BREAKING BIOFILMS: REGULATION OF TYPE II SECRETION SYSTEM IN *V. CHOLERAE* AND THE FORMATION OF THE HYPER-PSEUDOPILUS

By

Rudolph E. Sloup

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ABSTRACT

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Vibrio cholerae is the causative agent of the human disease cholera, it resides in aquatic resevoirs and forms biofilms, which are closely associated communities of bacteria embedded in polysaccharides, DNA, and proteins. In *V. cholerae* biofilm formation is regulated by the second messenger molecule cyclic di-GMP (c-di-GMP). A genetic screen for promoters regulated by the c-di-GMP revealed a novel promoter (P*epsG*) in the *eps* operon encoding the *V. cholerae* Type 2 secretion system (T2SS). The T2SS, which exports proteins from the periplasm to the extracellular space, is phylogenetically related to Type 4 pili. The major pseudopilin is encoded by *epsG* which forms a short piston like structure necessary for secretion. I hypothesized that differential regulation of the *eps* operon extends the pseudopilin forming a structure called a hyper-pseudopilus outside the cell where it promotes biofilm development.

In Chapter 2, I determined that the promoter upstream of the operon (P*epsC1*) is induced four fold by c-di-GMP and this induction is mediated by the c-di-GMP binding transcription factor VpsR directly. High levels of c-di-GMP were found to decrease the activity of extra cellular proteases secreted by the T2SS, however this effect was not a direct result of regulation of the T2SS as determined by mutation of the VpsR binding site in P*epsC1*. I was unable to establish a phenotype for the transcriptional control of the *eps* operon. This work establishes T2S as a new phenotype which is transcriptionally controlled by c-di-GMP and the biofilm associated transcription factor

VpsR. In Chapter 3, I show that overexpression of *epsG* in a continuous flow cell system increased *V. cholerae* biofilms while a Δ*epsG* strain showed no biofilm formation. However, there was no change in activity of T2S dependent serine proteases while *epsG* was over expressed indicating increased biofilms is not likely due to increased secretion. Polyclonal antibody stained EpsG was also detectable on the surface of WT cells and long pseudopili were visualized with over expression of *epsG*. This evidence suggests the T2SS forms a hyper-pseudopilus important for biofilm formation.

In Chapter 4, I present my work identifying novel anti-biofilm compounds. In 2011 Escherichia coli O104:H4 caused the deadliest E. coli outbreak in modern times resulting in 54 deaths and the highest rate of hemolytic uremic syndrome ever recorded. Subsequently, we showed a correlation between biofilm gene expression and virulence factor expression. I sought to identify small molecule compounds effective at inhibiting O104:H4 biofilms. I discovered at a concentration of 0.01% the nonionic surfactants polysorbate 80 (PS80) and polysorbate 20 (PS20) were found to inhibit biofilm formation by 90% and 91% respectively. These compounds were able to disperse preformed biofilms. Treatment of mice infected with E. coli O104:H4 resulted in high bacterial loads and inflammation. While addition of PS80 in the drinking water of the mice did not reduce bacterial loads, it completely abolished inflammation symptoms. PS80 is an FDA approved compound, well studied and effective at low nanomolar concentrations that reduces symptoms of infection in mice. which establishes it as an excellent candidate for further study as an anti-infective agent with anti-biofilm capabilities.

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CHAPTER 1: INTRODUCTION

Biofilms

Biofilms are networks of surface attached bacteria embedded in an extra cellular matrix (ECM). The ECM is a complex and variable substrate which is composed of extracellular polysaccharides, DNA, and proteins [3-5]. Biofilm formation is a widespread bacterial behavior and almost all bacterial species exhibit some form of biofilm lifestyle [6] which allows bacteria to maintain a position in the environment and offers protection from various stresses including mechanical perturbation, dehydration, predation, and antimicrobial agents. Bacteria in biofilms have a significantly different physiology and genetic expression than bacteria in a planktonic state [7, 8]. In the medical field, biofilm formation is particularly problematic, as it allows bacteria to be up to 1000 times more resistant to antibiotics, [9, 10]. Synthetic implants for medical use are susceptible to the persistent nature of biofilms [11-13]. Biofilm-based infection causes 550,000 deaths annually [14] and are estimated to be involved in 80% of all chronic infections according to the NIH [15]. Often the only effective way to overcome a chronic biofilm based infection is amputation of a patients limb [16]. While biofilms are well to cause negative consequences on health and industry, the study of biofilms for positive applications is also important. For instance, biofilms of *Geobacter sulfurreducens* are used in microbial fuel cells to produce electricity [17]. The development, structure and regulation of biofilms are important to our understanding of bacteria and their role in the environment and disease [18].

Bacteria initiate the biofilm lifestyle by contacting a surface where initial attachment is reversible for some time, then they switch to irreversible attachment. Bacteria grow and divide on the surface to form a monolayer of cells [19] or begin growing into clumps. Later bacteria form the three-dimensional structure of the biofilms which may be complex and form various structures such as mushroom-like structures. Finally, bacteria may disperse and leave the biofilm to become planktonic [6, 20-22].

Intense study of biofilms over many years has revealed a large number of regulatory elements controlling biofilm development. One such regulatory element is the second messenger molecule cyclic-di-GMP [23].

Cyclic di-GMP (c-di-GMP)

It is known that biofilm development is regulated by the second messenger molecule 3', 5'-cyclic diguanylic acid (c-di-GMP)[24, 25]. Second messengers are signaling molecules in cells that are used by bacteria to respond and appropriately adapt to their environment. C-di-GMP is found in 80% of gram negative bacteria. Specific enzymes in bacteria called diguanylate cyclases (DGCs) synthesize c-di-GMP and the molecule is degraded by c-di-GMP specific phosphodiesterases (PDEs) [26, 27]. Bacteria typically encode many DGCs and PDEs within their genomes. These proteins contain conserved C-terminal domains responsible for c-di-GMP synthesis and hydrolysis, and variable N-terminal domains which are responsible for sensing environmental signals and regulating a wide range of phenotypes. DGCs are characterized by GGDEF domains and synthesize c-di-GMP from 2 molecules of GTP while in a homodimer. Roughly half of GGDEFs also contain feedback inhibition site (I-

site) at a RXXD motif upstream of the GGDEF site which prevent enzymatically active homodimers therefore inhibiting c-di-GMP synthesis [28]. PDEs may have either EAL or HD-GYP domains and catalyze hydrolysis of c-di-GMP to pGpG or a pair of GMP, respectively. EALs are dependent on a conserved glutamate residue as well as a requirement for metal ions Mg^{2+} or Mn^{2+}. HD-GYP PDEs are not as ubiquitous as EALs. GGDEF and EAL proteins have been found to be nearly ubiquitous in bacteria [29, 30].

C-di-GMP regulates many bacterial processes from transcription to post-translational processes through interaction with effectors such as riboswitches, transcription factors and adapter protein domains [31]. There are two classes of c-di-GMP riboswitches, one of which are called GEMM [32]. The first c-di-GMP protein receptor discovered was the PilZ domain, which is named for the protein PilZ from *P. aeruginosa* involved in pilus formation, proteins with PilZ domains exist in many bacteria and examples include YcgR from *E. coli* and PlzC, and PlzD from *V. cholerae* [33-35]. PilZ domains are known to regulate a variety of functions such as polysaccharide synthesis, DNA binding, motility and translocation. Another type of c-di-GMP receptor are those that utilize c-di-GMP binding motifs found in DGCs and PDEs. I-sites can be used to sense c-di-GMP and if the GGDEF associated with them evolves a loss of function then they can be used exclusively for regulation of phenotypes independent of altering c-di-GMP concentrations [30]. One such example is CdgA, a protein involved in predation in *B. bacteriovorus* [36]. EAL domains which have lost catalytic activity are also known to be sensors for c-di-GMP regulation such as *P. aeruginosa* FimX, which is a factor in type 4 pilus motility [37]. Other c-di-GMP receptors include transcriptional regulators such as the NtrC-like enhancer binding protein FleQ from *P. aeruginosa*

which a regulator of flagellar genes, and VpsR of *V. cholerae* which is a regulator of polysaccharide genes as well as other genes [38-40]. Other transcription factors include FlrA and VpsT from *V. cholerae* and CRP-type activators from *Burkholderia* and *Xanthomonas* [41-44].

C-di-GMP appears to be a global regulator of bacterial biology and controls many phenotypes in addition to biofilm formation and motility and understanding the phenotypes controlled by this signal is one of the fundamental questions in the field. For example, c-di-GMP has been shown to regulate virulence, cell cycle, differentiation, secretion and an ever growing list of other functions including predation, heterocyst formation, and antibiotic production [24, 30, 45]. In addition, c-di-GMP regulates many forms of motility including flagellar swimming, swarming, and twitching motility mediated by type 4 pili [46-48].

Vibrio cholerae

Among the bacteria that utilize c-di-GMP, *V. cholerae* has a very high number of 63 DGCs and PDEs. *V. cholerae* is a human pathogen which is responsible for around 91,000 deaths each year [49, 50]. Certain strains that contain the phage encoding cholera toxin are the causative agent of cholera, an infectious disease which causes severe diarrhea and dehydration that can lead to death. These strains are identified as serogroup O1 which is divided into the Classical and El Tor biotypes, and serogroup O139 which was derived from the El Tor biotype. We are currently in the 7th worldwide pandemic of *V. cholerae,* which is caused by the El Tor biotype; the first six pandemics were caused by the Classical biotype [51]. Cholera infections are most problematic in

areas where there is poor sanitation and access to clean water. Cholera outbreaks in endemic areas such as such as Bangladesh and India occur seasonally and in non-endemic areas outbreaks can take place if there is a breakdown in the infrastructure for sanitation and delivery of drinking water such as in the outbreak in Haiti, which started in 2010. Cholera infections spread quickly during an outbreak in a fecal to oral manner due to the hype-infective state of *V. cholerae* in freshly shed cells in the feces. In its natural aquatic reservoirs, *V. cholera* is known to persist in biofilms in close association with chitinous marine organisms [52-56]. As such it makes an ideal model organism to study biofilm formation, development and regulation by c-di-GMP.

The major virulence factor of *V. cholerae* is cholera toxin (CT), which is the agent responsible for causing the profuse watery diarrhea which causes dehydration and may lead to death if not treated. CT is an AB_5 family ADP-ribosyltransferase which is secreted by the T2SS after which the B subunits attach to GM1 gangliosides on epithelial cells[57]. Subsequently endocytosis brings the complex into the target cell and the A1 subunit is released and then chaperoned into the endoplasmic reticulum. The A1 subunit is released to the cytoplasm, here it binds the host protein ADP-ribosylation factor 6 which causes a conformation change that activates its catalytic activity. Next A1 ribosylates Gs alpha subunit which increases cyclic AMP (cAMP). High levels of cAMP activate protein kinase A which phosphorylate the cystic fibrosis transmembrane conductance regulator. This leads to secretion of ions into the intestinal lumen which causes immense fluid loss from the intestine resulting in severe diarrhea and dehydration of the host. Another major virulence factor is the toxin coregulated pilus (TCP) which is a type IV pilus (T4P) required for intestinal colonization in humans.

Motility is also an important virulence factor in *V. cholerae* as mutants that are defective in motility are not able to traverse the mucosal layer above the epithelium to initiate disease[58]. *V. cholerae* motility is enabled by a single sheathed polar flagellum[59]. This flagellum is regulated by several transcription factors, one of which is FlrA, the master transcription factor that directs expression of the flagellar biosynthesis gene operon. Our laboratory has shown that c-di-GMP can bind to FlrA to inactive it ability to stimulate transcription, leading to a reduction in motility [44].

In *V. cholerae,* biofilms are an important lifestyle for survival in the environment and in the host. In the environment, biofilms attach cells to surfaces such a chitinous surfaces of marine organisms like copepods and other crustaceans. In these conditions, the biofilm aids in protection from environmental insults such as predation, mechanical disruption and chemical stresses[55, 56, 60]. During infection, biofilms aid in protection from bile acids and the acidic stomach. *V. cholerae* biofilms start when the cells attach to a surface and motility is repressed; the mannose sensitive hemagglutinin T4P (MSHA) is implicated in this step [53, 61]. Then, induction of genes required for synthesis of EPS, many of which are in the two *vps* gene clusters on the first chromosome encoded by the genes *vpsU*, *vpsA-K* and *vpsL-Q*. C-di-GMP induces biofilm formation by inducing the transcription of these genes[62]. A deletion of the *vpsL* gene is used extensively in this thesis when biofilm-free cells are desired. Additionally, the gene cluster *rbmA-F* encodes genes for proteins involved in biofilm formation as well as *bap1*[63-65]. Induction of biofilms by c-di-GMP is promoted by two transcriptional regulators, VpsR and VpsT, which both bind to and are activated by c-di-GMP [66, 67]. The transcription factors work together to induce the *vps* gene clusters as

well as a variety of other elements important for biofilms. Also important to *V. cholerae* biofilms is eDNA which is regulated by the nucleases *dns* and *xds*.[68]

There is a variety of evidence supporting the importance of biofilms in *V. cholerae* infections. Microscopic examination of infecting *V. cholerae* in a variety of systems has revealed biofilm-like clusters of cells. Furthermore, large aggregates of cells can be observed in the stool of infected humans [69, 70]. Clonal microcolonies have also been observed in the small intestine of infant mice [71]. Also, in rabbit models aggregates of *V. cholerae* were observed adhering to the villi and mucous coat [72, 73].

Type II secretion system

In this thesis, I will describe my results indicating that c-di-GMP induces expression of the genes encoding the Type II secretion system (T2SS) in *V. cholerae.* The T2SS is a general secretion system found in most if not all gram negative bacteria. It is unique from other secretion systems in that it can export fully folded proteins from the periplasm to the extracellular space [74] where other secretion systems, with the exception of the Type VI secretion system (T6SS), need to export unfolded proteins which mature after export. Because of this feature, the T2SS is the choice platform for exporting proteins which require multimeric states, folding under conditions found in the periplasm or cytoplasm, or the addition of other cofactors. The T2SS was discovered in the 1980s in the genus *Klebsiella* [75], and since then it has been studied in a variety of bacteria including but not limited to enterotoxigenic *E. coli* (ETEC) [76], *Pseudomonas* [77], *Erwinia* [78, 79], *Xanthomonas* [80] and *Vibrio* [81]. T2S proteins are predicted in a wide range of bacterial clades with hundreds of representatives in the Proteobacteria

from a wide range of environments from halophiles[82] to psychrophiles [83], there are also representatives in clades such as Chlamydia, Cyanobacteria, and Spirochetes. T2SS is necessary for pathogenesis for a number of bacteria including *V. cholerae* (cholera toxin)*, Burkholderia cenocepacia* AU1054, *P. aeruginosa* (exotoxin A) and Enterotoxigenic *Escherichia coli* (heat-labile enterotoxin) [84, 85]. My research has focused on the T2SS of *V. cholerae* which is encoded by the *eps* (extracellular protein secretion) gene cluster. In *V. cholerae, t*he T2SS is known to export proteases and toxins and biofilm effectors, as well as other enzymes, and is necessary for biofilm formation in *V. cholerae* and other bacteria [86, 87]. The T2SS is also necessary for healthy cells such as in *V. cholerae* T2SS mutants lead to cell envelope stress [88].

The T2SS is homologous to Type IV pili (T4P) [89, 90]. It is thought that the pilus structure helps to secrete folded proteins through the outer membrane pore. These pilins, which are encoded by the major pilus gene *epsG* and the minor pseudopilins *epsH-J* in *V. cholerae,* form a short piston-like structure necessary for secretion called the pseudopilus [74]. The pseudopilins share homology with the type IV pilins. However, the pseudopilus is not known to extend outside the cell in WT strains to form a pilus. The T2SS is also homologous to archaea pili as well as their flagellum which are also called archaellum [91-93]. Having highly similar structures present in such a diverse set of organisms suggest that T2SS has its evolutionary roots very deep in the tree of life.

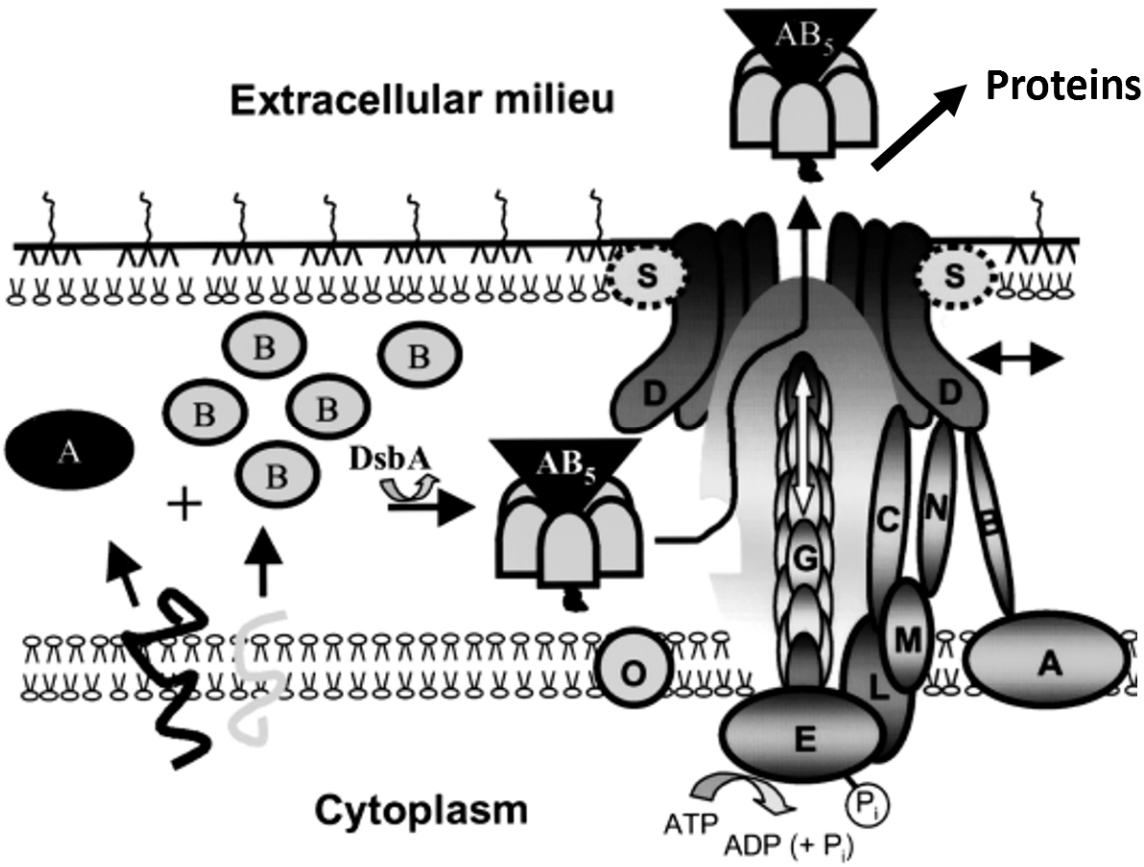


Figure 1.1. The T2SS. Image of T2SS adapted from review article [1]. Proteins A (*ctxA*) and B (*ctxB*) enter the periplasm by the Sec pathway. Subsequently they fold and assemble into their native conformation. The T2SS is then able to excrete the protiens to the extracellular milieu using ATP as the energy source driven by the APTase E (*epsE*). They exit through the secreton D *epsD*. The pseudopilus is composed of the pseudopilins with G (*epsG*) being the major constituent.

The entire T2SS complex is composed of up to 15 different proteins, which are typically multimeric. In *V. cholerae,* twelve of these proteins are encoded in the *eps* gene cluster beginning with *epsC* and going to *epsN* and there is also a prepilin peptidase *vcpD* (*pilD*) which is not near the *eps* gene cluster. EpsD is the secretin which sits in the OM and its multimeric assembly is aided by GspAB.[94]. The major pseudopilin EpsG, and the minor pseudopilins EpsH,I,J,K are imported to the periplasm through the Sec

translocase where the prepilin peptidase VcpD cleaves the leader sequence at a conserved glycine residue, then they are integrated into the T2SS apparatus. The hexameric ATPase EpsE powers assembly of the pseudopilus and secretion it sits in the cytoplasm where it interacts with the inner membrane protein complex. The inner membrane protein complex consists of EpsF, EpsL, and EpsM. EpsL has been shown to interact with EpsG suggesting it transmits the power from the EpsE to the pseudopilins for assembly [95-97].

While the T2SS is most widely recognized for its ability to secrete proteins to the extracellular space the complex is also used to secrete proteins destined to become outer membrane attached [98].

Regulation of T2SS

Until recently little was known about transcriptional regulation of the T2SS. A microarray of *V. cholerae* found a significant increase in *epsC-N* transcripts upon induction of a DGC [67]. Also, it was found that *epsC* and *epsE* transcripts were upregulated in knockouts of *hfq* and *rseA,* which the authors showed was due to increased expression of the sigma factor *rpoE.* However, there was only a modest decrease in transcripts in an *rpoE* mutant compared to the wild type strain [99]. As *rpoE* helps the cells to respond to periplasmic stress, it was hypothesized that increased expression of the T2SS by RpoE was a response to mitigate such stress. Concurrent with our experiments, new insights were gained into the regulation of T2S. Two promoters were predicted to be upstream of *epsC;* the downstream promoter was predicted to be σ^{70}-dependent while the upstream promoter was σ^{E} dependent [100].

Moreover, two *V. cholerae* DGCs were shown to induce an *epsC-lux* transcriptional fusion in *E. coli* [100]. The ATPase that drives pseudopilus synthesis of the T2SS from *P. aeruginosa* is positively activated by direct binding to c-di-GMP [101]. However, the authors of that study were unable to find the same result in *V. cholerae* as the T2SS ATPase EpsE did not interact with c-di-GMP.

Antibiofilm Strategies

The first antibiotic discovered was penicillin in 1940 which ushered in the modern era of antibacterial chemotherapy. Since then over 350 antimicrobial agents have been used from a wide range of sources including natural products, semi-synthetic compounds, and synthetic compounds [102, 103]. Approximately 100,000 tons of antibiotics are produced annually and used heavily to treat bacterial infections but also for other uses such as growth promotion in livestock [104]. Heavy and liberal use of antibiotics has increased the spread of antibiotic resistant bacteria [105, 106]. In addition, antibiotic discovery and development has slowed over time as we have observed diminishing returns making it more expensive to develop and harder to find novel antibiotics this has caused drug companies to slow or eliminate research in antibiotics [107]. However, emerging multi drug-resistant (MDR) pathogens are decreasing the pool of effective antibiotics. This is a major problem as infectious diseases are the second largest killer of humans in the world [107, 108]. It is also notable that antibiotics can also act as inducers of biofilm formation at subinhibitory concentrations [109, 110]. Therefore, there is an urgent need to develop new anti-microbial strategies to treat infectious disease.

In addition to studying c-di-GMP signaling and the regulation of T2SS in *V. cholerae,* my thesis also explored the discovery, characterization, and development of antibiofilm compounds. Due to the large global impact of biofilms, new classes of drugs are being investigated to target biofilms. These compounds are part of a larger class of compounds termed anti-infective compounds that do not kill bacteria but inhibit their ability to cause disease. Anti-infective compounds have the advantage that they will reduce selective pressure to evolve resistance as they are not lethal to cells. This allows the body to clear the infection by natural pathways while reducing disease severity. Anti-infective compounds are an important class of drugs because the traditional mode of treatment uses antibiotics, which have been an amazing advancement in medicine; however, antibiotics are losing efficacy as bacterial resistance evolves and spreads. In addition to medical application, antibiofilm compounds could have a wide variety of applications in industrial settings. For instance, in food processing contamination and transfer of pathogens such as *E. coli* O157:H7 from contamination sources to food and machinery, which is often dependent on biofilm formation, [111] could be an application for antibiofilm compounds in the sanitizers used to treat the production line. At the time of writing there are no traditional anti-biofilm drugs in clinical use and only a handful of antibiofilm targeted technologies in trials [112, 113]. While this shows the significant challenges in developing ant-biofilm compounds it also means the field is wide open for discovery. Multispecies biofilms are also of great concern there have been many studies showing that infections often consist of multiple species in the biofilm [114] and this necessitates broad spectrum antibiofilm compounds or cocktails of multiple antibiofilm compounds.

A number of antibiofilm compounds have been described. For instance, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, a QS inhibitor was used to treat *P. aeruginosa* cultures, the exoproducts of those cultures was then presented to human airway epithelial cells. Cells which received native exoproducts had decreased would healing, while those which were presented with the QS inhibitor treated exoproducts healed faster [115]. Related to QS inhibitors I contributed to the identification of a set of DGC antagonist which inhibit biofilm formation [116]. While the former are all small molecules larger proteins are also found to have antibiofilm activity such as the glycoside hydrolase dispersin B [117, 118].

There are variety of alternatives to traditional chemotherapy for combating biofilms as well. One antibiofilm vaccine is being developed against *Fusobacterium nucleatum* as the vaccine targets and outer membrane protein involved in bacterial aggregation [119]. Vaccines that target specific biofilm associated structures represent another strategy in fighting biofilms. It has also been shown that protozoan predation can reduce biofilms *in vitro* [120] and this may be an effective strategy in industry or non-medical applications. Another predatory system would be bacteriophages which could be used to control biofilms [121].

In this work I also reveal the efficacy of a set of nonionic surfactants polysorbate 80 and polysorbate 20 against E. coli O104:H4 biofilms.

Escherichia coli biotype O104:H4

In Chapter 3, I describe my discovery and characterization of anti-biofilm compounds of *E. coli* O104:H4. Originating in Germany in 2011, a newly evolved

pathogenic *Escherichia coli* biotype O104:H4 (hereafter referred to as O104:H4) infected 3842 people, 22% of which developed hemolytic uremic syndrome (HUS) leading to 54 deaths making this the deadliest *E. coli* outbreak ever recorded. O104:H4 is most closely related to the enteroaggregative *E. coli* (EAEC) as determined by genomic sequencing [122]. The virulence properties of O104:H4 that promoted such a high disease severity rate are not understood. However, it is known that O104:H4 acquired a prophage carrying the stx from an *E. coli* O157:H7 which is a protein produced by Stx-producing *E. coli* (STEC).

Stx is associated with severe disease symptoms in humans including diarrhea and HUS [123], and it is thought that the adherence properties of EAEC may help increase the severity of disease by allowing the bacteria to be closely associated with epithelia and cause increased transmission of Stx into the host bloodstream. There are 2 groups of Stx: Stx1 and Stx2 [124, 125]. Of those, the latter is associated with more severe disease symptoms in humans and is the group carried by O104:H4. Stx are similar to cholera toxin in that they are AB toxins composed of 5 B subunits and a single enzymatically active A subunit which is cleaved into a pair of fragments A1 and A2. The A1 subunit acts by N-glycosidase activity resulting in catalytic inactivation of 60S ribosomal subunits in the target host. The B subunit bind to a membrane glycolipid, globotriaosylceramide (Gb3).

EAEC are characterized by their stacked brick biofilm appearance on epithelial cells and are known to cause persistent or acute diarrhea in infants and young children as well as affecting adults with HIV and travelers termed travelers' diarrhea. Although many virulence factors have been identified in EAEC, none of them are common to all

pathogenic strains. These virulence factors include *aggA*, *aggR*, *set1*, *pic* and *app* loci. AggR is a transcriptional activator associated with increased diarrheal disease as determined by epidemiological studies [126, 127]. AggR regulates a number of genes including dispersin and the Aat secretion system, which is required to transport dispersin. Dispersin is thought to allow AAFs to escape the strong negative charge of LPS allowing them to extend out further from the cells so the cells can bind to objects further away. AggR also regulates the aggregative adherence fimbriae (AAF), which are encoded on the pAA plasmid. These fimbriae are responsible for the aggregative adherence that attach bacteria to the intestinal mucosa in the stacked brick pattern and induce inflammation. In O104:H4 AAF have also been shown to be important for biofilm formation on fresh produce and abiotic surfaces [128]. Long polar fimbriae (LPF) have also been shown to be necessary for biofilms and adhesion to epithelial cells [129]. O104:H4 was also shown to have high levels of curli and c-di-GMP two biofilm associated factors.

Summary of findings:

Chapter 1: Transcriptional regulation of type 2 secretion by c-di-GMP

Cyclic di-GMP (c-di-GMP) is a second messenger in *V. cholerae* that regulates biofilm formation and inhibits motility. A screen for promoters that are regulated by c-di-GMP led us to investigate the regulation and role of biofilms in the *V. cholerae* Type II secretion system (T2SS). I have characterized 2 transcription regulatory elements in the T2SS. The first, P*epsC,* preceded the first gene in the cluster *epsC* and the second, P*epsG,* precedes *epsG* in the middle of the gene cluster. The promoter I identified

upstream of P*epsG* is a completely novel promoter. We identified their transcriptional start sites and characterized the regions of DNA that are necessary for expression and regulation. We have also shown that the T2SS in *V. cholerae* is transcriptionally regulated at P*epsC* by the bacterial second messenger c-di-GMP. This regulation is mediated by the c-di-GMP binding transcription factor VpsR through direct interaction with the promoter P*epsC*. During these studies, I found that c-di-GMP decreased extracellular protease activity in a VpsR-dependent manner, but this decrease was not due to transcriptional regulation at P*espC.* I also demonstrated that transcriptional regulation of P*epsC* by c-di-GMP does not affect cholera toxin secretion or biofilm formation.

