesis, I performed a colonization assay. In these experiments, juvenile squids were incubated with a ΔsypE ΔcysK mutant strain of *V. fischeri*, either for 3 hours or for overnight. My results from these experiments revealed that *V. fischeri* cells were unable to colonize the squid when inoculated for 3 hours, whereas they successfully colonized the squid when inoculated for an extended period of time (overnight incubation). These results suggest that cysteine may be required in the initial stages of colonization. From these data, we can also speculate that the squid may secrete cysteine to aid the colonization by *V. fischeri*. There are studies suggesting that squid secretes amino acids like glycine and arginine (Graf and Ruby, 1998) to facilitate the colonization of *V. fischeri*. The Graf and Ruby paper also found that cys mutants colonized to only 5% of the wild-type strain after 3 days. Because my work indicates that cysteine is required for biofilm formation and early in the colonization, it provides an explanation for the colonization defect that Graf and Ruby reported.

**Significance**

This work provides further insight into biofilm formation by *V. fischeri* and the involvement of non-syp genes in this complex process. Importantly, this study demonstrates the role of cysteine in biofilm formation by *V. fischeri* for the first time, and
provides evidence that cysteine functions at a level distinct from *syp* transcription and Syp PS production. My work also determined that *cysK* is the most critical cysteine biosynthetic gene for biofilm formation by *V. fischeri*, and that CysK functions in biofilm formation in at least two ways, one of which is providing cysteine, and the other of which is currently unknown. Thus, this thesis also provides evidence for another function of CysK in *V. fischeri* apart from making cysteine, and provides the idea that CysK may be making a contact with other proteins to promote biofilm formation. Finally, this work also reveals three stages of wrinkled colony development, a finding that has not been observed before in studies of *V. fischeri* biofilm formation.
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VITA

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