Chapter 2: Investigating the role of the T2SS hyper-pseudopilus in biofilms

The T2SS exports proteases and toxins from the periplasm to the extracellular space and is functionally related to Type 4 pili which are implicated in biofilm development. The major pseudopilin of the T2SS is encoded by *epsG*. EpsG forms a short piston like structure in the periplasm necessary for secretion. We hypothesized that transcription from P*epsG* increases EpsG forming a pseudopilus outside the cell to promote biofilm development. Consistent with this hypothesis, overexpression of *epsG* in a continuous flow cell system increased biofilms while a Δ*epsG* strain showed no biofilm formation. EpsG was also detectable external to biofilm grown WT cells and present as long pseudopilus structures in biofilms upon over expression of *epsG*. I am still investigating the role of P*epsG* as nothing is currently known about its regulation or function. I have demonstrated that ectopic expression of EpsG can increase biofilms, I

have also shown this does not affect secretion. I hypothesize that there are conditions in which the P*epsG* is differentially regulated and then the T2SS acts as a biofilm structural component, although such conditions have not been identified.

Chapter 3: O104:H4 biofilm inhibition and dispersal by polysorbate 80

We collaborated with the laboratory of Shannon Manning to measure biofilm formation of O104:H4, and compare infection of O104:H4 to Enterohemorrhagic *Escherichia coli* O157:H7 in a germ-free mouse infection model. In this project, we demonstrated that O104:H4 forms robust biofilms *in vitro*, and the expression of genes hypothesized to be involved in biofilm formation was correlated with increased toxin production and disease severity *in vivo*. From this work we hypothesized that *in vivo* biofilm formation increases disease severity of O104:H4 infections.

Based on this study, I initiated a project to identify compounds that inhibit biofilm formation of O104:H4. I assayed 21 previously identified anti-biofilm compounds for inhibition of O104:H4 biofilms, and found only 2 of these to be effective in inhibiting *in vitro* biofilms. The surfactants polysorbate 80 (PS80) and polysorbate 20 (PS20) reduced biofilms 90% or greater at a concentration of 0.01%. Neither polysorbate affected cell growth. The effective concentration at 50% (EC_{50}) for PS80 was 0.0001% (0.81 μM) and PS20 was 0.00006% (0.54 μM). PS80 and PS20 also dispersed preformed biofilms. I measured a 50% attachment defect in cells treated with PS80. To gauge the *in vivo* efficacy of PS80, we collaborated with the laboratory of Alredo Torres to infect mice treated with PS80 with *E. coli* O104:H4 and measured disease outcomes compared to an infected but untreated control. Untreated mice had similar bacterial

colonization levels as mice treated with PS80. However, histopathological examination revealed disease severity was significantly reduced in mice treated with PS80. PS80 is a generally recognized as safe (GRAS) compound that is frequently found in foods. PS80 effectively inhibits O104:H4 biofilms at nanomolar concentrations and significantly reduces disease severity in mice infected with *E. coli* O104:H4. Our work has shown that PS80 exhibits significant potential for curbing future O104:H4 outbreaks.

CHAPTER 2: CYCLIC DI-GMP INDUCES THE EXPRESSION OF TYPE TWO SECRETION IN *VIBRIO CHOLERAE* WHILE INHIBITING EXTRACELLULAR PROTEASE ACTIVITY

Abstract

Vibrio cholerae is a human pathogen which reciprocates between growth in environmental reservoirs and infection of human hosts causing severe diarrhea. The second messenger cyclic di-GMP (c-di-GMP) mediates this transition by controlling a wide range of functions such as biofilms, virulence and motility. Here we report that c-di-GMP also impacts the *Vibrio cholerae* Type II Secretion System (T2SS) by induction of the *epsC-epsN* gene cluster that encodes for the T2SS. Analysis of the *eps* genes confirmed the presence of two promoters located upstream of *epsC*, the first gene in the operon, one of which (P*epsC*1) is induced by c-di-GMP. C-di-GMP induction of P*epsC*1 is observable throughout the *eps* genes, suggesting these genes are expressed as an operon. This induction is directly mediated by the c-di-GMP-binding transcriptional activator VpsR. We also discovered a novel second promoter region in the T2SS operon located upstream of *epsG* that is not regulated by c-di-GMP. Increased c-di-GMP leads to decreased extracellular activity of T2SS-dependent proteases; however, secretion of cholera toxin is not reduced. This reduction of protease activity is VpsR dependent but independent of transcriptional induction of the *eps* operon. These results place type II secretion and extracellular protease activity as new phenotypes controlled by c-di-GMP in *V. cholerae*.

Importance

Type II Secretion Systems (TTSS) are the primary molecular machines by which Gram-negative bacteria secrete proteins and protein complexes that are folded and assembled in the periplasm. The substrates of TTSSs include extracellular factors like proteases and toxins. Here, we show that the widely conserved second messenger cyclic di-GMP (c-di-GMP) upregulates expression of the *eps* genes encoding the TTSS in the pathogen *V. cholerae* via the c-di-GMP-dependent transcription factor, VpsR. To our surprise, induction of T2SS expression resulted in decreased extracellular protease activity while not impacting cholera toxin secretion. Our results describe T2SS and extracellular protease activity as new bacterial functions controlled by c-di-GMP in *V. cholerae*.

Introduction

Vibrio cholerae is a major bacterial pathogen responsible for the diarrheal disease cholera, causing an estimated 2.8 million cases each year resulting in approximately 91,000 deaths [50]. *V. cholerae* is endemic to coastal waterways in tropical countries where it persists in the environment as a biofilm on chitinous surfaces and periodically causes outbreaks in human populations. *V. cholerae* can rapidly spread and multiply in favorable environmental conditions as seen in recent outbreaks, including the 2010 Haiti outbreak [130]. A fundamental component of the ability of *V. cholerae* to cause disease is its ability to transition from environmental reservoirs to human hosts. This transition is in part regulated by the second messenger molecule cyclic di-GMP (c-di-GMP).

C-di-GMP is a nearly ubiquitous second messenger molecule in bacteria that controls a range of physiological functions including biofilm formation, motility, and virulence factor expression [30]. C-di-GMP is synthesized by diguanylate cyclases (DGCs) [131] and degraded by phosphodiesterases (PDEs) [132, 133]. Together these enzymes alter the concentration of c-di-GMP in the cell in response to environmental inputs. C-di-GMP has been shown to repress motility and virulence to promote a sessile, biofilm-associated lifestyle by stimulating the production of exopolysaccharide matrix substances (EPS) and adhesins while inhibiting flagellar activity or expression [25, 26, 134-136]. Intracellular c-di-GMP has been proposed to be high in environmental reservoirs, inducing biofilm formation to promote survival while it is reduced in human hosts allowing virulence factor gene expression [137], although recent results from our laboratory suggest *in vivo* levels of c-di-GMP may be dependent upon spatial localization within the intestine [138].

The Type II Secretion System (T2SS) of *V. cholerae* also contributes to environmental persistence and host disease. The T2SS is a sophisticated multi-protein complex that spans the inner and outer membrane of many Gram-negative bacteria. Proteins destined for export via the T2SS are first translocated across the cytoplasmic membrane via the SEC or TAT pathway [139, 140], where they assemble in the periplasm before being exported as fully folded proteins into the extracellular milieu [1]. In *V. cholerae*, the T2SS consists of 13 proteins, 12 of which are encoded by contiguous genes comprising the extracellular protein secretion (*eps*) gene cluster. Many T2SS-dependent proteins are degradative enzymes or toxins that contribute to bacterial pathogenesis; thus the T2SS is considered an important molecular machine

necessary for virulence [84]. Within the host, *V. cholerae* causes diarrhea by T2SS-dependent secretion of cholera toxin. In addition to cholera toxin*, V. cholerae* exports other extracellular factors including chitinases, proteases, DNase, and pilin via the T2SS, which aid in its ability to successfully occupy diverse ecological niches [141]. The T2SS also secretes the three proteins RbmA, RbmC, and Bap1 which are necessary for robust, shear-resistant biofilm formation [134, 142-144]. Other major bacterial pathogens such as *Escherichia coli* and *Pseudomonas aeruginosa* secrete virulence factors through the T2SS encoded by genes sharing considerable similarities to those of *V. cholerae* [1, 87, 145, 146].

Until recently little was known about transcriptional regulation of the T2SS. A microarray of *V. cholerae* found a significant increase in *epsC-N* transcripts upon induction of a DGC [67]. Concurrent with our experiments, two promoters were predicted to be upstream of *epsC;* the downstream promoter was predicted to be σ^{70} dependent while the upstream promoter was σ^{E} dependent [100]. Moreover, two *V. cholerae* DGCs were shown to induce an *epsC-lux* transcriptional fusion in *E. coli* [100].

In searching for genes in *V. cholerae* regulated by c-di-GMP*,* we identified a novel promoter upstream of the *epsG* gene in the middle of the *epsC-epsN* T2SS gene cluster. This discovery inspired us to examine transcription control of the T2SS in *V. cholerae*, and the impact of this control on T2SS activity. Although the P*epsG* promoter was not significantly regulated by c-di-GMP, we found that transcription from the promoter of *epsC,* the first gene of the putative *eps* operon, was induced by c-di-GMP. Indeed, induction of *epsC* by c-di-GMP extends through the *eps* operon in a coordinated fashion providing evidence that this gene cluster is indeed an operon. Further analysis

revealed two transcription start sites upstream of *epsC,* one of which is induced by c-di-GMP via the c-di-GMP-dependent transcription factor VpsR. This regulation appears to be direct as VpsR directly interacts with the *epsC* promoter at a putative VpsR binding site and site directed mutation of this site abolishes c-di-GMP induction of *epsC*. We further find that the activity of extracellular proteases is decreased at elevated c-di-GMP, but secretion of cholerae toxin is unaffected. Although decreased protease activity is dependent on VpsR, it does not require transcriptional regulation of *eps* by c-di-GMP. Thus, we find that type II secretion and extracellular protease activity are new bacterial behaviors in *V. cholerae* regulated by the global second messenger c-di-GMP.

Materials and Methods

Bacterial strains, culture conditions, and DNA manipulation

All experiments utilized *V. cholerae* El Tor biotype strain C6706str2 or mutant derivatives (Table A1). Plasmids were introduced into *V. cholerae* through biparental mating with *E. coli* S17-λpir as the donor and verified by antibiotic selection and culturing on Thiosulfate-citrate-bile salts-sucrose agar plates (Difco). Unless otherwise stated, bacteria were incubated at 35°C shaking at 220 RPM in Lauria-Bertani (LB) medium (Accumedia). Agar plates were made with 15 g/L Agar (Accumedia). Antibiotics were used at the following concentrations: kanamycin, 100 μg/mL, chloramphenicol 10 μg/mL, and polymyxin B 10 U/mL. Protein expression vectors were induced with 100 μM isopropyl-β-D-thiogalactoside (IPTG) unless otherwise stated. All compounds were purchased from (Sigma). Relevant plasmids and primers are shown in Table A2.

Polymerase chain reaction was performed using standard methods with Invitrogen HiFi Taq polymerase. Transcriptional fusions of test promoters were constructed in the pBBRlux plasmid SpeI and BamHI restriction sites using restriction endonucleases (Fermentas or New England Biolabs) and ligated with T4 DNA ligase (New England Biolabs). For gene expression studies, luminescence was measured in opaque white 96 well microtiter plates (Corning) following 6 hours of growth following a 1/1000 dilution on either a SpectraMax M5 plate reader (Molecular Devices) or Envision Multimode Plate Reader 2104-0020 (Perkin Elmer).

Identification of transcriptional start sites

RNA was prepared from CW2034 with the plasmids pAEKlv8 and p6f12. CW2034 is a Δ*vpsL* mutant of C6706str2 that does not flocculate at high c-di-GMP concentrations and was thus used for experiments such as RNA extraction and gene expression. Cultures were lysed using Trizol reagent according to the manufacturer's instructions (Invitrogen) from 2 mL of cells at an OD_{600} of 0.5. The transcriptional start sites were determined by 5'-rapid amplification of cDNA ends (5' RACE) according to the manufacturer's instructions (Invitrogen). Two rounds of nested amplification using the primers listed in Table A2 were used with a 0.1% dilution of the original PCR reaction.

Quantitative real-time PCR

Overnight cultures of CW2034 with the plasmids pCMW75 and pEVS141 were diluted 1:1000 in 3 mL LB, induced with 1 mM IPTG and grown to OD_{600} of 1.0. Cultures

were harvested, pelleted by centrifugation and the supernatant was discarded. Cells were lysed, and RNA was extracted with the RNeasy RNA extraction kit (Qiagen) per the manufacturer instructions. RNA was reverse transcribed to cDNA with GoScript Reverse Transcription System (Promega). Quantitative PCR was carried out with TaqMan reagents (Invitrogen).

Electro mobility shift assay (EMSA)

VpsR purification was carried out as previously described [40]. DNA probes were generated by PCR with FAM (6-carboxyfluorescein) labeled primers CMW234 and CMW235 which flank the SpeI and BamHI restriction sites of pBBRlux. DNA was purified using Promega SV Gel and PCR clean up kits. 10 nM probes were incubated with 50 μg/mL poly dI-dC at 30°C for 30 minutes with VpsR in a 20 μL total reaction volume balanced with VpsR buffer (20 mM Sodium phosphate, 250 mM NaCl, 20% glycerol, and 0.05% β-mercaptoethanol). Following incubation, 2 μL of 80% glycerol was added to each reaction and appropriate volumes of reaction were loaded onto 5% polyacrylamide-Tris-Acetic Acid-EDTA gels. Electrophoresis was carried out for 1 hour at 95 volts and visualized on a Typhoon FLA 9000 scanner (GE healthcare Life Sciences).

Protease Assay

Analysis of secreted protease was determined using the protocol adopted from [147]. Overnight cultures were made using three individual colonies of each strain with shaking at 37 degrees. Optical density was determined at 600 nm, and 1 mL was centrifuged at 16,000 g for 5 minutes. Supernatant was harvested and kept on ice. 20 μL of 1 mM substrate, BOC-Gln-Ala-Arg-7-Amino-4-methyl-coumarin, from Sigma was added to 80 μL of culture supernatant to achieve a 0.2 mM final substrate concentration. Fluorescence was recorded in real time using a Spectra Max M5 plate reader (Molecular Devices) every minute for 20 minutes with excitation at 350 nm and emission at 460 nm, and cutoff 455 nm and the average increase in slope is reported as a avg(dF/dT)/OD_{600}. A higher avg(dF/dT)/OD_{600} is indicative of higher protease activity.

GM1 ELISA

V. cholerae containing the EtxB expressing plasmid pWD615 [148] and pCMW75 were grown overnight, diluted 1:100 in LB, and grown shaking. Cells were harvested in late exponential phase and pelleted by centrifugation. The supernatant and cells were separated and kept on ice, cells were lysed by sonication then resuspended in equal volumes of PBS. Enterotoxin B was quantified by GM1 ELISA as previously reported [141].

Biofilm Assay

V. cholerae WT biofilms were grown on an MBEC plate where biofilms form on pegs attached to the lid. Overnight cultures in LB were diluted 1:500 then 160 μL was

incubated for 8 hours. The MBEC lid with attached biofilm was then transferred to a new microtiter plate containing 160 μL of PBS for 5 minutes to remove planktonic bacteria. Subsequently the lid was transferred to an opaque black 96 well plate (Perkin Elmer) containing 160 μL of 25% BacTiter-Glo (Promega) where it was incubated for 5 minutes and then luminescence was measured on a SpectraMax M5 plate reader (Molecular Devices).

Results

Transcription control of the *eps* T2SS operon of *V. cholerae*

We performed a genetic screen to identify promoters of *V. cholerae* that are induced by the second messenger molecule c-di-GMP [40]. While performing this screen, we identified a promoter located upstream of the *epsG* gene in the putative *epsC-epsN* operon that encodes the Type II Secretion System (T2SS) of *V. cholerae*. The promoter was identified in a genetic fragment containing 1176 base pairs located from 1052 base pairs upstream to 123 base pairs downstream of the putative *epsG* (VC2730 in strain C6706) translation start site and as such the DNA fragment was named P*epsG* (Fig. 2.1A). As internal promoters in the *eps* gene cluster have not been described, we further explored this finding.

To confirm these findings, we reconstructed the P*epsG-lux* vector and measured luminescence in *V. cholerae* with and without induction of the DGC *qrgB*, a heterologous protein from *Vibrio harveyi* that we have demonstrated is active in *V. cholerae*, encoded on a separate plasmid. Indeed, the P*epsG-lux* vector exhibited significant expression versus the promoterless *lux* vector, confirming that this fragment

of DNA encodes a functional promoter. However, the P*epsG* promoter did not show differential regulation in response to changing c-di-GMP levels (Fig. 2.1B).

The identification of the P*epsG* internal promoter led us to hypothesize that transcriptional control of the *eps* operon could impact T2SS function in *V. cholerae.* At the initiation of these experiments, no studies had examined this hypothesis. We constructed a transcriptional fusion of a 628 basepair region upstream of *epsC* (referred to as P*epsC*) to the *lux* operon on the plasmid pBBR-lux (Fig. 2.1A). Significant expression from P*epsC-lux* was observed in *V. cholerae*, confirming this region of DNA contained the promoter/s for the *eps* gene cluster. However, unlike P*epsG,* increased c-di-GMP led to 3.7-fold higher expression of P*epsC-lux* (Fig. 2.1B). These results show that transcription from the P*epsC* promoter region is induced by c-di-GMP.

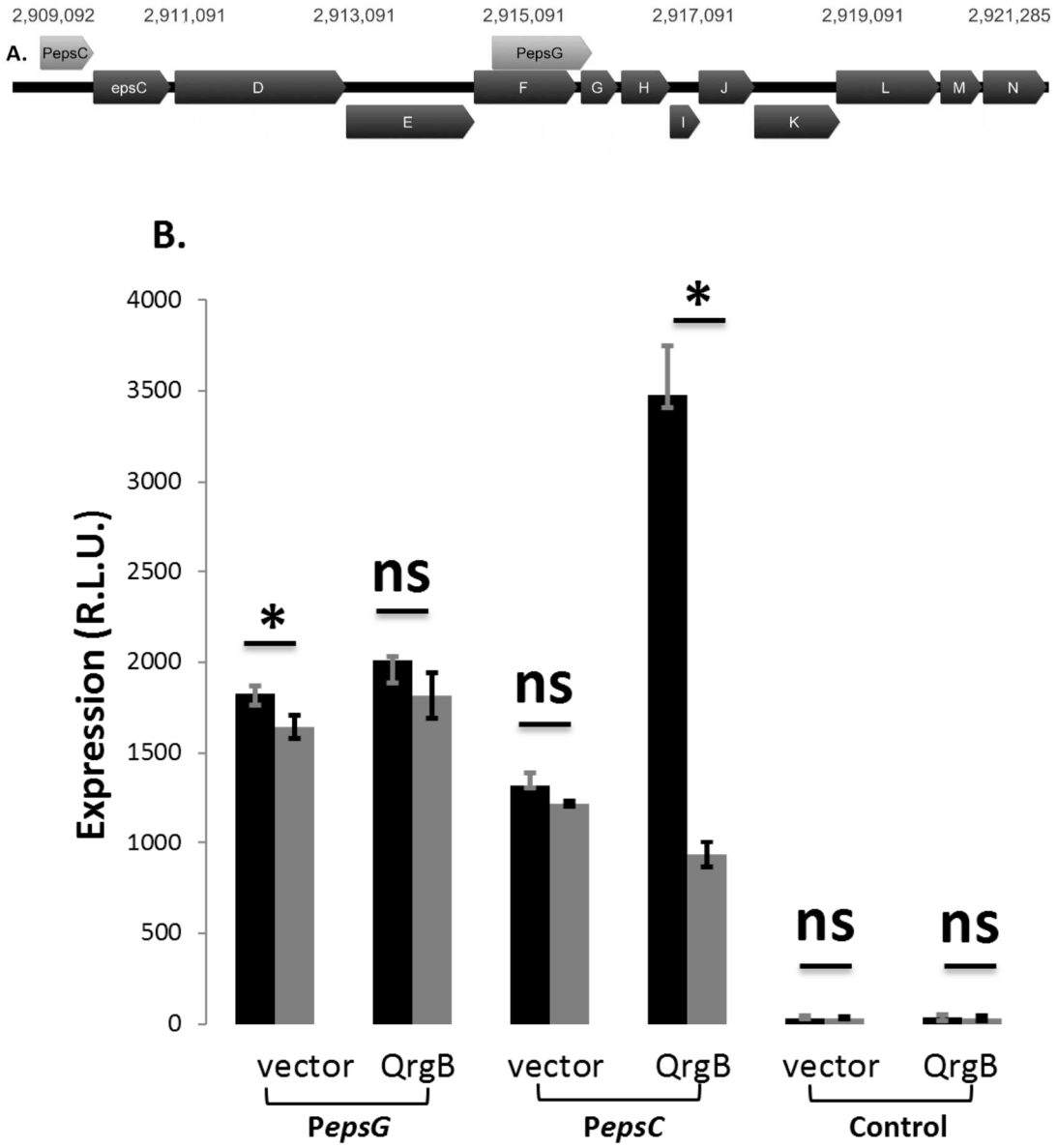
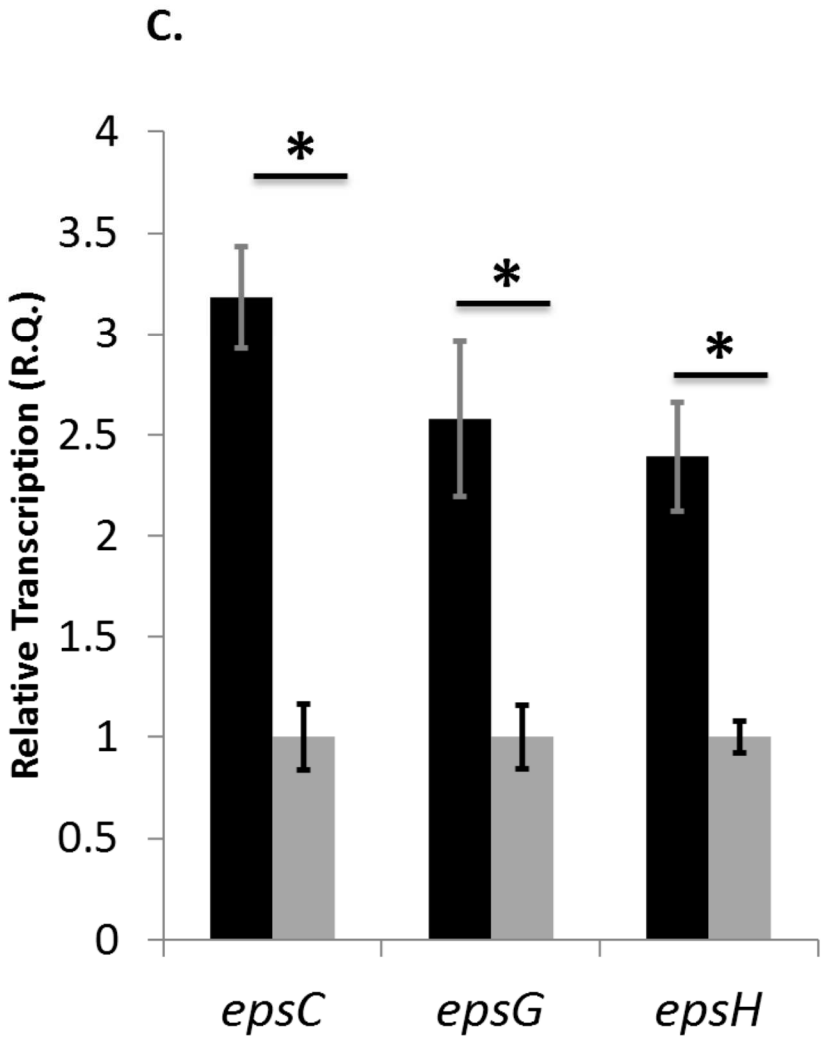


Figure 2.1. C-di-GMP induces the eps operon A. The *eps* operon. Open reading frames are shown in dark grey, sequences used in initial promoter analyses are shown light grey, and numbers indicate DNA bp number on the N16961 *V. cholerae* chromosome 1 as a reference sequence. **B.** P*epsG* and P*epsC* were transcriptionally fused to the lux operon. Luminescence production following overexpression of *qrgB* and the vector control was determined in the Δ*vpsL V. cholerae* mutant. The light bars indicate non-induced cultures; the dark bars indicate cultures induced by 100 μM IPTG. A promoterless pBBRlux plasmid was used (control). Relative Light Units (R.L.U) were calculated by dividing raw luminescence by the optical density at 595 nm. Error bars indicate standard deviations of three technical replicates. **C.** C-di-GMP induces transcription of *epsC, epsG,* and *epsH* as measured by Q-RT-PCR.

Figure 2.1. (cont'd)



Induction of P*epsC* by c-di-GMP drives expression of *epsC*, *epsG*, and *epsH*

The *eps* gene cluster appears to be structured in an operon although this has not been formally demonstrated. We therefore wondered if induction of P*epsC* by c-di-GMP leads to increased expression of the entire *eps* region including genes downstream of the *epsG* promoter. To determine this, we used quantitative real-time PCR (qRT-PCR) analysis of the *epsC*, *epsG*, and *epsH* genes to quantify relative RNA levels with and without *qrgB* induction. Indeed, relative transcription of *epsC*, *epsG*, and *epsH* was induced with increased levels of c-di-GMP 2 to 3-fold (Fig. 2.1C). No significant difference was observed with and without addition of IPTG for the vector control, corroborating that induction is due to c-di-GMP production (Fig. 2.1C.). These data suggest that c-di-GMP induces transcription of the entire *eps* T2SS region in *V. cholerae* via activation of the *epsC* promoter. Furthermore, these results show coordinate induction of *epsC, epsG* and *epsH* genes thus providing experimental evidence that the *eps* genes are indeed structured as an operon.

Characterization of the *epsC* promoter

To identify which upstream region of P*epsC* is necessary for expression and c-di-GMP induction, we constructed 5' promoter truncations of the P*epsC* DNA fragment that was analyzed in Fig. 2.1, and measured expression of these truncations at low and high c-di-GMP levels via QrgB expression in *V. cholerae*. All fragments of P*epsC-lux* that contained sequence from -155 and longer (numbered relative to the *epsC* translational start site) to the start of the *epsC* translation start site exhibited promoter activity and were stimulated by increased c-di-GMP equivalently to our original P*epsC*-lux fusion

(Fig. 2.2A). Alternatively, truncating to -124 virtually abolished all expression of the promoter and induction by c-di-GMP. To further understand this regulation, we determined potential transcription start sites upstream of *epsC* using 5'-Rapid Amplification of cDNA ends (5'-RACE). Two putative transcription start sites were identified in P*epsC* located at bases -203 and –88 relative to the *epsC* translation start site (Fig. 2.2A). These transcriptional start sites lie immediately downstream of the σ^E and σ^{70} promoters predicted by Zielke et. al. based on sequence analysis [100]. In that study, the σ^E 5' promoter was named P2 while the σ^{70} 3' promoter was named P1. For consistency, we will refer to the *epsC* promoters using this nomenclature. Based on these results, we conclude that the transcription start site located at -83, P*epsC*1 is the primary site for transcription initiation in the conditions we examined here, and c-di-GMP induction of the P*epsC*1 requires a sequence encoded 41-72 bps upstream of the P*epsC1* transcription start site.

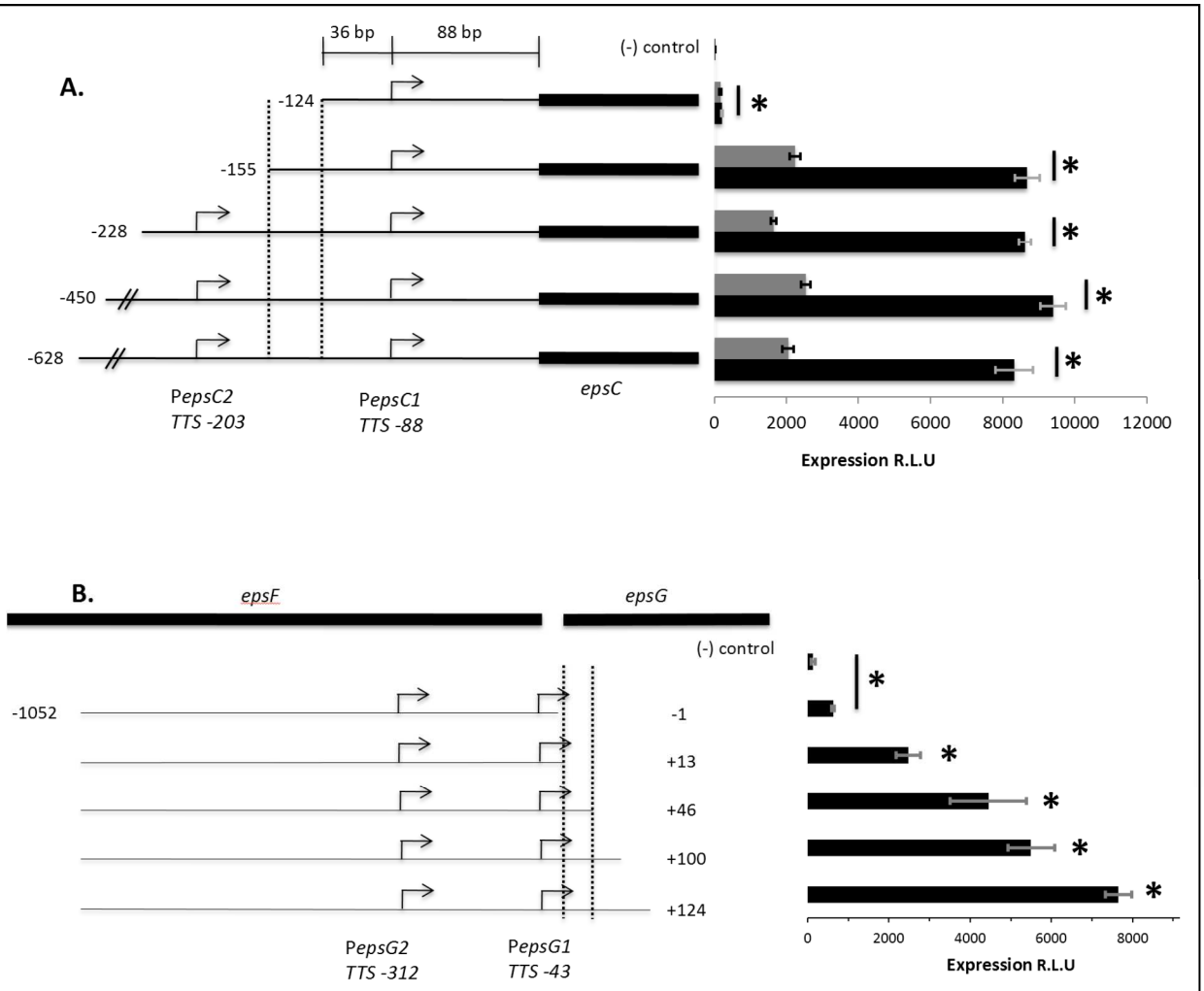


Figure 2.2. Functional analysis of the epsC and epsG promoter A. The *epsC* promoter was analyzed by generating 5' deletions. The numbers on the left indicate the 5' ends of the constructs relative to the *epsC* open reading frame. Arrows indicate transcription start site determined by 5'-RACE. Double forward slash indicates sequence is not to scale. Luminescence production from P*epsC*-lux following overexpression of *qrgB* was determined in the *V. cholerae* Δ*vpsL* mutant. The light bars indicate non-induced cultures; the dark bars indicate cultures induced by 0.1 mM IPTG. A promoterless pBBRlux plasmid was used as the negative control ((-) control). Dashed lines indicate critical region for promoter activity. **B.** The *epsG* promoter was analyzed by generating 3' deletions. The numbers on the right indicate the 3' end relative to the *epsG* translation start site with each having a 5'-end at -1052. Error bars indicate the standard deviation of three biological replicates.

Characterization of the *epsG* promoter

We performed a similar analysis of the *epsG* promoter by generating 3'-truncations of the original P*epsG-lux* fragment (Fig. 2.2B). 3'-truncations were generated as we wanted to determine if the identified promoter fragment controls transcription of the entire *epsG* gene. As this promoter is not regulated by c-di-GMP, we only measured overall expression in *V. cholerae*. Removal of bases +46 to +124 (relative to the *epsG* translation start site) did not significantly impact expression of this fusion; however, more significant decreased promoter activity was observed upon deleting the bases from +13 to +46. This decreased expression was further evident in the shortest fragment that has a 3' end located directly upstream of the *epsG* start codon as it produced only slightly higher luminescent values than the promoterless pBBRlux plasmid (Fig. 2.2B). 5'-RACE analysis of this region also identified two putative transcription start sites located at bases –43 and –312 relative to the *epsG* start site named P*epsG*1 and P*epsG*2, respectively. Therefore, we hypothesized that a promoter upstream of the –43 start site is the primary driver of transcription in the conditions we examined and that a region within the *epsG* open reading frame is necessary for promoter activity. As neither the function nor regulatory components of the *epsG* promoters are known we focused the remainder of our study on c-di-GMP induction of P*espC*1 and its impact on type II secretion.

Induction of P*epsC*1 requires the transcription factor *vpsR*

To further understand the c-di-GMP induction of P*epsC*1, we sought to identify transcription factors necessary for this regulation. Three c-di-GMP-dependent transcription factors have been identified in *V. cholerae;* FlrA, VpsT, and VpsR [40, 42,

44]. We measured expression of the *epsC-lux* fusion in mutants carrying deletions of these transcription factors. We observed P*epsC* was fully induced by c-di-GMP in an *flrA* deletion mutant. Deletion of *vpsT*, a c-di-GMP-dependent transcription factor induced by VpsR bound to c-di-GMP that stimulates biofilm formation [40], did not impact induction of P*epsC* by c-di-GMP either. However, we found that the increase in bioluminescence typically observed upon increased c-di-GMP was abolished in a Δ*vpsR* mutant, suggesting that VpsR is required for the c-di-GMP mediated regulation of *eps* genes (Fig. 2.3).

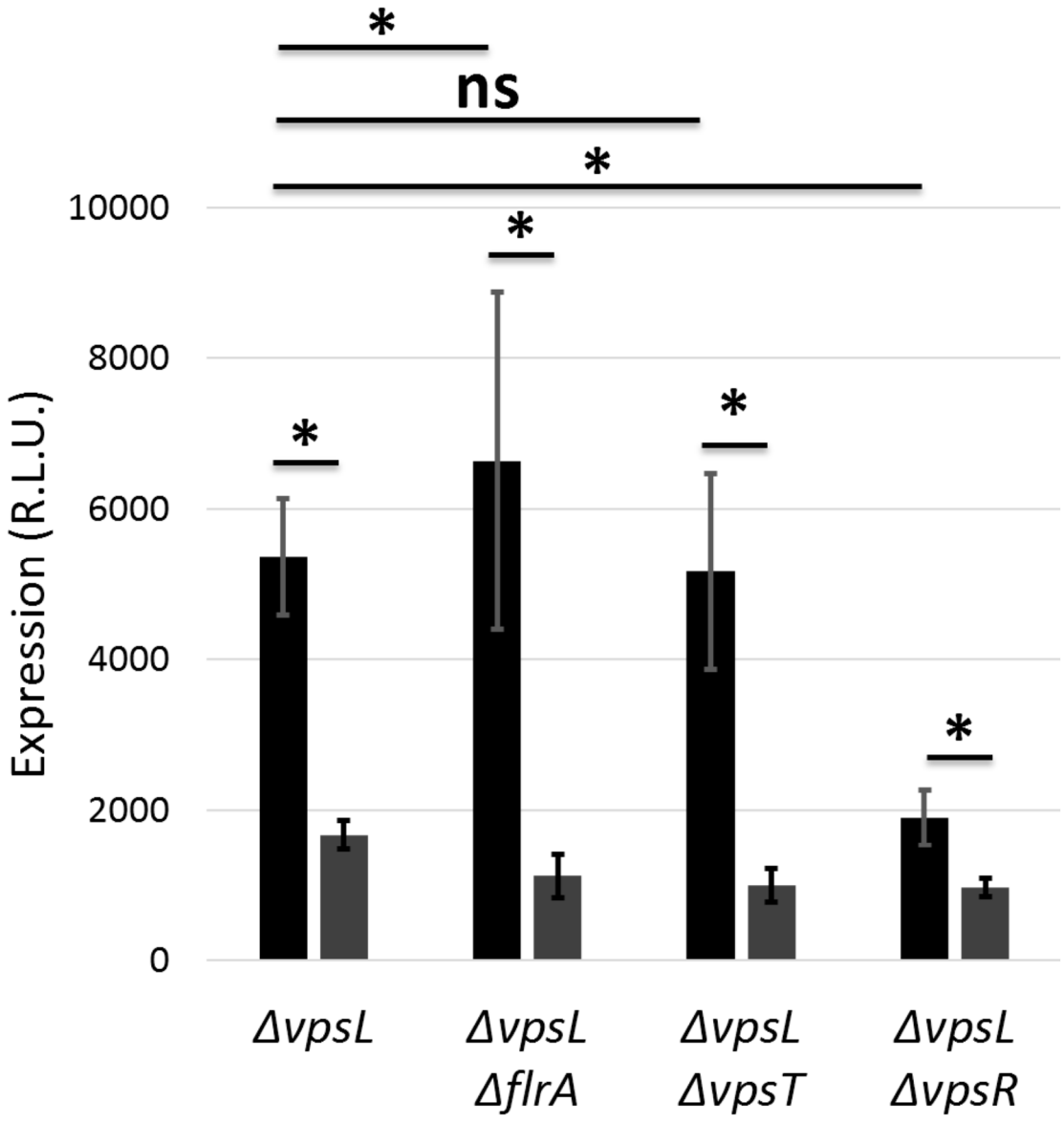


Figure 2.3. C-di-GMP induction of PepsC1 requires VpsR Luminescence production following induction of *qrgB* with 0.1 mM IPTG (dark bars) and in non-inducing conditions (light bars) was determined in Δ*vpsL*, Δ*vpsL*Δ*flrA*, Δ*vpsL*Δ*vpsT* and Δ*vpsL*Δ*vpsR*. Error bars indicate the standard deviation of three biological replicates.

We constructed a consensus VpsR binding motif based on previous work and used this to search for a sequence in P*epsC* [149]. Analysis of the *epsC* promoter sequence identified a 14 bp putative VpsR binding site TTTAACGTTTGAGA (Fig. 2.4C) located from -138 to -124, 36 base pairs upstream of the P1*epsC*. This binding site matches 10/14 of the bases in the recently described VpsR binding site [2]. Promoter truncation analysis indicated the region encoding this putative binding site is essential for c-di-GMP induction of *epsC* (Fig. 2.2A). To first determine if VpsR can bind to the *epsC* upstream region, we amplified the DNA encoded in the -228 and -124 *epsC-lux* fusions (see Fig. 2.2A) and performed EMSA analysis with purified VpsR. Increasing amounts of VpsR bound to and shifted the 228 bp fragment but did not shift the 124 bp DNA probe (Fig. 2.4A). Multiple bands of different sizes were observed suggesting VpsR can bind to the *epsC* promoters in different multimeric states.

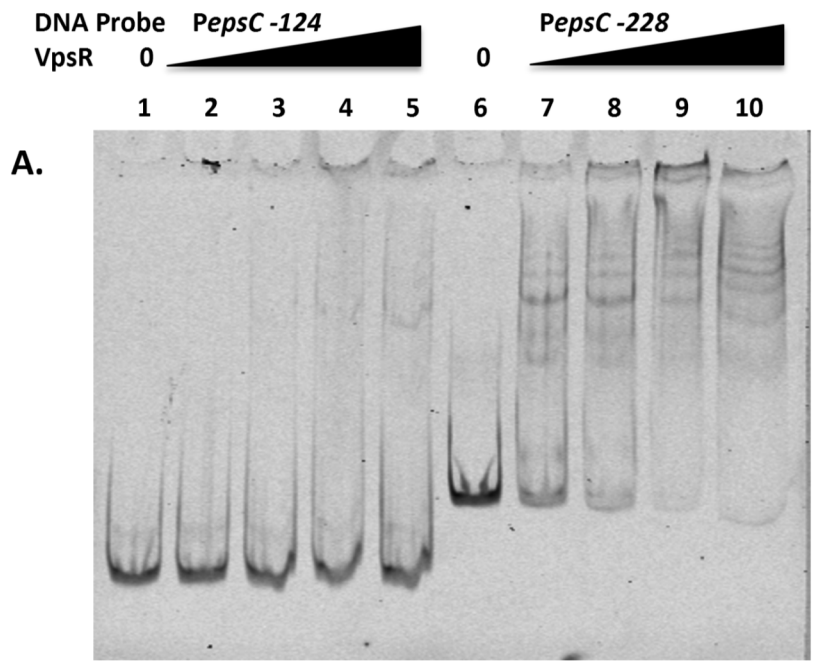
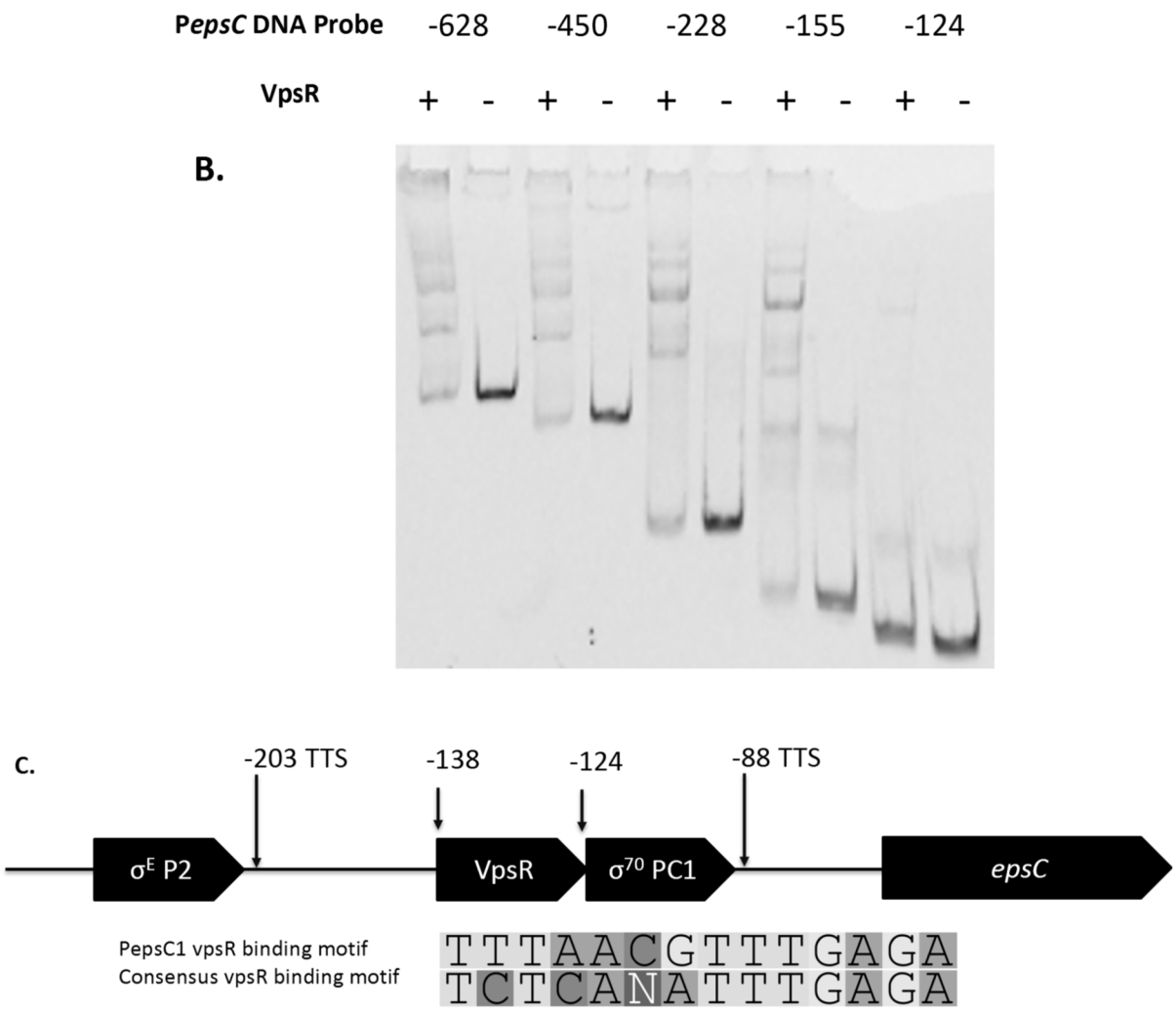


Figure 2.4. VpsR directly regulates epsC A. EMSA of P*epsC* containing the fragments indicated in Fig. 2.2A with purified VpsR at the following concentrations lanes 1 and 6, no protein; lanes 2 and 7, 0.9 μM; lanes 3 and 8, 1.7 μM; lanes 4 and 9, 3.1 μM; and lanes 5 and 10, 4.0 μM. **B.** EMSA of the 5' truncations in Fig. 2.2A with or without 0.9 μM VpsR. **C.** A map of P*epsC* indicating the σE dependent promoter (P2) and the σ70 dependent promoter (P1) with the putative VpsR binding site. The vpsR binding site sequence is shown as compared to the consensus sequence from Zamorano-Sánchez et. al.[2]

Figure 2.4. (cont'd)



We further confirmed the location of the VpsR binding site by amplifying all of the *epsC* promoter truncations shown in Fig. 2.2A and analyzed VpsR binding using EMSA. Consistent with our genetic results, VpsR bound to every fragment except the -124 (Fig. 2.4B). This is the only fragment that is not regulated by c-di-GMP and does not encode the putative VpsR binding site. These results indicate that VpsR binds directly to P*epsC* at a VpsR binding site located from -124 to -155 relative to the *epsC* translation start

site. We also attempted EMSA in the presence of excess c-di-GMP however we did not observe any change in binding affinity or binding patterns (data not shown). This is consistent with our previous results and a published study showing that c-di-GMP does not impact VpsR binding to target promoters [2, 40].

C-di-GMP decreases the activity of extracellular proteases

We hypothesized that c-di-GMP induction of the *epsC* promoter would increase the secretion of T2SS target proteins. Extracellular proteases are known to be secreted by the T2SS and they provide a sensitive, robust readout of secretion functions. Proteases secreted by the T2SS include the serine proteases VesA (VCA0864), VesB (VC1200), and VesC (VC1649) as well as the Hap (HA) protease, a metalloprotease known to activate cholera toxin [150]. To determine if secretion of proteases into the extracellular environment was impacted by c-di-GMP, we analyzed proteolytic activity of *V. cholerae* culture supernatants at high and low levels of c-di-GMP. This experiment was done in a Δ*vpsL* mutant to abolish extracellular matrix production induced by c-di-GMP that might interfere with the measurement of protease activity.

Protease activity was assayed using a standard approach that measures cleavage of a fluorogenic oligopeptide substrate by serine proteases resulting in increased fluorescence. The majority of activity in this assay is due to VesA, VesB, and VesC [151]. All cultures were supplemented with 2.5 mg/mL EGTA to inhibit the activity of Hap, a metalloprotease that is incapable of cleaving the substrate used in this assay; however, we previously observed that Hap can interfere with the functioning of the Ves proteases, presumably through their degradation (data not shown). Fluorescence was

monitored over time and the average slope of increase in fluorescence (dF/dT) normalized to optical density was calculated for the Δ*vpsL V. cholerae* mutant with and without induction of c-di-GMP.

Contrary to our hypothesis, we observed that protease activity was lower in strains containing high c-di-GMP (induced, black) at every time point examined compared with the same strain that has normal c-di-GMP levels (uninduced, gray) (Fig. 2.5A). The difference is particularly striking after 8 hours, when protease activity is greatest in *V. cholerae.* Growth of this strain is not significantly impacted by these concentrations of c-di-GMP. If these changes in activity are due to transcription induction of P*epsC*1 by c-di-GMP, we hypothesized that the c-di-GMP mediated reduction in extracellular protease activity would not occur in a Δ*vpsR* mutant as P*epsC*1 is no longer induced by c-di-GMP. While the average (dF/dT) was significantly reduced in the Δ*vpsL* mutant with increased levels of c-di-GMP as previously observed, no significant difference was observed in Δ*vpsL* Δ*vpsR* with and without induction of *qrgB* (Fig. 2.5B). To rule out that the difference we observed was due to c-di-GMP transcriptional regulation of the Ves proteases, we constructed transcriptional fusions of the promoters driving each *ves* protease to the *lux* operon. C-di-GMP did not significantly reduce transcription of any of the *ves* proteases (Fig. A2). These data suggest c-di-GMP reduces extracellular protease activity in *V. cholerae* in a VpsR-dependent manner.

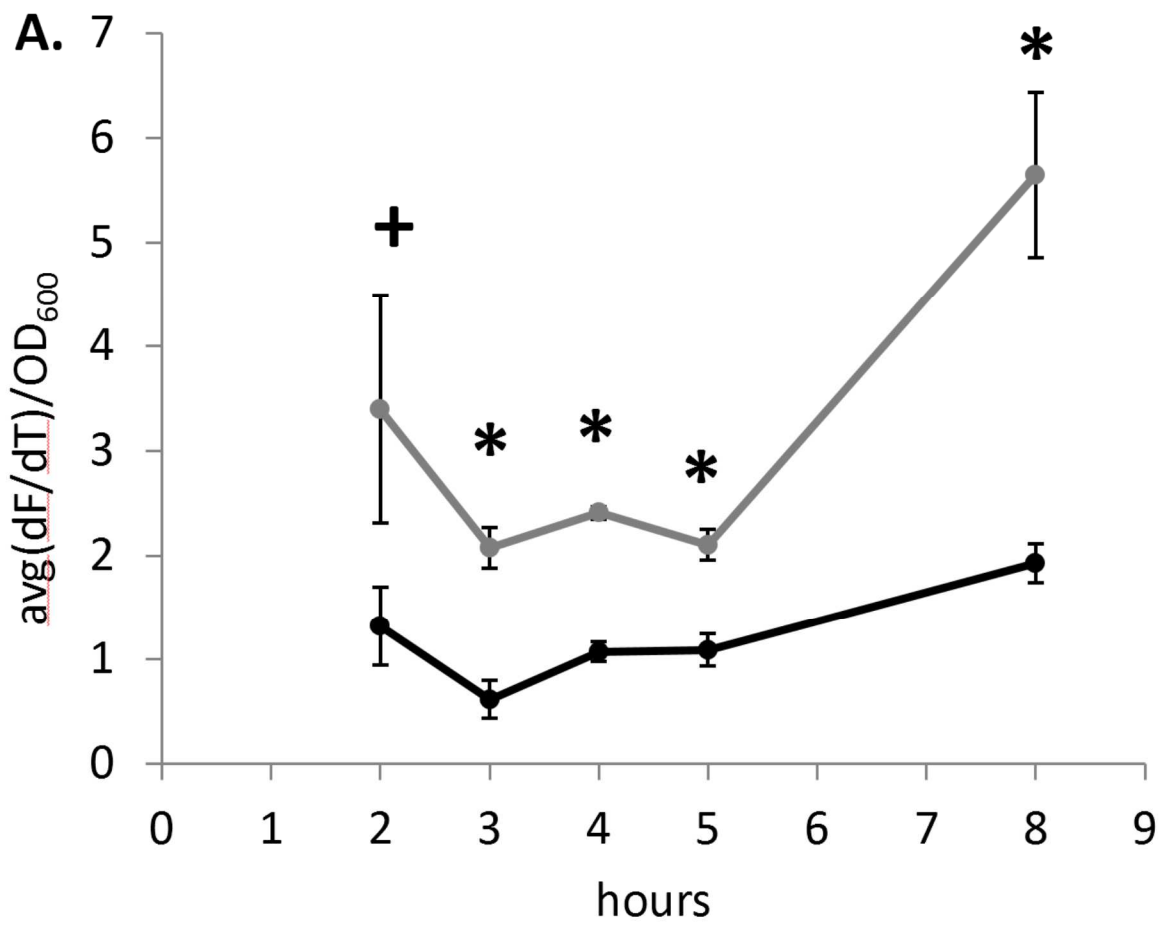
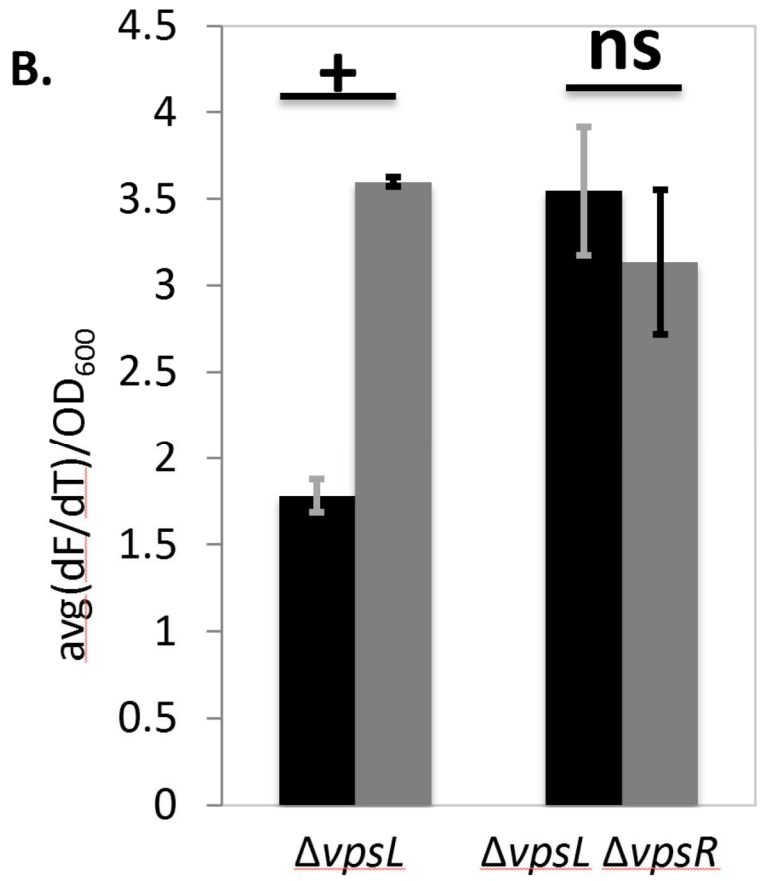


Figure 2.5. Increased c-di-GMP results in decreased extracellular protease activity A. Fluorescence was monitored following cleavage of a fluorogenic oligopeptide by proteases secreted via the T2SS. Following induction of *qrgB*, Δ*vpsL* cultures were allowed to incubate for 2,3,4,5, and 8 hours before supernatant was collected and mixed with substrate. Increased fluorescence is measured each minute for 20 minutes, and the average slope of increase in fluorescence over time is calculated (avg(dF/dT)). The dark line indicates cultures induced with 0.1 mM IPTG while light bars indicate non-induced cultures. Error bars indicate standard deviation of three biological replicates. **B.** Δ*vpsL* and Δ*vpsL*Δ*vpsR* supernatants were assayed after 6 hours following cyclic di-GMP overexpression. Dark bars indicate cultures induced with 100 μM IPTG, light bars indicate non-induced cultures. The error bars represent the standard deviation (n=3).

Figure 2.5. (cont'd)



C-di-GMP does not impact secretion of cholera toxin

We wondered if the decreased secretion mediated by c-di-GMP impacts all the substrates of the T2SS or if this inhibition is specific to certain effectors, such as proteases. To determine this, we investigated the impact of c-di-GMP on secretion of cholera toxin, the most well characterized target of the T2SS [87, 141, 152, 153]. We performed enzyme-linked immunosorbent assays (ELISA) to detect and quantify cholera toxin protein directly with and without *qrgB* induction. Because all of our

previous experiments were performed in LB media, we first wished to examine cholera toxin secretion in these conditions. As cholera toxin is not highly expressed in LB medium in the El Tor *V. cholerae* strain that we utilized, the plasmid pWD615 that constitutively expresses the *etxB* gene was used. *etxB* encodes the B subunit of enterotoxin which serves as a surrogate for cholera toxin. CtxB and the EtxB are both efficiently secreted by the T2SS at over 90% efficiency, and they also share 88% protein sequence similarity and have been used interchangeably in *E. coli* and *V. cholerae* [154].

EtxB secretion was examined by centrifuging the cells to separate protein that is in the supernatant versus protein that is cell associated. EtxB was detected using a standard GM1 ELISA assay [141]. This experiment was performed at multiple time points following subculturing of an overnight culture. We observed robust secretion of EtxB at all time points examined, but this secretion was unaffected by c-di-GMP levels in the cell (Fig. 2.6). Contrary to our findings for proteases, these data show that increased levels of c-di-GMP do not impact secretion of cholera toxin by the T2SS.

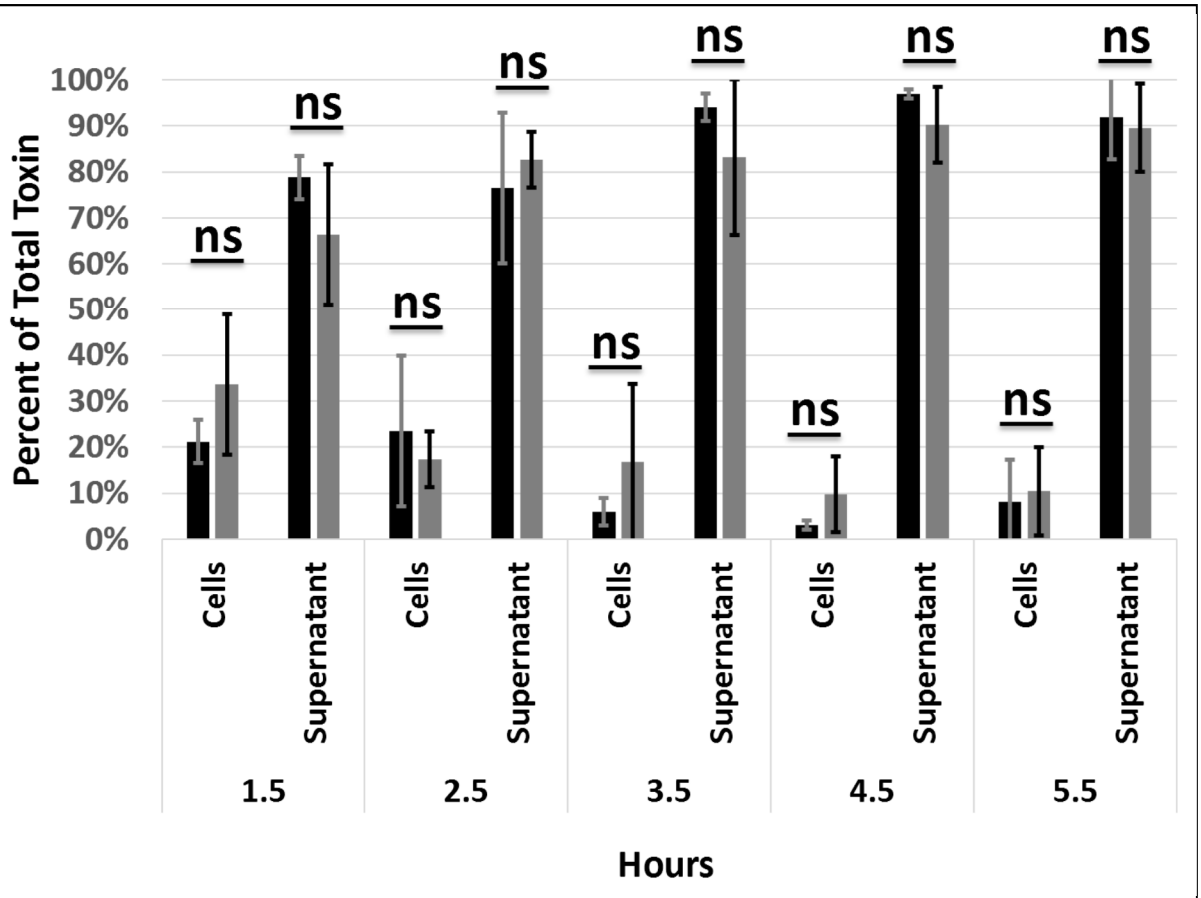


Figure 2.6. C-di-GMP does not impact secretion of EtxB Secretion of the *E. coli* toxin EtxB constitutively expressed from a plasmid was determined at high and low levels of c-di-GMP. Dark bars indicate cultures induced with 100 μM IPTG, light bars indicate non-induced cultures. Cultures were grown for the indicated time points following a 1/1000 dilution. The cells were removed by centrifugation and lysed. Both the supernatant and cell-associated EtxB was determined using a GM1 ELISA. The error bars represent the standard deviation (n=3).

C-di-GMP decreases extracellular protease activity even if *epsC* is not induced

Our results thus far showed that c-di-GMP decreased extracellular protease activity in a VpsR-dependent manner. We hypothesized this was due to the induction of the *eps* operon by c-di-GMP and VpsR. To test this hypothesis, we mutated the VpsR binding site upstream of P*epsC1* (labeled RBM, Supplemental Fig. 2.2) on the genome of *V. cholerae,* and performed a number of experiments to validate these mutations disrupted VpsR binding to the *epsC* promoter and subsequent induction by c-di-GMP. First, we constructed a transcriptional P*epsC*-RBM-*lux* fusion into the pBBRlux vector (labeled P*espC-RBM-lux*) . We observed that P*epsC-RBM-lux* had basal levels of expression lower than the native promoter P*espC* promoter, and it was not responsive to c-d-GMP (Fig. 2.7A). These results are consistent with disruption of VpsR binding to this promoter. We also observed greatly reduced VpsR binding to this mutant promoter fragment compared with an equivalent promoter fragment encoding the wild type VpsR binding site using an EMSA (Fig. 2.7B). Because *epsC* has multiple promoters, we confirmed that expression of *epsC* in the RBM strain, where the mutation to the VpsR binding site was encoded at the native locus, was uninducible by c-di-GMP using qRT-PCR. These results showed that expression of *epsC* in RBM was only mildly reduced in RBM as compared to WT and there was no induction of *epsC* when c-di-GMP was elevated in this mutant (Fig. 2.7C). We then quantified extracellular protease activity in the RBM strain at low and high levels of c-di-GMP. Contrary to our hypothesis, we observed there was no change in protease activity when c-di-GMP was high in RBM as compared to the WT strain (Fig. 2.7D). This indicated that the lowered protease

secretion activity we observed earlier was not due to the transcriptional regulation of the T2SS.

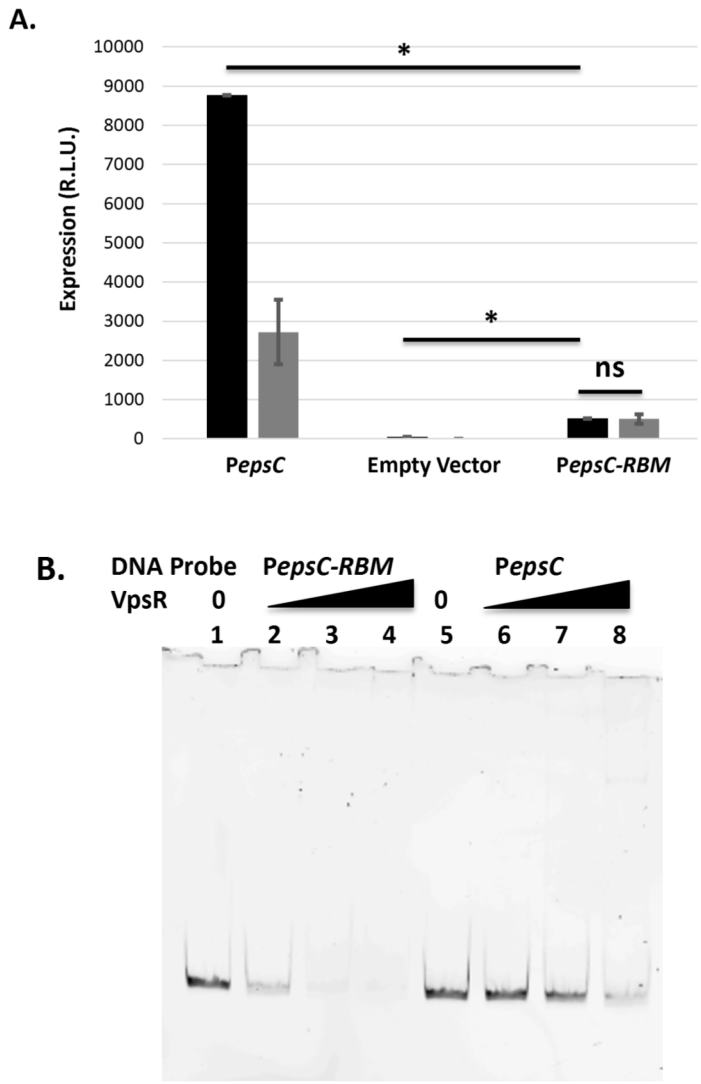
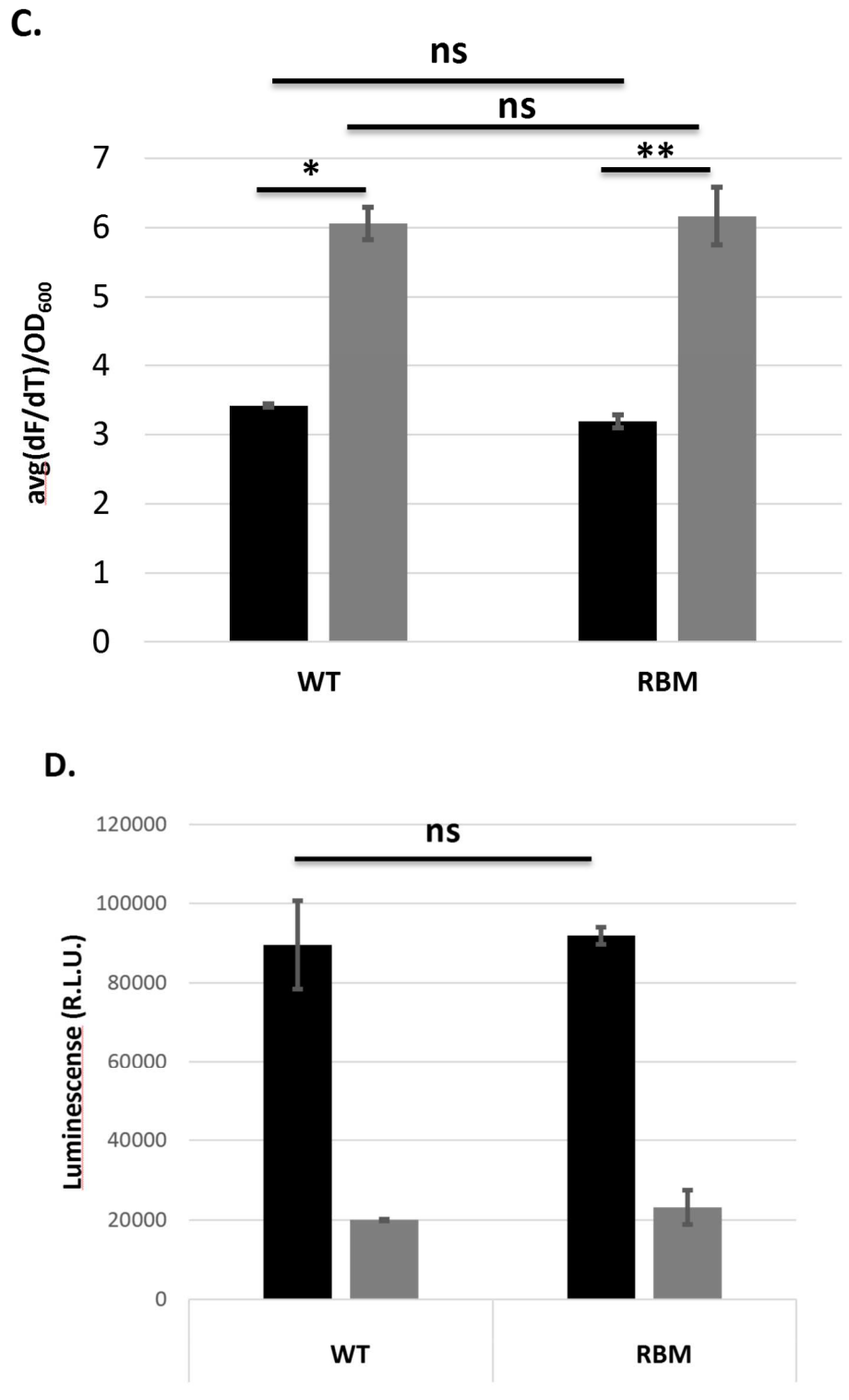


Figure 2.7. C-di-GMP induction of the eps genes is not required for decreased extracellular protease activity For all graphs, high c-di-GMP (black) was generated by inducing the GGDEF protein QrgB with 100 μM IPTG while normal levels of c-di-GMP (gray) is the uninduced control. **A.** The promoter upstream of *epsC* from strain RBM that encodes the mutation to the VpsR binding site was constructed as a *lux* transcriptional fusion. **B.** Binding of VpsR to the VpsR binding site promoter (lanes 1-4) or the WT *epsC* promoter (lanes 5-8) with no protein or VpsR concentrations of 0.45 μM (2,6), 0.9 μM (3, 7), or 1.8 μM (4, 8). **C.** Extracellular protease activity in the WT or RBM mutant strain. **D.** Biofilm formation as measured by quantifying viable bacteria (see material and methods) of the WT or RBM mutant at high (black) levels of c-di-GMP generated by inducing the QrgB GGDEF protein with 100 μM IPTG versus normal levels of c-di-GMP (gray) representing the uninduced samples is shown. For all graphs, the error bars represent the standard deviation (n=3) and * $p<0.05$ or ** $p<0.01$.

Figure 2.7. (cont'd)



Since T2SS, c-di-GMP, and VpsR are all implicated in biofilm formation, we tested if biofilm formation in the presence of high c-di-GMP was altered in the RBM mutant strain compared with the WT by measuring viable bacteria in static biofilms using the BacTiterGlo assay. However, both the RBM and WT strain exhibited equivalent biofilm formation in the presence of high or low c-di-GMP Fig. 2.8), showing that in the conditions we tested transcriptional induction of *epsC* by c-di-GMP does not impact biofilm formation.

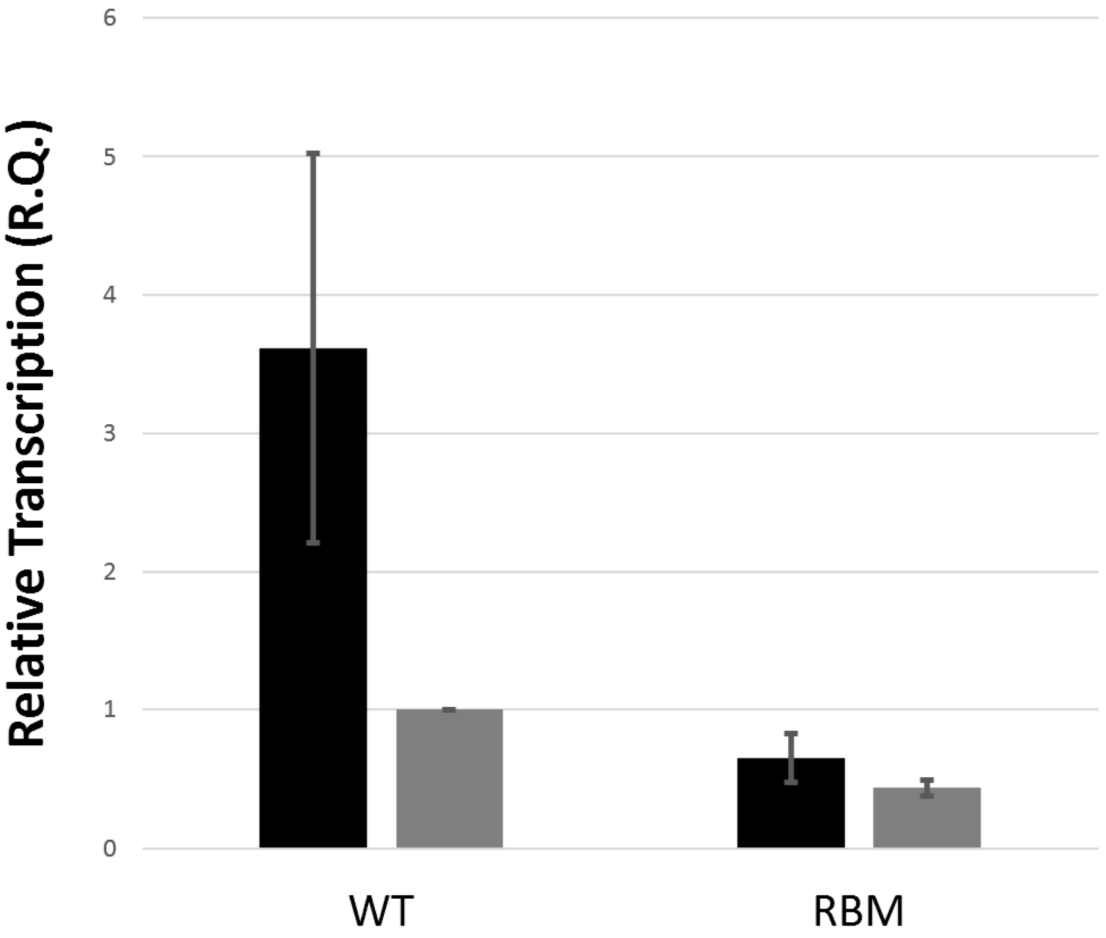


Figure 2.8. Disruption of VpsR binding at epsC does not impact biofilm formation Transcription of *epsC* as measured by Q-RT-PCR in the wild type or RBM mutant strain is indicated and normalized to the WT normal c-di-GMP sample. The error bars represent the standard deviation (n=3).

Discussion

Our results indicate that the transcription of the T2SS in *V. cholerae* is induced in a VpsR-dependent manner by c-di-GMP. We also provide evidence that the genes *epsC, epsG* and eps*H* are coordinately regulated by c-di-GMP and therefore behave like constituents of an operon. A previous microarray analysis and a study of transcriptional regulation of the *eps* operon from *V. cholerae* hinted at c-di-GMP regulation of these genes although this induction was not explored in detail [67, 100]. We hypothesized that high c-di-GMP would increase overall secretion; however, increased c-di-GMP led to decreased extracellular serine protease activity but did not impact secretion of cholera toxin.

Only recently it was thought the T2SS in *V. cholerae* was constitutively expressed. However, our research as well as a recent manuscript by Zielke et. al. demonstrated transcriptional control of the *eps* operon [100]. These findings raise a number of intriguing questions such as why regulate expression of this complex that is crucial for secretion of a variety of proteins? Moreover, does the number of secretion complexes on the cell change during different conditions, and do different protein effectors require different stoichiometries of the T2SS components? It has been shown in *V. cholerae* that T2SS mutants lead to cell envelope stress and the cell responds by induction of the σ^E stress response [88]. Recently it has also been shown that the more upstream promoter P*epsC*2 is induced by σ^E [100]. This regulation makes logical sense as increased synthesis of the *epsC-N* operon may be a response to periplasmic stress. However, in our studies we did not observe increased secretion of either proteases or

cholera toxin at high c-di-GMP when the *epsC-N* operon was induced, suggesting that overall *eps* gene expression does not necessarily directly correlate with T2SS activity.

It is interesting to note that in *V. cholerae* the quorum sensing master regulator HapR induces *epsC-N* expression [100]. HapR also reduces the intracellular concentration of c-di-GMP [155]. Therefore, these regulatory effects are conflicting as *hapR* mutants have high c-di-GMP levels but lower *epsC-N* expression. These observations suggest that the regulation of *epsC-N* by HapR must be through a c-di-GMP-independent mechanism that is epistatic to c-di-GMP induction of P*epsC*1. With our identification of a second promoter region upstream *of epsG*, and the observation that both *epsC* and *epsG* are each potentially expressed from two transcriptional start sites, it is clear that transcriptional control of *eps* expression is more extensive than previously appreciated and additional regulatory pathways must control expression of the *eps* operon.

The T2SS is necessary for proper biofilm development in *V. cholerae*, in part through secretion of three extracellular proteins [144], and c-di-GMP is similarly essential for biofilm formation [25, 30, 155, 156]. VpsR binds c-di-GMP and upregulates the *vps* gene clusters in a c-di-GMP-dependent manner [40, 157, 158]. Thus, VpsR appears to be a molecular link connecting the intracellular c-di-GMP concentration to both Vibrio Polysaccharide biosynthesis and type II secretion. Why *vps* gene expression is coupled with expression of the *eps* operon is intriguing. Given the growing appreciation of extracellular proteins as components of the *V. cholerae* biofilm matrix [134, 144], we hypothesize that induction of *epsC-N* by c-di-GMP, and the subsequent inhibition of extracellular protease activity, may provide a mechanism by which to

stabilize the extracellular proteins RbmA, RbmC, and Bap1 which are essential for driving the early stages of biofilm formation [134, 144]. This idea is conceptually similar to a prior study showing that the extracellular levels of the attachment factor GbpA in *V. cholerae* are modulated by quorum sensing regulation of the extracellular proteases HapA and PrtV, which is thought to promote dispersion from chitin surfaces or association with the host [159]. Increased protease activity is also implicated in dispersal of *Pseudomonas fluorescens* biofilms [160]. Therefore, if increased proteolytic activity is a mechanism for dispersal, then inhibition of protease secretion by c-di-GMP, which promotes biofilm formation, would be favored.

Extracellular protease activity of *Pseudomonas aeruginosa* PA01 was recently shown to be inhibited by the protein MorA, a protein that encodes both GGDEF and EAL domains, although both of these domains contributed to inhibiting protease secretion [161]. This regulation was postulated to be post-translational, although a mechanism was not described. The recent findings that the type II secretion ATPase PA14_29490 binds to c-di-GMP could be involved in this regulation [101]. These results combined with our findings suggest that c-di-GMP regulation of extracellular protease activity may occur in a number of bacteria.

The molecular mechanism by which c-di-GMP inhibits extracellular protease activity is not clear although we have uncovered several important clues. First, we showed that this inhibition requires high c-di-GMP and VpsR. Second, transcriptional regulation of the *ves* proteases by c-di-GMP does not occur. Third, direct transcriptional control from the T2SS is not involved. One possibility is that c-di-GMP bound to VpsR could impact expression of a factor that inhibits the activity of the extracellular

proteases. Of note, GspE, the ATPase of the *V. cholerae* T2SS, did not bind to c-di-GMP, unlike its *P. aeruginosa* ortholog, and thus modulation of GspE activity does not explain the reduction of extracellular protease activity we report here [101].

We report two novel promoters driving expression of *epsG*, and determining the function of these promoters will be important to understanding the T2SS of *V. cholerae*. It is possible that these promoters function to alter the stoichiometry of the proteins in the T2SS. The location of this internal promoter is provocative as it drives expression of the main pseudopilin *epsG* and the additional minor pseudopilins *epsHIJK* as well as the rest of the operon *epsM-N*. The promoter upstream of *epsG* could alter the stoichiometry of these pseudopilins in the T2SS complex, which may be important to regulate secretion after the T2SS complex has formed. Overexpression of major pseudopilin in *P. aeruginosa* that is homologous to *epsG* has been shown to produce a type 4-like pilus [162, 163], and our lab has observed the same for *epsG* in *V. cholerae* (unpublished data). As such it is possible that increased expression from P*epsG* could result in the T2SS behaving more like type 4 pili rather than a secretion system.

The T2SS is conserved in many bacterial species and is important in pathogenesis [84, 146]. Additionally, more than 75% of bacteria contain DGC and PDE domains which are involved in c-di-GMP synthesis and degradation [164]. A functional link between the T2SS and biofilms has been established in *V. cholerae* as well as other γ-proteobacteria such as *Escherichia coli* [165]. We therefore predict that c-di-GMP regulation of T2SS and extracellular protease activity is widespread in other important bacterial pathogens.

Acknowledgments

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CHAPTER 3: INVESTIGATING THE ROLE OF THE T2S HYPER-PSEUDOPILUS IN BIOFILMS

Abstract

Biofilms are complex multicellular communities of bacteria encased in a matrix of polysaccharides, nucleic acids, and proteins. Here, we investigate the role of a pseudopilin formed by the type II secretion system (T2SS) of *Vibrio cholerae* in biofilm formation. The *V. cholerae* T2SS, which is encoded by the *eps* operon, exports folded proteins, such as the cholera toxin and several proteases, from the periplasm to the extracellular environment. Protein export by the T2SS requires the pseudopilin EpsG that is predicted to function as a piston or gate for the T2SS. Recently, we identified a novel promoter in a DNA fragment which starts 1057 bp upstream of the translational start site of *epsG* and extends 124 bp downstream of the start site. At the start of this work, the promoter was observed to be induced by the second messenger cyclic di-GMP (c-di-GMP). As c-di-GMP induces biofilm formation, and the T2SS is structurally related to type 4 pili, we hypothesized that increased expression of EpsG could lead to formation of a hyper-pseudopilus involved in biofilm formation. A hyper-pseudopilus is a T2S based pseudopilus which has extended outside the bacterial cell to form a long structure that is very similar to a type 4 pilus. It is composed largely of the major pseudopilin. In the case of *V. cholerae* the major pseudopilin is EpsG. Here I show that indeed over expression of EpsG can form a structure outside the cell and I have preliminary evidence that the structure exists in WT cells. I also show that biofilms are increased with high levels of EpsG however secretion is not significantly affected by the same high levels of EpsG. These findings present strong evidence that the T2SS forms a hyper-pseudopilus involved in biofilms of *V. cholerae*.

Introduction

In Chapter 1, I described the discovery of a novel promoter in front of the gene *epsG* which is the major pseudopilin in the T2SS. The Type Two Secretion System (T2SS) of *V. cholerae* also contributes to environmental persistence and host disease. The T2SS is a sophisticated multi-protein complex that spans the inner and outer membrane of many Gram-negative bacteria. It is encoded in the operon *epsC-N.* Proteins destined for export via the T2SS are first translocated across the cytoplasmic membrane via the SEC or TAT pathway [139, 140], where they assemble in the periplasm before being exported as fully folded proteins into the extracellular milieu [1]. In *V. cholerae*, the T2SS consists of 13 proteins, 12 of which are encoded by contiguous genes comprising the extracellular protein secretion (*eps*) gene cluster. Many T2SS-dependent proteins are degradative enzymes or toxins that contribute to bacterial pathogenesis; thus the T2SS is considered an important molecular machine necessary for virulence [84]. Within the host, *V. cholerae* causes diarrhea by T2SS-dependent secretion of cholera toxin. In addition to cholera toxin*, V. cholerae* exports other extracellular factors including chitinases, proteases, DNase, and pilin via the T2SS, which aid in its ability to successfully occupy diverse ecological niches [141]. The T2SS also secretes the three proteins RbmA, RbmC, and Bap1 which are necessary for robust, shear-resistant biofilm formation [134, 142-144]. Other major bacterial pathogens such as *Escherichia coli* and *Pseudomonas aeruginosa* secrete virulence factors through the T2SS encoded by genes sharing considerable similarities to those of *V. cholerae* [1, 87, 145, 146].

In the initial promoter screen, I observed induction of P*epsG* by c-di-GMP; however, that induction was not reproducible. However, since the T2SS shares much functional and sequence homology with type 4 pili (T4P), I hypothesized that under the correct regulatory conditions the promoter P*epsG* would activate increasing expression of *epsG*. The overproduction of EpsG relative to the rest of the T2SS would allow the pseudopilus to extend outside the cell to form a structure like a T4P called a hyper-pseudopilus, and this structure could be involved in biofilm structure as T4P are known to be necessary for biofilm formation in various cases. T4P have been shown to be required for proper biofilm and microcolony formation in *P. aeruginosa* PA14 [166], and in *V. cholerae* the MSHA T4P is used for the transition to irreversible attachment before biofilm formation [61]. In *Vibrio parahaemolyticus,* however, the MSHA T4P is necessary for attachment to surfaces and other T4P, ChiRP, is used for agglutination [167]. T4P are also implicated the ability of cells to detach from surfaces [168].

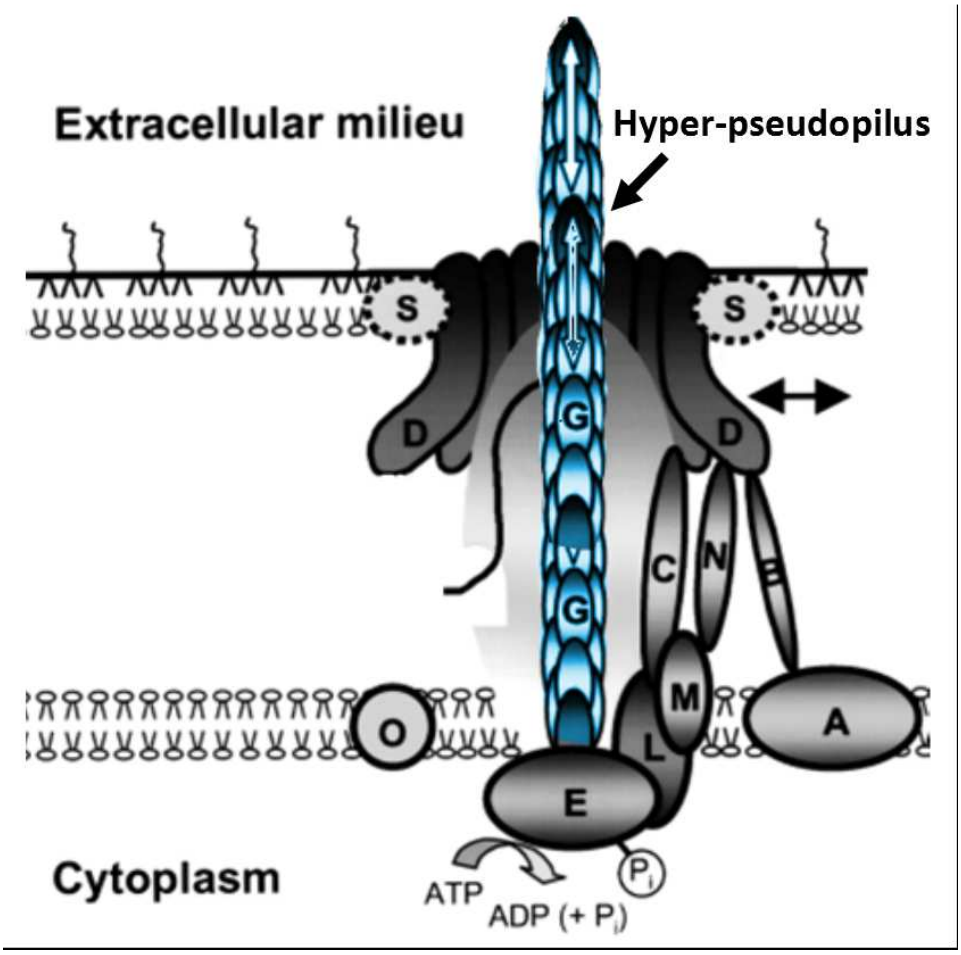


Figure 3.1. The Hyper-pseudopilus Image of hypothetical T2S hyper-pseudopilus adapted from review article [1]. EpsG extends outside the cell as compared to a standard T2S model in figure 1.1.

My hypothesis that regulation of *epsG* in *V. cholerae* could form hyper-pseudopili (Fig. 3.1) involved in biofilm formation is supported by the prior observations that the pseudopilin of the T2SS could form a hyper-pseudopilus when over expressed in another species. The T2SS of *Klebsiella oxytoca* was over expressed in *E. coli* which resulted in the synthesis of pilus-like structures [169], and these structures are

composed of the major pseudopilin PulG [170], which is the homolog of EpsG. Importantly, however, T2SS pseudopili have not been observed under physiological expression of the cognate genes, and a role for these in biofilm formation has not been described.

In sum, I discovered that overexpression of the *epsG* did indeed increase biofilm, and preliminary evidence suggests this is due to pilus formation on the outside of the cell. Importantly, expression of *epsG* did not impact secretion. In further support of the role of T2SS in biofilm formation, deletion of *epsG* significantly reduced biofilms, but this result is likely due to disruption of secretion, which is known to be required for the secretion of the proteins RbmA, RbmC, and Bap1, which are known to be important for biofilm formation [134, 142-144].

Materials and Methods

Bacterial strains, culture conditions, and DNA manipulation

All experiments utilized *V. cholerae* El Tor biotype strain C6706str2 or mutant derivatives (Table A.1). Plasmids were introduced into *V. cholerae* through biparental mating with *E. coli* S17-λpir as the donor and verified by antibiotic selection and culturing on Thiosulfate-citrate-bile salts-sucrose agar plates (Difco). Unless otherwise stated, bacteria were incubated at 35°C shaking at 220 RPM in Lauria-Bertani (LB) medium (Accumedia). Agar plates were made with 15 g/L Agar (Accumedia). Antibiotics were used at the following concentrations: kanamycin, 100 μg/mL, chloramphenicol 10 μg/mL, and polymyxin B 10 U/mL. Protein expression vectors were induced with 100 μM isopropyl-β-D-thiogalactoside (IPTG) unless otherwise stated. All

compounds were purchased from (Sigma). Relevant plasmids and primers are shown in Table A.2.

Polymerase chain reaction was performed using standard methods with Invitrogen HiFi Taq polymerase. Plasmids were constructed in by restriction digest cloning SpeI and BamHI restriction sites using restriction endonucleases (Fermentas or New England Biolabs) and ligated with T4 DNA ligase (New England Biolabs). For gene expression studies, luminescence was measured in opaque white 96 well microtiter plates (Corning) following 6 hours growth of a 1/1000 dilution on either a SpectraMax M5 plate reader (Molecular Devices) or Envision Multimode Plate Reader 2104-0020 (Perkin Elmer).

Biofilm measurement using crystal violet staining

Biofilms were measured on the pegs attached to the top of a minimum-biofilm-eliminating-concentration (MBEC) plates (Biosurface Technologies, Bozeman MT). Biofilms were grown by adding 150 µL of a 0.05 OD595 culture of bacteria to a well and allowing the incubate at 37C for 24 hours while rotating. After biofilms have formed the biofilm is quantified by crystal violet staining. All processing steps are carried out at room temperature and the lid containing the biofilms is transferred to a new 96 well plate with the wells containing aliquots of the treatment step. Biofilms were fixed with 160 µL of 95% ethanol and allowed to incubate for 5 minutes. Then 160 µL of a 0.41% crystal violet solubilized in 12% ethanol solution is added to stain the biofilms and allowed to penetrate for 10 minutes. The crystal violet is washed off by 3 washes in 200 µL of PBS. Then the crystal violet stain is eluted from the biofilms by incubation in 95%

ethanol for 30 minutes in a clear microtiter plate. Then the resulting solution is measured on a SpectraMax M5 plate reader (Molecular Devices) at 595 nm.

Flow cytometry detection of *V. cholerae* EpsG

V. cholerae were grown up overnight from freezer stocks then reinoculated 1:100 in LB and grown for 3 hours to log phase at ~ 0.8 OD600. 0.5 mL of culture was harvested and added to 1 mL of 3.7% formaldehyde in 0.22 µm filtered PBS. Cells were then spun down at 8000 rpm for 3 minutes in a tabletop centrifuge, which was used for all subsequent spin downs. The supernatant was decanted and 1 mL of 3.7% formaldehyde in 0.22 µm filtered PBS was added and allowed to incubate for 15 minutes at room temperature to fix cells. Cells were spun down again and the supernatant was decanted then 50 µL of primary EpsG rabbit IgG antibody was added and incubated on ice for 20-40 minutes. 1.0 mL of filtered PBS was added and then the cells were spun down again and the supernatant was decanted. A 1:100 diluted secondary antibody R-Phycoerythrin-conjugated AffiniPure F(ab')2 fragment goat anti-rabbit (H+L) (Jackson ImmunoResearch West Grove PA), which was used to for fluorescent labeling. 50 µL of secondary antibody was added and allowed to incubate on ice for 20-40 minutes. The cells were spun down again the supernatant was decanted and 1 mL of 0.22 µm filtered PBS was added. Cells were then diluted to approximately 10^6 cells per mL and run through a LSRII flow cytometer (BD Biosciences) fluorescence was achieved by excitation with a 488nm laser.

Flow cells

Disposable flow cells (Stovall Life Science, Greensboro, NC) were used to observe biofilm formation under flow conditions and provide a good platform for confocal imaging. A once flow-through system was constructed as previously described [171] with a reservoir of sterile LB media which was pumped through the system by a peristaltic pump at a rate of 0.2 mL / minute. The system was clamped just upstream of the flow cell to prevent liquid from moving upstream prior to inoculation. Overnight cultures of *V. cholerae* were used for inoculation, cells were diluted to OD600 of 0.05 and 0.5 mL was injected from the upstream tube into a flow cell that was inverted so bacteria would attach favorably to the top of the flow cell for imaging in an upright microscope. Then the tubes were unclamped and the pump was turned on. Incubation in the flow cell took place at 35C for a time period of 24 hours. Macroscopic images were captured by a 5 megapixel autofocus dual-LED flash EVO 3D (HTC Taoyuan, Taiwan).

Microscopy

Imaging of cells used in flow cytometry was performed on an epifluorescence compound microscope (DM5000, Leica Microsystems, Wetzlar, Germany) equipped with X-Cite120 Illumination System (EXFO Photonic Solutions Inc., Mississauga, Ontario) with dsRed filter (Leica). Images were captured with an attached Spot Pursuit 2 CCD camera (SPOT Imaging Solutions, Sterling Heights, MI). Differential interference contrast images were overlaid with red fluorescence images.

Biofilms were labeled with antibodies to EpsG and imaged in flow cells. For each step flow cells were stopped and the upstream tube was clipped to stop back flow and treatments were administered to fill the entire flow cell from the upstream tube, after each treatment the flow cell was turned on to wash out the current reagents for 3 minutes. First treatment was 3.7% formaldehyde in 0.22 μm filtered PBS for 15 minutes to fix the biofilms. The flow cell was washed out. Then 1:100 diluted primary EpsG rabbit IgG antibody was added and incubated on 20 minutes. The flow cell was washed. A 1:100 diluted secondary antibody R-Phycoerythrin-conjugated AffiniPure F(ab')2 fragment goat anti-rabbit (H+L) (Jackson ImmunoResearch West Grove PA), which was used for fluorescent labeling and incubated for 20 minutes while protected from light by tinfoil. The flow cell was washed a final time for 10 – 15 minutes and capped on both ends and disconnected from the flow system for imaging. Antibody labeled biofilms were imaged by confocal laser scanning microscopy (CLSM) using a Carl Zeiss Pascal laser scanning microscope (Carl Zeiss, Jena, Germany) equipped with a 40×/1.4 numerical-aperture Plan-Apochromat objective or an Olympus FluoView 1000 Spectral-based Laser Scanning Confocal Microscope configured on an Olympus IX81 automated inverted microscope platform (Olympus America Inc., Center Valley, PA). CLSM zstacks were generated in the Fluoview software suite.

Protease Assay

The protease secretion assay was carried out as described in Chapter 1.

Transmission Electron Microscopy

V. cholerae was grown overnight from freezer stocks then diluted 1:1000 and grown to log phase approximately 3 hours. All steps were carried out at room temperature and the parlodion-coated nickel grid FF-200-Ni (Electron Microscopy Sciences Hatfield, PA) was floated upside down on a drop of the treatment in an upside down top of a 96-well plate unless otherwise noted. A drop of culture was then placed on the upside down lid of a 96-well microtiter plate and a was floated upside down on the culture for 30 minutes to allow bacteria to stick to the grid. The grid was then moved to a drop of H_2O with fixative and allowed to incubate for 5 minutes. A blocking step was performed with 5% milk blocking solution for 60 minutes. The grid was incubated with primary antibodies to EpsG which were diluted 1:200 in PBS-BT (0.9%NaCl, 0.01 M phosphate buffer, 0.2% BSA, 0.02% Tween 20) for 60 min. Next a wash for 5 minutes in PBS-BT was performed. Grids were then moved to the secondary antibody solution containing 12 nm Colloidal Gold AffiniPure Goat Anti-Rabbit IgG H+L EM Grade (Jackson ImmunoResearch West Grove PA) diluted 1:100 in PBS-BT and incubated for 60 minutes. Two washes in H_2O were performed for 5 minutes each and then the remaining liquid was blotted off with filter paper and allowed to air dry. Negative staining was performed with 2% uranyl acetate at the electron microscopy facility and imaging was done on a JEOL 100CXII (Jeol Peabody, MA) transmission electron microscope at the MSU Center for Advanced Microscopy.

Results

Overexpression of EpsG increases biofilms

As c-di-GMP promotes biofilm formation and the c-di-GMP inducible promoter at *epsC* increases *epsG* expression, I hypothesized that *epsG* contributes to biofilm formation. Based on this hypothesis, I predicted that deleting the *epsG* gene would reduce biofilm formation, and overexpressing *epsG* would increase biofilm formation. To test this hypothesis, an unmarked *epsG* deletion was created in *V. cholerae* through homologous recombination by methods previously described [172]. The DNA created by overlap extension PCR was introduced into *V. cholerae* by natural transformation as previously described [173]. An EpsG expression vector was constructed with *epsG* expression under control of the P*tac* promoter. The biofilm formation capacity of each of these strains were quantified using a 96-well plate biofilm device [174] followed by staining with crystal violet, as previously described [175]. In *V. cholerae ΔepsG* minimal biofilm was formed that was equivalent to the Δ*vpsL* biofilm deficient *V. cholerae* mutant control (data not shown); however, in the *epsG* expression strain, biofilms were increased in both the WT and a Δ*hapR* mutant which is locked in the low-cell-density quorum sensing state and naturally exhibits enhanced biofilm (Fig. 3.2). Following this analysis, I examined biofilm formation of these strains in Stovall flow cells (Life Science, Inc., Greensboro, N.C.) where media is passed through a small chamber containing the bacterial biofilm to create a constant flow of resources and shear forces. Identical results were observed indicating that overexpression of EpsG increased biofilm formation while mutation of *epsG* inhibited biofilm formation (Fig. 3.3).

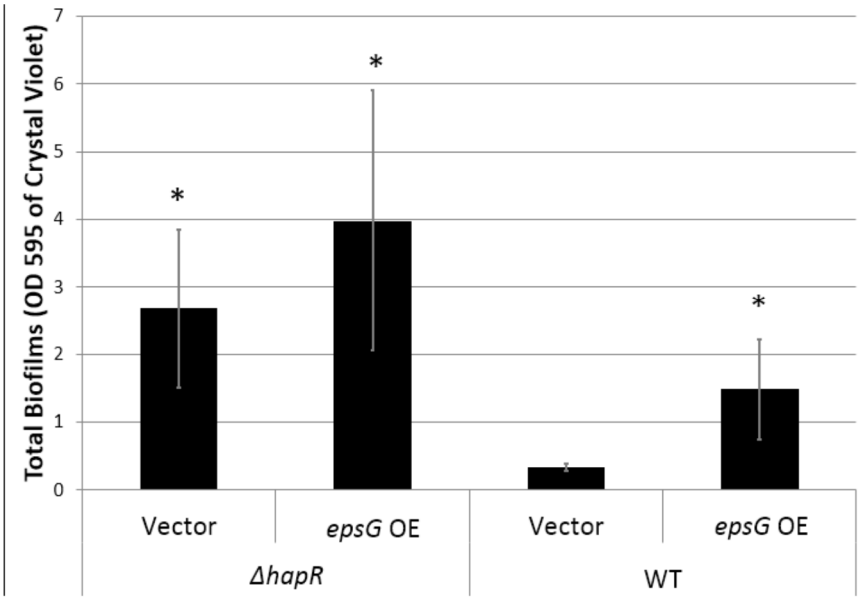


Figure 3.2. Overexpressing epsG increases biofilm biomass Biofilm formation assessed on minimum-biofilm-eliminating-concentration (MBEC) plates at 24 hours. The error bars represent the standard deviation (n=3). * indicates $P < 0.05$ based on students paired T test compared with the WT Vector control.

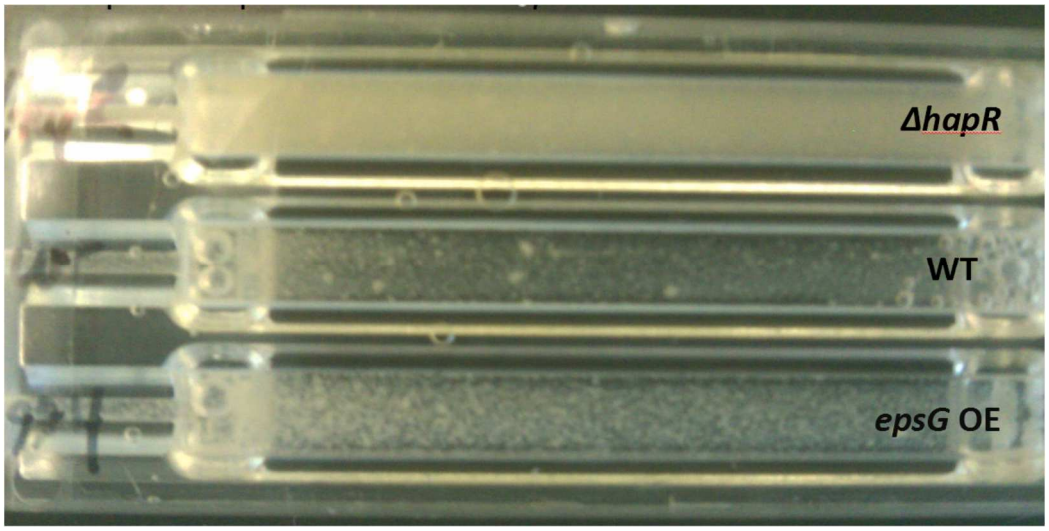


Figure 3.3. Flow cell biofilms are increased with ectopic expression of epsG Stoval flow cell with *V. cholerae* biofilms grown for 24 hours.

Visualization of EpsG on the cell surface

To determine if EpsG can be detected outside of the cells, a polyclonal antibody that binds to EpsG was used to label the pseudopilin, and an R-Phycoerythrin-labeled secondary antibody was used for imaging the pseudopilin with confocal laser scanning microscopy (CLSM) or a compound microscope. This method was similar to antibody labeling employed in flow cytometry except that the blocking step was replaced with a wash with LB, and LB media was used for all washes instead of water. Imaging showed that in the *ΔepsG* strain, no EpsG was detected (Fig. 3.4). Imaging of the WT strain of *V. cholerae* resulted in detection of EpsG in the biofilm, although this appeared to be in small patches located throughout the biofilm. *V. cholerae* ectopically expressing EpsG results in increased EpsG detection and a network of long resolvable hyper-pseudopili can be observed (Fig. 3.4, 3.5). This indicates that EpsG is capable of forming pili like structures, even though they are not resolvable in the WT strain.

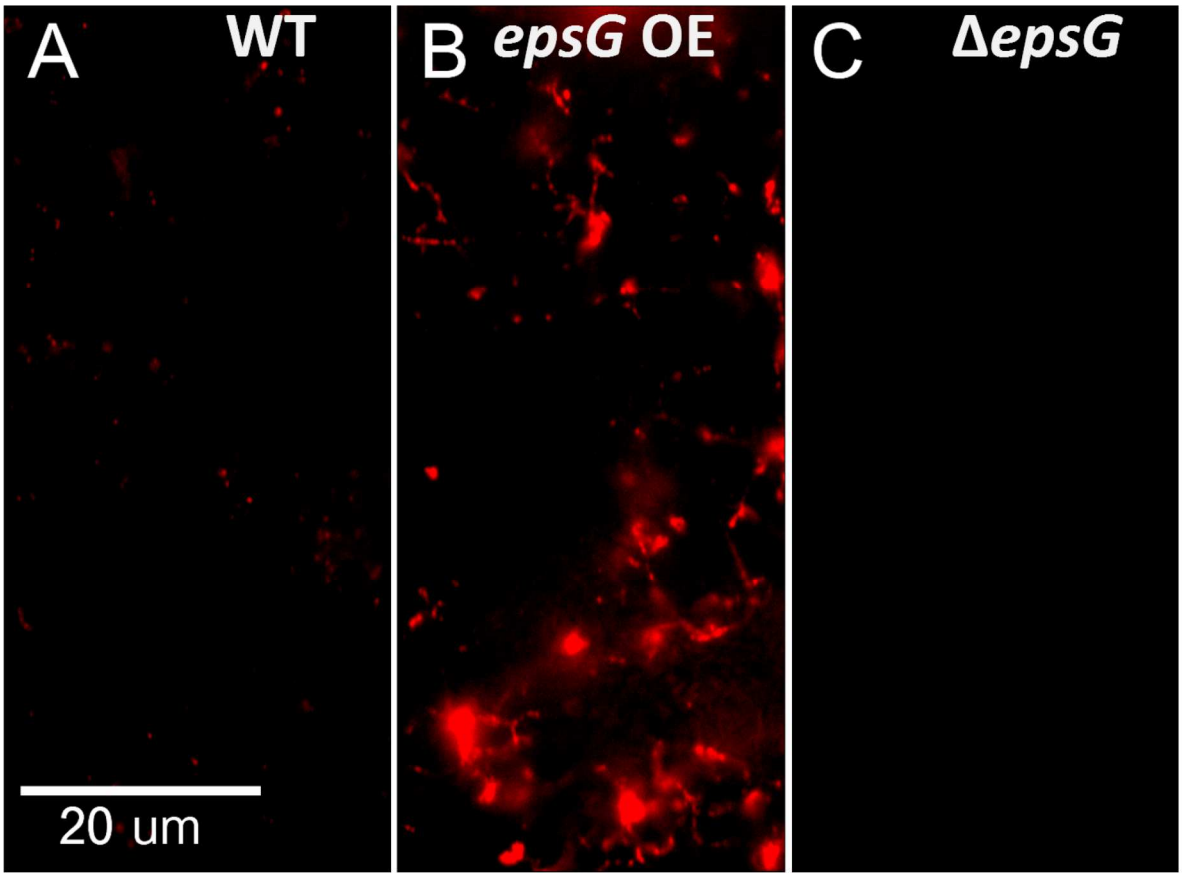


Figure 3.4. EpsG detected in biofilms of *V. cholerae* CLSM image of fluorescent labeled EpsG in a stoval flow cell at 400X magnification. The images are representative of multiple samples.

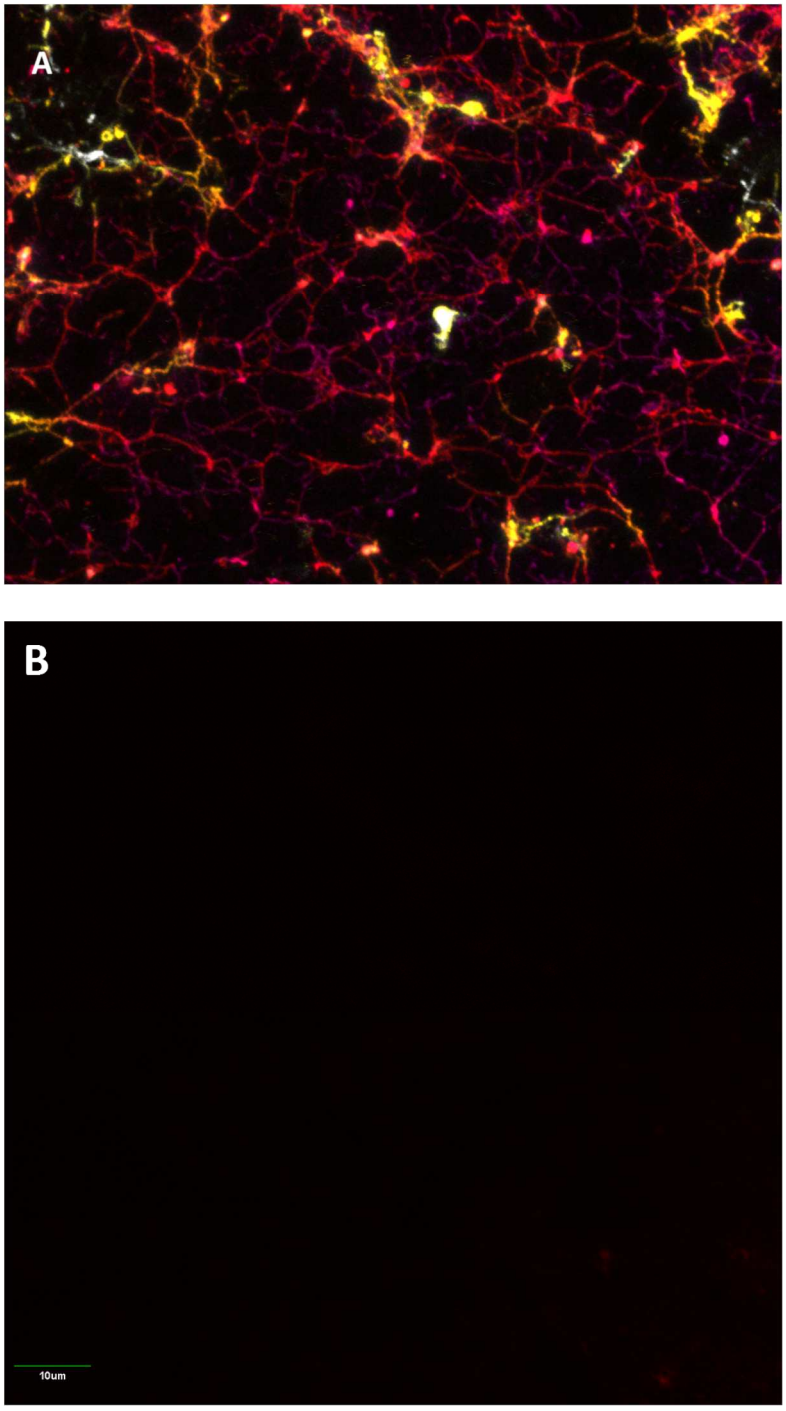


Figure 3.5. EpsG detected throughout the biofilm CLSM image stack of V. cholerae with EpsG over expressed. Color heat map indicates EpsG labeling at different heights in the confocal image stack. The entire image represents about 10 um of thickness of biofilm. A EpsG over expression vector pMMB994. B. EpsG knock out does shows that antibodies do not bind nonspecifically.

Cell shearing western blots

As a third method to detect external EpsG hyper-pseudopili, I performed Western blots on sheared surface components. Briefly, whole cells are pelleted to remove the supernatant and pili are sheared off the cell surface by pushing the cells through a syringe. The sheared pili are then separated from cells by ultracentrifugation. Western blot analysis of these samples detected EpsG sheared from the surface of WT cells. To our knowledge, this is the first observation of external T2SS pseudopili in the absence of overexpression of pseudopilin genes. In addition, in cells which form more biofilms such as those that had increased levels of c-di-GMP by a vector expressing the DGC *qrgB* and *hapR* mutant resulted in increased EpsG. Expression of *qrgB*-, an active site mutant of *qrgB* that cannot make c-di-GMP, showed decreased levels of EpsG compared with the *qrgB* overexpressing strain that were more similar to the WT, suggesting c-di-GMP levels can increase surface EpsG.

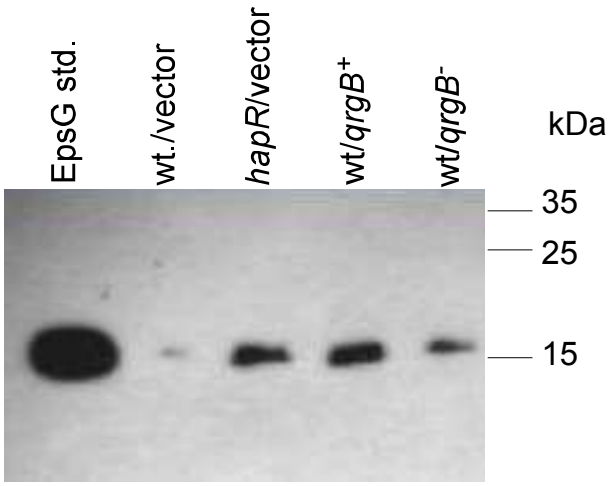


Figure 3.6. Western blots of sheared cells detect external EpsG More EpsG is detected in cells with high c-di-GMP (wt/*qrgB*+) than those with vector control or active site mutant of *qrgB* (wt/qrgB-). *hapR* a high biofilm forming mutant also has more EpsG than WT.

Measuring secretion of extracellular proteases

As EpsG overproduction greatly increased biofilm formation, it was not clear if this phenotype was due to increased pseudopili formation or alteration of its role in functional secretion. To determine if EpsG is playing a direct role in biofilm structure rather than modulating secretion in this condition, I quantified secretion of extracellular proteases of the *V. cholerae* WT, Δ*espG*, and *epsG* expression strains using a standard protease assay. This assay measures the activity of a pair of trypsin-like proteases in the media that are exported by the T2SS (Fig.3.5). Cells are grown over-night and separated from the media by centrifugation. A fluorogenic peptide substrate BOC-Gln-Ala-Arg-7-Amino-4-Methyl-Coumarin is added, which fluoresces when the amino acids are cleaved by proteases. This assay showed that the *ΔepsG* strain exhibits less

secretion than the WT strain as expected. This mutation could be complemented with a plasmid that expressed *epsG* from the pTac promoter, although basal levels of expression were sufficient for partial complementation. The lack of full complementation is likely due to polar effects of the *epsG* deletion on the rest of the T2SS operon. Importantly, secretion of protease in the *epsG* overexpression strain is not significantly different than the WT strain both in the uninduced and induced conditions. These results support the hypothesis that the increased biofilm formation of EpsG overexpression strain is due to its role as a structural component as opposed to indirectly involved as a secretor of biofilm components such as structural proteins or enzymes. However, I cannot conclude if the defect in biofilm formation of the *epsG* deletion mutant is due to secretion defects or the loss of the EpsG pseudopilus.

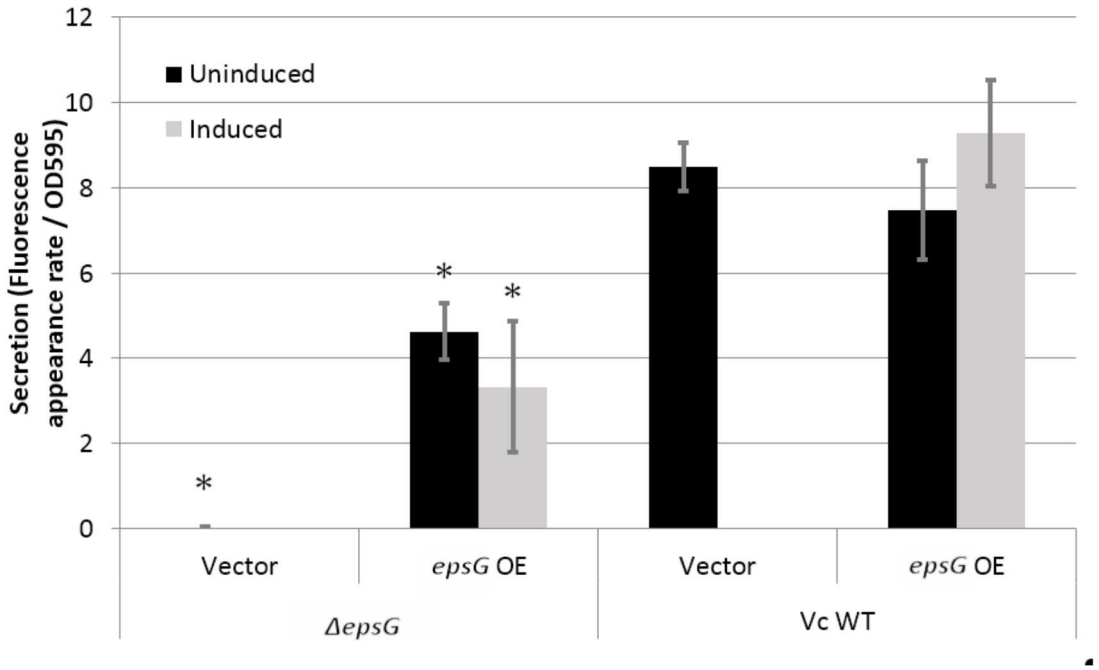


Figure 3.7. Ectopic expression of epsG does not increase secretion
Fluorescence was monitored following cleavage of a fluorogenic oligopeptide by proteases secreted via the T2SS. Following induction of *epsG* cultures were allowed to incubate overnight and supernatant was collected and mixed with substrate. Increased fluorescence is measured each minute for 20 minutes, and the average slope of increase in fluorescence over time is calculated (avg(dF/dT)). The dark line indicates cultures induced with 0.1 mM IPTG while light bars indicate non-induced cultures. The error bars represent the standard deviation (n=3). * indicates $P < 0.05$ based on students paired T test compared with the WT Vector control.

Transmission electron microscopy

I attempted to visualize the hyper-pseudopilus by transmission electron microscopy and labeling with gold labeled antibodies. I was unable to achieve a condition where EpsG was labeled in TEM. This may be due to the harsh conditions that are used in staining and fixing cells especially the uranyl acetate negative stain. The antibodies were nonspecifically bound to structures that resembled EPS. (Fig. 3.8)

Under high magnification long thread like structures that resemble pili could be observed in abundance in a strain ectopically expressing *epsG*. The same structures were rare or far less common when in *ΔepsG* strain. (Fig. 3.9) However, these could not be positively identified as EpsG hyper-pseudopili without antibody labeling or complete absence of the structures in the *ΔepsG* strain. These images do however provide a good base from which future attempts to antibody labeled can be based off of as we can see a structure that antibodies should bind to.

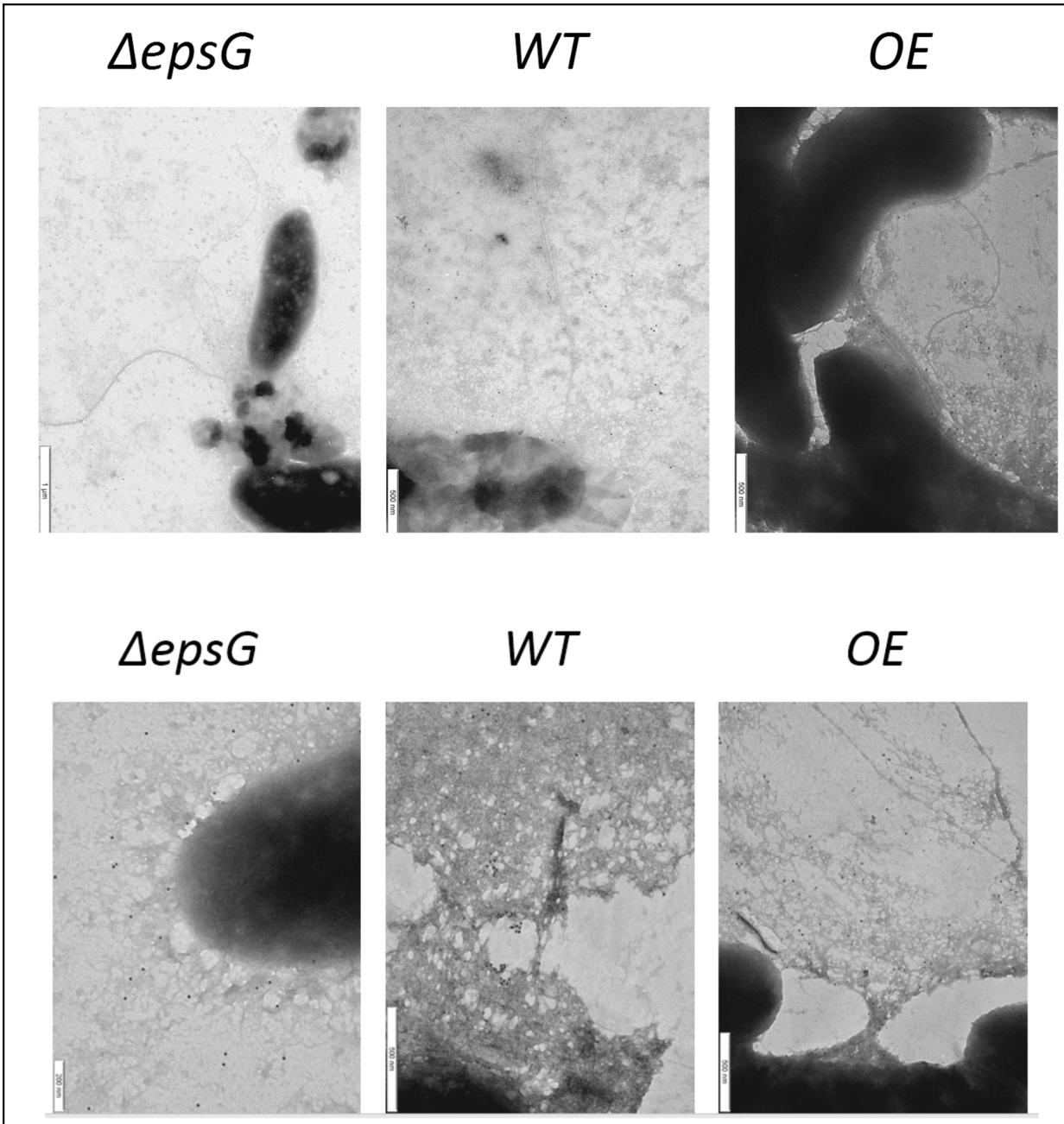


Figure 3.8. TEM imaging of V. cholerae Nonspecific binding of antibodies to eps like material. First column contains images of V. cholerae Δ*epsG*, second column is *V. cholerae* WT, third column is *V. cholerae* WT pMMB994 induced with 0.1mM IPTG which over expressed *epsG*.

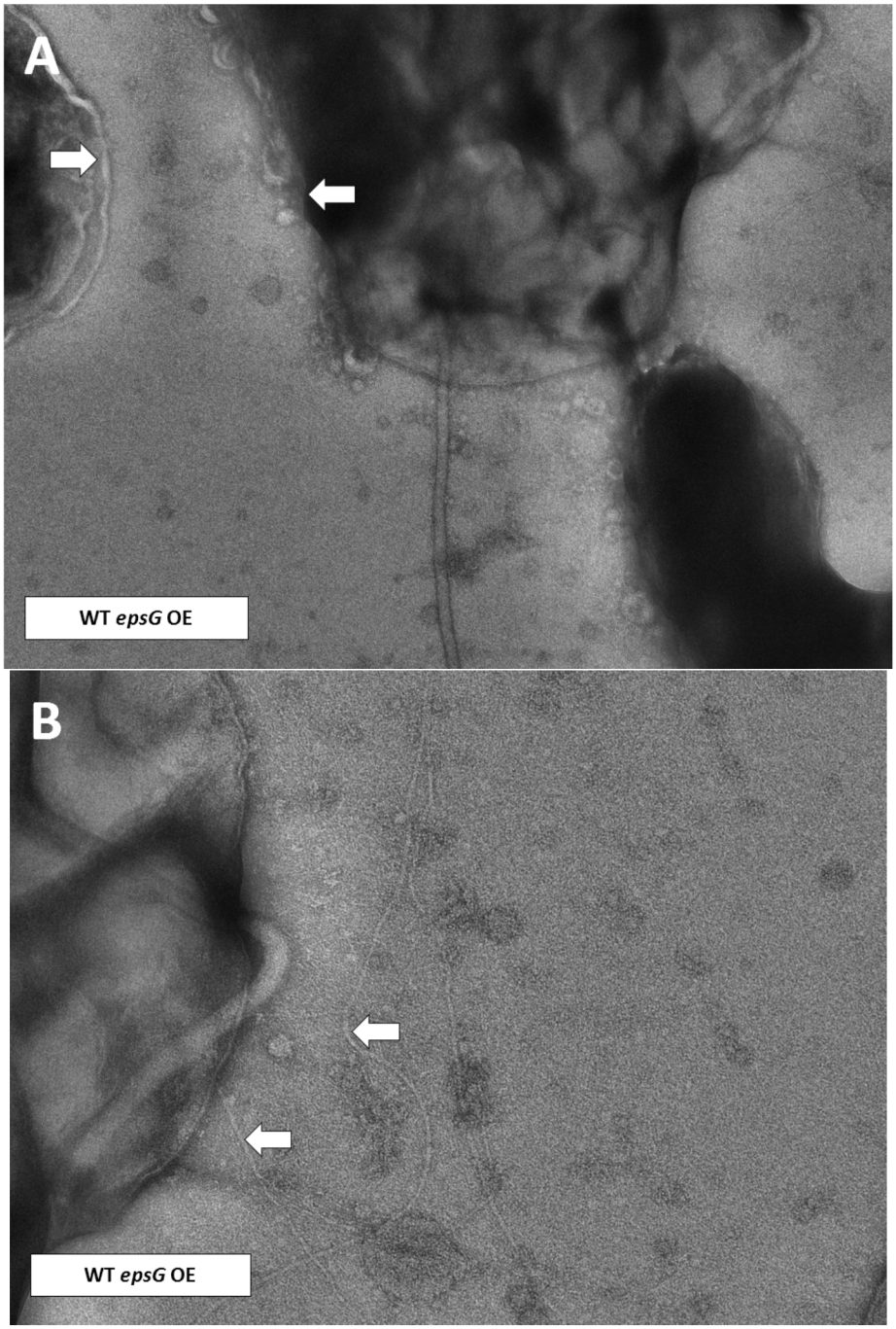


Figure 3.9. Close up TEM imaging of *V. cholerae* A-B. Structures that resemble pili are found in abundance in the high EpsG strain. *V. cholerae* WT pMMB994 induced with 0.1mM IPTG which over expressed *epsG*.

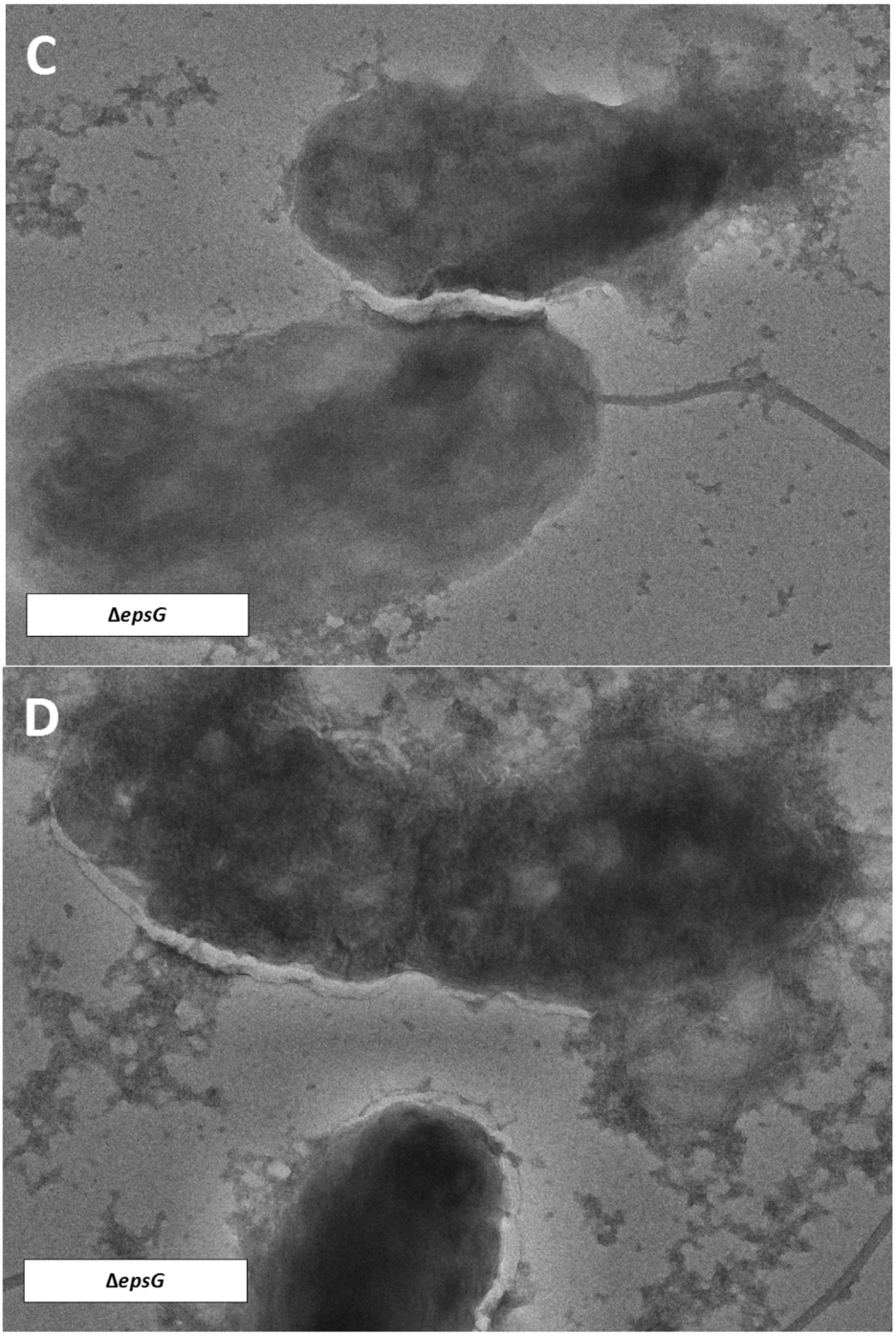


Figure 3.9. Close up TEM imaging of *V. cholerae* C-D. Structures that resemble pili are not found often in abundance in the *ΔepsG* strain however there were some visible which may have been another pilus system such as MSHA.

Flow cytometry detection of *V. cholerae* EpsG

In order to test this hypothesis and quantify extra cellular hyper-pseudopili, I used flow cytometry and immunofluorescence microscopy. I labeled *V. cholerae* strains with a primary polyclonal antibody specific for EpsG followed by incubation with the corresponding secondary antibody that is R-Phycoerythrin labeled. I then quantified the fluorescence of individual cells using flow cytometry (Fig. 3.10). I observed high levels of labeling of EpsG in a strain in which *epsG* was induced by the P*tac* promoter. I also observed high levels of labeling in a *hapR* mutant, which locks the cells in the low-cell-density quorum sensing state, that expresses high levels of biofilms. However, a strain in which c-di-GMP levels were increased by expression of a diguanylate cyclase did not have significantly more labeling than the WT control.

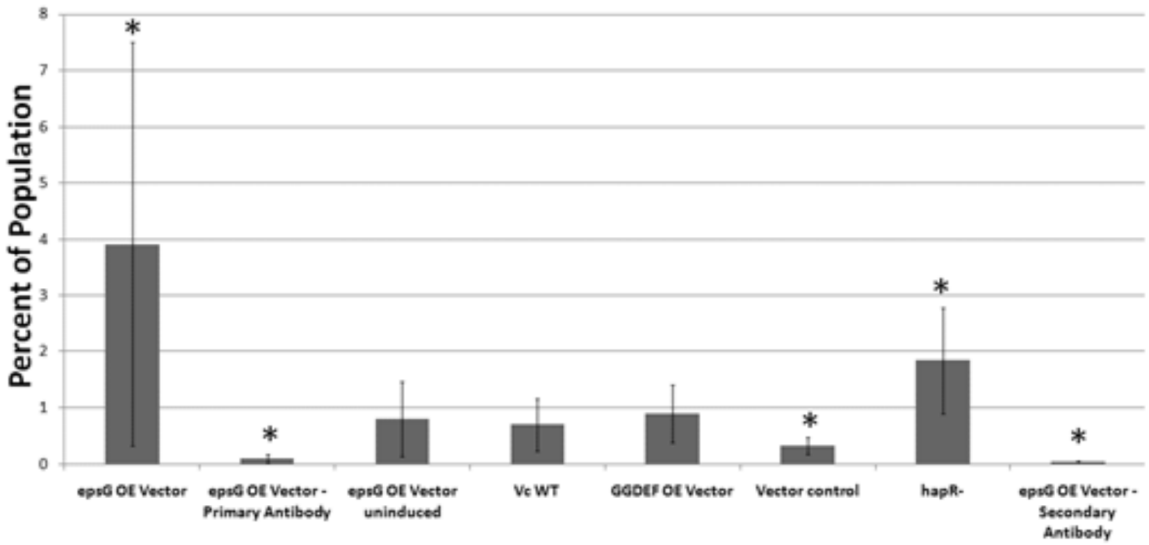


Figure 3.10. Flow cytometry detection of EpsG Cultures labeled with OE were induced with 0.1 mM IPTG. Cells were run through flow cytometer and populations were gated on the majority of cells in a WT control that was unlabeled to establish background fluorescence then the percent of the population labeled with R-Phycoerythrin was quantified. The error bars represent the standard deviation (n=3). * indicates $P < 0.05$ based on students paired T test compared with the Vc WT.

Discussion

The T2SS has a well-established role secretion apparatus and here I present evidence it has a structural role in biofilms of *V. cholerae*. I have found that ectopic expression of *epsG* results in increased biofilm formation and that the same expression does not result in significant changes in secretion. I hypothesized that the *V. cholerae* T2SS serves a dual function as both a secretion system and an extracellular biofilm component by formation of a hyper-pseudopilus.

My results indicate that a hyper-pseudopilus is formed and resolvable under CLSM when *epsG* is ectopically expressed. Unfortunately, CLSM has been unable to produce well defined images of the hyper-pseudopili in the WT strain. Also background fluorescence and nonspecific binding were not the source of fluorescence in WT cells during CLSM as indicated by lack of signal in the *ΔepsG* strain. EpsG was detected in sheared cell western blots indicating external hyper-pseudopili are very likely present. In order to positively identify the hyper-pseudopilus, I attempted to image the complex under TEM however antibody labeling was not effective in this method and is still an area that requires more experimentation. However, a structure that looked like T4P was present in the strain over expressing EpsG.

Previous studies with *P. aeruginosa* have shown that the major pseudopilin of the T2SS is capable of assembling on a T4P, indicating promiscuous function [163]. In addition, the major pseudopilin has been shown to assemble into a hyper-pseudopilus when expressed in other species of bacteria [163]. Other studies have also shown that pilins can be highly specific to a specific operon and that small differences in the amino acid sequence can result in major changes in the specificity of pseudopilins to a specific

T2SS operon [162]. Perhaps the pseudopilins of *V. cholerae* are not highly specific to the T2SS then it is possible that when EpsG is highly expressed it assembles on the T4P apparatus as well as the T2SS apparatus. *V. cholerae* utilizes at least 3 T4P including the chitin regulated pilus (ChiRP). the toxin co-regulated pilus (TCP) and mannose-sensitive hemagglutinin (MSHA) pili [176-178]. In this case it could simply be that the pseudopilus does not form a T2SS hyper-pseudopilus, but rather EpsG supplements an existing T4P. Experiments should be carried out to resolve this in the future. One way to approach this may be to knock out EpsD which forms the secretin. Removal of the secretin will eliminate the ability of the pseudopilus to form a T2SS external structure on the cells. If hyper-pseudopili are no longer able to form it would add evidence the hyper-pseudopilus is T2SS based.

I hypothesized that EpsG plays a specific structural role in biofilm development separate from its role as a secretion apparatus, and alternative regulation of the *eps* operon through P*epsG* leads to this function of the T2SS. The promoter P*epsG* could increase expression of *epsG* and downstream genes resulting in increased hyper-pseudopili. Alternatively, the promoter could also increase *epsH* and downstream genes. This could alter the ratio of minor pseudopilins to the major pseudopilin, which could reduce length of the hyper-pseudopilin. In addition, *epsK* is thought to destabilize the pseudopilus and control its length [179] and could be used to disassemble the hyper-pseudopilus when no longer needed for biofilm formation, such as during dispersion or when secretion needs to be increased.

The role of the promoter P*epsG* is also an area that requires further study. Although we attempted to find factors that alter the expression of the promoter (data not

shown) we were unable to find any in preliminary experiments. More investigation into the function of this promoter and uncovering any effectors influencing its expression could provide tools to aid in the understanding of what is taking place in the T2SS as well is if and under what conditions a hyper-pseudopilus may be formed in WT cells.

Since the T2SS is related to T4P, it is likely that any structural role the T2SS-based hyper-pseudopilus plays in biofilm development is the same as the role T4P play in biofilm development. The literature indicates T4P have multiple roles in the development of biofilms. Some T4P are known to be involved in initial attachment to surfaces. In other cases, T4P are known to be important for twitching motility in biofilms[166]. In addition, different T4P have been shown to attach to different surfaces to serve separate roles in bacteria, such as, surface adhesion versus cell-cell adhesion [180].

It is known that proteins and multi-protein complexes in bacteria can serve multiple functions and can be regulated by multiple different systems. For example, it is known that the T4P complex can function to secrete proteins [181]. It has also been shown that the flagellar system can also act as a secretion apparatus for non-flagellar proteins [182]. T2SS is known to be involved in membrane integrity as an extra function perhaps biofilms structure is yet another moonlighting capability of this system. The T2SS is widespread in gram negative pathogens and it is likely that other species, such as *E. coli* and *P. aeruginosa,* may also use the T2SS as a structural component of biofilms if it can be shown in *V. cholerae*. This is especially important in the case of cystic fibrosis, in which *P. aeruginosa* forms a persistent biofilm based infection.

CHAPTER 4: POLYSORBATES PREVENT *ESCHERICHIA COLI* O104:H4 PATHOGENESIS BY INHIBITING BIOFILM FORMATION

Accepted in Biofouling[183]

Abstract

Escherichia coli biotype O104:H4 recently caused the deadliest *E. coli* outbreak ever reported. Based on prior results, we hypothesized that compounds inhibiting biofilm formation of O104:H4 would reduce its pathogenesis. We determined that the nonionic surfactants polysorbate 80 (PS80) and polysorbate 20 (PS20) reduced biofilms by ≥ 90% at submicromolar concentrations and elicited nearly complete dispersal of preformed biofilms. PS80 did not significantly impact *in vivo* colonization in a mouse infection model; however, mice treated with PS80 exhibited virtually no intestinal inflammation or tissue damage while untreated mice exhibited robust pathology. As PS20 and PS80 are classified as “Generally Recognized as Safe” (GRAS) compounds by the FDA, these compounds have clinical potential to treat future O104:H4 outbreaks.

Keywords: *Escherichia coli*, biofilm, polysorbate, O104:H4

Introduction

Escherichia coli O104:H4 (hereafter referred to as O104:H4) is a newly evolved pathogenic strain of *E. coli* responsible for a massive 2011 European outbreak [122]. Genome sequencing of O104:H4 revealed that this strain evolved from an enteroaggregative *E. coli* (EAEC) that acquired an *E. coli* O157:H7 Stx2 phage [122]. The 2011 outbreak was the most severe *E. coli* outbreak ever recorded resulting in nearly 4,000 infections leading to 54 deaths. Over 22% of patients exhibited hemolytic uremic syndrome (HUS) suggesting that this *E. coli* isolate persists and expresses high levels of Stx toxin during infection that can enter the bloodstream and damage the kidneys [184-186]. Because O104:H4 is resistant to many clinical antibiotics, and antibiotics have been implicated with increased disease severity in Stx-producing *E. coli* infections [187], treating O104:H4 infections is difficult, necessitating the need for novel intervention strategies [188].

O104:H4 harbors a number of virulence factors including but not limited to the pAA plasmid encoding the aggregative adherence fimbriae (AAF), two distinct operons encoding long polar fimbriae (lpf), and a Stx2-producing Shiga toxin lamboid phage [122, 129] . It is hypothesized that the high colonization ability of the EAEC parent strain combined with toxin production by the Stx2-phage accounts for the high numbers of infections and HUS rate [189]. However, the specific O104:H4 virulence factors responsible for these disease outcomes requires further investigation.

Mouse infection models have implicated the siderophore aerobactin [190], *lpf* [129], biofilm formation [191], and Stx2 production [192] as important for disease. Moreover, naturally evolved O104:H4 strains lacking the pAA plasmid were isolated that

correlated with less severe disease symptoms [193]. The importance of the pAA plasmid in O104:H4 colonization was recently questioned as it had no significant impact in the colonization or disease symptoms in an infant rabbit model, but this model does not exhibit HUS [129, 194] . These same studies determined that Stx2 is critical for disease and autotransporters and Lpf drive colonization.

Biofilm formation, defined as a multi-cellular community of microorganisms encased in an extracellular matrix, of O104:H4 has been implicated as an important driver of severe sequela. The pAA plasmid and the AAF were critical for *in vitro* biofilm formation and promoted increased adherence to cultured epithelial cells [195]. One outcome of this adherence was increased transit of the Stx2 toxin across the epithelial cell barrier [195]. Deletion of *lfp1* fimbrial loci severely inhibited *in vitro* biofilm formation and adherence to both polarized and non-polarized epithelial cells, and this mutation had an impact on *in vivo* colonization [129]. Finally, infection of germ-free mice revealed the characteristic EAEC “stacked brick” morphology of O104:H4 in close proximity to the intestinal epithelium [191], which was also observed in other studies [190, 192, 194]. This same study described a correlation between *in vivo* toxin expression and the induction of biofilm genes, leading to the hypothesis that *in vivo* biofilm formation of O104:H4 promotes toxin expression for unknown reasons [191].

Because biofilms are implicated in the pathogenesis of O104:H4, we sought to identify new compounds that inhibit biofilm formation of O104:H4. We determined that the common food additives polysorbate 80 (PS80) and polysorbate 20 (PS20) inhibit and disperses O104:H4 biofilms *in vitro* at sub-micromolar concentrations without negatively impacting growth. Although PS80 had no significant impact on O104:H4

colonization levels in a mouse infection model, this compound completely abolished intestinal inflammation and tissue damage compared with the untreated control. Our research suggests that inhibiting O104:H4 biofilm formation is an attractive strategy to reduce the severity of these infections and identifies polysorbates as a potential treatment for future O104:H4 outbreaks.

Materials and Methods

Bacterial strains and culture conditions

E. coli O104:H4 strain TW16133 [191] was used in all *in vitro* experiments. O104:H4 was grown in Luria-Bertani (LB) medium (Accumedia, Lansing, MI) incubated at 35°C shaking at 220 RPM. For *in vivo* mouse studies the bioluminescent O104:H4 strain RJC001 was used [190].

In vitro biofilm assays

Biofilms were measured by staining with 0.41% crystal violet solubilized in 12% ethanol in 96 well microtiter plates. O104:H4 was inoculated 1 to 500 into LB from turbid overnight cultures and 160 µL of this suspension was placed into a well of a clear 96 well polystyrene CellStar microtiter plate (Greiner Bio-One, Monroe, NC). Cultures were grown at 35°C with rotation for 8 hours. The plate was washed with 200 µL phosphate buffered saline (PBS). Subsequently 200 µL of 95% ethanol was added for 10 minutes at room temperature to fix the cells. The ethanol was tapped out of the 96 well plate and 200 µL of crystal violet solution was added and allowed to incubate at room temperature for 2 minutes. The crystal violet solution was tapped out of the 96 well plate and then

the plate was washed with tap water 3 times to remove crystal violet not bound in biofilms. The water was removed by tapping and 200 □L of ethanol was added to each well to elute the crystal violet from the biofilms. The eluted crystal violet was then measured on a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA) at 595 nm. When necessary, 1:10 dilutions of the crystal violet solution were analyzed to prevent absorbance saturation. Bacterial dispersion from biofilms was measured in the same way as *in vitro* biofilm assays by crystal violet staining with replicated microtiter plates. One plate was fixed with ethanol at 5 hours while polysorbates were added to the other plate at 5 hours then allowed to incubate for 3 more hours before biofilms were measured.

Bacterial attachment assay

Turbid overnight cultures of O104:H4 were diluted 1 to 62.5 into fresh media with 0.01% polysorbates in treated samples. 100 µL of this solution was added to each well of a 96 well microtiter plate. Bacteria were allowed to attach during a 1 hour incubation at 35˚C shaking at 100 rpm in a Syrotory Shaker Model G76 (New Brunswick Scientific, Edison, NJ). Wells were washed 2 times with 150 µL of PBS to remove planktonic cells that were unattached and stained with 150 µL of SYTO 9 (Invitrogen, Waltham, MA) diluted 1:1000 in PBS for 15 minutes. Wells were washed again with 150 µL of PBS then fixed with 150 µL of 3.7% formaldehyde. Cells were enumerated by counting 3 fields of view in six wells per sample on an Nikon Eclipse TS100 equipped with X-Cite series 120 Q illuminator (Exfo) inverted epi florescent microscope.

In vivo bacterial infections in mice

Mice: Eight to ten week old female CD-1 (ICR) mice were purchased from Charles River Laboratories (Willimington, MA). Animals were housed in a specific pathogen-free barrier under biosafety level 2 conditions. 48 hours before the infection, mice were treated with streptomycin (5 g/L in their drinking water supplemented with 6.7% fructose) to reduce the normal flora [190]. The level of water consumption by mice was similar across all cages evaluated. Food was restricted for 12 hours prior to infection and cimetidine was also administered 2 hours prior to infection to reduce the acidity of the stomach at a concentration of 50 mg/Kg of body weight. All animal studies were performed in accordance with the Animal Care and Use Committee's guidelines at UTMB as recommended by the National Institute of Health (NIH).

Bacterial infection and treatment

For the bioluminescence experiments, animals were inoculated with a suspension of bioluminescent O104:H4 RJC001 as previously described [190]. The strain RJC001 is resistant to 100 □g/ml of streptomycin and 50 □g/ml of kanamycin. A total of 12 mice were infected with the strain RJC001 at a bacterial suspension of 1x 10^8 CFU resuspended in 400 µl of PBS via oral gavage as previously described [190]. The infected mice were divided in two groups of six mice each.

The group used to evaluate the effect of PS80 (Sigma Aldrich, St. Louis, MO) on O104:H4 infection was treated by diluting 0.01% of PS80 in their drinking water throughout the experiment. The remaining group of six mice was untreated and used to

monitor the regular course of infection by O104:H4 strain RJC001. There was no difference in the volume of water consumed by the treated and untreated groups.

Bioluminescent quantification of infection

For *in vivo* imaging, mice were anesthetized with 2-3% isoflurane in an oxygen-filled induction chamber. Once anesthetized, the mice were transferred to the in-chamber anesthesia delivery system were they were imaged. Bioluminescent images were acquired by using an IVIS Spectrum (Caliper Corp., Alameda, CA). Bioluminescent signal is represented in the images with a pseudo-color scale ranging from red (most intense) to violet (least intense) indicating the intensity of the signal. Signal intensities were obtained from regions of interest (ROIs), which are user-specified areas in an optical image. ROI of same size were drawn at the mouse abdominal cavity, and the same ROI was used for each time point evaluated to be able to establish a comparison between groups and different days. Signal from ROIs were expressed as photon flux (photons/s/cm^2/steradian), where steradian (sr) refers to the photons emitted from a unit solid angle of a sphere. Any value outside the scale ranging from $1.1x10^8$ to $8x10^5$ (photons/s/cm^2/steradian) was not used, since they fell outside our limit of detection [190]. All mice were monitored daily up to 6 days post-infection.

In order to correlate the bioluminescence readouts with bacterial counts, the number of bacteria was monitored in fecal pellets at 1, 2, 4 and 6 days post-infection. Feces were resuspended in PBS by vortexing, and the bacteria were plated for enumeration. For quantification of bacteria in tissues, sections of the cecum were collected at 6 days post-infection in 15 mL tubes containing PBS and homogenized using

the Covidien Precision Disposable Tissue Grinder Systems. The resuspended feces and tissue homogenates were then serially diluted and plated on MacConkey agar containing streptomycin (100 μg/mL) and kanamycin (50 μg/mL). After overnight incubation at 37 ºC, colonies were counted and expressed as either CFU per gram of feces or CFU per organ

.

Histopathology

Sections of mouse terminal small intestine (ileum), and cecum were excised at 6 days post-infection and washed with PBS. The sections were fixed in buffered 10% formalin, paraffin-embedded, sectioned into 5 μm slices and then stained with hematoxylin and eosin at the Histopathology Core at UTMB. The tissues were examined and scored by a pathologist that was completely blinded to any details of the study. Tissues were scored according to degrees of severity of inflammation and necrosis (0-not present, 1-minimal, 2-mild, 3-moderate, 4-severe). In addition, the inflammatory cell constituents were characterized for each subject.

Results

Polysorbate 20 (PS20) and Polysorbate 80 (PS80) inhibit O104:H4 biofilm formation

We assayed 21 anti-biofilm compounds that were either described in published literature or being developed in our laboratory for the ability to inhibit O104:H4 biofilm formation in a standard crystal violet microtiter biofilm assay (Table 4.1). Nineteen compounds that exhibit anti-biofilm activity against other bacteria exhibited no significant effect on O104:H4. However, the nonionic surfactants PS80 and PS20, significantly reduced O104:H4 biofilm formation at concentrations of 0.01%. (Fig. 4.1A).

Polysorbates were previously shown to inhibit biofilm formation in *Pseudomonas aeruginosa* and other pathogenic bacteria including six clinical *E. coli* isolates [196]. The nature of these *E. coli* were not described, making this work the first report of polysorbate inhibition of O104:H4 biofilm formation. We determined the *in vitro* efficacy of these compounds by measuring O104:H4 biofilm formation at doses ranging from 0.01% to 0.0000316% and determined that both PS20 and PS80 have low micromolar effective concentrations of 50% inhibition (EC_{50}) at 0.00006% (0.54 μM) and 0.0001% (0.81 μM), respectively (Fig. 4.1B). Polysorbates do not inhibit growth of O104:H4 at any concentration that we examined (Fig. A.3.).

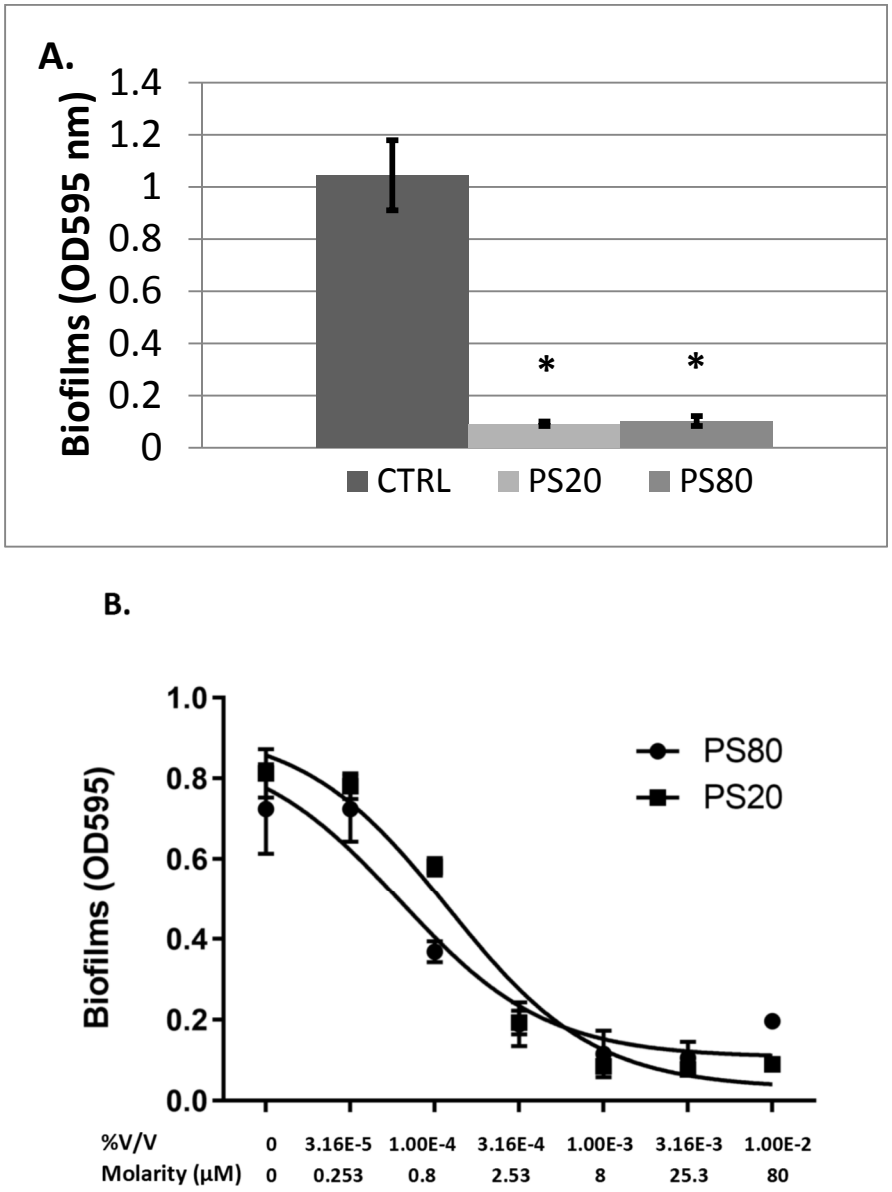
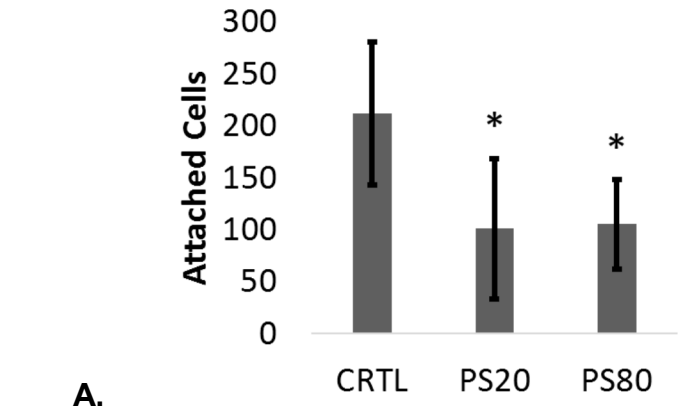


Figure 4.1. PS80 inhibits biofilm formation A. Antibiofilm activity was assessed for PS80 and PS20 at 0.01% (v/v) **B.** A dose response curve determined the EC50 values of 0.00006% (0.54 µM) for PS20 and 0.0001% (0.81 µM) for PS80. The EC50 curve was fit using PRISM (Graphpad) with a log-dose vs response, three parameters, nonlinear regression. * indicates $P < 0.05$ based on students paired t-test. The error bars represent the standard deviation (n=3).

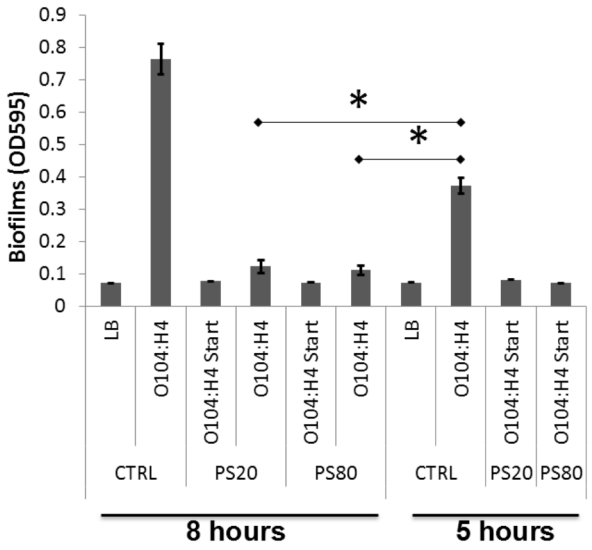
PS20 and PS80 reduce initial attachment and disperse preformed biofilms

We hypothesized that the polysorbates might negatively impact biofilms by blocking initial attachment to surfaces, the earliest process of biofilm formation. We incubated O104:H4 with the polystyrene microtiter plates for 1 hour in the presence and absence of PS20 and PS80 and measured attachment by quantifying attached cells stained with Syto 9 (Invitrogen). Total cells were counted in 3 fields of view chosen randomly from 6 wells. Our hypothesis was supported as both PS20 and PS80 significantly reduced attached cells by half (Fig. 4.2A).

To determine if PS80 can disperse a preformed biofilm, we grew biofilms in microtiter plates for 5 hours then exposed a subset of these biofilms to 0.01% PS20, 0.01% PS80, or the vehicle control. Biofilms were allowed to grow for an additional 3 hours. A control group was subjected to treatment with polysorbates at time zero, and a duplicate plate was used to measure biofilm accumulation at 5 hours. We found that treatment of PS20 and PS80 at five hours was able to disperse biofilms, leading to a significant inhibition of biofilms compared to both the 5 and 8 hour untreated controls (Fig. 4.2B). Indeed, treatment with PS20 and PS80 at five hours was nearly as effective at reducing biofilms as treatment with polysorbates at the initiation of the experiment. These results show that PS20 and PS80 can impact both initial biofilm formation and elicit dispersal of preformed biofilms.



A.



B.

Figure 4.2. PS80 disperses biofilms and inhibits attachment A. PS20 and PS80 at 0.01 % significantly reduce initial attachment. **B.** Biofilm dispersal was assayed with treatment of 0.01% PS20 or PS80. Biofilms were formed for 5 hours then treated for an additional 3 hours. O104:H4 Start indicates samples that were treated from time 0, and CTRL indicates addition of the vehicle dH2O. * indicates $P < 0.05$ based on a two tailed students paired T test. The error bars represent the standard deviation (n=3).

Nonionic surfactants inhibit biofilms

Polysorbates are nonionic surfactants that do not possess a charged head group. We wondered if all surfactants could inhibit O104:H4 biofilm formation. To explore this question, we tested the activity of other surfactants to inhibit O104:H4 biofilms. Six anionic surfactants, two additional nonionic surfactants, and a zwitterion surfactant were analyzed for O104:H4 biofilm formation. The nonionic surfactants Triton X-100 and tyloxapol also reduced O104:H4 biofilms similarly to PS20 and PS80. However, neither the zwitterion nor the anionic surfactants possessed antibiofilm activity (Fig 3). In fact, some of the anionic surfactants tested, which contain components of human bile, actually increased O104:H4 biofilms although this increase was not statistically significant.

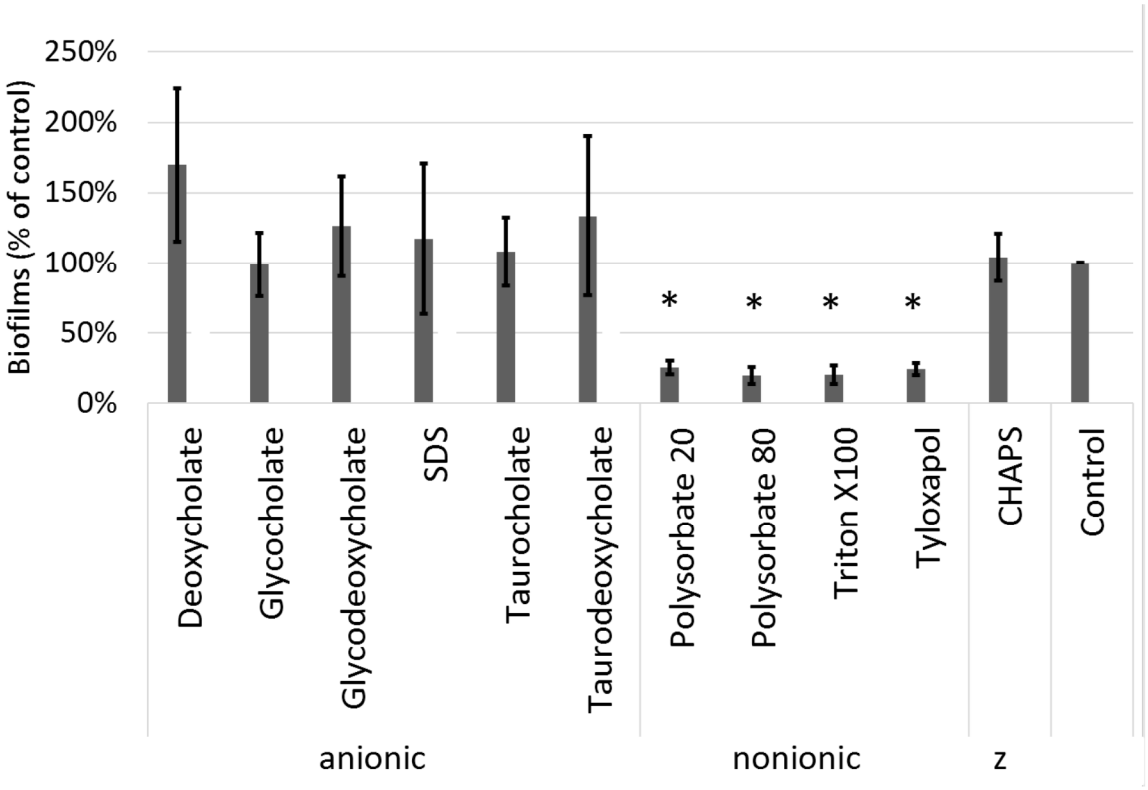


Figure 4.3. Nonionic surfactants inhibit biofilms A variety of surfactants were measured for biofilm inhibition with PS80 concentrations at 0.01% V/V. “z” indicates zwitterion. The error bars represent the standard deviation (n=3). * indicates $P < 0.05$ based on students paired T test compared with the control condition.

PS80 does not reduce colonization of O104:H4 in a mouse infection model

It had previously been hypothesized that *in vivo* biofilm formation correlates with virulence factor expression [191]. Thus, we determined the impact of PS80 upon infection of mice with bioluminescent O104:H4. For this experiment, PS80 was added to the drinking water of O104:H4 infected mice at a concentration of 0.01%, and this group was compared to an untreated infected control group. Mice were then monitored over a 6-day period using the *In Vivo* Imaging System (IVIS) for the number of viable bacteria in the intestinal tract. Additionally, viable O104:H4 in feces were quantified by selective plating on LB agar containing streptomycin at 100 µg/mL and kanamycin at 50 µg/mL which selects for O104:H4 strain RJC001. We observed no significant reduction in luminescence in the PS80 treated group of mice suggesting that PS80 did not impact bacterial colonization (Fig. 4.4A). In fact, the PS80 treated mice in days 3, 4, and 5 had modest but significant increases in luminescence. Similarly, bacteria recovered in feces did not show a significant reduction at day 2 or day 4 and a significant but marginal reduction at day 1 (Fig. 4.4B). At day 6 the fecal bacteria load was significantly lower in the treated group suggesting that polysorbate might assist in clearing the infection; however, this was not supported by equivalent *in vivo* luminescence at day 6 (Fig. 4.4B). At the end of 6 days the mice were euthanized and tissue samples were collected from the cecum. Viable O104:H4 was quantified in the cecum and there was no significant difference between the treated and untreated mice (Fig. 4.4C). The weight of the mice was also measured and normalized to the starting weight. The PS80 treated mice gained weight faster and had higher weight gains than the untreated mice during

every day of the experiment, but this difference was not statistically significant (Fig. 4.4D).

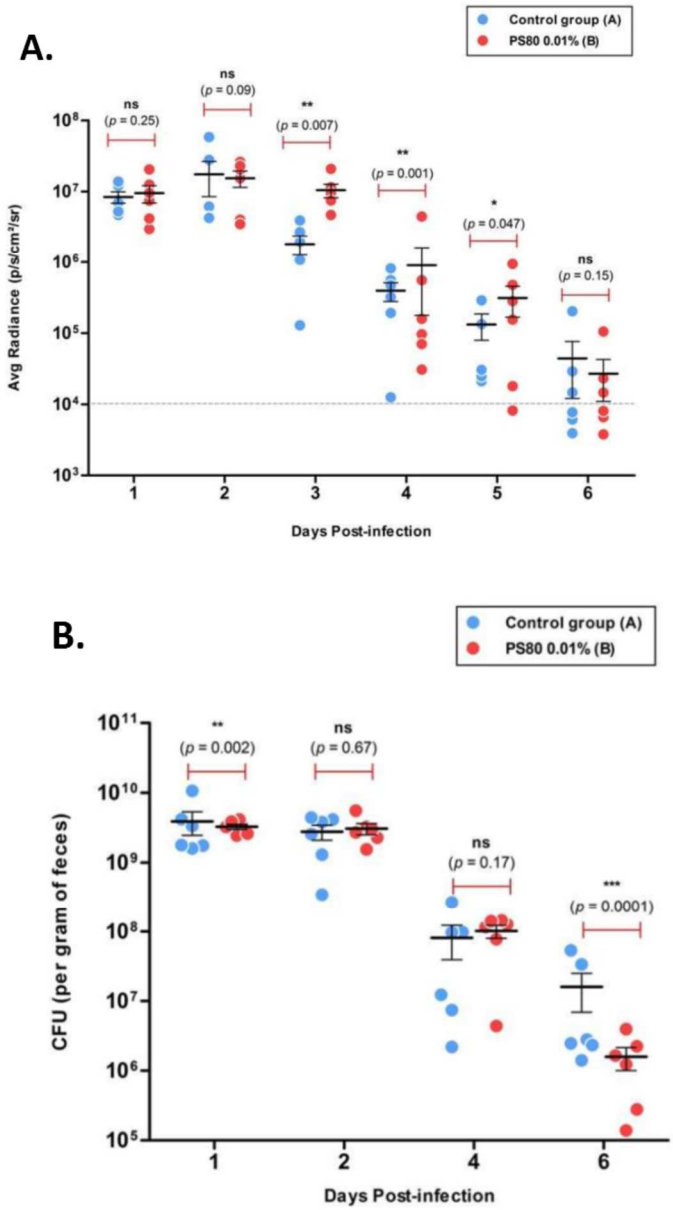
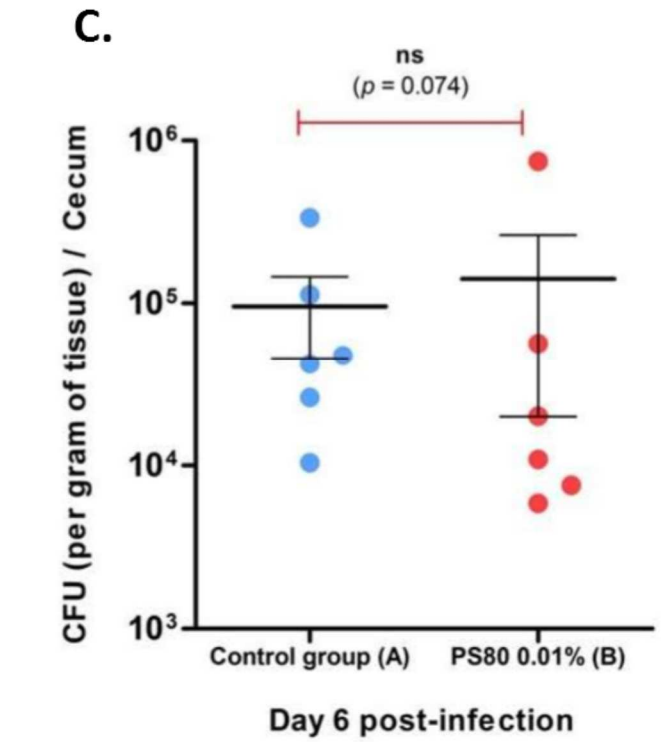
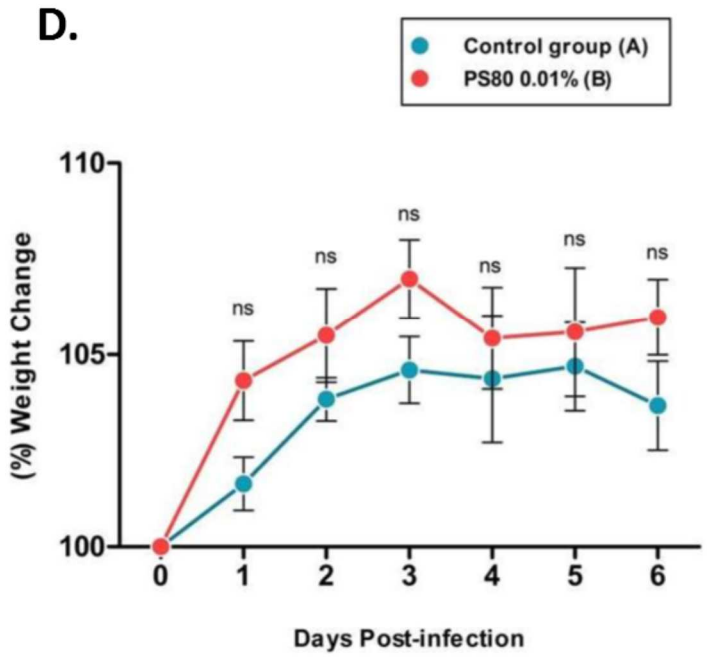


Figure 4.4. PS80 does not reduce O104:H4 colonization A. The number of colonizing bacteria was measured daily for six days by average radiance using the IVIS. **B.** The number of O104:H4 CFU in feces was quantified by serial dilution plating on selective media. **C.** At day 6, viable O104:H4 were quantified from harvested cecum by dilution plating. **D.** Mouse weight during the infection was measured daily and normalized by percent of starting weight. (n=6)

Figure 4.4. (cont'd)





PS80 inhibits virulence of O104:H4 in a mouse infection model

Although colonization was not significantly affected by PS80, we quantified pathology of the cecum to determine if there was a difference in severity of disease. Cecum samples were fixed and immobilized in paraffin and thin sections were mounted on slides followed by staining with hematoxylin and eosin. These sections were scored blindly for tissue pathology. All animals in the untreated group exhibited inflammation ranging from mild to severe, and in 5/6 animals the inflammation was neutrophilic (Table 4.2, Fig. 4.5A). By comparison, only 1/6 animals in the PS80 treated group exhibited any inflammation, which was scored as mild and was not neutrophilic (Table 4.2, Fig. 4.5A). Moreover, the only two animals that presented tissue necrosis were from the untreated group (A2 and A6), and these two animals exhibited the most severe inflammation, with the severity of inflammation proportional to the severity of necrosis (Table 3.2). Additional histopathologic findings in the untreated group included dilation of lamina propria lymphatic vessels and submucosal edema in animals with inflammation. The difference of the average in inflammation score between the two treatment groups was statistically significant (Fig 5B). These results indicate that treatment with PS80 did not significantly impact colonization levels but did significantly reduce pathology of infecting O104:H4.

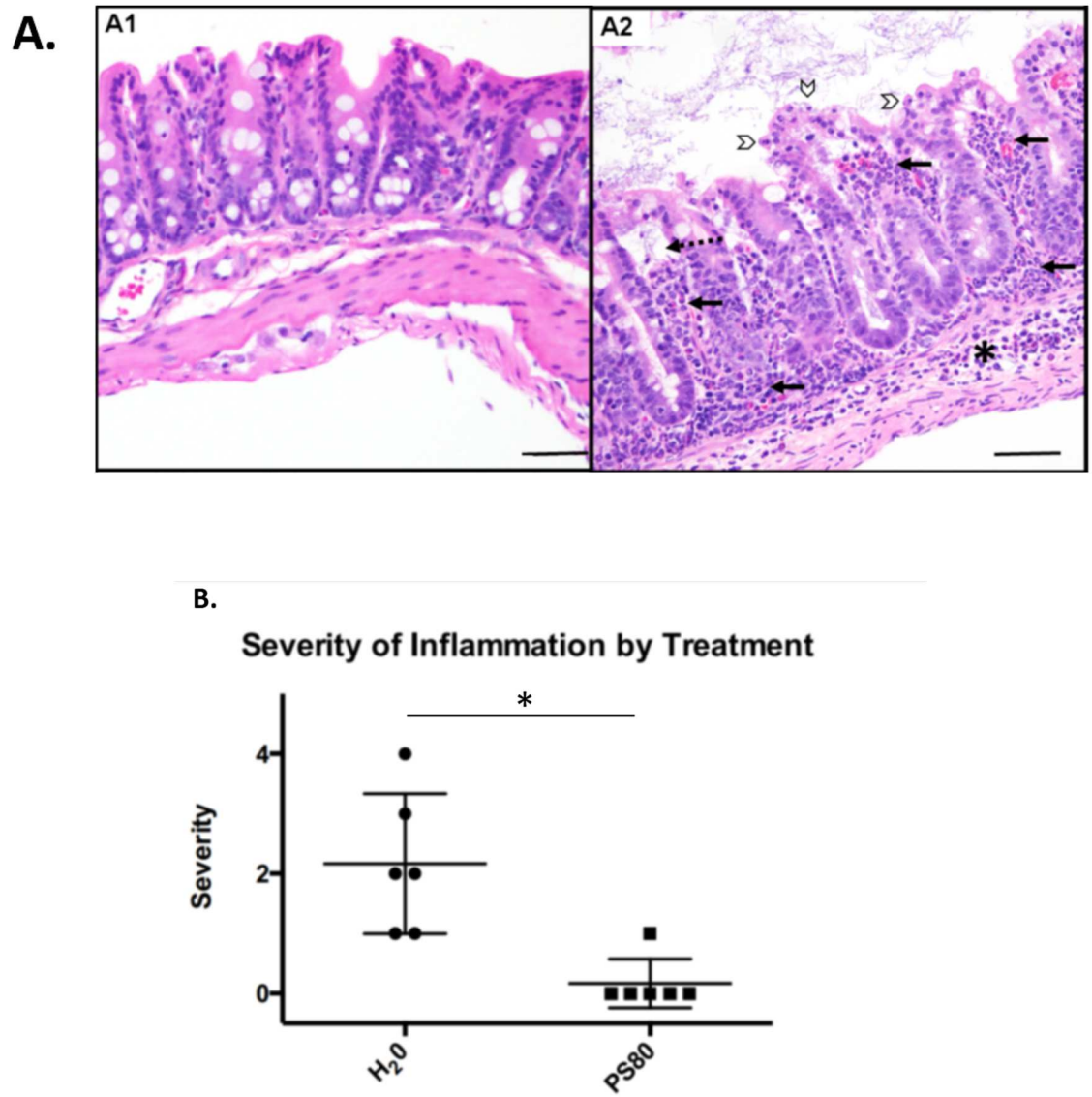


Figure 4.5. PS80 prevents O104:H4 pathology A. Hematoxylin and eosin stained slides that are representative of the group are shown. A1 shows a PS80 treated animal with normal serosa, muscularis, submucosa, lamina propria and epithelium (from bottom to top). A2 shows an untreated (H20 group) animal with neutrophilic and mononuclear inflammation of the submucosa (*), and lamina propria (solid arrows), dilation of lymphatic vessels (dashed arrow), and epithelial cell necrosis and sloughing (open arrowheads). **B.** The mean inflammation scores from cecum of mice sacrificed at day 6 as measured by histopathology is shown. * indicates $P < 0.05$ based on students paired T test. The error bars represent the standard deviation (n=6).

Discussion

The *E. coli* O104:H4 strain that led to the devastating outbreak across Europe in 2011 resulted from the evolution of a novel bacterial pathogen that combined the superior colonization and persistence abilities of EAEC with high toxin production from an O157:H7 *stx* phage. This outbreak led to the highest prevalence of HUS ever recorded. Studies utilizing a germ-free mouse model suggested that *in vivo* biofilm formation was correlated with high levels of virulence factor expression including toxin gene expression [191]. We hypothesized that this was a causative duality in that the high-cell density state of a biofilm induced toxin gene expression, and a disruption of *in vivo* biofilm formation would reduce disease severity. To test this hypothesis, we searched for compounds that disrupted biofilm formation of O104:H4 to determine if they impact *in vivo* disease outcomes. Our results indicated that O104:H4 was highly resistant to the vast majority of anti-biofilm compounds that we examined as they had no significant impact on *in vitro* biofilm formation. However, we determined that the surfactants PS20 and PS80 were potent O104:H4 anti-biofilm compounds, both inhibiting the formation of biofilms and dispersing preformed biofilms at sub-micromolar EC_{50} concentrations. In support of our hypothesis, treating O104:H4 infected mice with PS80 completely abolished clinical symptoms.

The mechanism of PS80 inhibition of biofilms remains to be determined, although our results offer some clues. PS20 and PS80 appeared to function at all levels of biofilm formation, impacting both initial adherence and dispersing mature biofilms. Thus, we hypothesize that the target(s) of PS20 and PS80 are essential for all stages of O104:H4 biofilm formation. Of note, other nonionic surfactants such as Triton X100 and Tyloxapol

also disrupted biofilms, but anionic and zwitterion surfactants had no effect. As the nonionic surfactants do not share significant atomic compositions, this result suggests that it is the physical properties of these molecules, rather than their specific chemical moieties, that inhibits O104:H4 biofilms. The biofilm matrix is generally anionic, and thus cationic surfactants have been shown to disperse biofilms. As an example, the cationic surfactant delmopinol is used to disrupt oral biofilms [197].

The molecular mechanism by which O104:H4 forms biofilms has not yet been determined. Cyclic di-GMP signaling has been described to promote biofilms of O104:H4 by inducing curli fibers, and it was hypothesized that the curli led to inflammation increasing the ability of toxin to access the bloodstream [198]. Interestingly, PS20 was recently shown to disrupt UPEC pellicle formation by inhibiting the ability of curli fibers to form an extracellular network [199] . We are currently exploring if nonionic surfactants disrupt O104:H4 biofilms by inhibiting curli function.

Treatment of infected mice with PS80 had no significant impact on *in vivo* colonization even though this compound was a potent inhibitor of biofilm formation and able to disperse preformed biofilms *in vitro*. Rather, major differences were seen in the pathological outcomes of colonization including virtually no tissue inflammation or necrosis in treated animals upon a blinded histopathological scoring of infected tissue. Of note, this reduction in pathology occurred even during days in which treated mice exhibited increased colonization as measured by *in vivo* luminescence. We hypothesize this increased luminescence was due to dispersal from biofilms as bacteria in biofilms may be oxygen limited which is a requirement for bioluminescence.

The mechanism by which PS80 inhibits O104:H4 pathology remains to be determined although the *in vitro* results presented here and in other studies would suggest that inhibition of biofilm formation is responsible. The severity of pathogenic *E. coli* infections derives from high levels of Shiga toxin entering the bloodstream resulting in HUS. Our results are consistent with a prior study that found the pAA plasmid promotes increased biofilm formation and translocation of Stx2 across an *in vitro* epithelial cell monolayer [195]. We hypothesize that inhibition of biofilm formation decreases disease severity by reducing Stx2 expression and/or decreasing Stx2 translocation. In this study, we attempted to quantify Stx2 production from the cecal contents of mice harvested at day 6, but were unable to detect *stx2* gene expression using quantitative RT-PCR. In the mouse model used here, the infection was nearly resolved by day 6 exhibiting a 3 to 4 $\log_{10}$ decrease in viable bacteria (Fig. 4.4B), which was likely responsible for our inability to detect *in vivo stx2* gene expression. Alternatively, PS80 treatment could function by modulating the immune response to O104:H4 or alter its ability to adhere to epithelial cells, although contrary to its known anti-biofilm activity, we could find no published literature to support such activity for PS80.

Regardless of the mechanism, our studies suggest that polysorbate administration is an attractive approach to alter an O104:H4 infection from a severe life threatening condition to a self-limiting mild enteritis. PS20 and PS80 classify as O104:H4 anti-infective compounds. Unlike traditional antibiotics, these compounds do not significantly impact growth, but rather they modulate an important virulence property [200]. Importantly, PS80 reduces virulence with the need for additional antimicrobials.

Anti-infective approaches are an attractive strategy to treat infectious disease as they convert dangerous pathogens into more mild infections that the body can naturally resolve. In addition, it is hypothesized that the selection for resistance to such compounds would be much lower than antibiotics [200]. Indeed, PS20 and PS80 treatment of O104:H4 would virtually exhibit no selection for resistance as these compounds do not significantly impact gut colonization. An additional advantage of these compounds is that they should be much less disruptive to the normal gut microbiota than traditional antibiotics as polysorbates are common, non-toxic food additives that our gut microbiota frequently encounters. Maintaining an intact gut microbiome should be beneficial in resolving O104:H4 infections and prevent other secondary enteric infections that rely on microbiota disruption such as *Clostridium difficile* [201].

Our research shows that polysorbates are potent anti-biofilm compounds of O104:H4 both *in vitro* and *in vivo.* PS20 and PS80 have been designated Generally Recognized as Safe (GRAS) compounds by the FDA and are thus common components of food. In fact, ice creams can contain 0.02% or higher PS80, a concentration that is orders of magnitude higher than the *in vitro* EC_{50} to inhibit O104:H4 biofilms determined here [202]. Interestingly, non-symptomatic O104:H4 infections were reported during the 2011 outbreak [203], and it is intriguing to speculate that one factor that may have contributed to these cases is consumption of polysorbates in food products. Pathogenic *E. coli* often cause disease by contaminating food products, and the 2011 O104:H4 epidemic was mediated by colonized fenugreek sprouts [204]. Because of the safety of polysorbates and their ability to disperse *in vitro* biofilms,

washes of contaminated food with polysorbates could reduce infection through colonized food. PS80 administration in the drinking water of mice prevented nearly all histopathology. If this result is confirmed in additional animal studies and is representative of human infections, PS80 could be effective at significantly reducing HUS occurrence in future O104:H4 outbreaks.

Acknowledgments

We are grateful to Robert Parker and Shannon Manning for efforts to quantify *in vivo stx* expression and for providing us with strain TW16133.

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Table 4.1: Antibiofilm compounds examined in this study

Compound	Reference
ABC-1*	[205]
ABC-1 Derivatives* 6,8,14,17,19,21,27,31,34,52,53,54,62	unpublished
Diguanylate cyclase inhibitors* 3, 10, 18	[206]
Dispersin B*	[207]
Norspermidine*	[208]
D-Tyrosine*	[209]
Polysorbate 80	[210]
Polysorbate 20	[196]

***** compounds did not exhibit significant reduction of O104:H4 biofilm formation (data not shown).

Table 4.2: Histopathology score

Animal ID	Inflammation*	Necrosis	Treatment
A1	2 (M>N)	-	none
A2	3 (N=M)	+ (individual cells)	none
A3	2 (N=M)	-	none
A4	1 (M>N)	-	none
A5	1 (M)	-	none
A6	4(N>M)	+++(erosion & ulceration)	none
B1	0	-	PS80
B2	1(M)	-	PS80
B3	0	-	PS80
B4	0	-	PS80
B5	0	-	PS80
B6	0	-	PS80

*M=mononuclear, N=neutrophil

CHAPTER 5: CONCLUSIONS AND FUTURE PERSPECTIVES

The T2SS has been studied heavily by the scientific community to establish its role in pathogenesis, and a large amount of work has been done characterizing the proteins involved in its assembly and function. Moreover, many attempts have been made to determine how proteins are targeted for secretion by the apparatus. However, prior to the beginning of this work the T2SS of *V. cholerae* was not known to be regulated at any level and was thought to be constitutively expressed. In addition, very little work had been done to investigate transcriptional control of the system. Since then the first major advances in regulation of the *V. cholerae* T2SS have manifested. The first is the revelation that sigma E induces transcription from the P*epsC2* promoter [100]. The other 2 major findings have been uncovered in this work. First, T2SS is transcriptionally induced by c-di-GMP through the transcriptional effector VpsR. VpsR is a biofilm regulator and this finding further links biofilms to the T2SS. However, this work was unable to positively associate a phenotype to the induction of the T2SS by VpsR and c-di-GMP. In addition, attempts to uncover the effects of this regulation were masked by compensation from the other promoter P*epsC2*. This work creates exposes new regulatory factors to the T2SS and opens the field up to a lot of interesting questions in addition to those discussed in Chapter 2. Such as why does the cell need to coordinate expression of the T2SS with c-di-GMP and VpsR which are both implicated in biofilm formation but there is as of yet no evidence that coordination of the T2SS with toxin production is needed even though toxin secretion is a major function of the T2SS. Investigation into the regulons of σ^E and VpsR to see what secretion products are being affected may yield insights in this area.

The second major advancement is the discovery of the existence of the second promoter set in the middle of the T2SS gene cluster that encodes 2 transcriptional start sites just upstream of *epsG*. There is as yet an undefined role for these promoters, and nothing is known about their regulation. This discovery should also spur interest in the prevalence and role of transcriptional regulation of the T2SS in other species of bacteria, and the search to see if this second promoter exists upstream of the pseudopilin gene in those species as well. Future work on identification of regulatory elements which effect this promoter is an important next step in research. The existence of PepsG suggests that stoichiometry of the T2SS proteins is important, it could simply be a constituative promoter that increases the number of pseudopilins, but it may also be determined that there are regulatory conditions where the promoter is turned on or off and this results in a phenotypic change of the T2SS. For instance, if PepsG was turned off and there were few or no pseudopilins then the T2SS may only serve as a pore to relieve membrane stress, then when P*epsG* is induced secretion resumes.

Because a phenotype has not been established for the regulation of the T2SS from P*epsC1* and because we do not know what is regulating P*epsG* another it may be valuable to control expression of the genes in the T2SS independent of any native regulatory elements. Inserting combinations of IPTG and arabinose inducible promoters in place of PepsC1, PepsC2 and PepsG and tuning their expression may help uncover a phenotype. This is especially true because currently the elements that regulate the T2SS regulate a large number of other genes in *V. cholerae* which increases the odds of a false positive phenotype. For instance, it may be useful to induce expression from P*epsG* on the genome and see if hyper-pseudopili form or biofilms are increased.

Together these discoveries of T2SS promoters and transcriptional control pose many questions about just how complicated regulation of the system may be, it is already far more complex than originally assumed. For example, we also show that induction of the T2SS, which should lead to more T2SS apparatus on the cell, does not results in increased secretion in the case of serine proteases nor cholera toxin. If this is the case what purpose does transcriptional control of the T2SS serve?. The T2SS has been observed in V. cholerea to have polar secretion in one study [211] and also to be distributed around the cell in another study [212]. Transcriptional regulation may have an effect on localization of the T2SS if under some conditions it is expressed highly, such as when c-di-GMP levels increase. If pseudopili are elaborated in these conditions, then polar localization could be important for mediating attachment and biofilm formation. This model, although speculative, is consistent with my results in Chapters 2 and 3 and the published literature and could provide a framework for future research.

Thus far, it has not been shown that a T2SS can form a hyper-pseudopilus in a WT genetic background. I have shown evidence that the T2SS of *V. cholerae* can form a hyper-pseudopilus by confocal microscopy and under wild type conditions external EpsG can be detected in flow cells under confocal microscopy and also through western blocks of sheared cells. Although these data are not yet conclusive, the existence of a naturally occurring hyper pseudopilus is exciting none the less as it is the first demonstration of the hyper pseudopilus in a *Vibrio* species as well as the first evidence of it in a WT strain where the major pseudopilin was not overexpressed.

Going forward, research efforts should concentrate on verifying that the T2SS is the base for hyper-pseudopilus. In order to determine this, the T4P genes should be

knocked out and the resulting strain should be examined under TEM. Alternatively, antibodies for T2SS secretin EpsD may work better in TEM and labeling the secretin then looking for hyper pseudopili associated with may be more effective since attempts to visualize the hyper-pseudopilus with antibodies for EpsG have so far been unsuccessful. Another major focus of study should be on the observation that biofilms were increased with ectopic expression of *epsG*. Recently a study has tracked biofilm development at the single cell resolution [213] and this technique may be valuable in determining a role for the hyper-pseudopilus. Direct observation of biofilm formation with over expression of EpsG compared to WT biofilm development may be useful, and additionally observation of mixed populations of WT and *epsG* over expressing strains could show us if the *epsG* over expressing strains favor a particular position in the biofilms such as at the top of stalks or near the base in attachment.

In Chapter 4, I report my successful identification of a novel anti-biofilm compound. Antibiotics have been a staple chemotherapy for bacterial infections for many decades now; however, the rise of antibiotic resistance has been threatening the success of this class of treatments. Biofilms are the most common form of lifestyle employed by bacteria which cause problematic infections. Antibiofilm compounds as well as other anti-infective agents offer an exciting alternative form of chemotherapy. Here I present not only a highly effective antibiofilm compound *in vitro* but also one that works *in vivo*. I demonstrated that PS80 inhibits O104:H4 biofilm formation by 90% and also disperses preformed biofilms *in vitro*. I also demonstrated that PS80 reduces disease symptoms in a mouse model *in vivo*, which is a major step forward as many treatments will never make it out of the *in vitro* stage of discovery. It is also already

approved by the FDA for use in food and is classified as Generally Recognized as Safe which reduces many of the barriers to bringing a drug to market. These properties make PS80 a very attractive candidate for treatment of *E. coli* diseases such as Travelers diarrhea.

The range of bacterial biofilms that PS80 is effective against also deserves further investigation. PS80 has, for example, is also a promising antibiofilm compound against *P. aeruginosa* [210]. Thus, its efficacy against other *P. aeruginosa* strains of clinical relevance, such as cystic fibrosis isolates, as well as other pathogenic strains of *E. coli* and other bacteria warrants investigation. The *P. aeruginosa* study also demonstrated that resistance to PS80 could evolve. Hence, it is important to gain inisights into the mechanisms of resistance. Moreover, my discovery that other nonionic surfactants with diverse chemical structures also inhibit biofilm formation expands the range of antibiofilm compounds that singly or in combinations could be used to minimize the evolution of resistance.

Another important area of study is in co-administration of PS80 with other drugs such as antibiotics. In the case of O104:H4 my initial testing of PS80 co-administered with a variety of antibiotics did not uncover any synergistic effects. (Fig. A1) However, these experiments involved the exposure of cells to the antibiofilm compound and antibiotic early on, and before the biofilms formed. Thus, future studies are necessary that examine that efficacy of the antibiotic treatment when co-administered with PS80 to treat pre-formed biofilms. Furthermore, many other combinations of antibiotics could be tested to investigate their efficacy when co-administered with PS80 to treat the bacterial biofilm infection.

Currently we do not know what the mechanism of action of PS80 is and this is an important future area of study. The antibiofilm properties of PS20, another nonionic detergent, against *E. coli* have been reported to involve the disruption of the biofilm curli fiber mediated networks at the air liquid interface [214]. A similar mechanism of action could explain the antiobiofilm properties of PS80 against O104:H4. However, there are a number of other possibilities and perhaps an untargeted approach to uncovering the mechanism of action such as evolution in the lab would be useful not only in discovering mechanism of biofilm inhibition but also in uncovering how resistance may evolve. Other possible targets that are known to be involved in biofilm formation in O104:H4 include c-di-GMP [215], long polar fimbriae [129] and AAF which has been implicated in biofilm formation on fresh produce [128]. The latter raises the intriguing possibility of using PS80 as a pre-treatment in food processing to reduce the possibility of an outbreak of EAEC.

The *in vivo* results show great promise however there is much work that needs to be done *in vivo*. Although PS80 is acting as an antibiofilm compound in vitro this still needs to be verified *in vivo,* sacrificing mice at an earlier time point when bacterial loads are higher and carefully sectioning the cecum so as not to disrupt bacteria we could look for the stacked brick morphology in close association with the epithelial cells [184, 216] and see if PS80 reduces or eliminates this. Because I found that other nonionic surfactants that did not have similar chemical makeup to PS80 inhibited biofilms it is likely that the antibiofilm activity is due modification of the physical properties of the liquid and this disrupts biofilm formation. If the PS80 is working through a mechanism that affects the physical properties of the media, then will it work in a larger animal

where it may be more difficult to alter the physical properties of the environment? In this case future in vivo experiments should be carried out in larger animal models unfortunately the largest animal model I am aware of is the infant rabbit model [217] as such there is a need for development of larger animal models of infection by O104:H4.

Here I have contributed a variety of new discoveries to the field of *V. cholerae*, T2SS and biofilms. New regulation of transcription and a link to a biofilms associated regulatory element has been uncovered. Demonstration of a hyper-pseudopilus as a phenotype, and the discovery of a new drug for application against biofilms. All of these discoveries may have much wider applications in many more distant species of bacteria.

APPENDIX

APPENDIX

Table A.1. Bacterial strains

Strain	Description and oligos (5'-3')	Reference
	Vibrio cholerae	
C6706str2	Wild Type	[58]
CW2034	Δ*vpsL*	[155]
RS01	Δ*epsG*	This study
WN310	Δ*vpsR* Δ*vpsL*	[40]
JP1195	Δ*vpsT* Δ*vpsL*	[40]
pDS54	Δ*vpsL* Δ*flrA*	[44]
	Δ*hapR*	
	Escherichia coli	
E. coli S17	λpir	[218]
TW16133	*E. coli* O104:H4 strain TW16133	[216]
RJC001	O104:H4 strain RJC001	[219]

Table A.2. Plasmids and primers

Plasmid	Description-all primers (5'→3')	Reference
p6f12	**-1052 to +123 *epsG* promoter cloned into pBBRlux** F-ATACACTAGTGATCACTCGCCAATTGGCG R-AGACGGATCCATCCGCTTTCTCTTTG	This study
pAEK1	**-1052 to +85 *epsG* promoter cloned into pBBRlux** F-ATACACTAGTGATCACTCGCCAATTGGCG R-AGACGGATCCCAAAGCTGGCCAGAAT	This study
pAEKv23	**-1052 to +45 *epsG* promoter cloned into pBBRlux** F-ATACACTAGTGATCACTCGCCAATTGGCG R-AGACGGATCCTACTTCGAGCAGGGTA	This study
pAEKvc5	**-1052 to +9 *epsG* promoter cloned into pBBRlux** F-ATACACTAGTGATCACTCGCCAATTGGCG R-AGACGGATCCTTTTTTCATAGTTACTC	This study
pRSepsGfull	-**1052 to -1 *epsG* promoter cloned into pBBRlux** F-ATACACTAGTGATCACTCGCCAATTGGCG R-ATACGGATCCAGTTACTCCACACTATGTCG	This study
pAEKlv8	**-628 *epsC* promoter cloned into pBBRlux** F ATAACTAGTCATAAGGAATAATCCGGC R ATACGGATCCAAATTTCCACGTTATTCC	This study
pAEK6	**-451 *epsC* promoter cloned into pBBRlux** F-AGACACTAGTGCGTTGGTCTGAGATC R-ATACGGATCCAAATTTCCACGTTATTCC	This study
pAEKsv1	**-228 *epsC* promoter cloned into pBBRlux** F-ATAACTAGTGCCACATTGCCTCTCTAAGC R-ATACGGATCCAAATTTCCACGTTATTCC	This study
pAEK5	**-153 *epsC* promoter cloned into pBBRlux** F-AGACACTAGTCAAGCAAGTCGAC R-ATACGGATCCAAATTTCCACGTTATTCC	This study
pAEKepsc2	**-124 *epsC* promoter cloned into pBBRlux** F-AGACACTAGTCACTTCGCTCCAC R-ATACGGATCCAAATTTCCACGTTATTCC	This study
pCMW75	*qrgB* expression vector	[155]
pCMW98	*qrgB* (GG→AA) active site mutant expression vector	[155]
pBBRlux	Promoterless reporter back bone and vector control expressing Lux operon	[220]
pWD615	*etxB* expression vector	[148]
pRH2	Promoter reporter for gene *ctxA* in pBBRlux	[40]
pRSPvesA1	**Promoter reporter for gene *vesA* (VCA0803) in pBBRlux** F- ACGTACTAGTGGAAGAGATCCAACTACCGC R- AGATGGATCCGCGTCACCTCATTGGTTGAATTG	This study
pMLK1A	**Promoter reporter for gene *vesB* (VC1200) in pBBRlux** F-ATTGAGCTCTAAAACGACGGTGAACCCCA R-ATTGGATCCTGTGGATTCCGCATAGAGCA	This study

Table A.2. (Cont'd)		
pMLK2A	**Promoter reporter for gene *vesC* (VC1649) in pBBRlux** F-ATTGAGCTCGGTGCGTAAAACCATGAGTTG R-ATTGGATCCGTGTTCCAACCACTCCTGAT	This study
pEVS141	Vector control for expression vectors	[221]
pDL1711	Promoter reporter for *vpsT* in pBBRlux	[222]
Primers to make RS01	USF-ATACACTAGTGATCACTCGCCAATTGGCG USR-TAAGGAGGATATTCATATGAGTTACTCCACACTATGTCG DSR-ATGTGTTGACTGACCGAGCG DSF-GAAGCAGCTCCAGCCTACACGCTTGGCTAATTAGCGGTAAC	This study
5'-RACE primers	GSP: GCGCATGCTCTACCGCCCAAT Primers used for nested amplification: CTGGCCCTCCCCAAGCGACAA GTCATTCAATATTGGCAGGT	This study

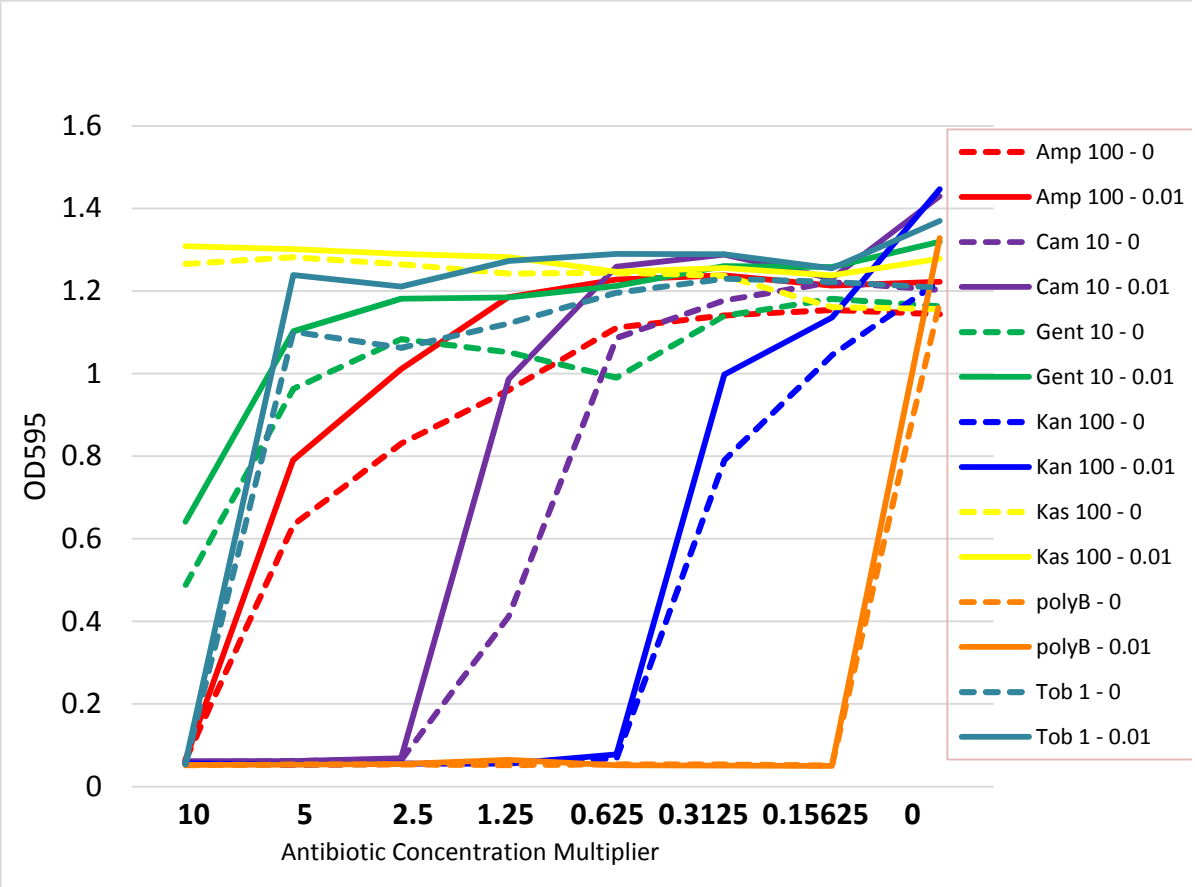

Figure A.1.: Antibiotic and Polysorbate Coadministration PS80 coadministration with antibiotics. Solid line represents the same colored antibiotic and PS80, solid line represents antibiotic administration alone. In all cases PS80 did not increase efficacy of antibiotic. Antibiotics are listed in short hand followed by the concentration multiplier in ug/ml then the PS80 concentration. For instance amp 100x 10 = 1000 ug/ml ampicillin at the highest concentration. Amp = Ampicillin, Cam = chloramphenicol, Gent = Gentamicin, Kan = Kanamycin, Kas = Kasugamycin, polyB = polymyxin B, Tob = Tobramycin.

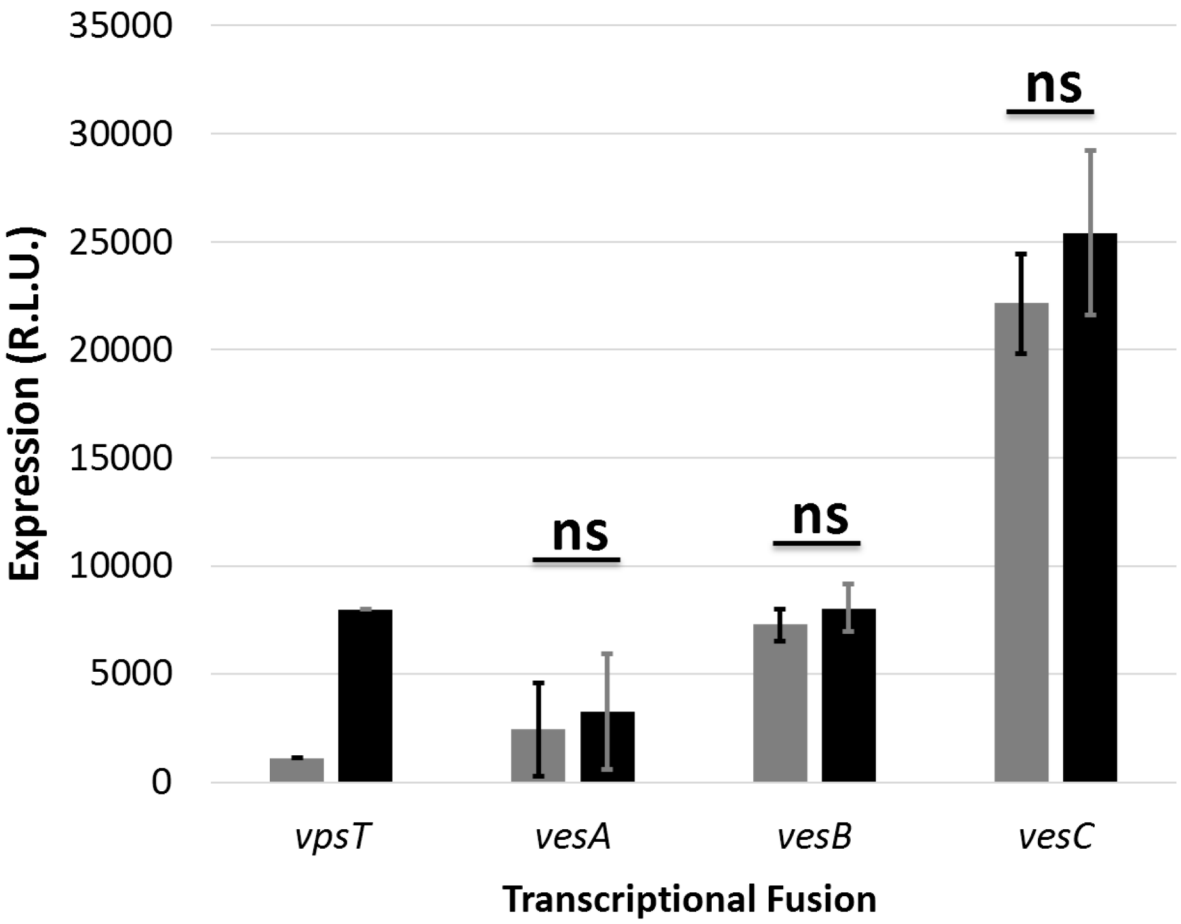

Figure A.2. C-di-GMP does not impact transcription of the ves proteases The promoter region (500 bases upstream of the coding sequence) of the *vesA*, *vesB*, and *vesC* genes was clones as transcriptional fusions with luciferase. Luciferase was measured at high (black bars) and low (grey bars) c-di-GMP via induction of *qrgB* with 100 mM IPTG. The fusions were expression in a V. cholerae *vpsL* mutant. Error bars represent the standard deviation (n=3).

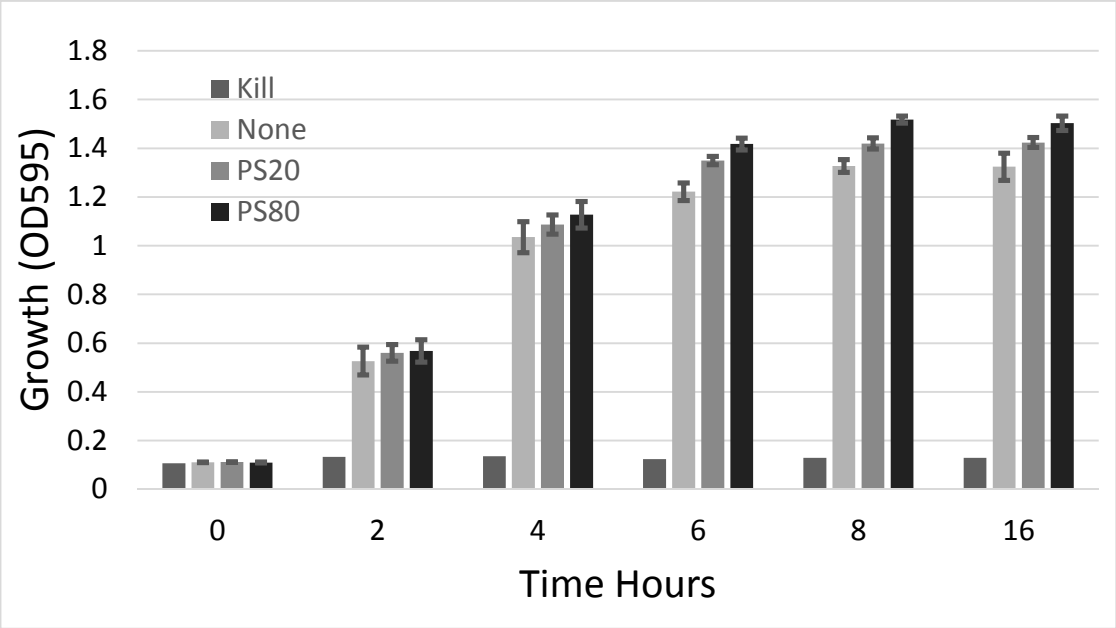

Figure A.3. Polysorbates do not inhibit growth of E. coli O104:H4 Growth of O104:H4 in LB media was monitored over time in the presence of 0.01 % PS20, PS80, or 50 µg/mL kanamycin and tetracycline 1 µg/mL (kill) as a positive control. The error bars represent the standard deviation (n=3).

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